The balancing effect between MAPK and NFκB pathways for the transcriptional regulation of Toll-like receptors



Xinyang Hong
Linacre College
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy
Hilary 2016

Abstract

Toll-like receptors (TLR) are a family of pattern recognition receptors crucial for pathogen pattern recognition. Upon activation, TLRs induce innate immune responses such as cytokine production. However irregular TLR activities can provide fatal, hence fine tuning of the TLR induced responses are necessary.

The TLR mediated immune responses are controlled by the positive/negative regulation of TLR signalling pathways, relocation of TLR proteins and modulation of TLR transcription. Systematic analyses of the agonist-induced transcriptional changes of TLRs were shown for the first time in my thesis. In my experiments, I have shown that each agonist induced a unique pattern of TLR transcription. Following PAM stimulation, mRNA levels of the cognate TLR1/2 increased whereas mRNA levels of the cross-regulating TLR4, 7/8/9 reduced in both cell lines and splenic macrophages from different mice strains. Through investigation of the signalling pathways responsible for mediating such TLR transcriptional changes, I then discovered the balancing effect between NFκB and MAPK signalling pathways. PAM induced TLR transcriptional changes were controlled by the additive and/or antagonistic interference between MAPK signalling cascades, ERK, JNK, P38 and NFκB signalling pathways. This was the first time that signalling synergy between MAPK and NFκB pathways were shown. Furthermore, PAM induced transcription of TLR1 and TLR8 may be partially regulated by the indirect feedback mediated by protein production. Importantly, the maintenance of the basal TLR mRNA expression also required activation of both MAPK and NFκB signalling pathways.

In addition, signalling control for TLR transcription induced by different agonists (PAM vs. LPS) or in different species (chicken vs. mice) was compared. LPS induced transcriptional changes of the cross-regulating TLR1/2 and 3 but not the cognate TLR4 in RAW cells. The LPS induced TLR transcriptional changes required activation of a combination of MAPK and NFκB signalling pathways which shared both similarities

and differences to the PAM induced signalling activation. In chicken, PAM induced more potent signalling activation, regulating the TLR transcriptional changes at a lower concentration than in mice. Overall, this thesis demonstrates that the transcriptional regulation of TLRs is complex, mediated by the coordination between MAPK and NFκB signalling pathways. These studies have significant implications in providing detailed insight of TLR transcriptional regulation which plays an important role in the regulation of TLR mediated innate immune responses.

Please watch the following videos that I made for:

A short introduction about TLR regulation - https://youtu.be/LTDdEZ3S97o

A short explanation about TLR signalling - https://youtu.be/51IY5XhdJR8

Acknowledgements

Over the past four years I have been privileged to work with a number of enthusiastic and dedicated scientists. I am particularly indebted to the following people:

My supervisors Dr. Adrian Smith and Dr. Sebastian Shimeld who have been incredibly supportive and encouraging throughout,

My colleagues, especially Amy Boyd, Patrik Flammer and Stephen Preston who have always been helpful and provided practical advice whenever needed,

My students Jae Suh, Melanie Panagi, Susie Gold and Steven Fiddaman whom I shared enjoyable lab experience and much friendship with for the best part of four years,

My friends Andy Tran and Harpreet Sandhu and my family who have supported me throughout my PhD.

Authors Declaration

I declare that the work in this thesis was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: ..... Date: .....

Table of Contents

Abstract 1
Acknowledgements 3
Authors Declaration 4
Table of Contents 5
List of Figures and Tables 9
Abbreviations 13
Chapter 1 Introduction 17
1.1 The innate and adaptive immunity 17
1.2.1 RIG-I like receptors (RLRs) 18
1.2.2 Nod-like receptors (NLRs) 19
1.2.3 C-type lectin receptor family (CLRs) 21
1.2.4 Toll-like receptor family (TLRs) 21
1.3 The Toll-like receptors 21
1.3.1 TLRs and their agonists 21
1.3.1.1 Discovery of Toll-like receptors 22
1.3.1.2 Mammalian TLR ligands 22
1.3.1.3 Conserved structure of TLRs 25
1.3.1.4 TLR Orthologues 26
1.3.2 Toll-like receptor signalling 28
1.3.2.1 Signalling pathways of TLRs 28
1.3.2.2 TLR signalling adaptor molecules 30
1.3.3 Regulation of TLR mediated immune responses 31
1.3.3.1 Negative regulators for TLR signalling 32
1.3.3.2 Negative regulators for TLR transcription 35
1.3.3.3 Circadian rhythm for immune responses 36
1.4 Aims and objectives of this thesis 37
Chapter 2 Materials and Methods 39
2.1 Experimental Animals 39
2.2 Isolation and Culture of Splenocytes and Preparation of Monocyte-Derived Macrophages 39
2.3 Preparation of Murine Intestine for Leptin Assay and Isolation of IELs and Epithelial Cells 39
2.4 Intestinal Leptin Assay 39
2.5 Cell Line Culture 41
2.6 TLR Agonists, NFκB & MAPK Signalling Inhibitors and Translation Inhibitors 43
2.7 Alamar Blue Assay 43

2.8 Griess Reaction Assay 43
2.9 Caspase-1 Colorimetric Assay 45
2.10 RNA Extraction 45
2.11 cDNA Synthesis 46
2.12 Polymerase Chain Reaction (PCR) 47
2.13 DNA Gel Electrophoresis 49
2.14 Sequencing 49
2.15 Quantitative Reverse Transcriptase PCR (q-rtPCR) Primer Design and Optimisation 50
2.16 Quantification of Gene Transcription 50
2.17 Transformation with Plasmid DNA 52
2.18 Endotoxin Free Maxi Prep 53
2.19 Plasmid Transfection 55
2.20 Transfection of small interfering RNA (siRNA) 55
2.21 Western Blot 56
2.22 Antibodies 56
2.23 Bioinformatics 58
Chapter 3 Transcriptional regulation of TLRs in murine cell lines 59
3.1 Introduction 59
3.2 Results 62
3.2.1 The TLR basal level changes over time in RAW264.7 cell line 62
3.2.2 The effect of agonists on expression of cognate TLRs in RAW264.7 cell line 65
3.2.3 Transcriptional regulation of non-cognate TLRs in RAW264.7 cell line 65
3.2.4 TLR activation induced pro-inflammatory responses in RAW264.7 cell line 69
3.2.5 TLR activation induced pro-inflammatory responses in P388D1 cell line 72
3.2.6 Transcriptional regulation of cytokines and TLRs in P388D1 cell line 74
3.3 Discussion 78
Chapter 4 Transcriptional regulation of TLRs in primary macrophages 82
4.1 Introduction 82
4.2 Results 86
4.2.1 TLR basal expression in murine splenic macrophages 86
4.2.2 Transcriptional regulation of cognate TLRs in murine splenic macrophage 86
4.2.3 Transcriptional regulation of non-cognate TLRs in splenic macrophage 87
4.2.4 Influence of day/night oscillation in response to TLR agonists 91
4.3 Discussion 96
Chapter 5 Signalling regulation of PAM induced TLR transcriptional changes 103
5.1 Introduction 103

5.2 Results 107
5.2.1 PAM induced MAPK signalling activation 107
5.2.2 MAPK signalling responsible for PAM induced cytokine production 109
5.2.3 Signalling pathways responsible for PAM induced TLR transcriptional regulation 112
5.2.3.1 Pharmacological inhibition of MEK/ERK pathway 112
5.2.3.2 Pharmacological inhibition of JNK pathway 115
5.2.3.3 Pharmacological inhibition of P38 pathway 117
5.2.3.4 Pharmacological inhibition of NFκB pathway 119
5.3 Discussion 123
Chapter 6 The effects of signalling synergy on the transcriptional regulation of TLRs 128
6.1 Introduction 128
6.2 Results 132
6.2.1 Multiple MAPK signalling cascades mediated immune responses 132
6.2.1.1 Multiple MAPK signalling cascades mediated IL1β mRNA changes. 132
6.2.1.2 Multiple MAPK signalling cascades mediated TLR mRNA changes. 134
6.2.2 Synergy between MAPK and NFκB pathways for TLR transcriptional regulation 139
6.2.2.1 Inhibition of individual MAPK signalling terminal cascades with NFκB. 139
6.2.2.2 Inhibition of complete MAPK signalling pathway with NFκB 141
6.2.3 Pharmacological inhibition altered phosphorylation status of MAPK terminal cascades 143
6.2.3.1 Phosphorylation status of ERK1/2 following inhibition of MAPK or NFκB 143
6.2.3.2 Phosphorylation status of JNK1/2 following inhibition of MAPK or NFκB 149
6.2.3.3 Phosphorylation status of P38 following inhibition of MAPK or NFκB 150
6.3 Discussion 162
Chapter 7 Inhibition of MAPK signalling pathways with small interfering RNAs 170
7.1 Introduction 170
7.2 Results 172
7.2.1 Targeted mRNA knockdown of ERK, JNK, P38 and off-target effect of siRNAs. 172
7.2.2 The effect of ERK1 knockdown on PAM induced transcriptional regulation of IL1β and TLRs 174
7.3 Discussion 180
Chapter 8 Indirect regulation of PAM induced TLR mRNA expression 183

8.1 Introduction 183
8.2 Results 185
8.2.1 Effect of secreted protein on PAM induced IL1β and TLR mRNA levels 185
8.2.2 High concentration of PAM is required for inducing the transcriptional changes of IL1β and TLRs in RAW cells 190
8.2.3 Effect of inhibiting translation, protein transportation and secretion on PAM induced IL1β mRNA level 190
8.2.4 Effect of inhibiting translation, protein transportation and secretion on PAM induced TLR mRNA levels 192
8.3 Discussion 199
Chapter 9 Comparative studies of TLR transcriptional regulation in different species or induced by different agonists 202
9.1 Introduction 202
9.2 Results 204
9.2.1 PAM induced IL1β and TLR transcriptional changes in HD11 cell line 204
9.2.2 Influence of day/night oscillation on agonist induced IL1β and TLR mRNA expression in chicken splenic macrophages 204
9.2.3 Signalling pathways required for LPS induced TLR transcriptional changes in murine RAW264.7 cell line 207
9.3 Discussion 214
Chapter 10 Conclusion 221
10.1 Agonist induced TLR transcription pattern in murine cell lines and splenic macrophages 221
10.2 Direct and indirect regulation of TLR transcriptional changes 223
10.3 Side effects of pharmacological inhibition and alternative signalling inhibition with siRNAs 225
10.4 Species-specific TLR transcriptional regulation 226
10.5 Future work 227
References 231
Appendixes 268

List of Figures and Tables

Table 1.1: TLRs and their ligands 24

Table 1.2: TLR orthologues between human, mouse and chicken 27

Figure 1.3: Signalling pathways following TLR4 activation 29

Table 2.1: Final treatment concentration for TLR agonists, signalling pathway inhibitors and translation inhibitors 44

Table 2.2: All plasmids names, catalogue numbers, lot number and their selective antibiotics 54

Figure 3.1: The cognate regulation of TLRs in RAW264.7 cells induced by agonists: PAM, LPS or FLA 64

Figure 3.2: Cross regulation of TLRs in RAW264.7 cells induced by TLR1/2 ligand PAM 67

Figure 3.3: Cross regulation of TLRs in RAW264.7 cells induced by TLR4 ligand LPS 68

Figure 3.4: PAM-induced pro-inflammatory responses in RAW264.7 cells 70

Figure 3.5: LPS-induced pro-inflammatory responses in RAW264.7 cells 71

Figure 3.6: PAM- and LPS-induced pro-inflammatory responses in P388D1 cells 73

Figure 3.7: PAM-induced TLR regulation in P388D1 cells 75

Figure 3.8: LPS-induced TLR regulation in P388D1 cells 76

Figure 4.1: The cognate regulation of TLRs in murine splenic macrophages induced by agonists: PAM, LPS, FSL, CL097 or ODN 88

Figure 4.2: The non-cognate regulation of TLRs in murine splenic macrophages induced by agonists: PAM, LPS, FSL, CL097 or ODN 89

Figure 4.3: Effect of Day/Night oscillation on agonist-induced pro-inflammatory responses in splenic macrophages 92

Figure 4.4: Effect of Day/Night oscillation on agonist-induced TLR transcriptional regulations in splenic macrophages 94

Table 4.5: Cognate- and cross-regulation of TLRs induced by agonist treatment PAM, LPS, FSL, CL097 or ODN in splenic macrophages 97

Table 4.6: Agonist-induced TLR transcriptional changes in RAW264.7 cell line vs in splenic macrophages 101

Figure 5.1: PAM-induced MAPK signalling activation in RAW264.7 cells 108

Figure 5.2: Effect of MAPK signalling inhibition on PAM-induced pro-inflammatory responses in RAW cells 110

Figure 5.3: Effect of inhibiting MEK/ERK signalling on PAM-induced TLR regulation in RAW cells 113

Figure 5.4: Effect of inhibiting JNK signalling on PAM-induced TLR regulation in RAW cells 116

Figure 5.5: Effect of inhibiting P38 signalling on PAM-induced TLR regulation in RAW cells 118

Figure 5.6: Effect of inhibiting NFκB signalling on PAM-induced TLR regulation in RAW cells 120

Table 5.7: Signalling pathways mediating PAM induced TLR responses in RAW cells 124

Figure 6.1: Effect of individual and multiple MAPK signalling inhibition on PAM-induced IL1 transcriptional expression in RAW cells 133

Figure 6.2: Effect of MAPK signalling inhibition on PAM-induced TLR1 family mRNA changes in RAW cells 135

Figure 6.3: Effect of individual and multiple MAPK signalling inhibition on PAM-induced TLR4 transcriptional regulation in RAW cells 137

Figure 6.4: Effect of MAPK signalling inhibition on PAM-induced TLR7 family mRNA changes in RAW cells 138

Figure 6.5: Effect of inhibiting individual MAPK signalling cascade with/without NFκB signalling on PAM-induced TLR transcriptional changes in RAW cells 140

Figure 6.6: Effect of complete inhibition of MAPK signalling pathway with/without NFκB signalling on PAM-induced TLR transcriptional changes in RAW cells 142

Figure 6.7: Effect of ERK and MEK inhibition on PAM-induced ERK1/2 signalling activation 144

Figure 6.8: Effect of JNK inhibition on PAM-induced ERK1/2 signalling activation 145

Figure 6.9: Effect of P38 inhibition on PAM-induced ERK1/2 signalling activation 146

Figure 6.10: Effect of NFκB inhibition on PAM-induced ERK1/2 signalling activation 147

Figure 6.11: Effect of JNK inhibition on PAM-induced ERK1/2 signalling activation .. 148

Figure 6.12: Effect of ERK and MEK inhibition on PAM-induced JNK1/2 signalling activation 151

Figure 6.13: Effect of JNK inhibition on PAM-induced JNK1/2 signalling activation .. 152

Figure 6.14: Effect of P38 inhibition on PAM-induced JNK1/2 signalling activation ... 153

Figure 6.15: Effect of NFκB inhibition on PAM-induced JNK1/2 signalling activation 154

Figure 6.16: Effect of JNK inhibition on PAM-induced JNK1/2 signalling activation .. 155

Figure 6.17: Effect of ERK and MEK inhibition on PAM-induced P38 signalling activation 157

Figure 6.18: Effect of JNK inhibition on PAM-induced P38 signalling activation 158

Figure 6.19: Effect of P38 inhibition on PAM-induced P38 signalling activation 159

Figure 6.20: Effect of NFκB inhibition on PAM-induced P38 signalling activation 160

Figure 6.21: Effect of JNK inhibition on PAM-induced P38 signalling activation 161

Table 6.22: Synergy between multiple signalling pathways which mediates PAM induced TLRs responses in RAW cells 163

Table 6.23: Effect of MAPK and NFκB signalling inhibition on phosphorylation status of each MAPK signalling cascades 167

Figure 7.1: Bioinformatics analysis of siRNA targeting ERK1/2, JNK1/2 and P38α ... 173

Figure 7.2: On-target and off-target knockdown induced by siRNA targeting ERK 1/2, JNK 1/2 and P38α 175

Figure 7.3: ERK1-targeting siRNA induced knockdown of ERK protein in time course and when treated with/without PAM agonist at 48hr post siRNA transfection 176

Figure 7.4: Effect of inhibiting ERK1 with siRNA on PAM-induced IL1β transcriptional expression in RAW cells..... 178

Figure 7.5: Effect of inhibiting ERK1 with siRNA on PAM-induced TLR transcriptional expression in RAW cells..... 179

Figure 8.1: Transcriptional expression of IL1β induced by 4hr PAM incubation followed by 4hr media incubation in RAW cells 186

Figure 8.2: Transcriptional expression of TLRs induced by 4hr PAM incubation followed by 4hr media incubation in RAW cells 187

Figure 8.3: Transcriptional expression of IL1β and TLRs induced by 4hr supernatant (SN) or last wash (LW) treatment in RAW cells 188

Figure 8.4: Transcriptional expression of IL1β and TLRs induced by 15mins PAM incubation followed by media incubation for 8 hrs in RAW cells 189

Figure 8.5: Transcriptional expression of IL1β and TLR following PAM titration in RAW cells..... 191

Figure 8.6: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced IL1 transcriptional expression in RAW cells..... 193

Figure 8.7: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced TLR1 family mRNA changes in RAW cells 195

Figure 8.8: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced TLR4 family mRNA changes in RAW cells 196

Figure 8.9: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced TLR7 family mRNA changes in RAW cells 197

Figure 9.1: Transcriptional expression of IL1β and TLR following PAM titration in HD11 cells..... 205

Figure 9.2: PAM-induced MAPK signalling activation in HD11 cells 206

Figure 9.3: Effect of Day/Night oscillation on agonist-induced lL1β responses in splenic macrophages in outbred and inbred chicken..... 208

Figure 9.4: Effect of Day/Night oscillation on agonist-induced TLR4 transcriptional regulations in inbred chicken line 6 splenic macrophages 209

Figure 9.5: Effect of inhibiting individual MAPK signalling cascade with/without NFκB signalling on LPS-induced TLR1 and TLR2 transcriptional changes in RAW cells.... 211

Figure 9.6: Effect of complete inhibition of MAPK signalling cascade with/without NFκB signalling on LPS-induced TLR1 and TLR2 transcriptional changes in RAW cells.... 212

Figure 9.7: Effect of inhibiting NFκB signalling on LPS-induced TLR1 and TLR2 transcriptional changes in RAW cells 213

Table 9.8: PAM-induced MAPK signalling activation in murine RAW264.7 cell line vs chicken HD11 cell line..... 215

Table 9.9: Signalling pathways mediating PAM induced TLR1/2 transcriptional regulation in HD11 cells vs RAW cells..... 216

Figure 9.10: An uncharacterised chicken amino acid sequence is closer related to ERK5 than ERK1 217

Table 9.11: Signalling pathways mediating PAM and LPS induced TLR1/2 transcriptional regulation in RAW cells 218

Abbreviation

AA	Arachidonic acid
AD	Transactivator domain
AhR	Aryl hydrocarbon receptor
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BCAP	B-cell adaptor for PI3K
BIR	Baculoviral inhibitor of apoptosis repeat domain
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
BTK	Bruton's tyrosine kinase
CARD	Caspase recruitment domain
CD14	Cluster of differentiation 14
CEL	Celastrol
CHX	Cycloheximide
CLR	C-type lectin receptor
Ct	Cycle value
CRD	Carbohydrate-recognition domain
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM3-grabbing non-integrin
DNGR-1	DC NK lectin group receptor-1
dsRNA	double-stranded RNA
DTT	DL-Dithiothreitol
DUSP	MAPK phosphatase dual specificity phosphatase

ECD	Ectodomain
ERK	Extracellular signal-regulated kinase
FLA	Flagellin
GOI	Gene of interest
GPI	Glycosylphosphatidylinositol
HSP	Heat shock protein
IFN	Interferon
IKK	Inhibitor of κB kinase
IL1	Interleukin-1
IL1-R	Interleukin-1 receptor
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase 1
IRF	IFN-regulatory factor
JNK	Jun n-terminal kinase
LGP2	Probable ATP-dependent RNA helicase DHX58
LNA	Locked nucleic acid
L-OspA	Lipidated outer surface protein A
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
LW	Last medium wash
MAL	MyD88-adaptor-like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signalling protein
MBL	Mannose binding lectin
MDA5	Melanoma differentiation associated gene 5
MDP	Muramyl dipeptide
MD-2	Myeloid differentiation factor 2

Mincle	Macrophage-inducible c-type lectin
miRNA	MicroRNA
MMR	Mannose receptor
MSU	Uric acid crystal
MyD88	Myeloid differentiation primary response 88
NEMO	NFκB essential modulator
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NK	Natural killer cell
NO	Nitric oxide
NOD	Nucleotide-biding oligomerisation domain
ODN	CpG-oligodeoxynucleotide
PAM	PAM_3CSK_4
PAMP	Pathogen associated molecular pattern
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PIR	Receptor interacting protein
PI3K	phosphoinositide 3-kinase
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
PYD	Pyrin domain
P38	P38 mitogen-activated protein kinase
RAW	RAW264.7
RIG	Retinoic acid-induced gene I
RIP2	Receptor-interacting protein 2

RLR	RIG-I like receptor
rRNA	Ribosomal RNA
RT	Reverse transcription
SARM	Sterile α- and armadillo-motif-containing protein
siRNA	Small interfering RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SENP	SUMO-specific protease 6
SHP	Small heterodimer partner
SIGIRR	Single immunoglobulin interleukin-1-related receptor
SN	Supernatant from the 4 hr medium incubation
SOCS	Suppressor of cytokine signalling
ssRNA	single-stranded RNA
SUMO	Small ubiquitin-like modifier
TAB	TAK-binding protein
TAK	Transforming growth factor-β-activating kinase
TB	Terrific broth
TIPE2	Tumour necrosis factor alpha-induced protein 8-like 2
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	Tumour necrosis factor type 1-associated death domain protein
TRAILR	TNF-related apoptosis-inducing ligand receptor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TM	Thioglycollate-elicited macrophage
VSV	Vesicular stomatitis virus
5’ppp	5’-triphosphate

Chapter 1 General Introduction

1.1 The innate and adaptive immunity

Induced by tissue damage or pathogen invasions, inflammation is the immunological response of a complex orchestration with cells from both the innate and adaptive immune system. The innate immunity is the frontline defence against host tissue injury and pathogens. Equipped with germline-encoded pathogen recognition receptors (PRRs), innate immune cells such as macrophages, neutrophils and dendritic cells (DCs) phagocytise pathogens and mediate the production of pro-inflammatory cytokines, chemokines and lipid mediators for leukocytes recruitment and migration to the site of inflammation. After phagocytising the exogenous pathogens, innate immune cells digest these pathogen-associated proteins into antigenic peptide while migrating to lymph nodes rich in naïve T cell population. They then present these antigenic peptides to T cells coupled with MHC class II molecules. Hence, these innate immune cells are also called antigen-presenting cells (APCs). This process also activates cell cytotoxicity, tissue re-modelling and adaptive immunity, which ultimately eliminate pathogens, restore tissue damage and terminate inflammation (Janeway, Travers et al. 2005, Seong, Matzinger 2004, Janeway 1989). While the innate immunity is robust and relatively non-specific, adaptive immunity is prolonged and characterised by specificity. This specificity comes from clonal selection from broad repertoires of antigen-specific receptors by gene re-arrangements (McCormack, Wade et al. 1991). With target specificity, adaptive immune cells then defeat pathogens at late phase of infection and generate long-term immunological memories.

Indeed, innate and adaptive immunity collaborate to protect the host, with the innate immune mechanisms being critical for the initiation, direction and recruitment of adaptive immune mechanisms (Medzhitov, Preston-Hurlburt et al. 1997, Medzhitov, Janeway 1997). This chapter will briefly outline the current knowledge of innate immunity with an overview of toll-like receptors (TLRs) and their signalling controls.

1.2 Pathogen recognition receptors

The innate immunity discriminates between host and foreign by innate recognition using PRRs. Expressed on both the immune (for example, macrophages, T cells) and non-immune cells (for example, epithelial cells), PRRs recognise both pathogen associated molecular patterns (PAMPs) and tissue derived danger associated molecular patterns (DAMPs) (Broz, Monack 2013, Greaves, Gordon 2009, Janeway, Travers et al. 2005, Seong, Matzinger 2004, Vabulas, Ahmad-Nejad et al. 2001, Gallucci, Lolkema et al. 1999).

The PRRs can be broadly divided into the transmembrane receptors and the cytoplasmic receptors. The transmembrane PRRs which bind to microbial components and tissue damage signals present on the surface of cell membranes and intracellular components whereas the soluble PRRs binds to exogenous molecules the cytosol. Based on the structural and functional differences, PRRs can be further classified into several major families such as the retinoic acid-induced gene I (RIG)-like receptors family (RLRs), the nucleotide-binding oligomerisation domain (NOD-like) receptor family (NLRs), the C-type lectin receptor family (CLRs) and the Toll-like receptor family (TLRs) (Brubaker, Bonham et al. 2015, Takeuchi, Akira 2010).

1.2.1 RIG-I like receptors (RLRs)

The cytoplasmic RLRs recognise viral RNAs with three main receptors: retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and probable ATP-dependent RNA helicase DHX58 (LGP2) (Takeuchi, Akira 2009, Yoneyama, Fujita 2008). Yoneyama et al. (2004) first identified the RLR-mediated type 1 interferons (IFNs) production following stimulation of viral dsRNA (Yoneyama, Kikuchi et al. 2004). Knockdown study using RIG-I deficient mice demonstrates that the production of antiviral interferon (IFN) is mainly utilised through the RIG-I pathways over the TLR pathways in most cell types except in plasmacytoid DC where the TLR signalling is used in preference to the RIG-I pathways (Kato, Sato et al. 2005).

Each RLR detects viral RNA at different length. The RIG-I recognises short dsRNA sequences in viruses such as vesicular stomatitis virus (VSV) and the MDA5 recognises long dsRNA in viruses such as *Picornaviridae* and *Caliciviridae*. The other RLR receptor LGP2, although binds viral RNA, can only induce IFN production indirectly through MDA5 signalling (Yoo, Kato et al. 2014, Kato, Takahasi et al. 2011, Kato, Takeuchi et al. 2006). The RLRs can distinguish the host from foreign by recognising the 5'-triphosphate (5'ppp) step-loop in single-stranded and double-stranded viral RNA (Rehwinkel, Tan et al. 2010, Hornung, Ellegast et al. 2006, Pichlmair, Schulz et al. 2006). Downstream of RIG-I and MDA5 activation, adaptor protein mitochondrial antiviral signalling protein (MAVS) which locate on the mitochondria is recruited for the activation of transcription factors IRF3, IRF7 and NFκB and the secretion of type 1 IFN (Saha, Pietras et al. 2006, Sasai, Shingai et al. 2006, Kawai, Takahashi et al. 2005).

1.2.2 Nod-like receptors (NLRs)

Also expressed in the cytosol, NLRs recognises both intracellular pathogens and stress signals. To date, 22 NLRs have been identified in human, 33 in mice. Although the putative *NLR* genes are found in invertebrates, NLRs are yet to be identified outside of vertebrates (Yuan, Ruan et al. 2015, Hibino, Loza-Coll et al. 2006). Receptors in the NLR family share structural similarities with the arrangement of a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding NACHT domain and a variable N-terminal domain (Kanneganti, Lamkanfi et al. 2007). Based on the N-terminal domain structure, NLRs can be divided into 4 subfamilies: the NLRC which contain the caspase recruitment domains (CARDs), the NLRP which contain the pyrin domains (PYDs), the NLRB which contain the baculoviral inhibitor of apoptosis repeat domains (BIRs), and the NLRA which contain the transactivator domains (ADs) (Williams, Flavell et al. 2010).

NOD1 and NOD2 from the NLRC subfamily recognise the meso-diaminopimelic acid in the peptidoglycan (PGN) and the muramyl dipeptide respectively, both of which are parts of bacterial cell wall (Chamaillard, Hashimoto et al. 2003, Girardin, Boneca et al. 2003). Upon activation, NOD1 and NOD2 oligomerise and signals through the NFκB-activating kinase receptor-interacting protein 2 (RIP2) with the CARD-CARD interaction, which in turn, activates the JNK, P38 pathways downstream of NOD2 and the NFκB pathways downstream of both NOD1 and NOD2 (Shaw, Lamkanfi et al. 2010, Kobayashi, Chamaillard et al. 2005, Mochizuki, Kobayashi et al. 2004).

Another NLRs function via the formation of inflammasome complexes which contain NLR, caspase-1 and the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC). Four types of inflammasomes have been defined according to the NLRs they contain (Guo, Callaway et al. 2015, Williams, Flavell et al. 2010). The NLRC4 inflammasome recognises cytosolic flagellin (Miao, Alpuche-Aranda et al. 2006); the NLRP1 inflammasome recognises anthrax lethal toxin and muramyl dipeptide (MDP) (Nour, Yeung et al. 2009, Faustin, Lartigue et al. 2007); the NLRP3 inflammasome recognises a range of PAMPs including bacterial nucleic acids, LPS, pore-forming toxins and danger signals such as extracellular adenosine triphosphate (ATP), uric acid crystals (MSU) and extracellular Ca^{2+} (Abderrazak, Syrovets et al. 2015, Rossol, Pierer et al. 2012, Schroder, Zhou et al. 2010); and the absent in melanoma 2 (AIM2) containing inflammasome recognises dsDNA sequences (Hornung, Ablasser et al. 2009).

Although inflammasomes activate by different stimuli, the mechanisms of inflammasome induced immune response is the same. The pro-caspase-1 proteins recruited by inflammasomes are cleaved into active caspase-1 via oligomerisation. The active caspase-1 protease then cleaves the precursor cytokines, pro-IL1β and pro-IL18, producing IL1β and IL18 in their active form (Guo, Callaway et al. 2015, Yang, Chang et al. 1998).

1.2.3 C-type lectin receptor family (CLRs)

CLRs are a large family of PRRs characterised by the conserved carbohydrate-recognition domains (CRDs) which bind to carbohydrates in a calcium-dependent manner. The CLRs can be divided into three subfamilies: the type 1 trans-membrane CLRs which include mannose receptor (MMR) and DEC-205; the type 2 trans-membrane CLRs which include the dectin-1, the dectin-2, the macrophage-inducible c-type lectin (Mincle), the dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) and the DC NK lectin group receptor-1 (DNGR-1); and the soluble CLR such as the mannose-binding lectin (MBL) (Kerscher, Willment et al. 2013, Graham, Brown 2009).

The CLRs play important roles in the regulation of host immunity by recognising fungal and mycobacterial components as well as detecting necrotic or tumorous cells. In addition, Dectin-1 contributes to the maintenance of homeostasis by sensing microbiota in the gut (Dambuza, Brown 2015).

1.2.4 Toll-like receptor family (TLRs)

The TLR family is one of the most extensively studied PRRs due to its function in the activation of innate defence and the recruitment of adaptive immune responses. The regulation of TLRs is the main focus of this project, hence its discovery, function, signalling and evolution is discussed in detail in the next section.

1.3 The Toll-Like Receptors

In 2011, Jules Hoffmann and Bruce Beutler were awarded the Nobel Prize for discovering the function of Toll in *Drosophila* immunity and the function of TLR in bacterial sensing. Over the past 30 years, our knowledge about the TLR family has been growing rapidly, with structural studies revealing how TLRs recognise PAMPs and signalling analysis indicating how TLRs regulate immune responses. Here I discuss some of the highlights of the TLR biology.

1.3.1 TLRs and their agonists

1.3.1.1 Discovery of Toll-like receptors

The Toll protein in *Drosophila*, encoded by a maternal gene, was initially identified to be involved in the dorso-ventral axis formation (Hashimoto, Hudson et al. 1988). Following two studies indicating the conserved functional domain, the Toll/interleukin-1 receptor (TIR) domain, between Toll and interleukin-1 receptor (IL1-R) (Gay, Keith 1991, Schneider, Hudson et al. 1991), a number of studies suggested the possible involvement of Toll in immunity. Ip et al. (1993) indicated the role of Toll in *Dif* mediated immune response. Lemaitre et al. (1995) presented the functional links between the Toll signalling and kappa B-related motifs in bacterial infection model. Rosetto et al. (1995) showed that overexpression of Toll can activate cecropin gene, which has antibacterial function. In 1996, Toll was found to regulate the *Drosophila* antifungal innate immune gene drosomycin (Lemaitre, Nicolas et al. 1996), defining the definite role of Toll in innate immunity.

The search of mammalian Toll-like molecule started as early as 1994, with the predicted TLRs share structural similarities of the leucine-rich repeats (LRR) and the TIR domains compared to Toll (Sims, Dower 1994). The first mammalian Toll homologues hToll (later renamed as TLR4) was cloned in 1997. Transfection with the constitutively active chimeric CD14-hToll induced activation of NFκB signalling, expression of the gene encoding the co-stimulatory protein CD80 and ultimately led to the activation of T cells (Medzhitov, Preston-Hurlburt et al. 1997). These results suggested the conservation of Toll among species, with both Toll and hToll play important roles in activating innate immune response and linking adaptive immunity. The human *LPS* gene (later identified as *TLR4*) was cloned in 1998, providing critical evidence for TLR4 being the signal receptor for LPS (Hoshino, Takeuchi et al. 1999, Qureshi, Lariviere et al. 1999, Poltorak, He et al. 1998). Since the identification of TLR4, a total number of 10 TLRs have been found in human, 12 in mice, 10 in chicken

and many more in other species (O'Neill, Golenbock et al. 2013). The TLR orthologues are discussed in section 1.3.3.1.

1.3.1.2 Mammalian TLR ligands

Along with the discovery of new TLRs, other microbial components were tested as possible ligands for TLRs. The main ligands for mammalian TLR1, 2, 3, 4, 5, 6, 7 and 9 were determined by gene knock-out between 1999 and 2003 (Beutler 2009, Akira, Takeda 2004). TLRs and their principal agonists are shown Table 1.1.

In addition to LPS, TLR4 recognise other PAMPs, such as mannan from yeast *Saccharomyces cerevisiae* and *Candida albicans,* and DAMPs, such as the heat shock proteins (HSP), inducing the secretion of cytokines (Ghosh, Sinha et al. 2015, Wheeler, Chase et al. 2009, Chase, Wheeler et al. 2007, Vabulas, Ahmad-Nejad et al. 2002, Asea, Rehli et al. 2002, Tada, Nemoto et al. 2002, Vabulas, Ahmad-Nejad et al. 2001, Ohashi, Burkart et al. 2000).

Similar to TLR4, TLR2 also recognise a wide spectrum of PAMPs, including diacyl lipopeptides, triacyl lipopeptides, peptidoglycan (PGN), lipoteichoic acid (LTA) and porins from bacteria, zymosan from fungi, hemagglutinin protein from virus, glycosylphosphatidylinositols (GPI) from protozoa and DAMPs such as HSP60 and HSP70 (Debierre-Grockiego, Campos et al. 2007, Akira, Uematsu et al. 2006b, Asea, Rehli et al. 2002, Vabulas, Ahmad-Nejad et al. 2001, Campos, Almeida et al. 2001).

In 2001, TLR5 was shown to recognise flagellin from bacteria, mediating cooperation between innate and adaptive immunity in the gut (Uematsu, Fujimoto et al. 2008, Uematsu, Jang et al. 2006, Hayashi, Smith et al. 2001). TLR3 was shown to recognise dsRNA from virus, inducing the activation of NFκB pathway and the production of type 1 IFNs (Alexopoulou, Holt et al. 2001).

Following the discovery of TLR3's antiviral function, TLR7 and 8 were shown to detect ssRNA while TLR9 was shown to detect both CpG motifs from bacteria and DNA from

Source	Ligand	TLR
Bacterial	Diacyl Lipopeptides	TLR2/TLR6
	Triacyl Lipopeptides	TLR1/TLR2
	Lipotechonic Acid	TLR2
	Lipoarabinomannan	TLR2
	Peptidoglycan	TLR2
	Porins	TLR2
	LPS	TLR4
	Flagellin	TLR5 & TLR11
	23S rRNA	TLR8
	CpG DNA	TLR9
Viral	Hemagglutinin	TLR2
	dsRNA	TLR3
	Envelope protein	TLR4
	ssRNA	TLR7 & TLR8
	CpG DNA	TLR9
Fungal	Mannan	TLR2 & TLR4
	Zymosan	TLR2/TLR6
	Phospholipomannan	TLR2
	Glycoinositolphospholipids	TLR4
	Profilin	TLR11/TLR12
Danger	HSP	TLR2 & TLR4
	Fibrinogen	TLR4

Table 1.1: TLRs and their ligands.

herpesvirus (Lund, Alexopoulou et al. 2004, Heil, Hemmi et al. 2004, Diebold, Kaisho et al. 2004, Krug, Luker et al. 2004, Krug, French et al. 2004, Lund, Sato et al. 2003, Hemmi, Takeuchi et al. 2000).

Functions of some TLRs were not addressed until more recent years. Both TLR11 and TLR12 were previously thought to recognise profilin-like proteins from apicomplexan parasites, such as *Toxoplasma gondii* and *Plasmodium falciparum (Raetz, Kibardin et al. 2013, Yarovinsky, Zhang et al. 2005)*. Recently, TLR11 was recently investigated for its role in the maintenance of gut immunity, due to its high expression on intestinal epithelial cells. Study of TLR11 knockdown mice demonstrated the role of TLR11 in sensing endolysosomal flagellin, independent of TLR5 signalling (Mathur, Oh et al. 2012). The TLR13 in mice was shown to recognise a conserved CGGAAAGACC motif of the 23S ribosomal RNA (rRNA) in both gram-positive bacteria *S.aureus* and gram-negative bacteria *E.coli*, inducing the production of pro-IL1β (Broz, Monack 2013, Oldenburg, Kruger et al. 2012, Hidmark, von Saint Paul et al. 2012, Li, Chen 2012). In human, although ligand for the orphan receptor TLR10 remains unknown, a recent study has indicated the role of TLR10 in recognising viral components from H5N1 influenza virus, inducing the secretion of IL8 (Lee, Kok et al. 2014).

1.3.1.3 Conserved structure of TLRs

TLRs are a family of type 1 transmembrane proteins which consist of an N-terminal agonist-recognition domain, a single transmembrane helix and a C-terminal cytoplasmic TIR domain (Bell, Mullen et al. 2003). The TIR domains are highly conserved between TLRs across species due to its function in signalling (O'Neill, Bowie 2007, Burch-Smith, Dinesh-Kumar 2007). The N-terminal ectodomains (ECDs) however, are more divergent across species (Werling, Jann et al. 2009, Jann, Werling et al. 2008). These ECDs consists of 19-25 leucine rich repeats (LRR), each of which contains a conserved LxxLxLxxN amino acid motif (Matsushima, Tanaka et al. 2007). To date, the ECD structures of TLR1, 2, 3, 4, 5, 6, 8 and 9 have been solved

(Matsushima, Miyashita et al. 2015, Ohto, Shibata et al. 2015, Marion 2014, Park, Lee 2013, Botos, Segal et al. 2011, Jin, Lee 2008). Each TLR binds to ligands at unique binding sites in ECDs and form homodimer (TLR3, 4, 5, 8, 9) or heterodimer (TLR2, 1, 6).

The ECD structures between TLR1, TLR2, TLR4 and TLR6 are similar, all of which contain 20-23 continues LRR modules with one or more α helices but lack asparagine ladders, forming ¾ circle shape. The intracellular TLRs, TLR3, TLR8 and TLR9 on the other hand, contain 26 LRR modules with intact asparagine ladders and Z-loops between module 14 and 15, forming more of complete circle shape (Botos, Segal et al. 2011, Jin, Lee 2008). Despite the diverse amino acid sequences for pattern recognition, structures of TLRs are generally conserved.

1.3.1.4 TLR Orthologues

TLRs are believed to have ancient origin and are present in animal phyla such as Nematoda, Arthropoda, Echinodermata and Chordata. Phylogenetic study identified 6 major vertebrate TLR families with genomic sequences of *Takifugu rubripes*. These families include the TLR1 family, the TLR3 family, the TLR4 family, the TLR5 family, the TLR7-9 family and the TLR11 family (Roach, Glusman et al. 2005). A list of TLR orthologues between human, mice and chicken is presented in Table 1.2.

Phylogenic sequence analysis of TLRs revealed positive selection for maintaining the functions of TLRs (Vinkler, Bainova et al. 2014, Areal, Abrantes et al. 2011). Most vertebrates have at least one orthologous gene in each TLR family. The TLR1 family includes TLR1, 2, 6, 10 and 14. Previously it was thought most TLR1 family members arose from independent gene duplication in different vertebrates, with the earliest took place 300 Mya producing TLR10 and the latest 65 Mya producing chicken TLR2 (TLR2a and TLR2b) (Roach, Glusman et al. 2005). However some argued that TLR10, TLR1/6 and TLR2 in mammal were thought to be the orthologues to avian TLR1a, TLR1b and TLR2a respectively, while the mammalian orthologues of avian TLR2b

<table>
<tr><td></td><td></td><td></td></tr>
<tr><td colspan="2">TLR1</td><td>TLR1.1, TLR1.2</td></tr>
<tr><td colspan="2">TLR2</td><td>TLR2.1, TLR2.2</td></tr>
<tr><td colspan="3">TLR3</td></tr>
<tr><td colspan="3">TLR4</td></tr>
<tr><td colspan="3">TLR5</td></tr>
<tr><td colspan="2">TLR6</td><td>N/A</td></tr>
<tr><td colspan="3">TLR7</td></tr>
<tr><td colspan="2">TLR8</td><td>N/A</td></tr>
<tr><td colspan="2">TLR9</td><td>N/A</td></tr>
<tr><td>TLR10</td><td colspan="2">N/A</td></tr>
<tr><td rowspan="4">N/A</td><td>TLR11</td><td rowspan="3">N/A</td></tr>
<tr><td>TLR12</td></tr>
<tr><td>TLR13</td></tr>
<tr><td rowspan="2">N/A</td><td>TLR15</td></tr>
<tr><td>TLR21</td></tr>
</table>

Table 1.2: TLR orthologues between human, mouse and chicken.

was thought to be lost. While most TLR1 family members are present in broad range of species some are not. TLR6 and 10 are only present in some land animals but not present in fish. The exception of gene duplication in this family is TLR14, which is only present in Xenopus but lost in on-land animals. (Huang, Temperley et al. 2011, Temperley, Berlin et al. 2008). The TLR7 family, which includes TLR7, 8 and 9, is present in both human and mouse. Chicken lack TLR8 and 9 due to gene deletion, however a functional homologue to human TLR9, the CpG-sensing TLR21 is present on chicken (Yeh, Liu et al. 2013, Keestra, de Zoete et al. 2010, Brownlie, Zhu et al. 2009). Other TLR families, TLR3, TLR4 and TLR5 are more conserved across species. In a similar manner, the main adaptor proteins in the TLR signalling pathways are conserved, with some genes, such as *IKK*, *IκB*, *NFκB* underwent parallel duplication (Roach, Racioppi et al. 2013, Song, Jin et al. 2012).

1.3.2 Toll-like Receptor Signalling

Ligand recognition by TLRs is followed by activation of signalling pathways via the recruitment of signalling adaptor proteins. In this section, I discuss the main signalling pathways and adaptor proteins used by TLRs and the regulators for TLR induced signalling.

The complexity of TLR signalling was established in previous studies. Oda and Kitano (2006) have published a comprehensive map based on 411 literature descriptions which summarised 397 signalling interactions between 340 proteins in 652 different species. Since then, more signalling adapter proteins were discovered (see 1.3.2.2) which further increased the TLR signalling complexity. A diagram indicating the simplified signalling pathways of TLRs are shown in Figure 1.3.

1.3.2.1 Signalling pathways of TLRs

Two main signalling pathways are recruited to activate transcription factors downstream of TLR-ligand binding. These two signalling pathways are the myeloid

differentiation factor 88 (MyD88)-dependent pathways and the TIR domain containing adaptor protein inducing interferon-β (TRIF)-dependent pathways (Akira, Takeda 2004).

The MyD88 protein was found to consist of a TIR domain, which binds to the TIR domain of the TLRs, and a death domain, which bind to the death domain of the IL-1R-associated kinase (IRAK) proteins (Wesche, Henzel et al. 1997, Muzio, Ni et al. 1997).

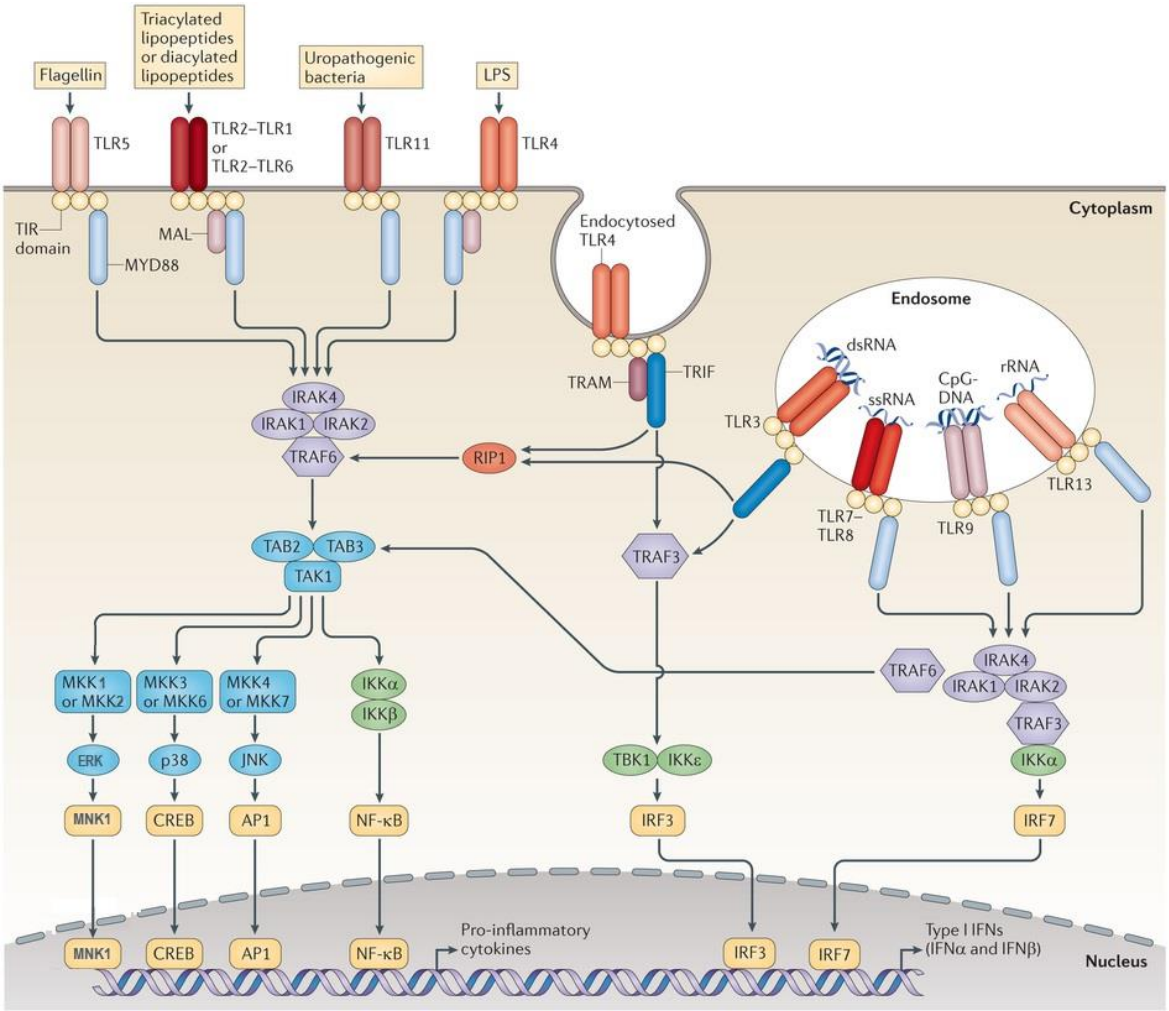


Figure 1.3: A simplified representation of the signalling activation following TLR-ligand binding. Figure adapted and modified from O'Neill et al. (2013).

The IRAK proteins, IRAK1 and IRAK2, then form a new complex by interacting with TNF receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase and an E2 ubiquitin enzyme complex consisting of Ubc13 and Uev1 (Kollewe, Mackensen et al. 2004, Jiang, Ninomiya-Tsuji et al. 2002). The E3 ligase is responsible for mediating the ubiquitination of TRAF6 and IRAK4, which then leads to the recruitment of another complex consisting of the transforming growth factor-β-activating kinase 1 (TAK1) kinase, TAK-binding protein 1 (TAB1), TAB2 and TAB3 (Ajibade, Wang et al. 2013). As TAK1 is a kinase for the inhibitor of κB kinase (IKK), it phosphorylates the IKK complex and induces activation of the transcription factor NFκB via degradation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα). In addition, TAK1 phosphorylates MAPKs which activates transcription factor AP1 via ERK, JNK and P38 signalling (Kawasaki, Kawai 2014, Kawai, Akira 2010, Kawai, Akira 2006, Akira, Uematsu et al. 2006b).

The TRIF protein was identified to interact with the endosomal TLRs in a MyD88-independent manner (Yamamoto, Sato et al. 2003a, Yamamoto, Sato et al. 2002b). TRIF connects with TRAF6 and TAK1 in the presence of adaptor protein receptor interacting protein 1 (PIR-1), the tumour necrosis factor receptor type 1-associated death domain protein (TRADD) and the E3 ubiquitin ligase pellino-1 (Jiang, Chen 2011, Chang, Jin et al. 2009). This in turn, activates both MAPK and NFκB signalling. TRIF signalling can induce the activation of antiviral transcription factor IRF3 via the formation of two different complexes, one consisting of TRAF3/TBK1/IKKi or the other consisting of TBK1/IRF3 and an inositol lipid PtdIns5P (Kawasaki, Kawai 2014, Kawasaki, Takemura et al. 2013, Kawai, Akira 2010, Akira, Uematsu et al. 2006a). Some TLRs interact directly with the TIR domain of TRIF whereas others require additional adaptor protein for binding (Yamamoto, Sato et al. 2003b). The TLR adaptor proteins are discussed in the following section.

1.3.2.2 TLR signalling adaptor molecules

To date, six TLR signalling adaptor molecules have been identified: MyD88, MAL, TRIF, TRAM, SARM and BCAP. The function of MyD88 was first characterised in 1997. MyD88 is use by all TLRs except TLR3, either directly or with the help with an additional adaptor protein. Mice with MyD88 deficiency were irresponsive to endotoxin and are highly susceptible to intestinal inflammation and colitis (Takeuchi, Hoshino et al. 2000, Kawai, Adachi et al. 1999). MAL was the second adaptor to be identified. It functions as a bridging receptor between TLR2/TLR4 and MyD88. Studies of MAL knockout in mice indicated impaired cytokine production downstream of TLR2 and TLR4 signalling whereas IL1 and TLR9 signalling remained intact (Horng, Barton et al. 2002, Yamamoto, Sato et al. 2002a, Fitzgerald, Palsson-McDermott et al. 2001). In addition, MAL may play a role in TLR8 signalling via the Bruton's tyrosine kinase (BTK) mediated signalling activation (O'Neill, Bowie 2007). The third adaptor TRIF was identified in 2003. The discovery of TRIF filled the gap of TLR3- and TLR4-mediated MyD88-independent activation of IRF3. Mice with TRIF deficiency indicated impaired IFN production via both TLR3 and TLR4 mediated pathway, but not via TLR2, TLR7 or TLR9 in macrophages (Yamamoto, Sato et al. 2003a). Following this, the TRIF-related adaptor TRAM was identified. TRAM is used by the endosomally located TLRs, TLR4, TLR7, TLR8 and TLR9 to connect with TRIF (Fitzgerald, Rowe et al. 2003, Yamamoto, Sato et al. 2003b). SARM1, identified in 2006, was shown to supress TRIF signalling. Knockdown of SARM with siRNA enhanced production of cytokines and chemokines downstream of TRIF while MyD88 signalling was not affected (Carty, Goodbody et al. 2006). The most recently identified adaptor protein is BCAP. It connects TLRs to the serine/threonine kinases PI3K/Akt via binding through the TIR domains. In B cells, BCAP was shown to supress the TLR4- and TLR9-mediated MyD88/MAL signalling (Troutman, Hu et al. 2012, Ni, MacFarlane et al. 2012).

1.3.3 Regulation of TLR mediated immune responses

Although TLR activity is critical for inducing immune responses, irregular TLR regulation, such as the LPS induced over-activation of TLR4, can cause fatal illness to the host. Hence tight regulation of TLR mediated immune responses is required.

In the 1940s, Beeston demonstrated that rabbits repeatedly treated with typhoid vaccine exhibited reduced fever symptom (BEESON 1946). This was the first piece of evidence indicating what was later known as “endotoxin tolerance”. Following this work, Freudenberg et al. (1988) demonstrated that the LPS-pre-primed mice were protected against lethal doses of LPS with macrophages responsible for mediating the tolerance effect (Freudenberg, Galanos 1988). Since the discovery of TLRs, extensive studies have refined tolerance as the hyporesponsiveness of TLRs towards repeated ligand stimulation. The TLR2 tolerance in human and TLR7 tolerance in mice were reported (Piao, Song et al. 2009, Lang, Mansell 2007). In recent years, cross-tolerance has been reported between TLR 2 and 4, TLR2 and TLR9, TLR2 and 5, TLR4 and 9, TLR7 and 9 as well as between TLRs and other PRRs (Kawai, Akira 2011, Marshall, Heeke et al. 2007, Dalpke, Lehner et al. 2005, Zarember, Godowski 2002, Haehnel, Schwarzfischer et al. 2002, Sato, Nomura et al. 2000). The TLR tolerance may be regulated by negative regulation of TLR signalling, suppression of TLR induced gene transcription or post-translational controls (Kondo, Kawai et al. 2012). These regulatory mechanisms are discussed in the sections below.

1.3.3.1 Negative regulators for TLR signalling

The negative regulators contribute to the fine-tuning of TLR signalling at several levels by: blocking TLR-ligand binding, supressing TLR signalling and blocking transmembrane adaptor molecules (Liew, Xu et al. 2005).

Several soluble decoy proteins which competitively bind to TLRs have been identified (Lang, Mansell 2007). The sTLR4 blocks the interaction between TLR4 with MD2 and CD14, which in turn, supresses the TLR4 downstream signalling and the activation of NFκB transcription factor (Hyakushima, Mitsuzawa et al. 2004, Iwami, Matsuguchi et al.

2000). Following the discovery of sTLR4, sTLR2 was discovered in human plasma and milk. The sTLR2 supresses the production of IL8 and TNFα induced by TLR2 (LeBouder, Rey-Nores et al. 2003).

The transmembrane negative regulators includes: CD33, IL1RL1, SIGIRR and TRAILR. These regulators target the adaptor proteins to prevent binding between TLR and adaptor molecules. The IL1RL1 protein contains an intracellular TIR domain which prevents TLR binding to the adaptive protein MyD88. Mice with IL1RL1 deficiency were hypersensitive against LPS and CpG challenges. Overexpression of IL1RL1 inhibited activation of NFκB via defected MyD88 but not TRIF signalling (Brint, Xu et al. 2004). Similar to IL1RL1, SIGIRR also contains a TIR domain which has shown to interact with IRAK1 and TRAF6 in TLR signalling. Mice with SIGIRR deficiency were more susceptible to endotoxin shock (Qin, Qian et al. 2005, Wald, Qin et al. 2003). TRAILR, which belongs to the TNF superfamily, does not contain a TIR domain. It inhibits TLR signalling via interaction with IκBα which in turn, reduce the nuclear translocation of NFκB (Wu, Burns et al. 1999). TRAILR is a selective negative regulator for TLR signalling. Mice with TRAILR deficiency produced excess cytokines when stimulated with TLR2, TLR3 and TLR4 agonists, but not with TLR9 agonist (Diehl, Yue et al. 2004). CD33 is a sialic acid-binding lectin which interacts with the cell surface protein CD14. Mice with mutant CD33 reduced phosphorylation of NFκB when challenged by LPS, indicating its negative regulatory role in TLR4/CD14 mediated signalling (Ishida, Akita et al. 2014).

In addition to the soluble decoy proteins and the transmembrane mediators, there are many intracellular negative regulators which target TLR signalling components, for instance: MyD88s, IRAKM, TOLLIP, SOCS1, NOD2, PI3K, A20, IRF4, TIPE2, TANK, SHP, DUSP, SENP6, SARM and BCAP. The MyD88s competitively bind to MyD88, forming heterodimer that inhibits the phosphorylation of IRAK1 via IRAK4 (Burns, Janssens et al. 2003, Janssens, Burns et al. 2002). Overexpression of MyD88s inhibits

IL1 and LPS induced NFκB activation (Janssens, Burns et al. 2002). The IRAKM inhibits TLR signalling by preventing the formation of IRAK1/IRAK4/TRAF6 complex. The IRAKM-deficient mice produced increased amount of cytokines following treatment with TLR4 and TLR9 agonists (Kobayashi, Hernandez et al. 2002). In addition, IRAKM play a role in the homeostasis of intestinal microbial immunity (Biswas, Wilmanski et al. 2011). The negative regulator TOLLIP, upon phosphorylation via IRAK1, ubiquitinates and degrades IRAK1. At the same time, the phosphorylation of TOLLIP releases itself from the TOLLIP/IRAK1 complex, suggesting a negative feedback loop between TOLLIP and IRAK1. Overexpression of TOLLIP supresses the activation of NFκB downstream of TLR2 and TLR4 (Zhang, Ghosh 2002, Bulut, Faure et al. 2001). Two members of the IRF family, the IRF5 and IRF7 interacts with MyD88 to induce the production of type 1 IFNs. The negative regulator IRF4 competitively binds to MyD88 instead of IRF5, supresses TLR signalling. Mice with deficient IRF4 exhibited hypersensitivity to DNA induced shock with increased production of pro-inflammatory cytokines (Negishi, Ohba et al. 2005).

The TLR signalling is mediated via phosphorylation and ubiquitination of signalling cascades, hence post-translational modulators such as A20, SHP and SENP6 play important roles in the regulation of TLR induced immune responses. The deubiquitinating enzyme A20 negatively regulates TLR signalling by cleaving the K63 polyubiquitin chain which is linked to either TRAF6 or IRF3 (Hitotsumatsu, Ahmad et al. 2008, Saitoh, Yamamoto et al. 2005, Boone, Turer et al. 2004). Mice with deficient A20 increased activity of NFκB following stimulation with TLR2, TLR3 and TLR9 agonists (Boone, Turer et al. 2004). Similar to A20, the Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP2) inhibits the polyubiquitination of TRAF6, in addition, it supresses TRIF dependent pathway by targeting TBK1. SHP-deficient mice were more susceptible to sepsis with increased production of IFNβ (Yuk, Shin et al. 2011, An, Zhao et al. 2006). The SUMO-specific protease 6 (SENP6) inhibits the

activity of NFκB by deubiquitinating NEMO. SENP6-deficient mice induced more pro-inflammatory cytokines and were more susceptible to endotoxic shock (Liu, Chen et al. 2013).

Other negative regulators, such as the suppressor of cytokine signalling (SOCSs) and the MAPK phosphatase dual specificity phosphatase (DUSPs) are also important negative regulators for TLR signalling for the extensive targets in regulation by multiple members in each family (Fey, Croucher et al. 2012, Liu, Shepherd et al. 2007, Chi, Barry et al. 2006, Baetz, Frey et al. 2004, Heeg, Dalpke 2003, Nakagawa, Naka et al. 2002, Kinjyo, Hanada et al. 2002).

Furthermore, the discovery of the oscillatory interaction between IKK, IκB and NFκB suggests that the TLR signalling may also be controlled via temporal negative feedback (O'Dea, Barken et al. 2007, Ihekwaba, Broomhead et al. 2004, Nelson, Ihekwaba et al. 2004, Hoffmann, Levchenko et al. 2002).

1.3.3.2 Negative regulators for TLR transcription

The transcriptional regulation of TLR signalling involves promoter modification by transcription factor selection, chromatin remodelling and histone modification, as well as the use of negative regulators for TLR mediated transcription (Medzhitov, Horng 2009).

The negative regulator Nurr1 supresses the NFκB transcriptional responses in microglia via recruitment of the NFκB-p65 located on the promoter region of the target inflammatory gene. This in turn, activates the CoREST repression complex and ultimately supress TLR induced immune responses (Saijo, Winner et al. 2009). The Aryl hydrocarbon receptor (AhR) supresses the production of IL6 and TNFα induced by LPS via binding with STAT1 and NFκB which lead to the inhibition of IL6 promoter activity (Kimura, Naka et al. 2009). The inhibitory histone methylation mark H3K27 supresses LPS induced pro-inflammatory responses by binding to the pro-

inflammatory gene promoters which lead to the inhibition of gene expression (Medzhitov, Horng 2009, Foster, Hargreaves et al. 2007). The ATF3 transcription factor inhibits LPS induced IL6 and IL12p40 gene expression by recruiting the histone deacetylase via binding to the acetylation site on the chromatin (Whitmore, Iparraguirre et al. 2007, Gilchrist, Thorsson et al. 2006).

The microRNAs (miRNAs) have been discovered in recent years. These gene regulators bind to the 3' untranslated region of target mRNA sequence and contribute to the regulation of a variety of genes. The miR-146a, miR-155 and miR-132 were the first few key regulators to be identified for the TLR mediated immune responses followed by the discovery of miR-199a, miR-21, miR-29 etc. (Kawasaki, Kawai 2014, Kondo, Kawai et al. 2012, Quinn, O'Neill 2011).

1.3.3.3 Circadian control of immune responses

In recent years, studies have shown that repeated changes in circadian rhythm could be harmful for human health, increasing risks for heart disease, diabetes and cancer in populations such as shift workers, cabin crews and pilots (Birky, Bray 2014). This may be mediated via the circadian-dependent immune responses following TLR activation (Zhou, Wang et al. 2015, Curtis, Fagundes et al. 2015, Nakao 2014, Mukherji, Kobiita et al. 2013, Silver, Arjona et al. 2012, Gibbs, Blaikley et al. 2012, Keller, Mazuch et al. 2009).

Early studies have indicated that circadian rhythm is the result of entrained biological clock in animals and plants with environmental factors such as light pulse, forced exercises and environmental oxygen level (Adamovich, Ladeuix et al. 2016, Dann 2000, Marchant, Mistlberger 1996). The circadian rhythm is controlled by auto-regulatory feedback loops which oscillate around 24 hrs and mediated by just a few genes and proteins. Transcription factor CLOCK and BMAL1 form heterodimers to activate transcription of genes by binding to the E-BOX sequences in the promoter region. Among the genes that are activated, there is *Per*, *Cry*, *Rev-Erbα* and *Rora*. The

Per and Cry proteins slowly form heterodimers and negatively regulates the CLOCK-BMAL1-dependent transcription. The REV-ERBα quickly inhibits the transcription of *Bmal1* while RORA then slowly activates the transcription of *Bmal1* to start the oscillation again (Lowrey, Takahashi 2011, Bell-Pedersen, Cassone et al. 2005).

Circadian rhythm can affect immune responses by influencing the expression of innate immune components. For instance, expression of TLR9, as well as signalling components IκB, p65 NFκB, *Jun* and *CD14* increased during the active phase of mice (Labrecque, Cermakian 2015, Scheiermann, Kunisaki et al. 2013). Mice injected with TLR9 ligand-adjuvanted vaccine when TLR9 expression is at the highest produced more IFNγ than ones immunised when TLR9 expression is at the lowest (Silver, Arjona et al. 2012). Hence understanding the effects of circadian rhythm on immune systems can be beneficial for vaccination and treatment of diseases.

1.4 Aims of thesis

Over the past 30 years, extensive studies have led to the characterisation of TLR induced immune responses. The regulation of TLR activation and TLR mediated responses are tightly controlled by numerous negative regulatory mechanisms. TLR tolerance is crucial for the maintenance of host health. Most tolerance studies have focused on the modulation of TLR expression in relation to their cognate agonists whilst only a few studies have indicated tolerance effect of the cross-regulating TLRs. The first aim of this study was to systemically investigate the agonist induced cross-regulation of TLRs in murine cell line and primary splenic macrophages. Results from these studies were presented in Chapter 3 and Chapter 4.

The second aim of this thesis was to identify the direct signalling control for the cross-regulation of TLR transcription. Chapter 5 set out to investigate effect of individual MAPK and NFκB pathways using pharmacological inhibition approach. Chapter 6 set out to investigate the signalling collaboration between the direct MAPK and NFκB pathways using pharmacological inhibition. Due to the well-known side effects of the

pharmacological inhibition, a second approach, siRNA inhibition was used to confirm selected findings. This part of the study was presented in Chapter 7.

In tolerance model, TLR signalling is largely regulated by the feedback mechanisms. The third aim of this thesis was to investigate the indirect feedback for the regulation of TLR transcription. This part of the study was presented in Chapter 8.

The final aim of this thesis was to compare the similarities and differences for agonist induced TLR transcription between species. One of the reasons which may cause the species-species variation is the physiological differences such as the animal's natural circadian rhythm. The regulation of TLR transcription by day/night oscillation was investigated. Results for these studies were presented in Chapter 9.

Chapter 2 Materials and Methods

2.1 Experimental Animals

7 to 8 week-old female mice with C57BL/6 or BALB/c genetic backgrounds were obtained from Charles River.

6 week-old Rhode Island Red or inbred line 6(1) chickens were obtained from the Poultry Production Unit at the Institute for Animal Health (IAH, Compton Laboratory).

Schedule 1 killing was conducted on experimental animals prior to dissection.

2.2 Isolation and Culture of Splenocytes and Preparation of Monocyte-Derived Macrophages

Murine splenocyte isolation method was adapted from Current Protocols in Immunology (Kruisbeek 2001). In brief, spleens were removed from mice and immediately transferred to ice-cold phosphate buffered saline (PBS) (pH 7.4, Gibco, Life Technologies, UK) with 5% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies, UK). Spleens were expelled into 50 ml centrifuge tubes (Centristar, Corning, UK) through 100 µm nylon cell strainers (Falcon, Fisher Scientific, UK) and centrifuged at 300 x g for 5 mins at 4 °C with Heraeus Multifuge® X3R centrifuge (Thermo Scientific, UK). Cell supernatant was discarded and cell pallets were re-suspended in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK), 10% heat-inactivated FBS (Gibco, Life technologies, UK), 2 mM L-glutamine (Gibco, Life Technologies, UK) and 0.1% penicillin-streptomycin (Sigma-Aldrich, UK). An aliquot of re-suspended splenocytes was stained with 0.4% trypan blue solution (Sigma-Aldrich, UK) and counted with haemocytometer to determine viability. Cells were then made up to 1 x 10^7 cells/ml to 1.6 x 10^7 cells/ml with complete culture medium and seeded in 0.5 ml/well for 24 well tissue culture clusters (Costar, Corning, UK). Cells were incubated at 37 °C, 5% CO_2.

Adhesion of murine splenic monocytes and dendritic cells to polystyrene surfaces requires approximately 2 hrs, however dendritic cells then detach from culture plate after 4 hrs of incubation. Hence, a change of medium with sterile Pasteur pipettes (Fisher Scientific, UK) after 24 hr incubation was required to isolate splenic macrophage population prior to experiments.

Chicken splenic macrophage isolation method was adapted from (Wigley, Hulme et al. 2002). Spleens were removed from birds and mashed through cell strainers as in mouse. Splenocytes from Rhode Island Red chickens were counted with haemocytometer and made up to 1 x 10^7 cells/ml in complete culture medium consisting of RPMI (Roswell Park Memorial Institute) -1640 (Sigma-Aldrich, UK), 10% heat-inactivated FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin. Cells were then seeded in 0.5 ml medium/well in 24 well tissue culture clusters and incubated for 48 hrs at 41 °C, 5% CO_2 with twice change of medium: one after 24 hrs of incubation, one at 48 hrs after incubation.

Splenocytes from inbred line 6_1 chickens were mixed with ice-cold PBS supplemented with 10% heat-inactivated FBS and overlaid onto an equal volume of Histopaque 1083 (Sigma-Aldrich, UK) in 50 ml centrifuge tubes. The mixture was then centrifuged at 1200 x g, room temperature for 40 mins with minimum deceleration speed. Cells in the interface layer were transferred to new 50 ml centrifuge tubes and washed 3 times with ice-cold PBS and pelleted by centrifugation at 400 x g for 5 mins at 4 °C. Pelleted cells were then re-suspended in complete culture medium consisting of RPMI-1640, 10% heat-inactivated FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin. An aliquot of cells was stained with 0.4% trypan blue stain and counted with haemocytometer, then adjusted to 5 x 10^6 cells/ml and plated at 1ml/well for 24 well tissue culture plates. Cells were incubated for 48 hrs at 41 °C, 5% CO_2 to allow adhesion and differentiation from monocytes to macrophages. A change of medium was required after 24 hrs of incubation.

2.3 Preparation of Murine Intestine for Leptin Assay and Isolation of IELs and Epithelial Cells

Protocol for isolating murine intestine was adapted from Current Protocols in Immunology ((Sheridan, Lefrancois 2012). The small intestine was cut from just below the pyloric sphincter (below the stomach) and above the cecum. The small intestine was then separated into approximately 1.5 cm length per piece for duodenum, jejunum and ileum. The large intestine was separated into caecum and colon. Each piece was flushed with ice-cold PBS supplemented with 2% FBS and sealed at the two ends with surgical threads. The Intestine pieces were then plated in 24 well plates, equilibrated at 37 °C, 5% CO_2 for 30 mins in 0.5 ml/well complete culture medium consisting of RPMI-1640, 10% heat-inactivated FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin prior to experiment.

After leptin assay, each intestinal piece was cut open and placed in 700 ul ice-cold PBS with 2 mM EDTA (Gibco, Life Technologies, UK) for 10 mins. After incubation, intestinal pieces were removed and PBS solutions were centrifuged at 400 x g for 5 mins. Cell pellets consisting mainly of epithelial cells and IELs were lysed in 60 µl RLT buffer (Qiagen, UK) and stored in -80 °C freezer.

2.4 Intestinal Leptin Assay

Recombinant murine leptin was purchased from PeproTech EC (UK) and re-suspended according to manufacturer's recommendation. Leptin solution was further diluted to 100 ng/ml final treatment concentration in complete medium consisting RPMI-1640, 10% heat-inactivated FBS, 2 mM L-glutamine and penicillin-streptomycin. Up to 100 µl of leptin was injected into each piece of intestine with a 30 gauge needle attached to a 1 ml syringe. Intestinal pieces were then incubated at 37 °C, 5% CO_2 for appropriate period until harvest.

2.5 Cell Line Culture

RAW264.7 cell line (murine macrophage of BALB/c background, Abelson murine leukaemia virus transformed, ATCC® TIB-71™) was cultured in DMEM with 10% heat-inactivated FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin. At 80% confluency, cells were detached from polystyrene culture surfaces with cell scrapers (Fisher Scientific, UK), pelleted by centrifugation at 400 x g for 5 mins, re-suspended in 15 ml complete culture medium and passaged 1:3 into fresh T75 tissue culture flasks (Corning, UK). Cells were incubated at 37 °C, 5% CO_2. RAW264.7 cells were seeded at 5 x 10^4 cells/well in 96 well tissue culture plates (BD Biosciences, UK) or 2.5 x 10^5 cells/well in 24 well tissue culture plates and incubated overnight prior to experiments.

P388D1 cell line (murine monocyte/macrophage, derived from lymphoid neoplasm of a DBA/2 mouse, ATCC® CCL-46™) was passaged and plated for experiments with the same protocol. Cells were cultured in RPMI-1640 with 10% FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin.

CMT-93 cell line (murine rectum epithelial cells of C57BL/icrf background, ATCC® CCL-223™) was cultured in complete medium consisting of DMEM, 10% heat-inactivated FBS, 4 mM L-glutamine and 10 units/ml penicillin-streptomycin. Cells were passaged with the following protocol. At 90% confluency, medium was removed from the adherent cells and cells were washed with 37 °C PBS. 2 to 3 ml of 0.25% trypsin-0.53 mM EDTA solution (Gibco, Life Technologies, UK) were then treated on cells for 10 mins with gentle agitation at room temperature. After detaching from tissue culture flasks, cells were transferred to 50 ml centrifuge tubes with complete medium containing serum to prevent further trypsin activity. The trypsinised cells were then pelleted by centrifugation at 400 x g for 5mins, washed 3 times with PBS, re-suspended in 15 ml complete culture medium and plated at 1 x 10^6 cells in T75 tissue culture flasks. CMT-93 cells were seeded at 1 x 10^5 cells/well in 24 well tissue culture plates or 4 x 10^5 cells/well in 6 well plates for experiments two days later.

HD11 cell line (chicken macrophage-like cells transformed with avian myelocytomatosis virus (MC29) (Beug, von Kirchbach et al. 1979)) was cultured in complete medium consisting of RPMI-1640, 10% heat-inactivated FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin. HD11 cells were passaged as RAW264.7 cells. These cells were plated at 1.5 x 10^5 cells/well in 96 well plates for experiments in 16 hrs or at 1.5 x 10^6 cells in T75 tissue culture flasks and grown at 41 °C, 5% CO_2.

THP-1 cell line (human peripheral blood monocytic leukaemia cells, ATCC® TIB-202™) was cultured in RPMI-1640, 10% heat-inactivated FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin. To subculture, cells were centrifuged at 400 x g for 5 mins and re-suspended at 5 x 10^5 viable cells/ml in fresh complete culture medium every 4 days. THP-1 cells were seeded at 5 x 10^4 cells/well in 96 well plates for experiments.

HEK293T cell line (human embryonic kidney epithelial cells, ATCC® CRL-3216™) was cultured in DMEM, 10% heat-inactivated FBS, 2 mM L-glutamine and 10 units/ml penicillin-streptomycin. HEK293T cells were passaged following the same protocol as of CMT-93 cells. Cells were seeded at 1.5 x 10^5 cells/well in 24 well plates for transfection 24 hrs after.

All cell line cultures were regularly screened for mycoplasma contamination (protocols according to (Uphoff, Drexler 2013)

2.6 TLR Agonists, NFκB & MAPK Signalling Inhibitors and Translation Inhibitors

Cells were plated for experiments as described and treated with agonists and inhibitors at concentrations described in Table 2.1. TLR agonists and signalling pathway inhibitors were obtained from InvivoGen (Bioscience-Autogen, UK) or Tocris (Bio-Techne, UK), translation inhibitors were obtained from BD Biosciences and Sigma-

Table 2.1: Final treatment concentration for TLR agonists, signalling pathway inhibitors and translation inhibitors

Property	Abbreviation	Full name	Final concentration
TLR1/2 agonist	PAM	triacylated lipopeptides (PAM_3CSK_4)	1.6 µg/ml (mouse); 1 µg/ml (chicken)
TLR4 agonist	LPS (Ultrapure LPS-EK)	lipopolysaccharide	1 µg/ml
TLR5 agonist	FLA	Flagellin	100 ng/ml
TLR7 agonist	CL097	Imidazoquinoline	10 µg/ml
TLR9 agonist	ODN1826	CpG oligonucleotide	10 µg/ml
TLR21 agonist	ODN M362	CpG oligonucleotide	10 µg/ml
ERK1/2 inhibitor	PD059	PD98059	30 µM
MEK1/2 inhibitor	U0126	N/A	30 µM
JNK1/2 inhibitor	SP125	SP600125	10 µM
JIP1 inhibitor	JIP	JIP-1 (153-163)	10 µM
P38α/β inhibitor	SB580	SB203580	30 µM
NFκB inhibitor	CEL	Celastrol	1 µM
Protein synthesis inhibitor	CHX	cycloheximide	10 µg/ml
Protein transportation inhibitor	Monensin (GolgiStop™)	N/A	1 µl/ml
Protein transportation inhibitor	Brefeldin A (GolgiBlock™)	N/A	1 µl/ml

Aldrich, UK. All agonists and inhibitors were re-suspended in appropriate medium according to manufacturer's recommendation. Inhibitors were incubated with cells for 1 hr prior to agonists' treatments for required time (see result chapters). For qPCR analysis, cells were harvested in 350 µl RLT buffer (Qiagen, UK) per well for 24 well plates and frozen at -80 °C; for western blot analysis, cells were harvested in 90 µl Tris-NP40 lysis buffer (see appendix) with a mixture of 1:100 Halt^{TM} protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, UK) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, UK) per well for 12 well plates, flash frozen on dry ice and stored at -80 °C. Supernatants were also collected for Griess assay (Promega, UK).

2.7 Alamar Blue Assay

Alamar Blue (resazurin) assay was performed to assess cell cytotoxicity (O'Brien, Wilson et al. 2000). In brief, cells seeded in 96-well tissue culture plates were incubated with 10% Alamar Blue with complete medium under standard conditions of 37 °C, 5% CO_2 for 8 hrs. Fluorescence wavelengths at excitation of 530 nm and emission of 590 nm were measured with FLUOstar Omega (BMG Labtech, UK).

2.8 Griess Reaction Assay

The amount of nitrite (NO_2^-) in cell culture supernatant was quantified using Griess reaction kit (Promega, UK) according to manufacturer's recommendations. Briefly, 50 µl sulfanilamide solution was added to equivalent volume of cell culture supernatant in 96 well plates. The plates were incubated at room temperature for 5 mins in the dark before 50 µl of NED solution was added. Colour changes of the solution mixture may be observed instantly. Absorbance was measured at wavelength 540 nm with FLUOstar Omega (BMG Labtech, UK) and quantity of nitrite was determined by comparison to a nitrite standard reference curve.

2.9 Caspase-1 Colorimetric Assay

Caspase-1 activity was quantified as described by Thornberry (Thornberry, Bull et al. 1992). Cells were harvested in Tris-NP40 lysis buffer and flash frozen on dry ice. 200 µg total protein from cell lysate was added to reaction buffer (see appendix), together with 50 µM Z-YVAD-pNa substrate to make up 100 µl final reaction volume/well in 96 well plates. Cell lysate and substrate was incubated in reaction buffer for 30 mins at 37 °C and absorbance was measured at 405 nm.

2.10 RNA Extraction

RNA extraction and genomic DNA removal was performed using RNeasy® mini kit with on-column DNase digestion (Qiagen, UK). RNeasy® kit contains silica columns which bind to RNA sequences longer than 200 bp in ethanol solution. Samples were processed according to manufacturer's instruction. In brief, cell lysate in RLT buffer were mixed with equal volume of 70% ethanol (Sigma-Aldrich, UK) and transferred immediately to an RNeasy column. The column was centrifuged briefly and washed with 350 µl RW1 buffer prior to a15 mins incubation with 80 µl DNase 1 and RDD solution. The column was then washed once more with 350 µl RW1 buffer and twice with 500 µl RPE buffer. After centrifugation in a new collection tube to remove any buffer, the column was transferred to a 1.5 ml Eppendorf tube (Sigma-Aldrich, UK) and RNA was eluted with 30 to 50 µl of RNase free water. Eluted RNA samples were stored at -80 °C.

RNA concentration and purity was determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, UK) at 260 nm and 280 nm (A_{260}/A_{280}). RNA integrity was determined by RNA gel electrophoresis.

2.11 cDNA Synthesis

Synthesis of cDNA was performed with the high-capacity cDNA reverse transcription (RT) kit (Life technologies, UK) according to manufacturer's instruction. Only intact

RNA with high purity of A_{260}/A_{280} ratio between 1.9 and 2.1 was used to generate cDNA. Reactions were set up with the following components:

Component	Volume (µl)
10x RT buffer	2
25x dNTP (100 mM)	0.8
Anchored Oligo dT(22)	2
MultiScribe™ Reverse Transcriptase	1
RNase Inhibitor	1
RNA samples	variable
Nuclease-free H_2O	variable (make up to 50 µl)

For negative controls (no RT), water was added instead of RNA samples. Reactions were performed in Veriti™ Dx 96-well thermal cycler (Thermo Fisher Scientific, UK) at the following conditions:

Step	Temperature	Time (mins)
1	25 °C	10
2	37 °C	120
3	85 °C	5
4	4 °C	∞

The cDNA was either used immediately after completion of RT or stored in -80 °C to avoid possible degradation.

2.12 Polymerase Chain Reaction (PCR)

Most Polymerase chain reactions were performed with One*Taq*® hot start quick-load® 2x master mix with standard buffer (NEB, UK), with the following reaction set up:

Component	Volume (µl)
10 µM forward primer	0.5
10 µM reverse primer	0.5

Hot start quick-load 2x master mix	12.5
Template cDNA	variable
Nuclease-free water	variable (make up to 25 µl)

Generally, 3 µl of template cDNA is sufficient for one PCR reaction. PCR reactions were performed with Veriti^{TM} Dx 96-well thermal cycler (Thermo Fisher Scientific, UK) with the following conditions:

Step	Temperature	Time (mins)	
Initial Denature	94 °C	0.5	
Denature	94 °C	0.5	35 cycles
Anneal [1]	60 °C	0.5	
Extension[2]	68 °C	1	
Final Extension	68 °C	5	
Hold	4 °C	∞	

[1] Annealing temperature varies depend on the primer pair.

[2] A general rule for extension time is 1 minute per kb, dependent of product size.

When screening for transformants, a small number of cells from a single colony was transferred to PCR master mix using a 10 µl sterile pipette tip, hence DNA from a single colony was amplified, with the following thermal cycle conditions:

Step	Temperature	Time (mins)	
Initial Denature	94 °C	4	
Denature	95 °C	0.5	35 cycles
Anneal [1]	60 °C	0.5	
Extension[2]	72 °C	1	
Final Extension	72 °C	7	
Hold	4 °C	∞	

[1-2] See above footnote.

2.13 DNA Gel Electrophoresis

PCR amplification products were analysed by electrophoresis of 1% agarose (Sigma-Aldrich, UK) gels at 120 V for 1 hr. Gels were prepared with 1x TBE (see appendix) buffer, containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich, UK) and visualised under ultra violet light with BopDpc-IT™ imaging system (Ultra-Violet Products, UK).

2.14 Sequencing

PCR products were sequenced to ensure amplification of correct target sequence by each primer pair.

PCR products were incubated with 60 µl of 20% PEG/2.5M NaCl solution for 30 mins at room temperature before pelleted by centrifugation at 2750 x g for 1hr at 4 °C. Supernatant was discarded by centrifuging the PCR plates upside down at 500 x g for 1 minute. DNA pellet was washed twice with 150 µl of cold 70% EtOH and pelleted by centrifugation at 2750 x g for 5 mins. Dried PCR product pellets were stored at -20 °C until required.

To set up sequencing reactions, PCR product pellets were re-suspended in 30 µl nuclease-free water, 2 µl re-suspended DNA template was added into each reaction. Reactions were composed as follows:

Component	Volume (µl)
BigDye® V3.1 (Applied Biosystems, UK)	0.25
5x dilution buffer V3.1	2
Nuclear-free water	1.75
10 µM primer (diluted 1:15)	4
DNA template	2

Reaction was performed in thermal cycler at the following condition:

Step	Temperature	Time (secs)
1	96 °C	10]

2	50 °C	5	30 cycles
3	60 °C	120	
4	4 °C	∞	

Upon programme completion, a mixture of 1 µl of 3M sodium acetate and 25 µl EtOH was added into each reaction. The 96-well plates were vortexed to mix, then spun at 500 x g for a few seconds and incubated at room temperature for 30 mins. The plates were spun for 1 hr at 2750 x g, 4 °C. Supernatant was discarded and the plates were spun at 500 x g for 1 min upside down on paper roll. Pellets were then dried at room temperature, sealed and analysed with 3730xl DNA analyser (Life Technologies, UK) at the Zoology Department in-house sequencing facility (University of Oxford, UK).

2.15 Quantitative Reverse Transcriptase PCR (q-rtPCR) Primer Design and Optimisation

Primer sets for q-rtPCR were designed with Primer3Web software (Untergasser, Cutcutache et al. 2012, Koressaar, Remm 2007) with the following criteria: (a) primer size between 18 to 23 bp in length, (b) GC content between 30% to 70%, (c) Tm between 58 °C to 62 °C, (d) avoid runs of 4 or more identical nucleotides, (e) span intron when possible, (f) amplicon sizes range between 70 to 150 bp. Primer pairs were produced by Integrated DNA Technologies (UK). Details of all qPCR primer pairs are listed in appendix.

Gradient PCR was performed with q-rtPCR primer pairs and PCR products were sequenced to ensure the correct targets were amplified (see 2.14). A range of primer concentrations from 50 nM to 900 nM were tested to determine the optimal primer concentration for q-rtPCR assay according to Fast SYBR Green (Applied Biosystems, UK) protocol.

2.16 Quantification of Gene Transcription

Transcriptional levels of target genes were quantified by q-rtPCR using the fast SYBR® green master mix (Life Technologies, UK). Quantification was achieved by measuring the increase in fluorescence intensity produced by the SYBR Green I dye binding to double stranded DNA during PCR. Q-rtPCR reactions were set up with optimised primer concentrations (see 2.15) and performed using the StepOnePlus™ Real-Time PCR system (Applied Biosystems, UK) at the following condition:

Step	Temperature	Time (secs)	
Enzyme activation	95 °C	20	
Denature	95 °C	3	
Anneal/Extend	60 °C	30	40 cycles
Melt curve step 1	95 °C	15	
Melt curve step 2	60 °C	60	Melt Curve Cycle
Melt curve step 3	95 °C	15	

Melt curve analysis was added at the end of qPCR amplification to ensure that a single, specific amplicon was produced by each qPCR reaction. Melt curves were produced by measuring reduced fluorescence signal in associated with dsDNA denaturing during temperature increase. QPCR results were presented as cycle value (Ct), at which a significant amount of fluorescence crosses the threshold was generated. Thresholds were set in the linear phase of exponential amplification and were set to the same level for every single gene of interest (GOI).

Ct value for housekeeping genes such as 28S or GAPDH were used to adjust for input RNAs among different samples. The slope of Ct against $\log_{10}$ of 10-fold serial dilution of a single preparation of RNA sample was calculated for every single gene on every plate. This slope of Ct represents the efficiency of the primers on a particular run. Sample Ct values were adjusted as follows and results were represented in 40-Ct value:

$$\text{Corrected Ct value} = \text{Ct of GOI}^{1} + (\text{M of Ref}^{2} - \text{Ct of Ref}^{3}) \times \frac{\text{S of GOI}^{4}}{\text{S of Ref}^{5}}$$

[1] Average Ct value of GOI

[2] Medium Ct value of reference gene for all samples within an experiment

[3] Average Ct value of reference gene for each treatment group (triplicates)

[4] Slope of GOI

[5] Slope of reference gene

Analysis of variance (ANOVA) was used to statistically analyse the qPCR results by using the linear mixed effects model functions *lme()* in statistical software package R V3.2.1 (2013) (Chambers 1992). Assumptions of which ANOVA is based on were tested with the following functions in R:

Testing residual normality	*qqnorm(resid(model))*
Test variance of residual	*plot(fitted(model),residuals(model))*
Test homoscedasticity	*plot(rnorm(100),rnorm(100))*
Test absence of influential data point	*dfbeta(model)*

Each biological replicate came from a different individual animal. Each technical replicate came from a different well of cell line culture which is treated independently. This proves the assumption of data point independence.

Bonferroni post hoc test was used for correction, in cases where multiple comparisons were conducted.

2.17 Transformation with Plasmid DNA

TLR plasmids (InvivoGen, UK) and transfection control plasmids (Table 2.2) were transformed into 5α competent *E. coli* cells (New England Biolabs, UK) according to manufacturer's recommendation. In brief, 1 ng of plasmid DNA was added to 50 µl

competent cells and mixed by inverting the tubes several time. The mixture was incubated on ice for 30 mins, heat shocked at 42 °C for exactly 30 secs and further incubation on ice for 5 mins. 750 µl SOC medium was then added to each tube and cells were incubated at 37 °C with 250 rpm gentle shaking for 1 hr. Transformation mixture was then plated on terrific broth (TB) agar plates (see appendix) with selective antibiotics of either blasticidin (1:1000, InvivoGen, UK) or kanamycin (1:1000, Sigma-Aldrich, UK) and incubated overnight at 37 °C.

2.18 Endotoxin Free Maxi Prep

GenElute™ HP endotoxin-free plasmid maxiprep kit (Sigma-Aldrich, UK) was used for growing all plasmids used in transfection experiments. Protocol for this kit is based on alkaline-SDS lysis followed by binding of plasmid DNA to silica column. Contaminants are removed by twice medium-salt washes.

A single colony was picked from the selective TB agar plate and incubated in starter culture consisting 3 to 5 ml TB medium with the appropriate selective reagents. Starter culture was incubated at 37 °C for 8hrs with vigorous shaking at 250 to 300 rpm before transferred to 400 ml of TB medium for 16 hr incubation at the same conditions. Bacteria cells were harvested by centrifugation at 5000 x g for 10 mins. Supernatant was removed and Cell pellets were stored at -80 °C until further processing.

Cell pellets were re-suspended in 12 ml of RNase A solution and lysed in 12 ml of lysis solution. Contents were mixed by gentle inverting and the mixture was incubated at room temperature for 5 mins to allow complete lysis. The lysis process was then neutralised by adding 12 ml cold neutralisation solution and mixed by gentle inverting. Lysate was then filtered through VacCap by applying vacuum. After adding 9 ml of binding solution to filtered lysate, the mixture was then transferred to the prepared binding column with vacuum on. Following two 12 ml washes and drying of columns for at least 20 mins, plasmid DNA was eluted in 3 ml of endotoxin-free water by

Table 2.2: All plasmids names, catalogue numbers, lot number and their selective antibiotics

TLR\Species	**Human**			**Mouse**		
	Cat No.	Lot No.	Selection Antibiotics	Cat No.	Lot No.	Selection Antibiotics
TLR1	pUNO-hTLR01	L0706-161	Blasticidin	pUNO-mTLR1	L2806-17	Blasticidin
TLR2	pUNO-hTLR02	L0707-191	Blasticidin	pUNO-mTLR2	L0607-271	Blasticidin
TLR4	pUNO-hTLR4a	L2811-31	Blasticidin	pUNO-mTLR4	L2706-17	Blasticidin
TLR6	pUNO-hTLR6	L2811-28	Blasticidin	pUNO-mTLR6	L2903-07	Blasticidin
TLR7	N/A	N/A	N/A	pUNO-mTLR7	L2704-10	Blasticidin
TLR8	pUNO-hTLR08a	L2907-16	Blasticidin	pUNO-mTLR8	L0607-271	Blasticidin
TLR9	pUNO-hTLR9	L2911-22	Blasticidin	pUNO1-mTLR9	360209-12	Blasticidin

Transfection control

Plasmid name	Company	Selection antibiotics
pCMV-eGFP	Nepa Gene, Japan	Kanamycin

centrifugation at 3000 x g for 5 mins.

2.19 Plasmid Transfection

HEK293T cells were at 90% confluency at the point of transfection. 1.5 µl TransIT2020 transfection reagent (Mirus Bio LLC, UK) was diluted in 50 µl OptiMEM™ I reduced serum medium (Invitrogen, UK) and 0.5 µg plasmid DNA was also diluted in 50 µl OptiMEM™ medium. Plasmid DNA/medium solution was then mixed with transfection reagent solution and incubated for 20 mins at room temperature to form transfection complex. During this time, cells were washed and replaced with 400 µl fresh medium. 100 µl of transfection complex was added to each well and cells were incubated at 37 °C, 5% CO_2 for 24 hrs prior to harvest in Tris-NP40 buffer.

2.20 Transfection of small interfering RNA (siRNA)

Ambion® *Silencer*® Select Small interfering RNAs (Life Technologies, UK) targeting genes encoding signalling components ERK, JNK and p38 were used for mRNA knockdown. These siRNAs are 19 mer dsRNA sequences with locked nucleic acid (LNA) modification and dTdT overhang for potency improvement (Bramsen, Kjems 2012).

RAW264.7 cells were in their active growth phase (50% confluency) at the point of transfection. 10 nM or 30 nM siRNAs were diluted in 10 µl OptiMEM™ medium and 0.4 µl TransIT2020 transfection reagent (Mirus Bio LLC, UK) was also diluted in 10 µl OptiMEM™ medium. SiRNA/medium solution was then incubated with TransIT2020/medium for 20 mins at room temperature. Cells were treated with 20 µl siRNA/transfection complex per well in 96 well plates. Transfection efficiency for TYE™ 563 DS dye-labelled transfection control (Integrated DNA Technologies, UK) was determined by fluorescence microscopy. Cells were incubated with TLR agonists for appropriate time (see chapter 7.2) starting from 48 hrs post siRNA transfection and were harvested in RLT or Tris-NP40 lysis buffer.

2.21 Western Blot

Cell lysates were thaw on ice and spun at 13,000 rpm for 25 mins on 4 °C to separate protein from cell debris and DNA contamination. 90 µl supernatants were transferred into new 1.5 ml Eppendorf tube (Sigma-Aldrich, UK) and mixed with 22.5 µl 4x NuPAGE® LDS sample buffer and 50 mM DL-Dithiothreitol (DTT) (Sigma-Aldrich, UK). Protein samples were denatured at 95 °C for 5 mins prior to loading.

Protein was separated according to their molecular mass (Da) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted on 5% stacking gels and 10% resolving gels (see appendix). Protein concentration was determined by Bradford colorimetric assay. 15 µl protein samples were loaded into each lane of a stacking gel, against blue pre-stained protein standard (New England Biolabs, UK). Stacking gels were run at 90 V for 30 mins and resolving gels were run at 120 V for 90 mins. Protein on resolving gels was then transferred to Imm-Blot® polyvinylidene difluoride (PVDF) membrane (Bio-Rad, UK) with wet transfer (see appendix) protocol. The transfer sandwich was placed in wet transfer chamber cassette and was run at 60 mA for 2 hrs. Membrane was then blocked with StartingBlock™ blocking buffer (Thermo Fisher Scientific, UK) for 1 hr with gentle shaking prior to overnight incubation with primary antibody. Membrane was washed three times with PBS-0.1% Tween or TBS-0.1% Tween and incubated in appropriate secondary antibodies for 1 hr. Membrane was washed five times after incubation to reduce excess antibody binding. Protein bands were visualised by treating the membrane with Novex® ECL chemiluminescent substrate (Thermo Fisher Scientific, UK) and imaged with G:Box chemiluminescence imager (Syngene, UK).

Quantification of western blot bands was achieved by densitometric analysis with ImageJ software v1.50a.

2.22 Antibodies

Antibodies were obtained from Abcam, Cell Signalling Technology (CST) and Jackson ImmunoResearch. Detail of each antibody is listed as follow:

Antibody	Company	Concentration	Isotype/label
Anti-TLR1	Abcam (ab108966)	1:1000	rabbit IgG
Anti-TLR2	Abcam (ab108998)	1:1000	rabbit IgG
Anti-TLR4	Abcam (ab22048)	1-6 µg/ml	mouse IgG
Anti-TLR6	Abcam (ab71429)	1:100-1:500	rabbit IgG
Anti-TLR7	Abcam (ab24184)	1-2 µg/ml	rabbit IgG
Anti-TLR8	Abcam (ab85859)	1-3 µg/ml	mouse IgG
Anti-TLR9	Abcam (ab134368)	1-5 µg/ml	mouse IgG
Phospho-ERK1/2	CST (4094)	1:1000	biotin
Phospho-SAPK/JNK	CST (5136)	1:1000	biotin
Phospho-P38	CST (4092)	1:1000	biotin
Anti-ERK1/2	CST (5013)	1:1000	biotin
Anti-SAPK/JNK	CST (9252)	1:1000	rabbit IgG
Anti-P38	CST (2387)	1:1000	biotin
Anti-MAPKAPK2	CST (3007)	1:1000	rabbit IgG

2.23 Bioinformatics

Ensembl genome browser and NCBI genome database were used to identify mRNA and amino acid sequences of signalling components in human, mouse and chicken. ClustalW software with standard parameters (Larkin, Blackshields et al. 2007) was used to align both amino acid and nucleotide sequences. When necessary, the amino acid alignments were manually adjusted in BioEdit sequence alignment editor first, and then the nucleotide open reading frame sequences were updated based on the amino acid alignments. Both amino acid sequences and nucleotide sequences were imported into MEGA6 (Tamura, Stecher et al. 2013) for phylogenetic analysis. The most fitting model was calculated for each alignment set when maximum likelihood tree was constructed in MEGA6 based on the following parameters:

Model/Method	WAG with Freqs. (+F) model
Rates among Sites	Gamma Distributed (G)
No. of Discrete Gamma Categories	5
Gaps/Missing Data Treatment	Partial deletion
Site Coverage Cut-off (%)	95%
ML Heuristic Method	Nearest-Neighbour-Interchange (NNI)
Test of Phylogeny	Bootstrap method
No. of Bootstrap Replications	100

Chapter 3 Transcriptional regulation of TLRs in murine cell lines

3.1 Introduction

TLRs are crucial pattern recognition receptors (PRR) specialised in the recognition of bacterial, viral and fungal patterns. Several TLRs detect bacterial surface structures: the TLR1/2/6/10 family recognises bacterial cell wall component lipopeptides, TLR4 recognises gram-negative bacterial membrane component lipopolysaccharide and TLR5 recognises flagella. Two TLR families detect bacterial and viral nuclear structures: TLR3 recognises sequence-independent dsRNA mainly from viruses, The TLR7 and 8 from the TLR7 family recognise ssRNA and TLR9 from the TLR7 family recognises un-methylated nucleic acid motifs present in both viruses and bacteria. The less-well characterised TLR11/12/13 family has shown to recognise uropathogenic bacteria, parasite and fungal components (Lewis, Obbard 2014, Boyd, Peroval et al. 2012, van der Vaart, Spaink et al. 2012, Kumar, Kawai et al. 2011, Kawai, Akira 2009, Gribar, Anand et al. 2008, Akira, Uematsu et al. 2006, Roach, Glusman et al. 2005). A detailed explanation of TLRs with their cognate agonists can be found in chapter 1 (section 1.3.1.2).

Upon activation by the cognate agonists, TLRs induce pro-inflammatory responses as well as substantial changes in the levels of mRNA encoding TLRs which can be referred to as the cognate and the cross regulation of TLRs (Peroval, Boyd et al. 2013, Kawai, Akira 2010, Kumar, Kawai et al. 2009, Takeda, Akira 2005). The ability to respond to TLR agonists and induce immune responses largely depends on the repertoire of TLRs present on cells. Previous studies have focused on TLR expression on monocytes, macrophages and dendritic cells as these cells play important roles in innate immunity as well as facilitating adaptive immunity through antigen presentation. Each cell type expresses a distinct pattern of TLRs which impacts on the capability of the cells to respond to TLR agonists. Cell lines which display phenotypes and

genotypes similar to primary cells *in vitro* and *in vivo* have been widely used to address cellular behaviour under various stimuli.

The monocyte/macrophage cell line, RAW264.7 (RAW) originated from BALB/c murine peritoneal macrophages, was transformed by Abelson Leukaemia Virus (Raschke, Baird et al. 1978, Ralph, Nakoinz 1977). Applequist et al. (2002) investigated the repertoire of TLR mRNA present on RAW cells with 30 cycles of PCR amplification. This study described the strong presence of TLR1, 2, 3, 4, 6, 7 and 9 mRNA in RAW cells. While TLR5 mRNA was undetectable in this study, Palazzo et al. (2008) measured the low expression of TLR5 in RAW cells by both PCR and western blot. The presence of TLR8, TLR11, 12 and 13 in RAW cells was also confirmed by later studies (Raetz, Kibardin et al. 2013, Li, Chen 2012, Zhu, Yang et al. 2011, Shi, Cai et al. 2009).

RAW cells were used extensively in studies of TLR induced responses due to the presence of a complete repertoire of TLR on these cells. Jones et al. (2001) investigated cytokine production induced by TLR2 agonist (LAM) and TLR4 agonist (LPS) in RAW cells. LPS induced expression of mRNA encoding IL1β and iNOS, as well as the secreted IL1β, TNFα and NO. LAM induced mRNA expression of IL1β but not iNOS. Secreted TNFα was also measured following stimulation with LAM. Similar observation was also reported by Carl et al. (2002). Malmgaard et al. (2004) investigated viral infection induced TLR-dependent cytokine production in RAW cells. Stimulation with viral components induced TNFα secretion downstream of TLR9. The TLR7/8 agonist R848 induced production of secreted type 1 IFNs downstream of TLR7 signalling through the IRF5 mediator in RAW cells (Schoenemeyer, Barnes et al. 2005).

Having similar phenotype and functional characteristics to RAW cells, P388D1 is a monocyte/macrophage cell line derived from mouse with DBA/2 background (Coleman, Forest et al. 1999, Snyderman, Pike et al. 1977). P388D1 was commonly used in early studies of LPS induced immunity. Treatment of LPS induced expression of IL1, IL6 and

TNFα but not IFNs in P388D1 cells (Bretz, Williams et al. 1994, Martin, Dorf 1990, Nishihara, Koga et al. 1988, Salo, Bleam et al. 1985). In recent studies, P388D1 was used to investigate TLR-mediated arachidonic acid (AA) mobilisation in response to variable TLR agonists. Stimulation of TLR1/2 agonist (PAM), TLR2 agonist (LTA), TLR3 agonist (Poly I:C), TLR4 agonist (LPS), TLR6 agonist (FSL-1) and TLR7 agonist (R848) all enhanced AA releases (Ruiperez, Astudillo et al. 2009, Ruiperez, Casas et al. 2007). Shibata et al. (2012) identified the absence of TLR5 and TLR9 on P388D1 cells. These studies suggest that P388D1 has a repertoire of functional TLRs similar to RAW cells.

Although agonists induced immune responses mediated through cognate TLR activation in cell lines was addressed in previous research (Walloschke, Fuhrmann et al. 2010, Spiller, Elson et al. 2008, Rhee, Jones et al. 2003), most studies focused on one or two TLRs in selected cell types. Furthermore, studies of non-cognate TLR regulation remain limited. The present work aimed to investigate the cognate- and cross-regulation of TLRs in murine cell lines systemically. TLR transcriptional changes induced by agonists derived from bacterial surface structure: PAM, LPS and FLA, was documented in RAW cells and P388D1 cells.

3.2 Results

TLR mRNA levels were measured by qPCR assay with specifically designed qPCR primer sets which span across an intron where possible. Detailed method for primer design and qPCR assay quantification can be found in chapter 2 (section 2.15 and 2.16). In brief, primer sets were designed with Primer3Web software (Untergasser, Cutcutache et al. 2012, Koressaar, Remm 2007). Conventional PCR were performed with each primer pairs at a range of concentrations to select optimal working concentration and PCR reaction products were sequenced to confirm amplification of accurate targets. Following validation, a series of 4 10-fold RNA dilutions (10^{-1} to 10^{-4}) from RAW cells were amplified in qPCR assays with the optimal primer concentrations. This gives Ct values across the RNA dilution range for each gene that primer sets target. These Ct values were then used to calculate the slopes for the standard curve method used for quantifying TLR mRNA level. The slopes of all TLR qPCR assays are shown in Appendix Figure 1. The standard curve method (Pfaffl 2001) compares the relative gene of interest (GOI) level to the reference gene (in this case, GAPDH) level in unknown samples by taking into the account of the primer slopes for GOI and the reference gene. Ct values for all qPCR assays were determined by StepOne V2.3 software (Life Technologies, UK).

QPCR results in this chapter were analysed using the statistical software package R V3.2.1 (2013) with a linear model (Chambers 1992) as each data point represents a different sample.

3.2.1 The TLR basal level changes over time in RAW264.7 cell line

Regulation of murine TLR mRNA levels was assessed at 2 hr, 8 hr, 24 hr and 48 hr post agonist stimulation. Expression of TLR transcripts vary in non-stimulated RAW cells. In TLR1/2 family, the average transcript level of TLR1 was expressed at +/- 13 cycles across the 4 chosen time points in non-stimulated RAW cells. Over time, TLR1 mRNA level peaked at 8hr at +/- 15 cycles, with a 2-cycle difference from 2 hr time

point. Its transcript level reduced back to +/- 13 cycles at 24 hr time point and further declined to +/- 11 cycles at 48 hr time point (Figure 3.1A). The average expression of TLR2 and TLR6 transcripts were both measured at +/- 16 cycles across the 4 chosen time points. Over time, TLR2 mRNA level decreased marginally in non-stimulated RAW cells. Expression of TLR6 transcript increased from 2 hr time point and peaked at 24 hr time point (Figure 3.1B, E). The average transcript level of TLR3 in non-stimulated cells was +/- 14 cycles across the 4 time points. Pattern for time-induced mRNA changes of TLR3 was similar to TLR1 in non-stimulated RAW cells. Its mRNA level increased from 2 hr time point and peaked at 8 hr. From 8 hr onwards, a reduction of TLR3 mRNA level was measured up to 48 hr time point (Figure 3.2A). Unstimulated RAW cells expressed TLR4 at +/- 16 cycles for all 4 time points. Its mRNA level was lowest at 24 hr time point and highest at 48 hr time point in non-stimulated RAW cells (Figure 3.1C). Expression of TLR5 in RAW cells has been a controversy in previous studies (see section 3.1). In this study, TLR5 transcript was detected in non-stimulated RAW cells across the 4 time points (Figure 3.1D). In TLR7/8/9 family, expression of TLR7 mRNA level in non-stimulated RAW cells was the highest, at +/- 16 cycles throughout the time points. TLR8 was expressed at +/- 15 cycles and TLR9 was expressed at +/- 14 cycles in unstimulated cells. Same pattern of TLR transcriptional changes according to time was also observed in the TLR7 family. Transcriptional expression of the TLR7/8/9 mRNA was highest at 8 hr time point. The mRNA levels declined from 8 hr time point to 48 hr time point over time (Figure 3.2 D-F). In TLR11 family, the average transcript levels of TLR11 and TLR12 were the lowest among all TLRs in non-stimulated RAW cells (Figure 3.2G). Although previously detected in RAW cells with PCR (data not shown), TLR11 failed to amplify with qPCR. TLR13 on the other hand, expressed at +/- 16 cycles and was among the highest expressed TLRs in non-stimulated RAW cells (Figure 3.2H). Transcript levels of TLR11 family did not change according to time.

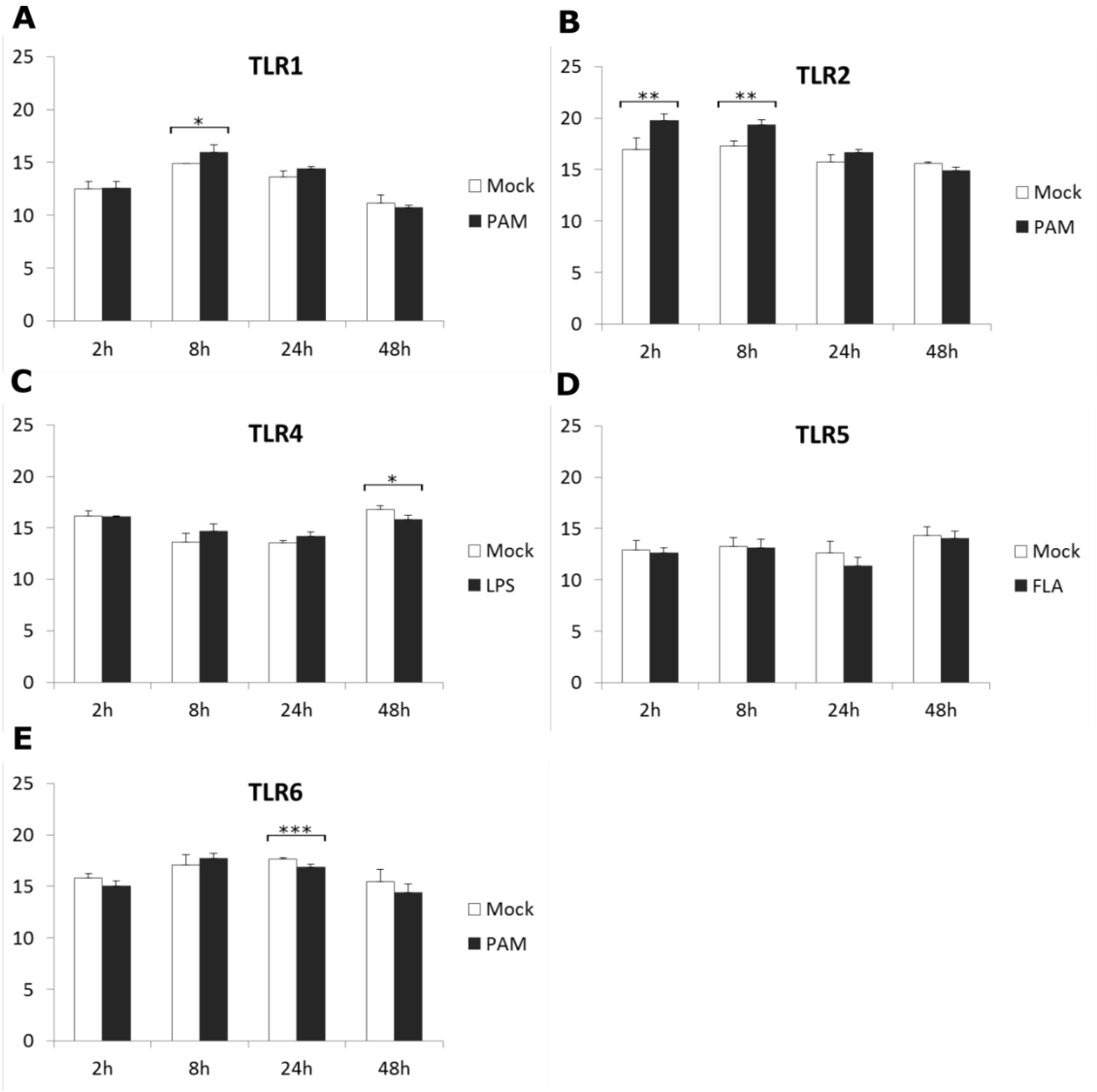


Figure 3.1: The cognate regulation of TLRs in RAW264.7 cells induced by agonists: PAM, LPS or FLA. The x axis indicates time points post agonist treatment; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated RAW cells; the black bars represent TLR expression in agonist-stimulated RAW cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels between adjacent bars: *p< 0.05, **p< 0.01, ***p< 0.001.

3.2.2 The effect of agonists on expression of cognate TLRs in RAW264.7 cell line

TLR1 and TLR2, known to form heterodimers following triacylated lipopeptide binding (Kang, Lee 2011, Jin, Lee 2008), were both upregulated by PAM, an synthetic triacylated lipopeptide agonist. Significant up-regulation ($p<0.05$) of TLR1 was measured at 8 hr post stimulation compared to TLR1 transcript level in non-stimulated RAW cells (Figure 3.1A). The up-regulation of TLR2 mRNA peaked from as early as 2 hr post agonist treatment and remained high until returning to the resting level after 8 hr post stimulation ($p<0.01$) (Figure 3.1B). TLR6, the other member of the TLR1/2 family, remained unaffected by PAM stimulation at these early time points. Expression of TLR6 was down-regulated at 24 hr post stimulation ($p<0.001$) (Figure 3.1E).

TLR4 forms homodimers when bound to LPS with the help of myeloid differentiation factor 2 (MD-2) (Park, Lee 2013, Bryant, Spring et al. 2010). Down-regulation of TLR4 was observed at 48 hr post LPS stimulation in RAW cells ($p<0.05$) (Figure 3.1C).

Low level of TLR5 transcriptional expression was observed in the resting and agonist stimulated RAW cells across all 4 time points (Figure 3.1D). Although TLR5 binds to flagellin, treatment of FLA-ST, a TLR5 agonist made of purified flagellin failed to alter transcriptional expression of TLR5 in RAW cells (Figure 3.1D). Nor was any pro-inflammatory response such as IL1β, IL6, NO or TNFα detected following FLA stimulation (data not shown). This may suggest that although TLR5 is expressed in RAW cells, its level is insufficient to initiate downstream signalling. Hence activation of TLR5 induced non-cognate TLR regulation and signalling modulation in RAW264.7 cells was omitted from later studies.

3.2.3 Transcriptional regulation of non-cognate TLRs in RAW264.7 cell line

PAM induced transcriptional modulation of most non-cognate TLRs in RAW cells (Figure 3.2). Members of the TLR4 family, TLR5 family, TLR7 family and TLR11 family

underwent cross regulation following activation of TLR1/2 with PAM. Significant down-regulation of TLR4 appeared in 2 phases, the initial phase of down-regulation was observed from 2 hr to 8 hr post PAM stimulation ($p<0.05$) and the second phase of down-regulation was measured at 48 hr post stimulation ($p<0.001$) (Figure 3.2B). Transcript level of TLR5 was down-regulated from 8 hr ($p<0.001$) to 24 hr ($p<0.01$) post stimulation through PAM induced signalling (Figure 3.2C). The pattern for transcriptional changes of TLR7 after PAM treatment was similar to TLR4. TLR7 underwent rapid down-regulated from 2 hr to 8 hr post PAM stimulation ($p<0.05$). At 24 hr post stimulation, a declining trend of TLR7 mRNA was observed although not significant ($p=0.098$), followed by the second phase of TLR7 down-regulation at 48 hr time point ($p<0.001$) (Figure 3.2D). TLR8 mRNA was down-regulated throughout the whole time course ($p<0.01$) (Figure 3.2E) while the other member of the TLR7/8/9 family, TLR9, was down-regulated only at 8 hr time point ($p<0.05$) (Figure 3.2F). TLR12 of the TLR11 family underwent PAM induced transcriptional regulation. Expressed at the lowest basal level among all TLRs, TLR12 was the only non-cognate TLR up-regulated by PAM treatment ($p<0.05$) (Figure 3.2G). PAM stimulation did not induce substantial transcriptional modulation for either TLR3 or TLR13, although both showed a trend towards down-regulation at 48 hr post stimulation (Figure 3.2A, H).

LPS also induced changes in transcript level of most non-cognate TLRs in RAW cells (Figure 3.3). Members of the TLR1/2 family, TLR3 family, TLR5 family, TLR7 family and TLR11 family underwent cross regulation following cognate activation of TLR4. Both TLR1 and TLR2 were up-regulated following LPS treatment. The increase of TLR1 mRNA level in LPS treated cells was constant from 2 hr to 48 hr post stimulation ($p<0.05$) (Figure 3.3A). TLR2 mRNA underwent rapid up-regulation from 2 hr ($p<0.05$) to 8 hr ($p<0.01$) post stimulation before returning to the basal level after 8 hr post stimulation ($p=0.074$) (Figure 3.3B). TLR6, the other member of the TLR1/2 family, was not affected by LPS stimulation throughout the time course (Figure 3.3E). TLR3 was

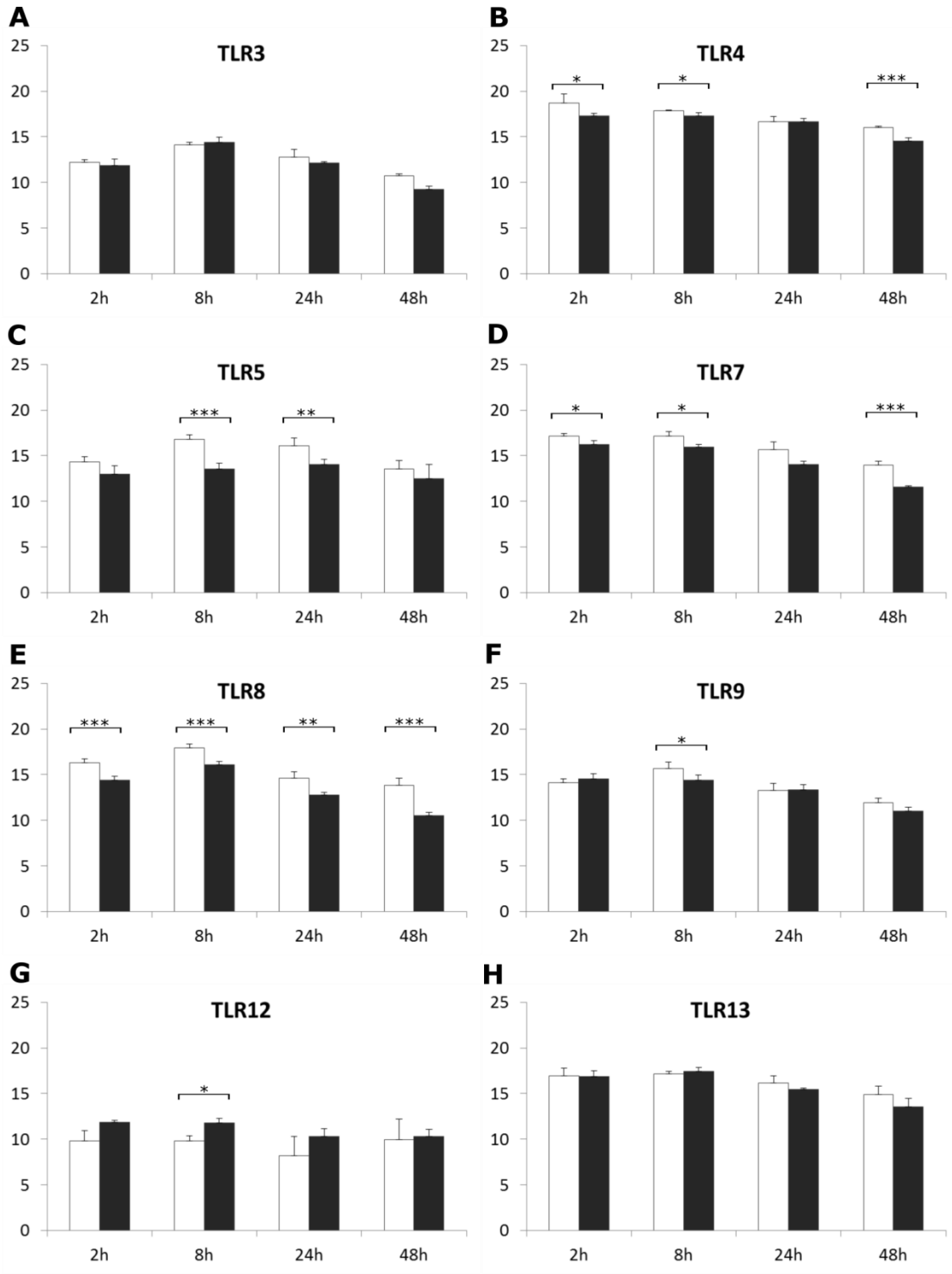


Figure 3.2: Cross regulation of TLRs in RAW264.7 cells induced by TLR1/2 ligand PAM. The x axis indicates time points post agonist treatment; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated RAW cells; the black bars represent TLR expression in PAM-stimulated RAW cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels between adjacent bars: *p< 0.05, **p< 0.01, ***p< 0.001.

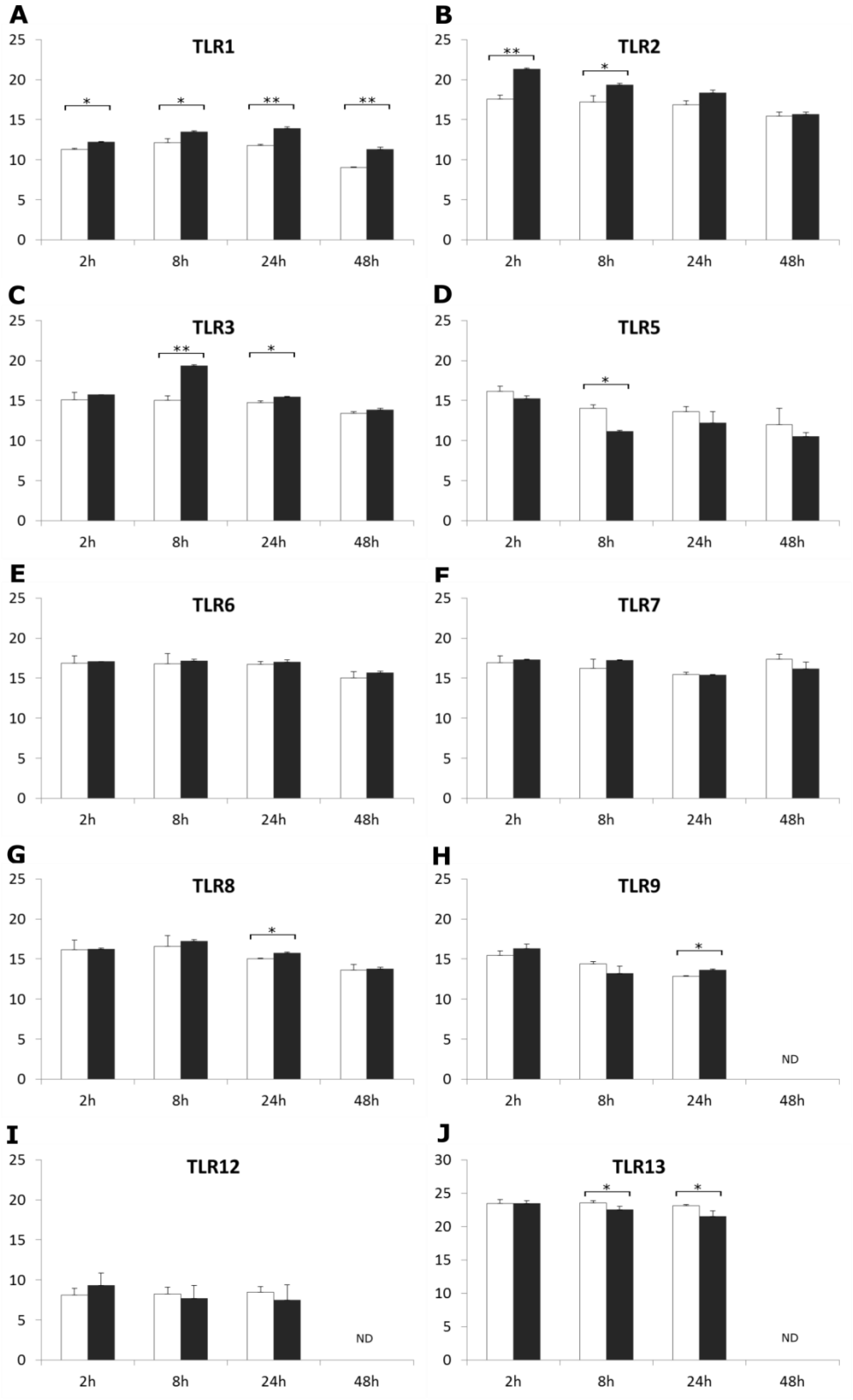


Figure 3.3: Cross regulation of TLRs in RAW264.7 cells induced by TLR4 ligand LPS. The x axis indicates time points post agonist treatment; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated RAW cells; the black bars represent TLR expression in LPS-stimulated RAW cells; ND: not done. Error bars and significance levels denoted by asterisks are the same as previous figures.

also up-regulated following LPS stimulation with increased transcript levels detected at 8 hr ($p<0.01$) and 24 hr time points ($p<0.05$) (Figure 3.3C). Despite the low transcriptional expression level in resting RAW cells, TLR5 was down-regulated at 8 hr post LPS stimulus ($p<0.05$) (Figure 3.3D). Transcript levels of TLR8 and 9 were up-regulated at 24 hr time point ($p<0.05$) (Figure 3.3G-H). On the other hand, TLR7 mRNA levels were not affected by LPS stimulation (Figure 3.3F). In TLR11 family, TLR13 mRNA levels were modulated following LPS treatment with down-regulation at 8 hr time point ($p<0.05$) and remained low at 24 hr ($p<0.05$) post stimulation (Figure 3.3J). No changes to TLR12 transcript were induced by LPS (Figure 3.3I).

3.2.4 TLR activation induced pro-inflammatory responses in RAW264.7 cell line

Robust pro-inflammatory responses induced by activation of TLR1/2 and TLR4 through respective cognate PAM and LPS exposure were consistent with previous studies (see section 3.1). Transcriptional up-regulation of IL1β, IL6, TNFα as well as inducible nitric oxide synthase (iNOS) were measured at 2 hr, 8 hr, 24 hr and 48 hr following PAM stimulation and at 2 hr, 8 hr and 24 hr following LPS stimulation (Figure 3.4 and 3.5). Nitric oxide (NO), the secreted product of iNOS, was also measured in cell culture supernatant with the Griess reagent system (see section 2.8).

All pro-inflammatory compounds measured were up-regulated following exposure to PAM, although temporal pattern for each cytokine was distinct (Figure 3.4). IL1β expression increased from 2 hr ($p<0.001$) and peaked at 8 hr time point ($p<0.001$). It then returned to the non-stimulated level by 48 hr time point (Figure 3.4A). The rapid increase of TNFα mRNA level was measured from 2 hr post PAM stimulation ($p<0.01$). This elevated transcript level then slowly reduced over time until returning to basal level by 48 hr post stimulation (Figure 3.4B). The expression pattern for IL6 was similar to IL1β except the magnitude of up-regulation was less ($p<0.01$) (Figure 3.4C). On the other hand, the transcript level of iNOS was slow to peak, with up-regulation beginning at 8 hr post stimulation ($p<0.01$) and stayed constant throughout the time course

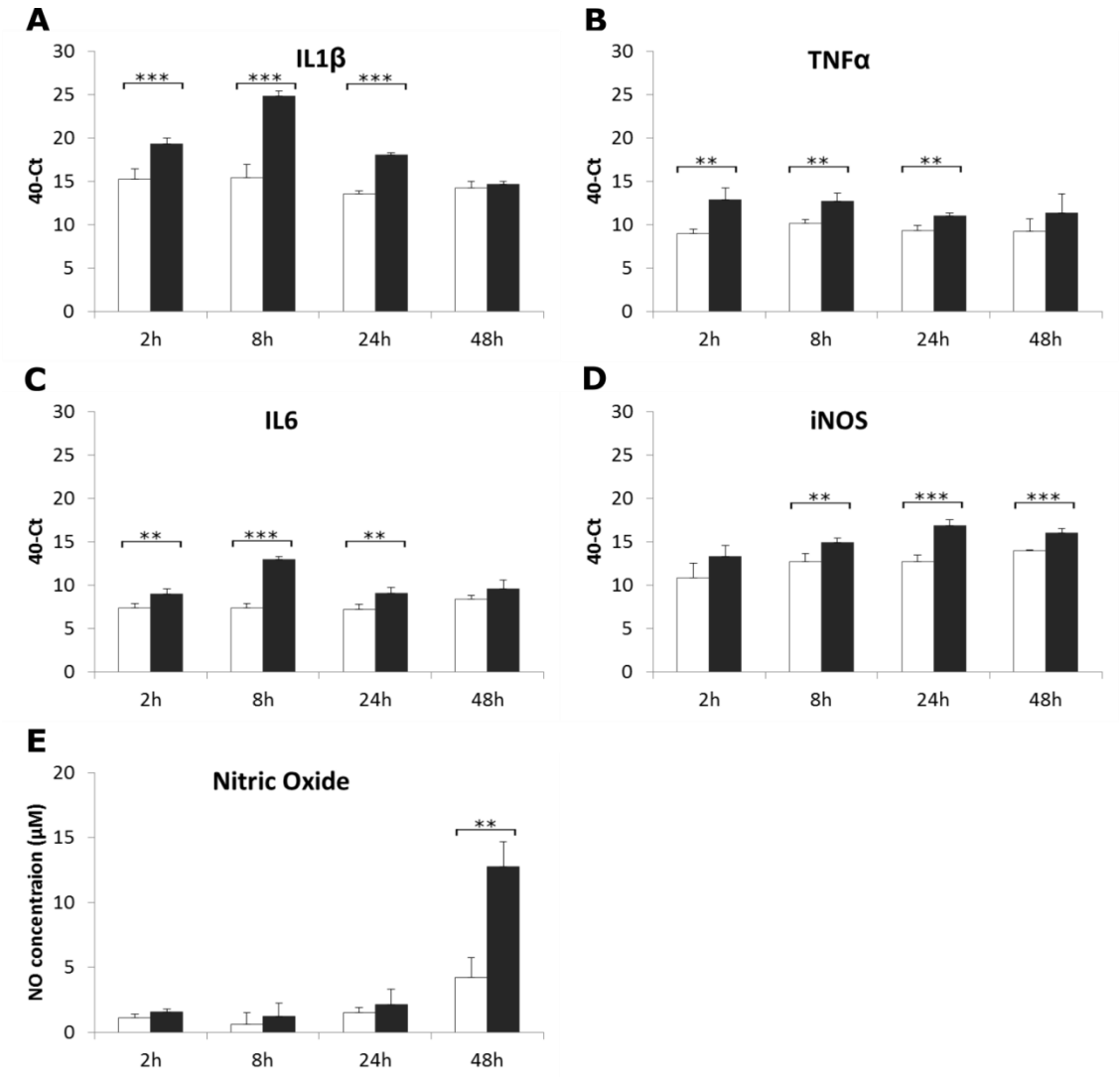


Figure 3.4: PAM-induced pro-inflammatory responses in RAW264.7 cells. The x axis indicates time points post agonist treatment; the y axis of A-D indicates mean 40 – Ct values adjusted for GAPDH, the y axis of E indicates NO concentration present in cell supernatant. The clear bars represent expression in non-stimulated RAW cells; the black bars represent expression in PAM-stimulated RAW cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

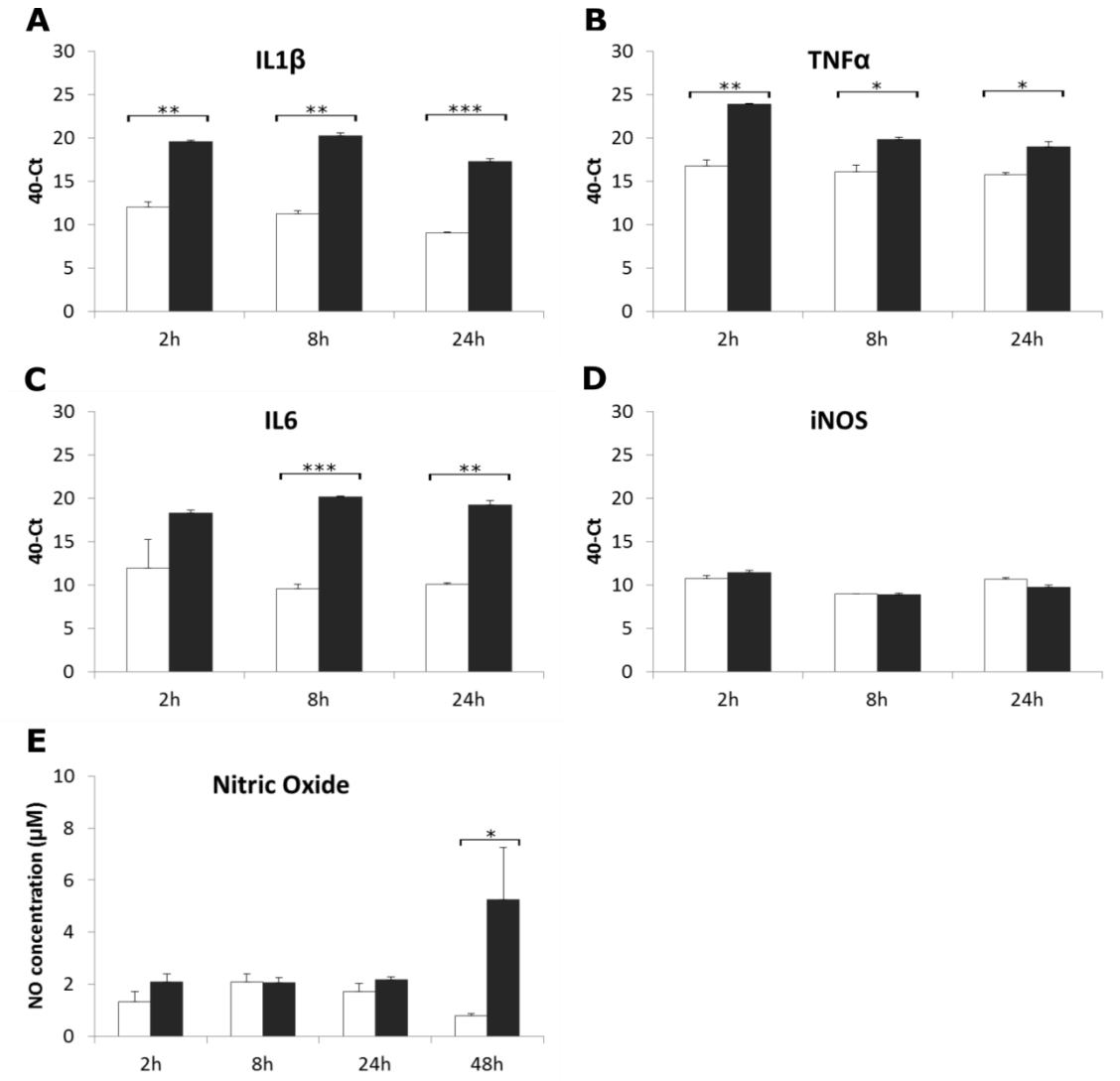


Figure 3.5: LPS-induced pro-inflammatory responses in RAW264.7 cells. The x axis indicates time points post agonist treatment; the y axis of A-D indicates mean 40 – Ct values adjusted for GAPDH, the y axis of E indicates NO concentration present in cell supernatant. The clear bars represent expression in non-stimulated RAW cells; the black bars represent expression in LPS-stimulated RAW cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

($p<0.001$) (Figure 3.4D). Low concentrations of NO were measured in cell culture supernatant at early time points. PAM treatment did not alter the NO concentrations at early time points, however a substantial increase in NO production was observed at 48 hr post stimulation ($p<0.01$) (Figure 3.4E). This corresponds to the PAM induced iNOS mRNA modulations as the increase of NO production was after the up-regulation of iNOS mRNA between 8 and 24 hr time points ($p<0.001$) (Figure 3.4D).

LPS stimulation up-regulated expression of mRNAs encoding pro-inflammatory mediators IL1β, TNFα and IL6, but did not affect iNOS (Figure 3.5). Expression patterns of IL1β and TNFα following LPS treatment were similar. Transcript level increased rapidly at 2 hr time point ($p<0.01$) and remained elevated throughout the time course (Figure 3.5A, B). The up-regulation of IL6 on the other hand, was slow to peak. Significant increase in IL6 transcript level was recorded at 8 hr ($p<0.001$) and 24 hr time points ($p<0.01$) although a non-statistically significant increase was present from early time point (Figure 3.5C). Production of NO was low in early time points. However substantial up-regulation of NO was detected at 48 hr following exposure to LPS ($p<0.05$) (Figure 3.5E) although no change to iNOS mRNA was observed at 2, 8 or 24 hr time points (Figure 3.5D). As we did not harvest LPS stimulated cells at 48 hr time point, it is possible that iNOS transcript level may increase between 24 and 48 hr time point following LPS stimulation.

3.2.5 TLR activation induced pro-inflammatory responses in P388D1 cell line

To compare the immunological effects of agonist treatment in different cell line models, PAM and LPS induced pro-inflammatory responses were examined in P388D1 cell line which derived from a mouse with different genetic background DBA/2.

Expression of IL1β and TNFα was measured at 2 hr, 4 hr, 6 hr, 8 hr and 24 hr post PAM stimulation. The increase in IL1β transcript level started from 4 hr after PAM stimulation ($p<0.01$), peaked at 8 hr time point ($p<0.001$) then returned to non-stimulated level by 24 hr time point ($p=0.071$) (Figure 3.6A). The up-regulation of TNFα

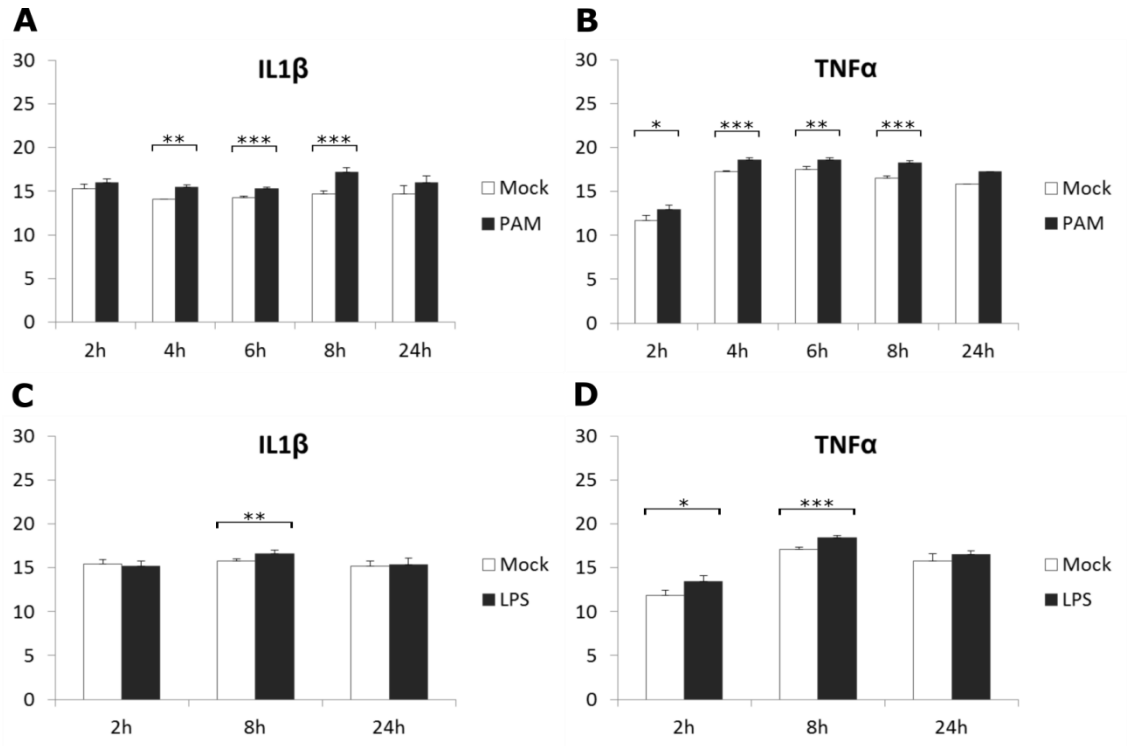


Figure 3.6: PAM- and LPS-induced pro-inflammatory responses in P388D1 cells. The x axis indicates time points post agonist treatment; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars in A-D represent cytokine expression in non-stimulated P388D1 cells; the black bars in A-B represent cytokine expression in PAM-stimulated P388D1 cells and the black bars in C-D represent cytokine expression in LPS-stimulated P388D1 cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels between adjacent bars: *p< 0.05, **p< 0.01, ***p< 0.001.

mRNA was detected from 2 hr post PAM treatment ($p<0.05$), remaining elevated through 4 hr ($p<0.001$), 6 hr ($p<0.01$) and 8 hr ($p<0.001$) time points and returned to non-stimulated levels by 24 hr time point ($p=0.097$) (Figure 3.6B).

LPS induced IL1β and TNFα expression was measured at 2 hr, 8 hr and 24 hr post stimulation. IL1β mRNA level increased at 8 hr ($p<0.01$) post LPS stimulation (Figure 3.6C). TNFα transcript level rapidly up-regulated at 2 hr ($p<0.05$) post stimulation and stayed up at 8 hr time point ($p<0.001$) (Figure 3.6D). Overall, agonist induced IL1β and TNFα mRNA up-regulation in P388D1 cells were weaker in magnitude of change and shorter in modulation period compared to RAW cells.

3.2.6 Transcriptional regulation of cytokines and TLRs in P388D1 cell line

Up-regulation of IL1β and TNFα mRNA levels induced by PAM stimulation were observed from 4 hr to 8 hr time point and up-regulation of IL1β and TNFα mRNA levels induced by LPS stimulation were observed at 8 hr time point (Figure 3.6). Hence the study of agonist induced TLR regulation in P388D1 cells focused on early time points when transcriptional modulation of cytokines occurred. The transcript levels of TLRs were assessed at 4 hr, 6 hr and 8 hr time points following PAM treatment and at 8 hr and 24 hr time points following LPS treatment. The initial study only included TLRs which were modulated by agonist stimulation in RAW cells. Analysis of TLR5 was excluded due to the lack of its presence in P388D1 cells (see section 3.1).

PAM induced transcriptional up-regulation of the TLR1/2 family in P388D1 cells. The mRNA levels of the cognate TLR2 increased at 4 hr after PAM treatment ($p<0.001$) then quickly returned to non-stimulated level by 6 hr time point ($p=0.072$) (Figure 3.7A). The mRNA level of TLR6 was up-regulated at 8 hr ($p<0.05$) after PAM treatment but not affected at earlier time points (Figure 3.7C). PAM treatment did not affect mRNA expression of TLR4 or TLR7, 8, 9 in P388D1 cells as they did in RAW cells (Figure 3.7B, D-F).

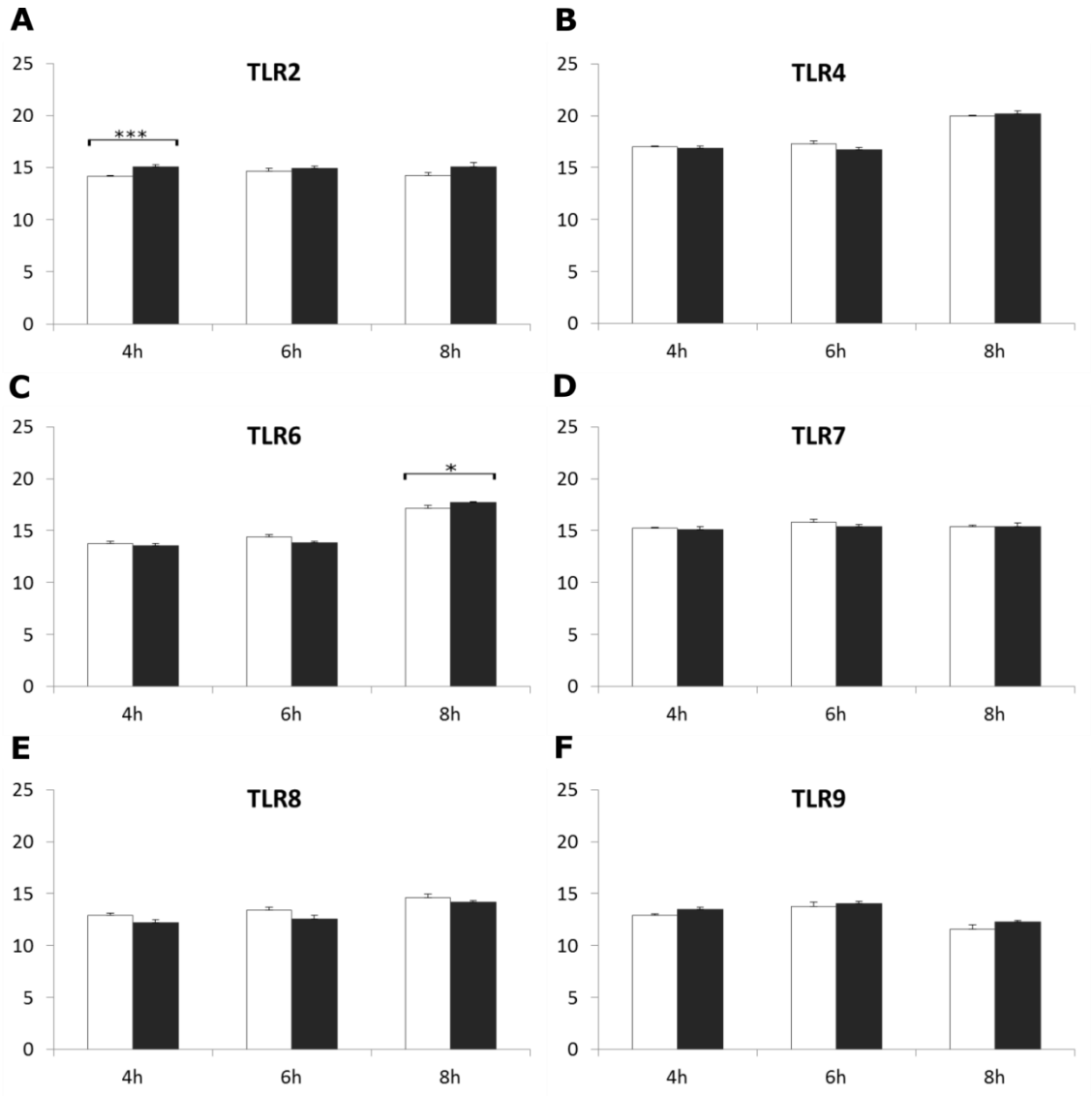


Figure 3.7: PAM-induced TLR regulation in P388D1 cells. The x axis indicates time points post agonist treatment; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in PAM-stimulated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

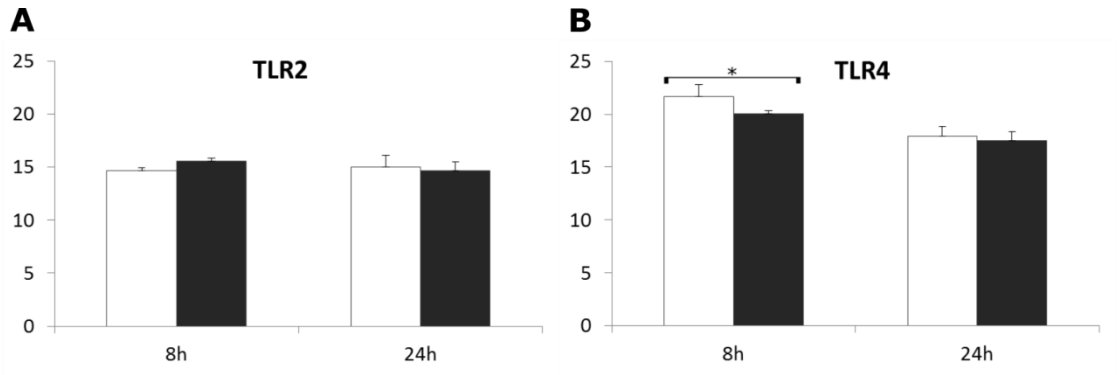


Figure 3.8: LPS-induced TLR regulation in P388D1 cells. The x axis indicates time points post agonist treatment; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in LPS-stimulated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

Present study of LPS induced TLR transcriptional changes in P388D1 cells focused on the regulation of cognate TLR4 and one of the cross-regulating TLRs (TLR2). The transcript level of TLR4 decreased at 8 hr time point after LPS stimulation ($p<0.05$) then restored to non-stimulated level by 24 hr time point ($p=0.56$) (Figure 3.8B). The same treatment failed to modulate the mRNA levels of non-cognate TLR2 in P388D1 cells (Figure 3.8A). Overall, the cognate TLR regulations were measured in PAM and LPS treated P388D1 cells. However transcriptional modulation of cross-regulating TLRs was not detected (Figure 3.7B, D-F, and Figure 3.8A).

3.3 Discussion

TLR are regulated for sensing pathogens in host immunity, but hypersensitivity or hyposensitivity of TLRs can also be associated with life threatening diseases such as sepsis and autoimmune disorders (Richez, Blanco et al. 2011, Fischer, Ehlers 2008, Marshak-Rothstein 2006). TLR activation induced transcriptional regulation of TLRs is a mechanism by which the hypersensitivity or dysfunction can be recorded. Regulation of TLRs is mediated by different mechanisms, including disassociation of signalling adaptor complex, degradation of signalling proteins and modification of promoter binding (Kondo, Kawai et al. 2012, Medzhitov, Horng 2009, Cabral, Gelderblom et al. 2006, Liew, Xu et al. 2005). Transcriptional regulation of cognate TLRs was documented in previous studies, especially in the case of LPS induced TLR4 hyposensitivity in order to avoid septic shock (Biswas, Lopez-Collazo 2009, Karima, Matsumoto et al. 1999). A few studies identified transcriptional modification of cross-regulation between certain TLRs, however these studies did not address all TLRs (Palazzo, Gariboldi et al. 2008, Marshall, Heeke et al. 2007, Cabral, Gelderblom et al. 2006, Sato, Linehan et al. 2006, Dalpke, Lehner et al. 2005, Heinz, Haehnel et al. 2003, Sato, Nomura et al. 2000). The present study aimed to comprehensively determine the TLR cognate- and cross-regulation in murine cell line models.

Activation of TLRs following PAM or LPS stimulation induced pro-inflammatory responses in murine cell lines. The up-regulation of cytokine transcript level and NO production was consistent with previous studies (Barrenschee, Lex et al. 2010, Carl, Brown-Steinke et al. 2002, Jones, Means et al. 2001b, Wadsworth, Koop 1999, Wang, Rossignol et al. 1994).

PAM stimulation induced a substantial degree of TLR cognate- and cross-regulation in RAW cells. TLRs from the same families shared similar patterns of agonist induced transcriptional regulation, which may be due to duplication of regulatory elements alongside duplication of TLR genes during vertebrate evolution. TLR1, 2 and 6 from

the TLR1/2 family were up-regulated following PAM treatment, suggesting that PAM may induce hypersensitivity in RAW cells. Interestingly, treatment with a different TLR2 agonist, LTA, induced down-regulation of these TLRs in the same cell line (Dalpke, Lehner et al. 2005). These results suggest that transcriptional modulation of TLR2 is agonist-specific in RAW cells. TLR7, 8 and 9 from the TLR7 family were all down-regulated following PAM treatment. This is consistent with previous research where dual ligand stimulation, R848 (TLR7 agonist) following PAM (TLR2 agonist), induced tolerance of cytokine production in RAW cells (Suet Ting Tan, Lin et al. 2013). On the other hand, only 1 out of 3 members from the TLR11 family, TLR12, was modulated following PAM treatment. TLR12 appeared to be the only cross-regulating TLR with increased mRNA level after stimulation of PAM. The TLR3 family, the TLR4 family and the TLR5 family each has 1 TLR member. Transcriptional regulation of TLR3 was not affected by PAM stimulation. TLR4 underwent down-regulation following PAM stimulation, supporting previous finding of PAM induced endotoxin tolerance in mice indicated by Sato et al. (2000). The present study confirmed low level of TLR5 mRNA expression in RAW cells, similar to previous observation by Palazzo et al. (2008). Following PAM stimulation, transcript level of TLR5 was reduced in RAW cells.

LPS induced TLR regulation in RAW cells was documented in a few studies. Heinz et al. (2003) indicated up-regulation of TLR3 following LPS stimulation. Palazzo et al. (2008) demonstrated up-regulation of TLR4, 5 and 9 as well as down-regulation of TLR7 following LPS stimulation. De Nardo et al. (2009) and McCoy et al. (2004) both indicated increased signalling and cytokine production down-stream of TLR9 following LPS stimulation. The present study confirmed previous finding of LPS treatment increased mRNA levels of TLR3 and 9 in RAW cells. However, not all observations were consistent with previous studies. LPS stimulation reduced transcript levels of both TLR4 and TLR5 in the present study, which differ to previous findings. These results suggest that LPS tolerance and LPS hypersensitivity states modulate TLR regulation

differently in RAW cells. LPS induced transcriptional modulation of the TLR1 family, TLR8 and the TLR11 family was also measured in the current study. TLR1, 2 from the TLR1/2 family and TLR8 from the TLR7/8/9 family was up-regulated following LPS stimulation. TLR13 from the TLR11 family was down-regulated following LPS stimulation in RAW cells.

PAM and LPS induced different TLR regulation pattern In P388D1 cells compared to RAW cells, despite similarities in TLR repertoires present on both cell lines. Only TLR2 and 6 underwent up-regulation following PAM stimulation and only TLR4 underwent down-regulation following LPS stimulation in P388D1 cells. These results suggest TLR regulation is cell type specific. Similar observations were made in previous studies. Walloschke et al. (2010) indicated a broad-range of cytokine secretion in RAW cells but not in P388D1 cells following dual stimulation of LPS and IFNγ. Liu et al. (2004) demonstrated LPS induced NO production in RAW cells but not in P388D1 cells. Snyderman (1977) studied chemotactic C5a stimulus induced immune responses in P388D1 cells and J774.1 cells. While J774.1 cells responded effectively to the stimulation, P388D1 cells were not responsive. Similar to RAW cells, J774.1 also derived from murine macrophage with BALB/c background. These findings suggest the difference of TLR regulation between RAW cells and P388D1 cells may be due to genetic background. In addition, mRNA expressions of most TLRs were lower in non-stimulated P388D1 cells than in RAW cells. Among all tested TLRs, expression of TLR2, TLR6 and TLR8 were about 4 times lower (2 cycles less by qPCR) in P388D1 cells. Expression of TLR9 was half (1 cycle less by qPCR) in P388D1 cells than in RAW cells. These results suggest the amount of TLR present on non-stimulated cells may affect the ability to respond to agonist and to induce transcriptional changes.

Overall, systematic analysis of TLR transcriptional regulation in cell line models represents the first step towards understanding agonist induced TLR modulation. Both cognate- and cross-regulation of TLR was induced by PAM and LPS in RAW cells

however only cognate-regulation of TLRs was induced in P388D1 cells, suggesting TLR regulation vary in different cell line models. The question remains, whether either of the cell lines is similar to primary murine macrophages. The next chapter will investigate TLR transcriptional regulation in murine splenic macrophages following stimulation of TLR agonists.

Chapter 4 Transcriptional regulation of TLRs in primary macrophages

4.1 Introduction

Primary macrophage populations reside in the majority of tissues in the body. These macrophage populations are extremely heterogeneous and exhibit their tissue-specific functionality. The major primary macrophage populations include: the adipose-associated macrophages in the adipose tissue, the bone marrow macrophages (BMDM) in the bones, the microglia in the central nerve system, the intestinal macrophages in the gastrointestinal tract, the kupffer cells in the liver, the alveolar macrophages in the lung, the peritoneal macrophages in the serous tissue, the Langerhans cells in the skin and the splenic macrophages from the spleen. Each of these macrophage populations can be identified by their specific phenotypic markers and functions (Amit, Winter et al. 2015, Davies, Taylor 2015, Davies, Jenkins et al. 2013). For instance, the adipose macrophages, identified by CD45+ markers, are involved in insulin regulation (Davies, Jenkins et al. 2013, Kang, Reilly et al. 2008, Odegaard, Ricardo-Gonzalez et al. 2007). The BMDMs, identified by CD169+ and ER-HR3+ markers, are responsible for supporting erythropoiesis and maintaining hematopoietic stem cells (Davies, Jenkins et al. 2013, Chow, Huggins et al. 2013, Nagata 2007). The peritoneal macrophages, identified by Gata6+ and Arg1+ markers, are also important for maintaining homeostasis (Davies, Taylor 2015, Rosas, Davies et al. 2014, Okabe, Medzhitov 2014, Gautier, Ivanov et al. 2014, Davies, Rosas et al. 2011). The splenic macrophage, identified by CD68+ and CD206+ markers, are responsible for the clearance of erythrocytes and maintaining iron metabolism (Davies, Taylor 2015, Ganz 2012, den Haan, Kraal 2012, Kohyama, Ise et al. 2009). Most importantly, all primary macrophages are best known for their roles in tissue immune defences against pathogens or stress signals(Ginhoux, Schultze et al. 2015, Glass, Natoli 2015, Okabe, Medzhitov 2015, Perdiguero, Geissmann 2015).

Gautier et al. (2012) analysed distinct TLR expression patterns on 4 tissue macrophage populations: the splenic macrophages, the alveolar macrophages, the peritoneal macrophages and the microglia. The expression of TLR4, 6, 7 and 13 was relatively constant across these macrophage populations however not all. The splenic macrophages expressed all TLRs. Transcript expression of TLR4, 7, 8, and 13 was high while TLR3, 11 and 12 was low in these cells. The peritoneal macrophages expressed all TLRs except TLR11 and 12. Gene expression of TLR13 was the highest while TLR1, 3 and 9 was low in these cells. The microglia expressed all TLRs except TLR8 and 11. The alveolar macrophages expressed TLR2, 3, 4, 6, 7, 8 and 13, but not TLR1, 9, 11 or 12. Transcript expression of TLR2 was extremely high in the alveolar macrophages. Its mRNA level was higher than any other TLRs in any macrophage types examined in this study. This study suggests that each tissue macrophage type expresses a specific TLR repertoire.

The TLR repertoires present on primary macrophages may induce differential immune modulations in these cells. Wang et al. (2013) investigated TLR4 activation induced regulations of immune mediators in three tissue macrophages: the peritoneal macrophages, the splenic macrophages and the BMDMs. A high level of pro-inflammatory mediators IL6, IL12 and TNFα were expressed by non-stimulated splenic macrophages. Upon LPS stimulation, expression of these pro-inflammatory mediators further increased while expression of anti-inflammatory mediator TGF-β decreased in splenic macrophages. In contrast, a high level of anti-inflammatory mediators IL10 and TGF-β were expressed by non-stimulated BMDMs. Upon LPS stimulation, expression of IL10 and TGF-β reduced while expression of pro-inflammatory mediators IL6, IL12 and TNFα increased in BMDMs. The non-stimulated peritoneal macrophages displayed characters of both splenic macrophages and BMDMs. Expression of pro-inflammatory mediators in non-stimulated peritoneal macrophages were low, similar to BMDMs and expression of anti-inflammatory mediators in non-stimulated peritoneal

macrophages were also low, similar to splenic macrophages. Upon LPS stimulation, peritoneal macrophages regulated immune mediators similar to BMDMs. Expression of anti-inflammatory mediators IL10 and TGF-β reduced while expression of pro-inflammatory mediators IL6, IL12 and TNFα increased in peritoneal macrophages (Wang, Yu et al. 2013).

Cross-talk between TLRs in primary tissue cells are increasingly described in the literature. Lin et al. (2000) investigated expression of TLR2 following LPS treatment. The TLR4 agonist induced dose-dependent TLR2 expression in murine adipocytes. While TLR2 was not present in non-stimulated adipocytes, protein expression of TLR2 was measured from 1 hr post stimulation. Cabral et al. (2006) studied expression of TLR5 following TLR2 agonist treatment in peripheral blood mononuclear cells (PBMCs). Three TLR2 agonists: PAM, lipidated outer surface protein A (L-OspA) and *Borrelia burgdorferi* lysate (BL) all induced dose-dependent reduction of TLR5 protein level. Furthermore, blocking of TLR2 with anti-TLR2 antibody significantly rescued the down-regulation of TLR5 in PBMCs. Similar observations were reported by Van Aubel et al. (2007). Treatment of TLR2 agonist PAM reduced TLR5 mRNA level in murine intestinal epithelial cells. The same study also reported other cross-talk between TLR2, 4 and 5. Similar to PAM induced TLR5 changes, treatment with TLR4 agonist LPS also reduced the transcriptional expression of TLR5. In addition, treatment with TLR4 agonist LPS and TLR5 agonist FLA both increased mRNA level of TLR2. Treatment with TLR2 agonist PAM down-regulated TLR4 transcriptional expression while treatment with TLR5 agonist FLA up-regulated TLR4 transcriptional expression. Jagannathan et al. (2009) investigated cross-talk between TLR2, 4 and 9 in B cells. Treatment of TLR2 agonist PAM or TLR9 agonist ODN both increased protein expression of TLR4 on the surface of B cells. The TLR4 agonist LPS induced transcriptional change of TLR9 was measured in a separate study. Reduced TLR9

mRNA level was measured from 2 hr time point and remained constant for more than 12 hrs after LPS stimulation in BMDMs (De Nardo, De Nardo et al. 2009).

Expression of TLRs and TLR induced immune responses in primary cells may be influenced by different factors, one of which being the animal's natural circadian pattern. Silver et al. (2012) studied the effect of circadian rhythm on some TLRs in murine peritoneal and splenic macrophages. Transcriptional expression of TLR9, but not TLR4 or 7, followed circadian oscillations for 24 hrs in *in vivo* culture. Knock down of the circadian genes *Per2* or *Clock* both terminated the oscillation of TLR9. Furthermore, mice immunised when TLR9 expression was at highest level led to stronger TNFα and IFNγ production. Sidhu et al. (2015) studied the effects of circadian oscillation on TLR responses in murine macrophages. Following stimulation with TLR2 agonist, production of IL6 and IL12p40 were regulated with circadian oscillation. Similarly, following stimulation with TLR7 agonist, production of IL12p40 was also regulated with circadian oscillation. Other studies investigated the effects of circadian oscillation on LPS induced immune responses in particular (Nakao 2014, Gibbs, Blaikley et al. 2012, Keller, Mazuch et al. 2009). Circadian rhythms in TNFα and IL6 secretion following LPS stimulation in murine splenic macrophages were dependent on circadian gene *Bmal1*.

This chapter of the present study comprised two parts, firstly to investigate the cognate- and cross-regulation of TLRs in murine tissue macrophages and to validate the results between RAW cells and primary macrophages, and secondly to analyse the influence of circadian rhythm on TLR regulation.

4.2 Results

TLR transcript levels in murine splenic macrophages were measured by qPCR and results were analysed with the linear mixed effects model (Lindstrom, Bates 1990, Hothorn, Bretz et al. 2008) using statistical software R V3.2.1 (2013). This approach accounts for systematic variation between samples from different mice within a data set by treating individual mouse as a random effect. Tukey's HSD test which compares all possible pairs to identify significant differences and includes multiple testing corrections was used for statistical analysis.

4.2.1 TLR basal expression in murine splenic macrophages

Expression of TLRs in non-stimulated splenic macrophages was largely similar in macrophages from C57Bl/6 and BALB/c mice. In the TLR1/2 family, expressions of TLR1 and TLR6 were both detected at +/- 10 cycles. There was no difference in the transcript levels between the two mouse strains (Figure 4.1A, E). Expression of TL2 mRNA level was detected at +/- 11 cycles in C57Bl/6 mice and +/- 12 cycles in BALB/c mice (Figure 4.1B). The splenic macrophage expressed TLR3 mRNA at +/- 12 cycles and TLR4 at +/- 10 cycles in mice from both C57Bl/6 and BALB/c backgrounds (Figure 4.1C, Figure 4.2C-D). Expression of TLR5 was low in both mouse strains. TLR5 transcript level was detected at +/- 8 cycles in BALB/c mice and +/- 10 cycles in C57Bl/6 mice (Figure 4.1D, Figure 4.2D). In the TLR7 family, TLR7 and TLR8 mRNA levels were very similar between C57Bl/6 and BALB/c mice, at +/- 11 cycles for TLR7 and +/- 12 cycles for TLR8 (Figure 4.1F, Figure 4.2C-D). On the other hand, expression of TLR9 was different between C57Bl/6 and BALB/c mice. TLR9 mRNA level was detected at +/- 15 cycles in C57Bl/6 mice and +/- 9 cycles in BALB/c mice. With 6-cycle differences, TLR9 mRNA levels were most different among all TLRs in non-stimulated splenic macrophages between the two mouse strains (Figure 4.1G).

4.2.2 Transcriptional regulation of cognate TLRs in murine splenic macrophage

Agonist induced cognate TLR regulations in murine splenic macrophages were assessed at 8 hr post stimulation. PAM, LPS and ODN induced modulations on the cognate TLR transcript levels were measured in macrophages from both C57Bl/6 and BALB/c mouse strains. FLA induced TLR5 mRNA changes were measured in BALB/c macrophages and CL097 induced TLR7 mRNA changed was measured in C57Bl/6 macrophages only.

In the TLR1/2 family, TLR1 and TLR2 mRNA levels were both up-regulated following PAM exposure in murine splenic macrophages from C57Bl/6 ($p<0.01$) and BALB/c ($p<0.01$) genetic backgrounds (Figure 4.1A-B). On the other hand, TLR6 mRNA level was not changed at 8 hr post PAM stimulation (Figure 4.1 E). Differential regulation of TLR4 between the two genetic backgrounds was observed following LPS stimulation. TLR4 transcript levels increased in C57Bl/6 mice ($p<0.001$) but decreased in BALB/c mice ($p<0.001$) following LPS stimulation at the same concentration (Figure 4.1C). The transcriptional expression of TLR5 was only measured in BALB/c macrophages. Although not modulated by FLA in RAW cell line (Chapter 3.2.2), TLR5 was down-regulated in murine splenic macrophages ($p<0.05$) (Figure 4.1D). In the TLR7 family, the regulation of TLR7 was only measured in C57Bl/6 mice. Transcriptional expression of TLR7 increased in splenic macrophages following CL097 stimulation ($p<0.001$) (Figure 4.1F). TLR8 was omitted from this study as its functionality in mice remains a controversy. TLR9 transcript levels were measured in both BALB/c and C57Bl/6 macrophages. Up-regulation of TLR9 mRNA levels were induced following ODN stimulation in both genetic backgrounds (BALB/c: $p<0.01$; C57Bl/6: $p<0.001$), despite differences in its basal expression levels (Figure 4.1G).

4.2.3 Transcriptional regulation of non-cognate TLRs in splenic macrophage

PAM, LPS, FLA and ODN induced TLR cross-regulations were detected in macrophages from BALB/c mouse strain. CL097 induced TLR cross-regulations were assessed in macrophages from C57Bl/6 mouse strain. While all tested agonists

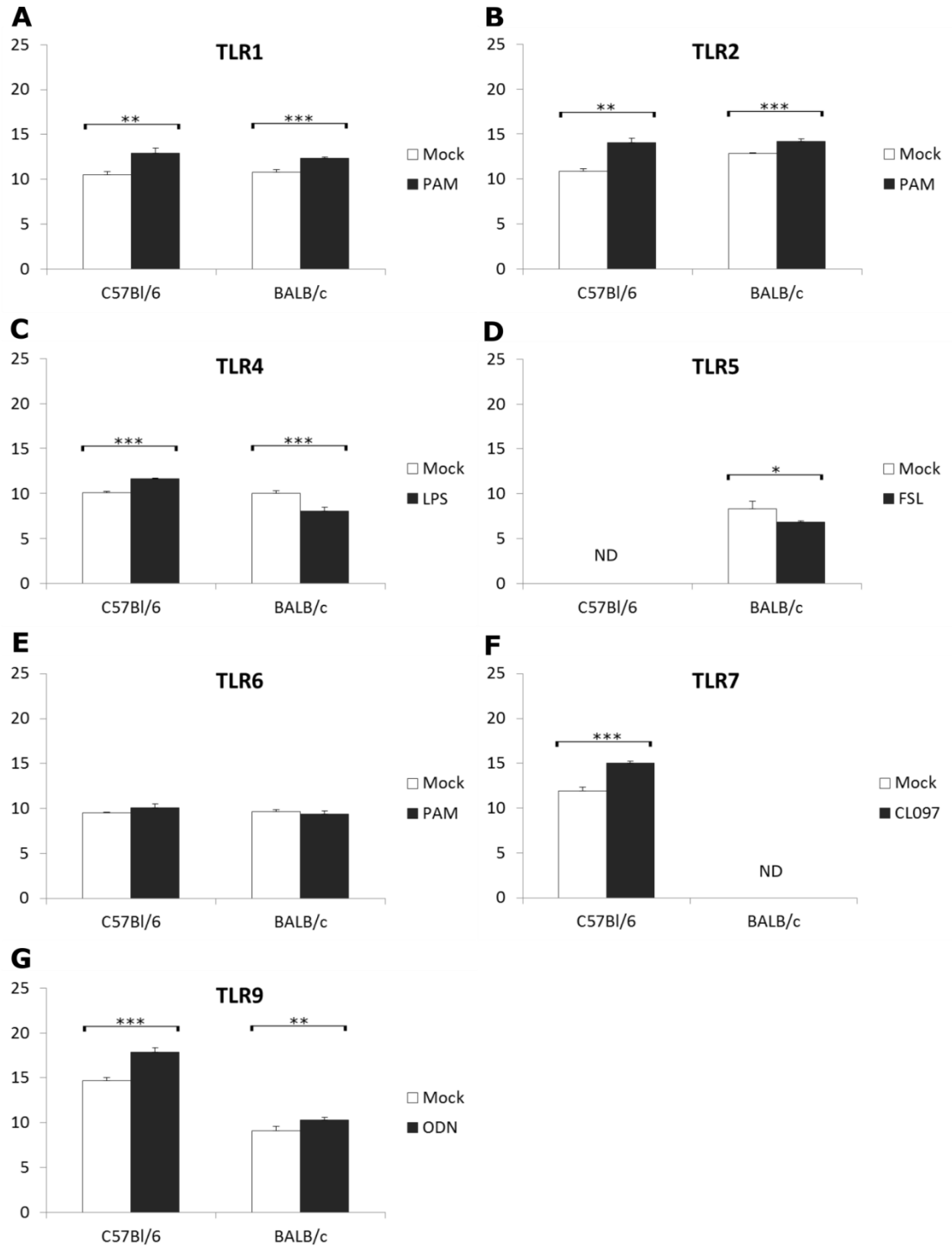


Figure 4.1: The cognate regulation of TLRs in murine splenic macrophages induced by agonists: PAM, LPS, FSL, CL097 or ODN. The x axis indicates the genetic background of the mice; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated splenic macrophages; the black bars represent TLR expression in agonist-stimulated macrophages; ND: not done. Error bars presented are at 95% confidence intervals and asterisks signify significance levels between adjacent bars: *p< 0.05, **p< 0.01, ***p< 0.001.

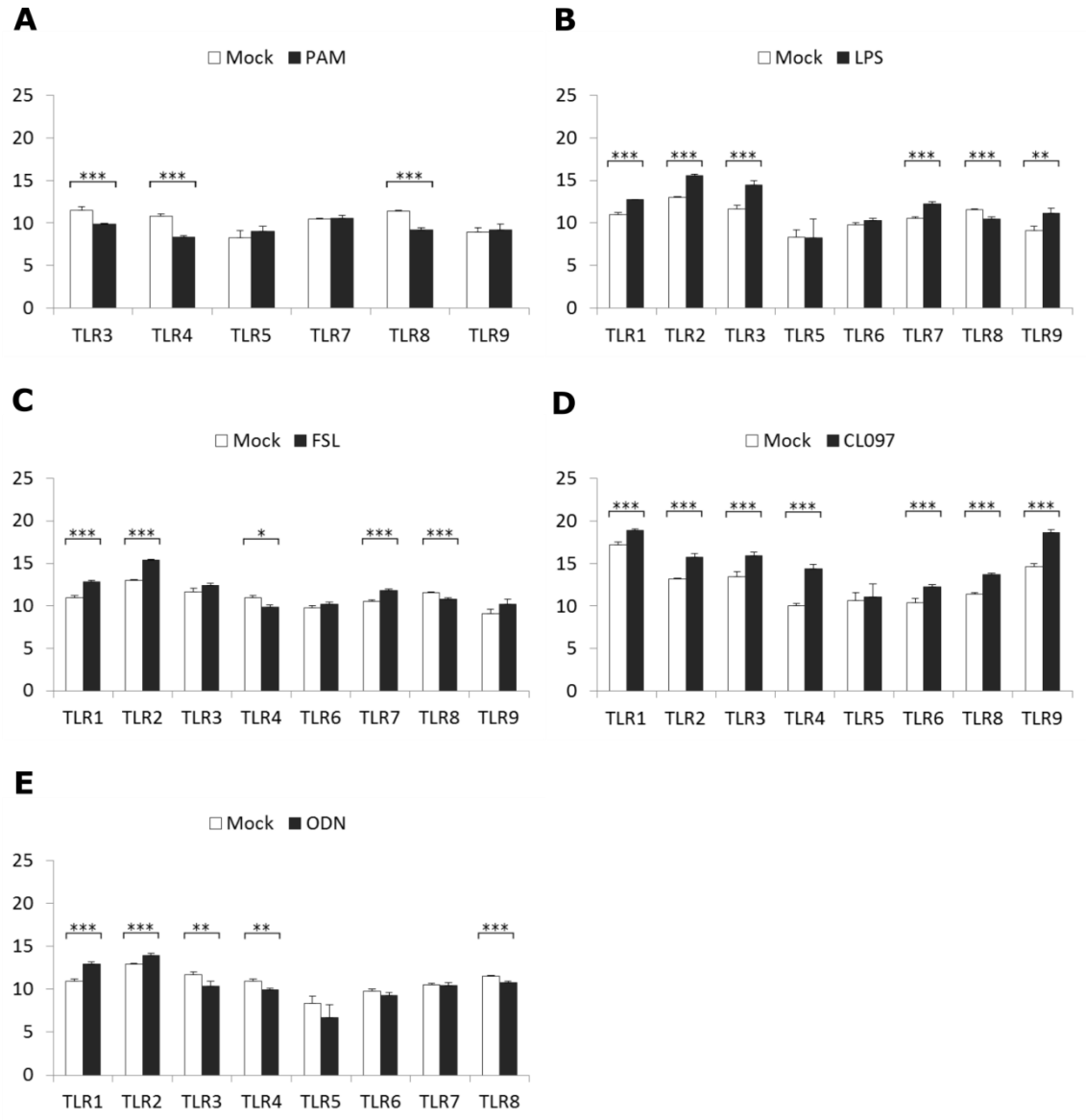


Figure 4.2: The non-cognate regulation of TLRs in murine splenic macrophages induced by agonists: PAM, LPS, FSL, CL097 or ODN. The x axis indicates the non-cognate TLRs; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated splenic macrophages; the black bars represent TLR expression in agonist-stimulated splenic macrophages. PAM, LPS, FSL and ODN stimulation was measured in macrophages from BALB/c background; CL097 stimulation was measured in macrophages from C57Bl/6 background. Error bars and significance levels denoted by asterisks are the same as previous figures.

induced transcriptional modulation of non-cognate TLR, distinct patterns were seen following treatment of each agonist.

PAM treatment induced down-regulation of non-cognate TLRs in splenic macrophages from BALB/c mice. Transcriptional expression of TLR3 ($p<0.001$), 4 ($p<0.001$) and 8 ($p<0.001$) reduced following PAM stimulation. Whereas mRNA levels of TLR5 ($p=0.24$), 7 ($p=0.46$) and 9 ($p=0.61$) remained unchanged (Figure 4.2A).

LPS induced both up- and down-regulation of non-cognate TLRs in splenic macrophages from BALB/c mice. In the TLR1/2 family, the transcript levels of TLR1 ($p<0.001$) and TLR2 ($p<0.001$) were both up-regulated following LPS stimulation whereas TLR6 mRNA level was unaffected ($p=0.53$). LPS treatment increased TLR3 mRNA level at 8 hr after stimulation ($p<0.001$). On the other hand, expression of TLR5 remained unchanged ($p=0.95$). LPS induced complex regulatory pattern in TLR7/8/9 family. TLR7 ($p<0.001$) and TLR9 ($p<0.01$) mRNA levels were both up-regulated following LPS stimulation whereas TLR8 mRNA levels were down-regulated ($p<0.001$) (Figure 4.2B).

FLA treatment also induced both up- and down-regulation of non-cognate TLRs in splenic macrophages from BALB/c background, although the regulatory pattern was somewhat different to LPS. In the TLR1/2 family, TLR1 ($p<0.001$) and 2 ($p<0.001$) mRNA levels increased following FLA stimulation whereas TLR6 mRNA level did not change ($p=0.55$). The transcript level of TLR3 was also unaffected by the same treatment ($p=0.17$). Stimulation of FLA reduced transcript level of TLR4 ($p<0.05$). In the TLR7/8/9 family, TLR9 transcript level was not modulated by FLA treatment ($p=0.19$), whereas TLR7 mRNA level was up-regulated ($p<0.001$) and TLR8 was down-regulated ($p<0.001$) (Figure4.2C).

CL097 induced only up-regulations of non-cognate TLRs in splenic macrophages from C57Bl/6 background. Transcript levels of cross-regulating TLRs, TLR1, 2, 3, 4, 6, 8

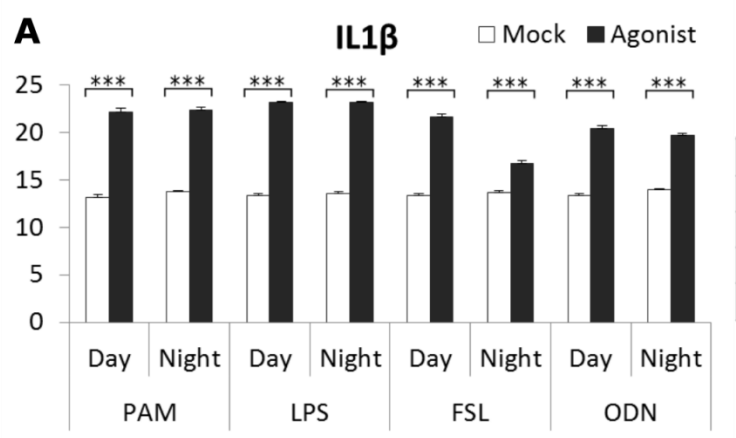
and 9 were all up-regulated following CL097 stimulation except TLR5 ($p=0.72$) (Figure 4.2D).

ODN induced both up- and down-regulation of non-cognate TLRs. Transcript levels of TLR1 ($p<0.001$) and 2 ($p<0.001$) increased following ODN stimulation whereas TLR6 mRNA level did not change ($p=0.065$). TLR3 ($p<0.01$) and TLR4 ($p<0.01$) were both down-regulated at 8 hr post ODN stimulation. TLR5, expressed at a low level in BALB/c splenic macrophages, was not modulated following ODN treatment ($p=0.15$). In a similar manner, TLR7 mRNA level was also not affected by ODN stimulation ($p=0.51$) whereas expression of TLR8 from the same TLR7 family was down-regulated ($p<0.001$) (Figure 4.2E).

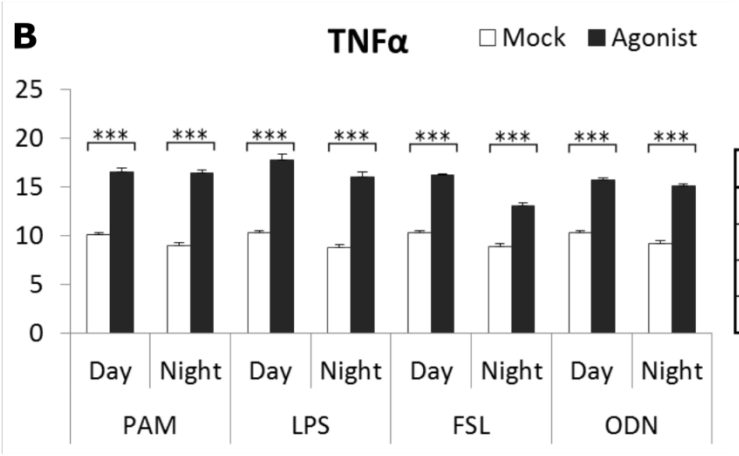
4.2.4 Influence of day/night oscillation in response to TLR agonists

To investigate the effect of animals' natural circadian rhythm on TLR regulation, a day/night oscillation experiment was conducted. Primary splenic macrophages from 4 different mice were isolated at 10am and allowed incubation in complete darkness for 48 hrs prior to the oscillation experiment. Agonists were treated at ZT19 (1am), the time point whenTLR9 expression was at the highest (defined as "night") and ZT7 (1pm), the time point when TLR9 expression was at the lowest (defined as "day"). (Silver, Arjona et al. 2012) (see Chapter 4.1). The pro-inflammatory mediators as well as cognate and non-cognate TLRs mRNA levels were measured at 8 hr post agonist stimulation.

Day/night oscillation affected expression of pro-inflammatory cytokines IL1β and TNFα differently. In non-stimulated macrophages, expression of IL1β was not affected by time. In stimulated splenic macrophages, all tested agonists PAM ($p<0.001$), LPS ($p<0.001$), FLA ($p<0.001$) and ODN ($p<0.001$), induced substantial up-regulation of IL1β, irrespective of the treatment time. However, the magnitude of IL1β up-regulation was significantly less during night than day when treated with FLA ($p<0.001$) and ODN ($p<0.01$) but not with PAM ($p=0.14$) or LPS ($p=0.27$) (Figure 4.3A). On the other hand,



IL1β	Day	Night
PAM	415.83	306.58
LPS	717.41	599.91
FSL	257.28	7.52
ODN	118.64	46.18



TNFα	Day	Night
PAM	73.84	150.57
LPS	155.43	130.59
FSL	53.38	16.52
ODN	38.89	53.44

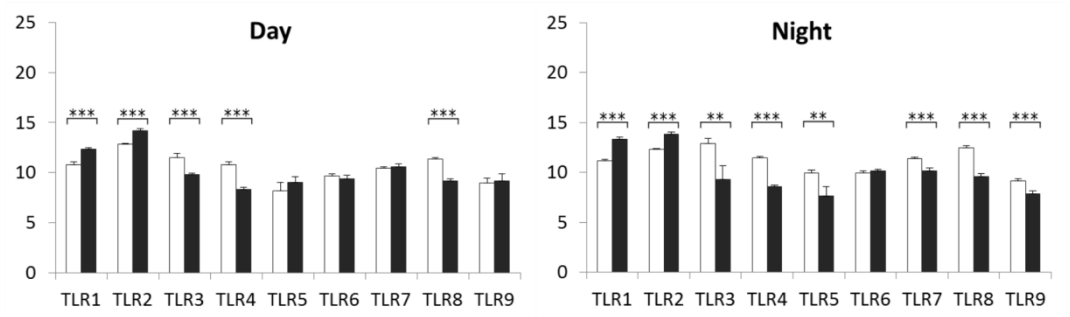
Figure 4.3: Effect of Day/Night oscillation on agonist-induced pro-inflammatory responses in splenic macrophages. The x axis indicates the treatment time (Day vs Night) and the agonist used: PAM, LPS, FSL, CL097 or ODN; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated splenic macrophages; the black bars represent TLR expression in agonist-stimulated splenic macrophages. Error bars and significance levels denoted by asterisks are the same as previous figures. Values in the tables depict average fold increase of mRNA levels in agonist-stimulated cells compared with non-stimulated cells. Green highlight colour indicates statistically significant differences of the fold changes between samples measured at day and night.

both basal level expression and agonist induced regulation of TNFα was affected by time. Transcript level of TNFα was less during the night than during the day in non-stimulated macrophages. Upon agonist stimulation, up-regulation of TNFα mRNA levels were induced following treatment of all tested agonists PAM ($p<0.001$), LPS ($p<0.001$), FLA ($p<0.001$) and ODN ($p<0.001$). However, the magnitudes of TNFα up-regulation were different between day/night time points with some agonist stimulations. PAM stimulation induced larger increase in TNFα transcript level during night than day ($p<0.05$) (Figure 4.3B). FLA treatment on the other hand, induced smaller magnitude of TNFα up-regulation during the night than day ($p<0.01$), despite the reduced TNFα mRNA expression at night (Figure 4.3B). Treatment of LPS ($p=0.61$) or ODN ($p=0.14$) did not affect the magnitude of TNFα up-regulation in splenic macrophages. These results suggest that day/night oscillation have an effect on PAM, FLA and ODN induced transcriptional regulation of pro-inflammatory mediators in BALB/c splenic macrophages. On the other hand, day/night oscillation did not affect mRNA expressions of pro-inflammatory mediators following LPS treatment.

Activation of cognate TLRs following agonist treatment are essential for the production of pro-inflammatory responses as well as transcriptional regulation of TLRs. As the magnitude of cytokine up-regulations were affected by agonist treatment time, transcriptional regulation of TLRs may also be affected by day/night oscillation. The cognate and non-cognate TLR mRNA levels were assessed at 8 hr following treatment with PAM, FLA or ODN at day or at night. LPS induced TLR transcriptional modulations were omitted from this analysis as LPS treatment failed to up-regulate IL1β or TNFα differently between day and night (Figure 4.3), suggesting that LPS induced TLR regulation is unlikely to be modified by day/night oscillation.

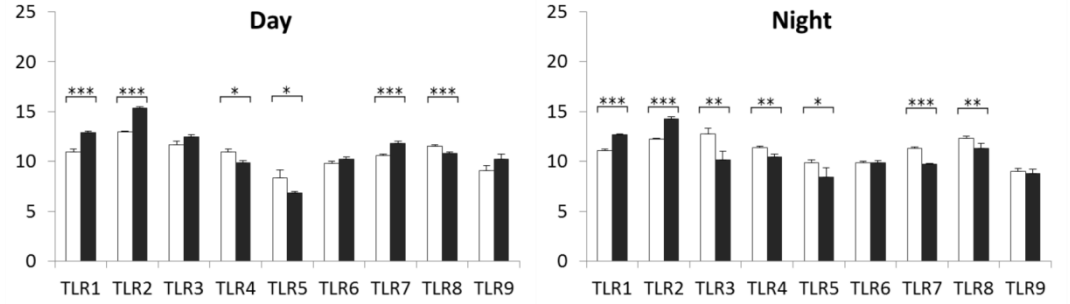
The expression of most TLRs mRNA levels remained the same between day and night in non-stimulated splenic macrophages, but not all. Expression of TLR3 ($p<0.01$), TLR4 ($p<0.01$), TLR7 ($p<0.001$) and TLR8 ($p<0.001$) were substantially higher at night

A



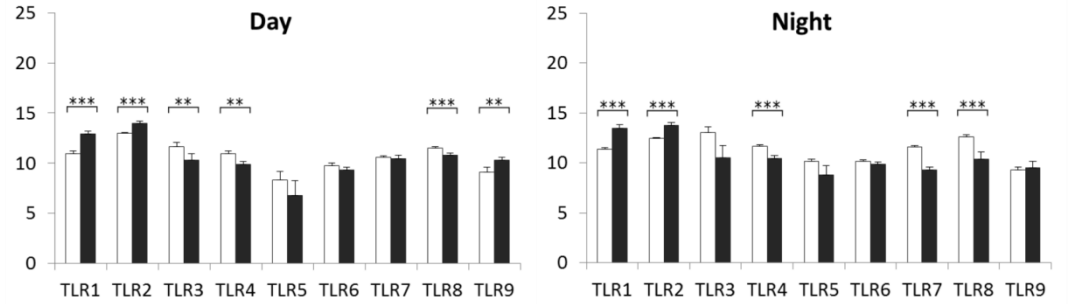
PAM	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9
Day	2.84628	2.6258	-2.95561	-5.66229	1.73249	-1.17805	1.09092	-4.3372	1.16398
Night	4.48615	2.85915	-10.0676	-7.26141	-5.27687	1.15394	-2.27944	-6.65178	-2.32833

B



FSL	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9
Day	3.69823	5.34076	1.67236	-2.15397	-3.0137	1.35156	2.31788	-1.63057	2.12451
Night	3.03741	4.18337	-5.4781	-1.87335	-3.3443	1.02611	-2.9078	-1.95749	-1.14538

C



ODN	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9
Day	3.86904	1.98977	-3.24457	-2.05363	-5.14517	-1.41206	-1.10491	-1.61687	2.16732
Night	4.27814	2.48372	-5.35014	-2.25421	-2.56682	-1.18495	-4.67057	-4.3388	1.14581

Figure 4.4: Effect of Day/Night oscillation on agonist-induced TLR transcriptional regulations in splenic macrophages. The x axis indicates TLR; the y axis indicates mean 40 – Ct values adjusted for GAPDH. The clear bars represent TLR expression in non-stimulated macrophages; the black bars represent TLR expression in agonist-stimulated macrophages. Error bars and significance levels denoted by asterisks are the same as previous figures. Values in the tables depict average fold increase (positive value) or fold decrease (negative value) of mRNA levels in agonist-stimulated cells compared with non-stimulated cells. Green highlight colour indicates statistically significant differences of the fold changes between samples measured at day and night.

than at day in non-stimulated macrophage (Figure 4.4). Agonists PAM, FLA and ODN induced cognate- and cross-regulations of TLRs during day time was discussed in earlier part of this chapter (see Chapter 4.2.2 and 4.2.3). Cells treated with agonists 12 hrs apart indicated different regulatory patterns for some TLRs. After PAM stimulation, TLR5 ($p<0.01$), 7 ($p<0.001$) and 9 ($p<0.001$) underwent down-regulation during night time whereas their transcriptional regulation were not modulated during the day (Figure 4.4A). Following FLA stimulation, TLR3 mRNA level was down-regulated during the night ($p<0.01$) whereas no transcriptional changes was measured during the day. TLR7, which was up-regulated at day by FLA ($p<0.001$), was down-regulated at night ($p<0.001$) (Figure 4.4B). Following ODN treatment, TLR3 and the TLR7/8/9 family members underwent different transcriptional regulations between day and night. ODN induced down-regulation of TLR3 during the day ($p<0.01$) but not during the night ($p=0.53$). TLR7 mRNA level was not altered following ODN stimulation during the day, was down-regulated substantially during the night ($p<0.001$). TLR9 transcript level, which was up-regulated by ODN during the day ($p<0.01$), remained unchanged following the same agonist treatment during the night (Figure 4.4C).

The effects of day/night oscillation on agonist induced differences in TLR transcription were then assessed. The changes in TLR3 mRNA levels were significantly different between day and night following FLA or ODN treatment. TLR7 mRNA levels were also differentially modulated at day time and at night time following stimulation of PAM, FLA or ODN. In addition, TLR8, another member of the TLR7 family, although down-regulated both during the day and night following ODN stimulation, had substantial larger decrease in its mRNA level at night time than at day time.

4.3 Discussion

The cognate- and cross-regulation of TLRs between certain TLRs was documented in some murine tissue types (De Nardo, De Nardo et al. 2009, Jagannathan, Hasturk et al. 2009, van Aubel, Keestra et al. 2007, Cabral, Gelderblom et al. 2006, Lin, Lee et al. 2000). However none of the studies conducted systematic analysis of TLR transcriptional regulation even in the most researched areas, for instance, sepsis. Using murine splenic macrophages as the study model, the agonist induced cognate- and cross-regulation of TLRs were comprehensively analysed and compared with results from the cell line model (chapter 3).

In this chapter, transcriptional regulation of TLR1 to 9 was documented following agonist stimulation of PAM, LPS, FSL and ODN in splenic macrophages from BALB/c or CL57Bl/6 mice (Table 4.5). Stimulation of agonists induced diverse TLR transcriptional expression patterns of which the cognate TLR regulation were mostly similar between the two strains of mice. In the TLR1/2 family, patterns for agonist induced transcriptional regulation of TLR1 and TLR2 were very similar. Both TLR1 and 2 underwent up-regulation following treatment of PAM, LPS, FLA or ODN in macrophages from BALB/c mice. This result is consistent with previous findings in murine adipose tissue and intestinal epithelial cells (van Aubel, Keestra et al. 2007, Lin, Lee et al. 2000). TLR6, the other member of the TLR1/2 family, remained unaffected by the same agonist stimulations. In C57Bl/6 macrophages, mRNA levels of all TLR1/2 family members increased following CL097 stimulation. More complex regulatory patterns were observed for TLR3. The TLR3 transcript levels reduced following PAM or ODN stimulation, whereas its mRNA levels increased after LPS or CL097 treatment. Treatment of FLA did not alter TLR3 transcriptional regulation in BALB/c splenic macrophages. In BALB/c splenic macrophages, TLR4 was down-regulated following agonist treatment of PAM, LPS, FLA or ODN, suggesting that stimulation with these agonists can induce TLR4 tolerance in murine BALB/c splenic macrophages. In

Splenic Mϕ	PAM	LPS	FSL	CL097	ODN
TLR1	**2.85**	3.45	3.70	3.25	3.87
TLR2	**2.63**	6.34	5.34	6.15	1.99
TLR3	-2.96	6.03	1.67	4.82	-2.38
TLR4	-5.66	**-3.88**	-2.15	20.92	-2.05
TLR5	1.75	-1.05	**-2.93**	1.34	-3.10
TLR6	**-1.18**	1.40	1.35	3.86	-1.41
TLR7	1.09	3.11	2.32	**8.07**	-1.10
TLR8	-4.34	-2.03	-1.63	4.43	-1.62
TLR9	1.16	3.82	2.12	13.30	**2.17**

Table 4.5: Cognate- and cross-regulation of TLRs induced by agonist treatment PAM, LPS, FSL, CL097 or ODN in splenic macrophages. Values in the tables depict average fold increase (positive value) or fold decrease (negative value) of TLR mRNA levels in agonist-stimulated cells compared with non-stimulated cells. Red highlight colour indicates statistically significant up-regulation of the fold changes; blue highlight colour indicates statistically significant down-regulation of the fold changes; bold font indicates the respective cognate TLR for each agonist.

C57Bl/6 splenic macrophages, TLR4 mRNA level was up-regulated following CL097 treatment. Cross-regulation of TLR4 induced by PAM were also documented in previous research. Down-regulation of TLR4 was induced following PAM stimulation in murine intestinal cells whereas up-regulation of TLR4 was induced in B cells (Jagannathan, Hasturk et al. 2009, van Aubel, Keestra et al. 2007). These results suggest regulation pattern of TLR4 can be distinct between different cell types. The cognate up-regulation of TLR5 was induced following FLA treatment, however cross-regulation of TLR5 after exposure of PAM, LPS, CL097 or ODN was not observed in splenic macrophages. In the TLR7 family, TLR7 and 9 were generally up-regulated whereas TLR8 was down-regulated following agonist treatments in BALB/c macrophages. TLR7 mRNA levels increased following stimulation with LPS or FLA, whereas its mRNA levels were not affected by treatment of PAM or ODN. TLR9 transcript levels were also up-regulated following stimulation with LPS or ODN but not with PAM or FLA. On the contrary, down-regulation of TLR9 was reported following stimulation with LPS in BMDM (De Nardo, De Nardo et al. 2009) suggesting transcriptional regulation of TLR9 can differ between cell types. TLR8 underwent down-regulation following treatment with PAM, LPS, FLA and ODN. In C57Bl/6 macrophages, treatment of CL097 induced up-regulation of the TLR7 family members TLR7, 8 and 9.

Production of cytokines and transcriptional regulation of certain TLRs following agonist treatments were both modulated by day/night oscillation in murine splenic macrophages. FLA treatment induced larger magnitude of cytokine up-regulation during the day whereas PAM treatment induced larger magnitude of cytokine up-regulation during the night. Furthermore, ODN stimulation induced a larger magnitude of TNFα transcriptional up-regulation at day time than at night time. This result differ from a previous report that ODN induced protein secretion of TNFα was higher at night than at day (Silver, Arjona et al. 2012). This may be due to the fact that splenic

macrophages were incubated with agonists for shorter period of time in the current study with the specific purpose of transcriptional analysis instead of protein analysis. The qPCR analysis could only measure the mRNA changes of TNFα at a particular time point whereas ELISA analysis is a measurement of accumulation of secreted TNFα protein throughout the time course. Although the effect of circadian rhythm on TLR9 regulation was examined in a previous study (Silver, Arjona et al. 2012), none of the cross-regulating TLRs were assessed. The present study identified that expression of TLR3, 7 and 8 mRNA levels were modulated differently between day time and night time following agonist stimulation of PAM, FLA or ODN. These cross-regulating TLRs underwent more severe down-regulation during the night than during the day, suggesting that TLRs may be more effectively regulated in murine splenic macrophages during the active phase of this nocturnal species.

Maurya et al. (2007) compared gene expression profiles in RAW264.7 cells, thioglycollate-elicited macrophages (TM) and BMDM using microarray technology. Roughly 650 genes were expressed differently between the 2 non-stimulated primary macrophage types and around 1400 genes were differentially expressed comparing non-stimulated RAW cells to primary macrophages. Unsurprisingly, closer analysis of gene functions revealed higher expression for genes responsible for immune cell migration and phagocytosis function in primary macrophages than in RAW cells whereas higher expression for genes associated with mitosis and DNA metabolism in RAW cells than in primary macrophages. Yang et al. (2011) investigated TLR3, 4 and 9 agonist induced gene expression in RAW cells. The study revealed genes, pathways and transcriptional factors expressed differently in response to poly(I:C), LPS or ODN treatment. However, at 1% false discovery rate and 2-fold cut-off threshold, small but significant gene expression changes such as the TLR transcriptional regulations could be overlooked. Maurya et al. (2007) also compared LPS induced gene expression in RAW cells with primary macrophages. Among the 5 to 600 genes that LPS induced

transcriptional regulation of, 243 were commonly regulated between cell line and primary cells, suggesting similarities in agonist induced gene regulations between RAW cell line and primary cells. However, this study mainly focused on genes responsible for signalling pathways and none of the cross-regulating TLRs were examined.

In the present study, agonists induced TLR transcriptional regulation was compared between murine splenic macrophages and RAW cell line (Table 4.6). PAM induced transcriptional regulation of the TLR1/2 family, TLR4 and TLR7/8/9 family were similar between cell line and primary macrophages. In the TLR1/2 family, up-regulation of the cognate TLR1 and 2 mRNA levels and the lack of TLR6 mRNA changes following PAM treatment were consistent between RAW cells and splenic macrophages. TLR4 underwent down-regulation in both RAW cell and in primary cells following PAM treatment. In the TLR7/8/9 family, transcript levels of TLR7, 8 and 9 reduced in RAW cells after PAM stimulation and mRNA levels of TLR8 also reduced in splenic macrophages following PAM treatment. Regulation of TLR3 and TLR5 mRNA levels induced by PAM treatment were different between cell line and splenic macrophages. The TLR3 mRNA levels, which were not affected by PAM stimulation in RAW cells, were down-regulated in splenic macrophages. Transcript levels of TLR5, although massively down-regulated following PAM stimulation in RAW cells, were not affected in primary cells. LPS induced up-regulation of the TLR1/2 family and TLR3 were consistent between RAW cells and splenic macrophages. On the other hand, TLR4, TLR5 and the TLR7/8/9 family were differentially regulated following LPS treatment between the cell line and splenic macrophages. TLR4 mRNA levels which were not changed in RAW cells following LPS treatment, were down-regulated in splenic macrophages. TLR5 transcript levels on the other hand, were down-regulated in RAW cells following LPS stimulation but not altered in splenic macrophage. LPS treatment did not induce transcriptional changes of the TLR7/8/9 family members in RAW cells

Agonist	PAM		LPS		ODN	
Cell type	RAW264.7	Splenic Mϕ	RAW264.7	Splenic Mϕ	RAW264.7	Splenic Mϕ
TLR1	**2.11**	**2.85**	2.56	3.45	1.23	3.87
TLR2	**4.21**	**2.63**	4.43	6.34	2.04	1.99
TLR3	1.19	-2.96	16.62	6.03	1.54	-2.38
TLR4	-1.43	-5.66	**2.09**	**-3.88**	-2.65	-2.05
TLR5	-9.89	1.75	-7.56	-1.05	-2.14	-3.10
TLR6	**1.57**	**-1.18**	1.28	1.40	-1.22	-1.41
TLR7	-2.23	1.09	1.91	3.11	-1.51	-1.10
TLR8	-3.51	-4.34	1.52	-2.03	-3.09	-1.62
TLR9	-2.32	1.16	-2.13	3.82	**1.14**	**2.17**

Table 4.6: Agonist-induced TLR transcriptional changes in RAW264.7 cell line vs in splenic macrophages. Values in the tables depict average fold increase (positive value) or fold decrease (negative value) of TLR mRNA levels in agonist-stimulated cells compared with non-stimulated cells. Red highlight colour indicates statistically significant up-regulation of the fold changes; blue highlight colour indicates statistically significant down-regulation of the fold changes; bold font indicates the respective cognate TLR for each agonist.

whereas in splenic macrophages, TLR7 and 9 mRNA levels were up-regulated while TLR8 mRNA levels were down-regulated after LPS treatment. ODN treatment induced consistent up-regulation of TLR2 transcript levels as well as down-regulation of TLR4 and 8 transcript levels in both RAW cells and splenic macrophages. Transcriptional expression of TLR6 and 7 were unaffected by ODN stimulation in both cell types. In contrast, TLR1 and 9 both underwent up-regulation whereas TLR3 underwent down-regulation following ODN stimulation in splenic macrophages. None of these three TLRs were modulated in RAW cells.

In summary, majority of agonist induced TLR transcriptional modulations were consistent between the cell lines and primary splenic macrophages from BALB/c and C57Bl/6 mice. Hence RAW cells were used instead of primary macrophages to examine aspects of TLR signalling and regulation in more detail (chapter 5, 6, 7 and 8).

Chapter 5 Signalling regulation of PAM induced TLR transcriptional changes

5.1 Introduction

Following agonist interactions with TLRs, complex signalling cascades are activated which results in the induction of pro-inflammatory responses. The cytoplasmic intracellular C-terminal domain of the TLRs (TIR domain) recruits signalling adaptors to mediate TLR signalling to downstream pathways (Sheedy, O'Neill 2007, Yamamoto, Sato et al. 2003a, Yamamoto, Sato et al. 2003b, Horng, Barton et al. 2002, Horng, Barton et al. 2001, Kopp, Medzhitov 1999, Burns, Martinon et al. 1998). A detailed description of TLR signalling adaptors and their roles in recruitment of kinases, ligases and transcription factors can be found in chapter 1 (section 1.3.2). In brief, 6 adaptor proteins: MyD88, MAL, TRIF, TRAM, SARM1 and BCAP was discovered (Nilsen, Vladimer et al. 2015, Petnicki-Ocwieja, Chung et al. 2013, O'Neill, Golenbock et al. 2013, Lim, Staudt 2013, Newton, Dixit 2012, Kenny, O'Neill 2008, O'Neill, Bowie 2007, Miggin, O'Neill 2006, Kawai, Akira 2006, Akira, Yamamoto et al. 2003). MyD88 is used by all TLRs except TLR3. Most TLRs (TLR1, 2, 5, 6, 7, 8, 9, 11) can engage MyD88 directly, some (TLR1, 2, 4, 6) may also recruit a bridging adaptor MAL in addition to MyD88 to mediate downstream signalling. TRIF protein is used by directly by TLR3 or in conjunction with a bridging adaptor TRAM by the TLRs locating on the endosomes (TLR2, 4, 7, 8 and 9). Adaptor SARM1 serves as a negative regulator for TRIF, however its full functionality remains to be elucidated. BCAP, which has a TIR binding domain, is thought to negatively regulate MyD88 and MAL downstream of TLR4 and 9 in B cells.

Depend on the adaptor protein that is recruited, different signalling pathways are activated through engagement of various kinases and ligases. The MyD88 adaptor was shown to bind to the IRAK kinases, IRAK1, IRAK2 and IRAK4, which then interact with TRAF6 (Akira, Takeda 2004). The ubiquitinated TRAF6 binds to TAK1-TAB1/2/3

complex. Activation of TAK1 in turn stimulates a number of downstream signalling cascades, for example, NFκB, P38 MAPK, JNK MAPK and ERK MAPK which are critical for immune regulations through transcriptional factor activation (Tarantino, Tinevez et al. 2014, Hacker, Tseng et al. 2011, Tseng, Matsuzawa et al. 2010, Israel 2010, Chen 2005, Sato, Sanjo et al. 2005, Wang, Deng et al. 2001). In cases of MyD88-independent regulation, TRIF adaptor was also shown to induce NFκB signalling through interaction with RIP1, TRAF6 and TRAF3 (Oganesyan, Saha et al. 2006). TRAF3 is critical for IFN production downstream of TLR3, 4, 7 and 9. Upon activation, TRAF3 activates IRF3 through the noncanonical IKKs, TBK1 and IKKi, which then induce IRF7 and forms a positive feedback loop for type 1 IFN production (Hacker, Tseng et al. 2011, Hacker, Redecke et al. 2006, Oganesyan, Saha et al. 2006, Kawai, Akira 2006). Both RIP1 and TRAF6 induce NFκB signalling but not IRF3 activation following recruitment with TRIF. In B cells, TRAF6 was shown to activate TAK-TAB complex downstream of TRIF and, in turn, induce MAPK signalling and transcription factor activation (Cusson-Hermance, Khurana et al. 2005, Meylan, Burns et al. 2004, Jiang, Mak et al. 2004, Gohda, Matsumura et al. 2004). In macrophage cells, RIP1 was also shown to induce TRIF-dependent MAPK signalling through recruitment of cytoplasmic adaptor TRADD (Chen, Chio et al. 2008, Ermolaeva, Michallet et al. 2008, Michallet, Meylan et al. 2008, Pobezinskaya, Kim et al. 2008).

A number of studies addressed signalling recruitment downstream of TLR2 activation in various cell types. Leng et al. (2010) investigated TLR1/2 agonist induced signalling activation in bone marrow derived dendritic cells (BMDC). PAM induced phosphorylation of MAPK signalling components ERK1/2, JNK1/2 and p38 with highest phospho level at 40, 40 and 90 mins respectively. Furthermore, PAM activated NFκB signalling in HEK293T cells transfected with hTLR2. These results suggest that TLR2 activation triggers a combination of MAPK and NFκB signalling pathways following PAM stimulation. Similar observations were reported by Berube et al. (2009) who

studied signalling control for PAM induced cytokine productions in pulmonary epithelial cells. PAM stimulation induced the phosphorylation of ERK1/2 and P38, as well as degradation of IKKβ. In addition, inhibition of ERK1/2, P38 or IKKβ reduced PAM induced IL8 mRNA and protein production. In murine BMDM, both TLR1/2 (FSL-1) and TLR2/6 agonist (PAM2) stimulation induced activation of MAPK signalling components ERK, JNK and P38 as well as degradation of IκB at the same time points (Farhat, Riekenberg et al. 2008). Peng et al. (2014) assessed PAM induced signalling activation in peripheral blood leukocytes (PBL). Phosphorylation of ERK, JNK2 and STAT3 was detected following treatment of PAM, which positively feedback on the STAT3 signalling through the increased IL6 expression. Kopp et al. (2010) investigated PAM induced differential signalling activation in mature adipocytes versus undifferentiated pre-adipocytes. In mature adipocytes, phosphorylation of ERK but not JNK was detected whereas phosphorylation of both ERK and JNK were observed in pre-adipocytes. In human eosinophils, stimulation of TLR2 agonist PGN phosphorylated ERK1, Akt and IκBα but not P38 (Wong, Cheung et al. 2007). Together, these results suggest that signalling pathways activated following stimulation with TLR2 agonists may be cell-type specific.

Activation of signalling cascades downstream of TLRs could also be agonist specific. MEK1/2 was responsible for TLR1/2 agonist PAM induced IL6 production whereas JNK1/2 alone was responsible for TLR2/6 agonist MALP-2 induced IL6 production in adipocytes (Kopp, Buechler et al. 2010). This difference in signalling activation may be due to sequence differences of BB loops in the TLR TIR domain. A mutation in TLR2 BB loop significantly reduced phosphorylation of ERK induced by TLR1/2 agonist PAM3 whereas the same mutation did not affect ERK signalling induced by TLR2/6 agonist PAM2 (Toshchakov, Fenton et al. 2007).

Phosphorylation of signalling pathways downstream of TLR activation in tolerance models were documented in a few previous studies. Reduced activation of ERK, JNK

and P38 was measured following repeated LPS treatment in intestinal epithelial cells, peritoneal macrophages and BMMs (Perkins, Polumuri et al. 2013, De Nardo, De Nardo et al. 2009, Otte, Cario et al. 2004). In a similar manner, LPS stimulation following PAM pre-treatment significantly reduced phosphorylation of ERK and P38, as well as completely inhibited JNK activity in peritoneal macrophages (Perkins, Polumuri et al. 2013). Interestingly, stronger and prolonged phosphorylation of P38 was measured in a human macrophage cell line U937 co-incubated with LPS and PAM. However, phosphorylation of ERK, JNK or NFκB was not altered compared to cells treated with LPS or PAM alone (Jin, Samuvel et al. 2011). In addition, stimulation of TLR9 agonist CpG DNA following LPS pre-treatment significantly increased activation of ERK1/2, JNK and P38 as well as degradation of IκBα in BMM (De Nardo, De Nardo et al. 2009).

Matsuguchi et al. (2000) investigated signalling cascades responsible for LPS induced TLR2 transcriptional regulation in RAW cells. Activation of MAPK signalling pathway was not essential for TLR2 up-regulation following LPS stimulation although ERK signalling cascade may have a negative regulatory effect on LPS induced TLR2 expression. Inhibition of NFκB completely blocked the LPS induced TLR2 up-regulation, indicating its key mediating role.

Previous research mainly focused on the signalling pathways responsible for the production of immune mediators following TLR agonist stimulation whereas the signalling pathways responsible for the regulation of TLRs were neglected. Hence the current study aimed to address signalling pathways responsible for PAM induced self- and cross-regulating TLRs by using pharmaceutical inhibitors in this chapter.

5.2 Results

5.2.1 PAM induced MAPK signalling activation

The phosphorylation status of MAPK signalling terminal components ERK, JNK and P38 was measured to investigate the signalling cascades induced by PAM induced TLR2 activation in the RAW64.7 cell line. The western blot images and the results from densitometry analysis are displayed in figure 5.1. The phosphorylation status of the three MAPK signalling components, ERK, JNK and P38 was recorded across 11 time points (0 min, 5 mins, 10 mins, 15 mins, 20 mins, 30 mins, 40 mins, 50 mins, 1 hr, 2 hrs, 4 hrs) over 4 hrs following PAM stimulation. In non-stimulated cells, phospho status for all MAPK terminal components was also measured at two time points prior to PAM stimulation, at pre-15 mins and pre-30 mins. In addition, a positive control sample and a negative control sample were included on every western blot. These controls were used to compare the antibody binding to a specific protein across different images. Intensities of all bands were normalised in relation to the positive control band and presented in densitometry graphs.

ERK1/2 protein was phosphorylated in non-stimulated RAW cells (Figure 5.1). The phosphorylation of ERK1/2 protein in non-stimulated cells peaked at 30 mins prior to PAM stimulation then quickly returned to a level similar to the negative control by 0 min time point. The second increase in basal phosphorylation of ERK1/2 was observed from 0 min time point. The phosphorylation peaked at 40 mins and remained constant til 4hr time point in non-stimulated cells (Figure 5.1A). This result indicates that cells underwent a stress response, which may activate ERK1/2 signalling due to changes in environmental CO_2 level. JNK and P38 were not phosphorylated in non-stimulated macrophages (Figure 5.1 B-C).

PAM stimulation induced phosphorylation of all three MAPK terminal signalling components, ERK, JNK and P38. PAM induced rapid phosphorylation of both ERK1 (p44) and ERK2 (p42) protein. ERK1/2 phosphorylation in PAM treated cells peaked at

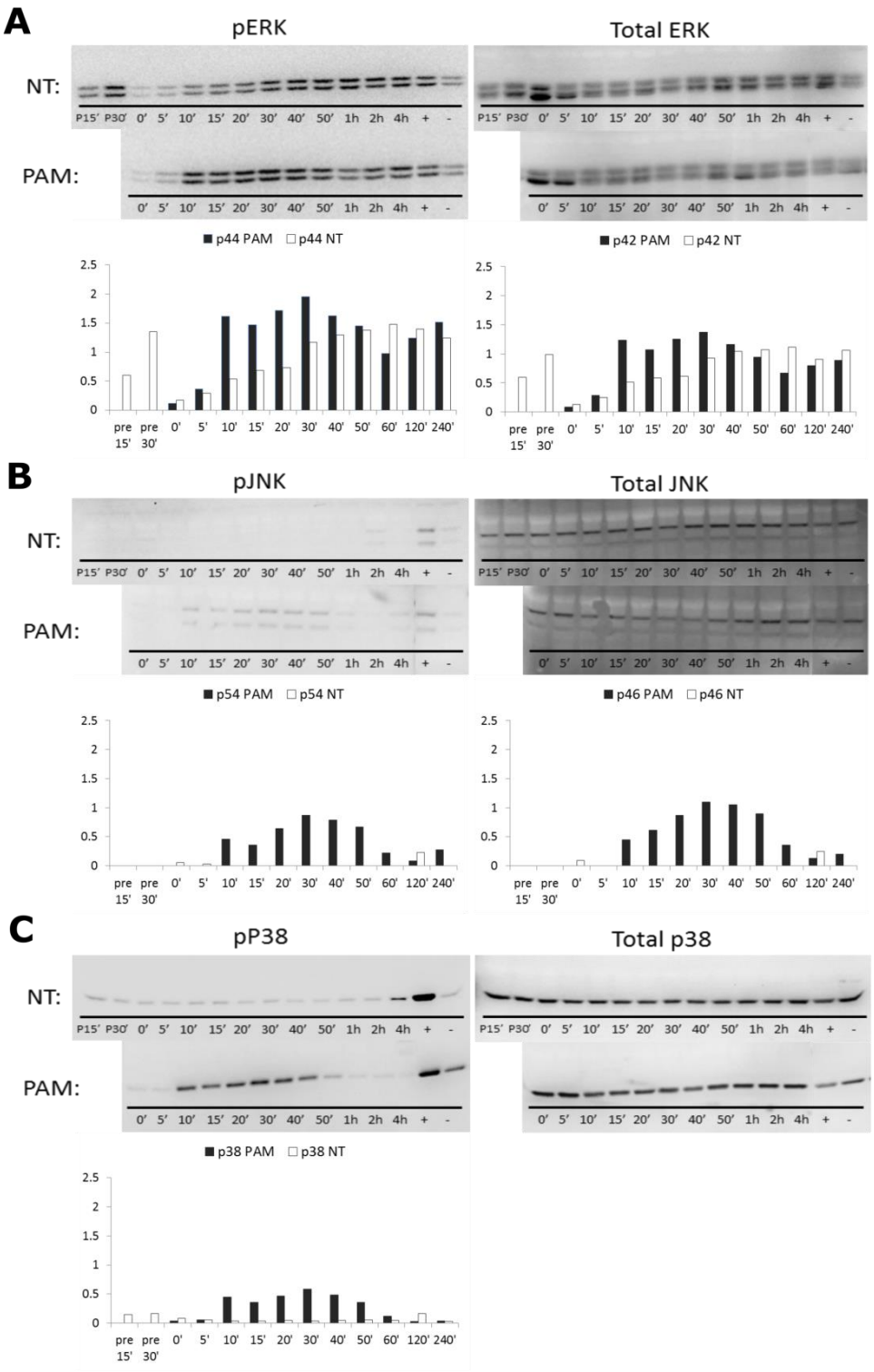


Figure 5.1: PAM-induced MAPK signalling activation in RAW264.7 cells. Images represent the phosphor- or total- status of protein ERK1/2 (A), JNK1/2 (B) or P38 (C) in PAM treated and non-treated cells. Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent protein phosphorylation status in non-stimulated cells; the black bars represent protein phosphorylation status in PAM-stimulated cells.

10 min and remained up until basal ERK phosphorylation matched up at 40 min (Figure 5.1A). JNK1 (p54), JNK2 (p46) and P38 also underwent phosphorylation following PAM stimulation. Phosphorylation of JNK1/2 started from 10 mins post PAM stimulation. The phospho intensity was slow to peak (at 30 mins) and quick to returned to basal level (at around 50 mins) (Figure 5.1B). Phosphorylation of P38 also started from 10 mins, peaked at 30 min then returned to the basal level by 60 mins. These results indicate that stimulation with TLR2 agonist PAM induced signalling activation through all MAPK pathway components ERK, JNK and P38 in RAW cells.

5.2.2 MAPK signalling responsible for PAM induced cytokine production

Having established the activation of all three MAPK signalling pathways, I then investigated the importance of each of the three MAPK pathway segregations ERK/MEK, JNK and P38 in PAM induced immune regulations by using pharmacological inhibitors PD059/U126, SP125/JIP-1 and SB580 respectively. QPCR results are represented in both relative units for 40-Ct and relative units for magnitude of change. For 40-Ct relative quantity calculation, the average 40-Ct value for cells with no treatment was defined as 0 unit and the average 40-Ct value for agonist PAM treated cells was defined as 100 units. 40-Ct values for cells incubated with inhibitor treatment only as well as cells incubated with both inhibitor pre-treatment and PAM agonist treatment were converted into relative units accordingly. For the magnitude of change relative unit calculation, the difference in Ct value between PAM and no treatment cells was defined as 100 units. Cycle value differences between inhibitor treated samples with and without PAM agonist were converted into relative units accordingly. Gene expression in cells with no treatment was considered as the basal mRNA level for PAM treated cells. Similarly, gene expression in cells pre-incubated only with inhibitors but no agonist stimulations was considered as the basal level for PAM and inhibitor co-incubated cells.

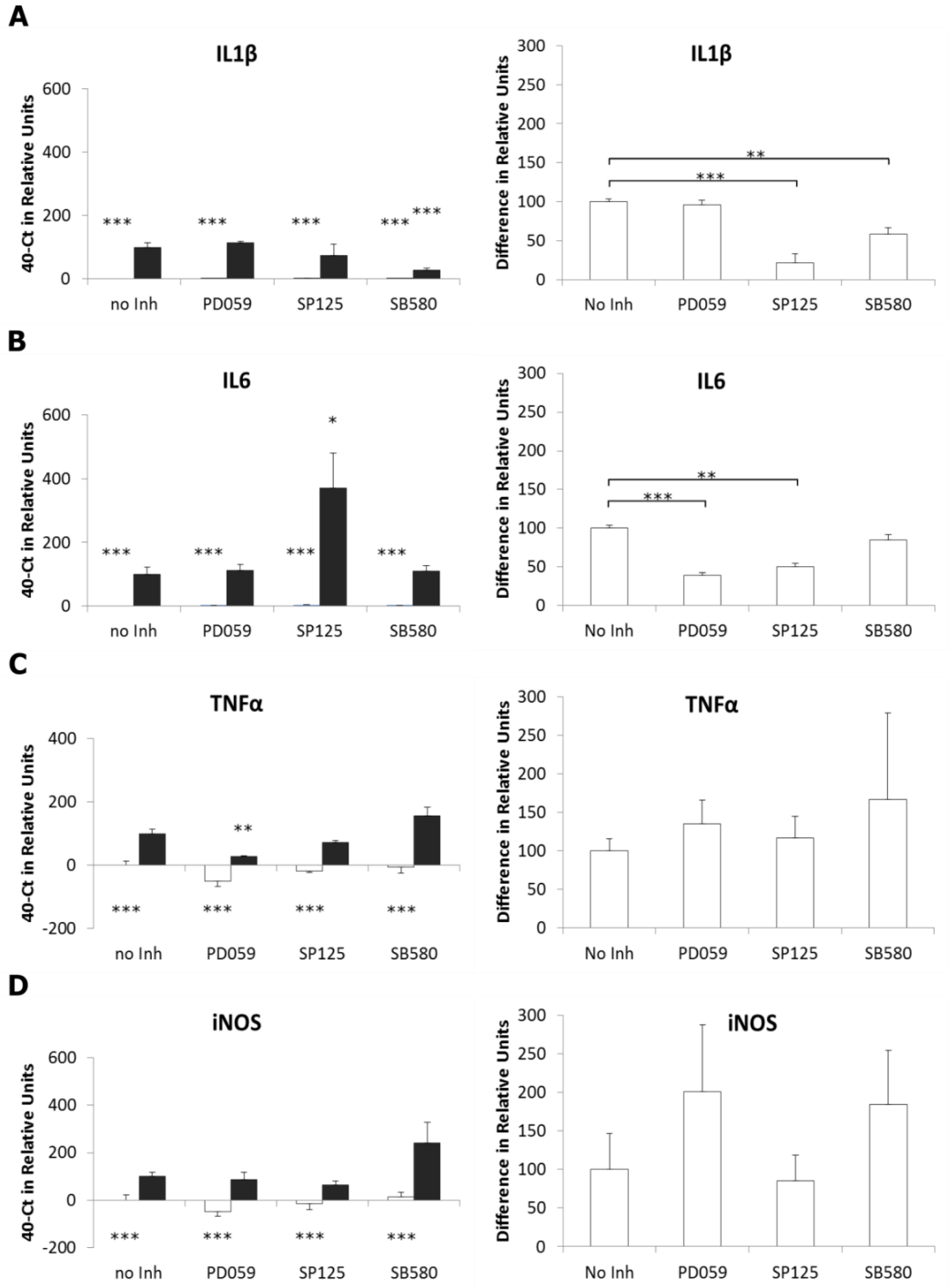


Figure 5.2: Effect of MAPK signalling inhibition on PAM-induced pro-inflammatory responses in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative units graphs, the clear bars represent cytokine expression in non-stimulated or inhibitor-treatment only cells; the black bars represent cytokine expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels of the differences between the bar over which they are placed and PAM treated cells: *p< 0.05, **p< 0.01, ***p< 0.001.

PAM stimulation resulted in robust pro-inflammatory responses compared to untreated cells (see Chapter 3.2.3). In inhibitor-only treated cells, pre-treatment of inhibitors PD059, SP125 or SB580 did not substantially affect mRNA level of IL1β. In PAM stimulated cells, IL1 up-regulation was observed in following PAM stimulation which was inhibited by inhibition of P38 with SB580 treatment ($p<0.001$) (Figure 5.2A). When magnitude of IL1β up-regulation was compared between agonist and inhibitor treatments, JNK inhibition and P38 inhibition both reduced the IL1β up-regulation. This is because the pre-treatment of SP125 induced a trend of IL1β up-regulation (although not significant) in inhibitor-only cells (Appendix Figure 2A) which may be due to the environmental shock that the cells went through. Similar to IL1β, IL6 underwent up-regulation following PAM stimulation. Pre-treatment of MAPK inhibitors did not substantially affect IL6 mRNA levels in inhibitor-only treated cells (Figure 5.2B). Although an increase trend of IL6 basal expression was observed when cells were pre-treated with ERK and JNK inhibitors, PD059 and SP125 respectively (Appendix Figure 2B). In PAM treated cells, inhibition of JNK further increased IL6 mRNA level compared to PAM induced IL6 mRNA up-regulation ($p<0.05$). However, if we take in to account the IL6 basal mRNA increase induced by JNK inhibition, the magnitude of PAM induced IL6 up-regulation was reduced by inhibiting JNK ($p<0.01$) (Figure 5.2B). While inhibition of ERK did not alter IL6 up-regulation in PAM treated cells, the magnitude of PAM induced IL6 up-regulation was reduced ($p<0.001$) due to the IL6 basal level increase following PD059 treatment (Figure 5.2B). TNFα and iNOS mRNA levels were up-regulated following PAM stimulation. In inhibitor-only treated cells, the basal mRNA expression of both TNFα and iNOS were not substantially affected by inhibition of MAPK signalling although a trend of down-regulation was observed for both TNFα and iNOS following exposure to ERK and JNK inhibitors, PD059 and SP125 respectively (Figure 5.2C-D). In PAM stimulated cells, PD059 treatment

substantially reduced mRNA expression of TNFα ($p<0.01$). However, ERK inhibition did not change the magnitude of TNFα up-regulation due to the reduced TNFα basal mRNA expression in PD059-only treated cells. Inhibition of JNK or P38 did not affect PAM induced TNFα up-regulation in RAW cells (Figure 5.2C). Unlike the other cytokines, the up-regulation of iNOS was not affected by inhibition of any of the MAPK terminal cascades (Figure 5.2D).

5.2.3 Signalling pathways responsible for PAM induced TLR transcriptional regulation

Having established the importance of MAPK signalling for cytokine production, I continued investigating the degree to which each TLR depended on MAPK signalling.

5.2.3.1 Pharmacological inhibition of MEK/ERK pathway

Two inhibitors, U126 and PD059, were used to investigate the effect of MEK/ERK signalling for PAM induced TLR transcriptional regulation. Inhibitor PD059 targets ERK1/2, 1 of the 3 terminal components in MAPK signalling and Inhibitor U126 targets MEK1/2, a signalling component directly upstream of ERK1/2. The data presented refer to the inhibition effect at 8 hours post PAM treatment, the time point at which TLR up- or down-regulation was most pronounced. Only the TLRs that were modulated following PAM treatment were selected for the signalling inhibition study. The raw 40-Ct values for the ERK/MEK inhibition effect on TLR regulation are shown in Appendix Figure 3.

In the TLR1/2 family, treatment with U126 up-regulated basal expression of TLR1 in inhibitor-only cells ($p<0.05$), treatment of PD059 on the other hand, did not significantly alter the basal expression of TLR1. In PAM stimulated cells, TLR1 mRNA level increased following PAM treatment. Treatment with either PD059 or U126 both substantially further increased TLR1 mRNA level compared to PAM-induced up-regulation level ($p<0.01$). However, when taking into account the effect of ERK/MEK

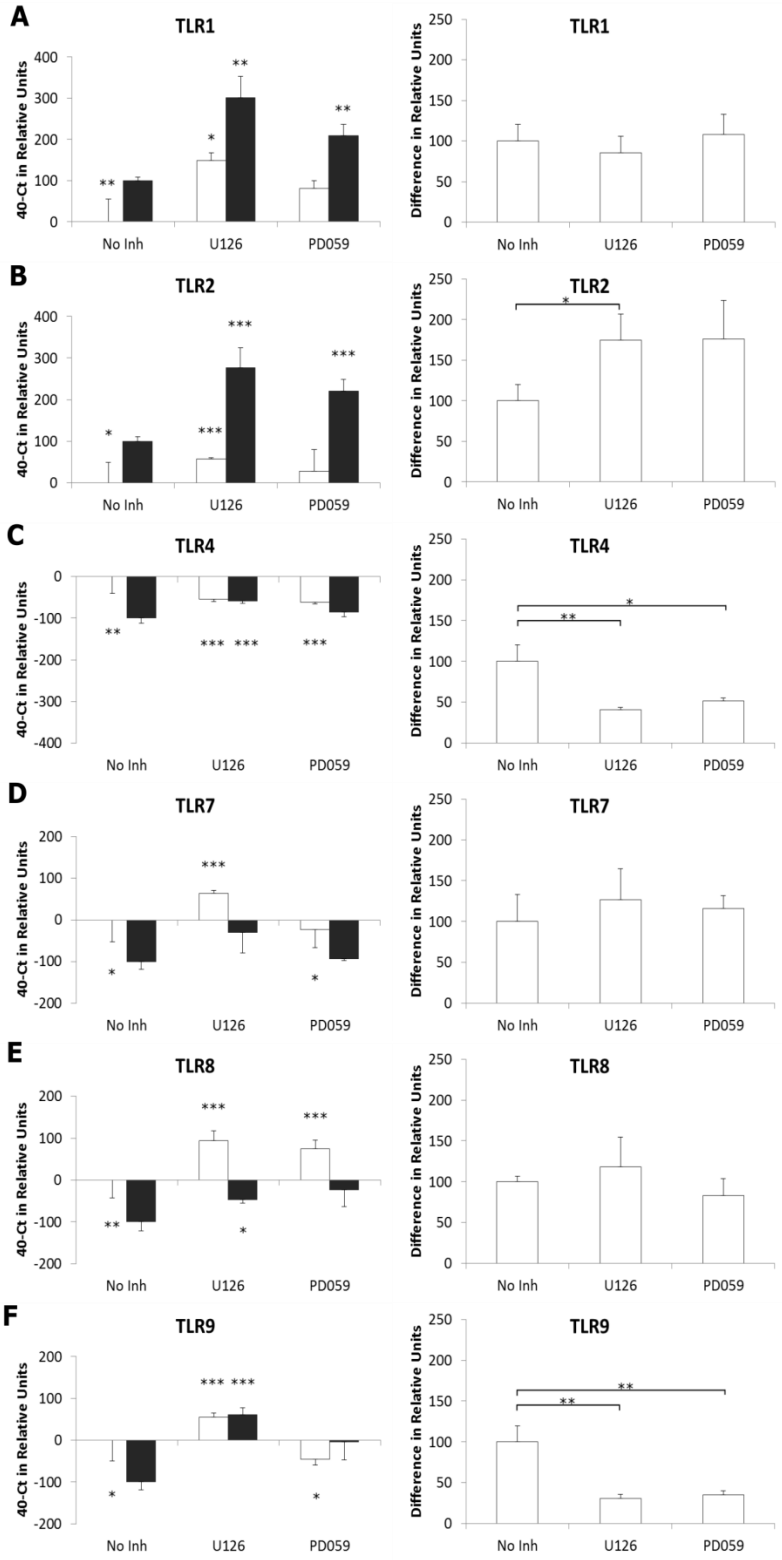


Figure 5.3: Effect of inhibiting MEK/ERK signalling on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In relative unit of 40-Ct graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

inhibition on inhibitor-only treated cells, the magnitude of PAM induced TLR1 up-regulation was not altered by treatment with U126 or PD059 due to increased TLR1 basal expression in the inhibitor-only treated cells (Figure 5.3A). Similar to TLR1, TLR2 basal mRNA level also showed a trend of increase in U126-only and PD059-only treated cells. In PAM stimulated cells, treatment of the two inhibitors both individually further up-regulated TLR2 transcript levels compared to the PAM-induced level ($p<0.001$). Overall, exposure to U126 increased the magnitude of TLR2 up-regulation induced by PAM ($p<0.05$) while PD059 treatment also presented a similar trend although not statistically significant ($p=0.12$) (Figure 5.3B).

Treatment with U126 and PD059 both reduced TLR4 mRNA level in inhibitor-only treated cells. In PAM treated cells, the TLR4 mRNA level reduced following PAM stimulation and U126 pre-treatment partially rescued PAM induced down-regulation of TLR4 ($p<0.001$), whereas PD059 pre-treatment did not result in the same effect. Comparing the effect of ERK/MEK inhibition on PAM treated and non-agonist treated cells, stimulation with inhibitors, U126 and PD059, both reduced the magnitude of PAM induced TLR4 down-regulation due to the modified TLR4 basal expression in inhibitor-only treated cells (Figure 5.3C).

In the TLR7 family, treatment with U126 or PD059 did not significantly alter the basal level of TLR7 mRNA in inhibitor-only stimulated cells. In PAM stimulated cells, TLR7 was down-regulated following PAM stimulation. Pre-treatment with either of the inhibitors did not affect the PAM induced TLR7 down-regulation (Figure 5.3D). TLR8 mRNA expression was also down-regulated following PAM stimulation. In inhibitor-only treated cells, both U126 and PD059 stimulation induced a slightly increased but not significant TLR8 basal levels compared to the non-stimulated cells (U126: $p=0.12$, PD059: $p=0.34$). In PAM stimulated cells, pre-treatment with U126 significantly rescued PAM induced down-regulation of TLR8 ($p<0.05$). Although exhibiting the same trend as U126 stimulation, PD059 pre-treatment did not induce significant rescue of TLR8

down-regulation due to variation between samples (p=0.84). Overall, neither U126 nor PD059 stimulation affected PAM induced TLR8 mRNA down-regulation due to the increased TLR8 basal mRNA expression in inhibitor-only treated cells (Figure 5.3E). On the contrary, the magnitude of PAM induced TLR9 down-regulation was rescued by treatment with either U126 or PD059 ($p<0.01$). In inhibitor-only treated cells, neither of the two inhibitors altered the TLR9 basal mRNA expression. In PAM treated cells, stimulation with U126 not only fully rescued PAM induced TLR9 down-regulation, but also up-regulated the TLR9 transcript level compared to its basal level in non-stimulated cells ($p<0.001$). Treatment of PD059 did not substantially modulate the PAM induced TLR9 down-regulation (Figure 5.3F).

5.2.3.2 Pharmacological inhibition of JNK pathway

Two inhibitors, SP125 and JIP-1, were used to investigate the effect of JNK signalling in the regulation of TLR transcript following PAM stimulation. Inhibitor SP125 targets at the ATP-binding site on the JNK protein which has structural similarity among all MAPK proteins. Hence, SP125 may also inhibit other MAPK signalling component. To ensure the accuracy of the results, a second and more specific JNK inhibitor JIP-1, which targets at the JNK-binding domain (JBD) on the JNK protein was also used. The raw 40-Ct values for the effect of JNK inhibition on PAM induced TLR regulation are shown in Appendix Figure 4.

Both TLR1 and 2 were up-regulated following PAM stimulation. In inhibitor-only treated cells, a slight but non-significant trend of TLR1 basal mRNA expression up-regulation was observed following treatment with either SP125 or JIP-1. When treated with PAM agonist, stimulation with JNK inhibitors both failed to modulate the PAM induced transcriptional up-regulation of TLR1. Overall, stimulation of SP125 substantially reduced the magnitude of PAM induced TLR1 up-regulation due to the TLR1 basal mRNA level increase following treatment with SP125. Although exhibiting the same trend as SP125, stimulation of JIP-1 did not alter the magnitude of PAM induced

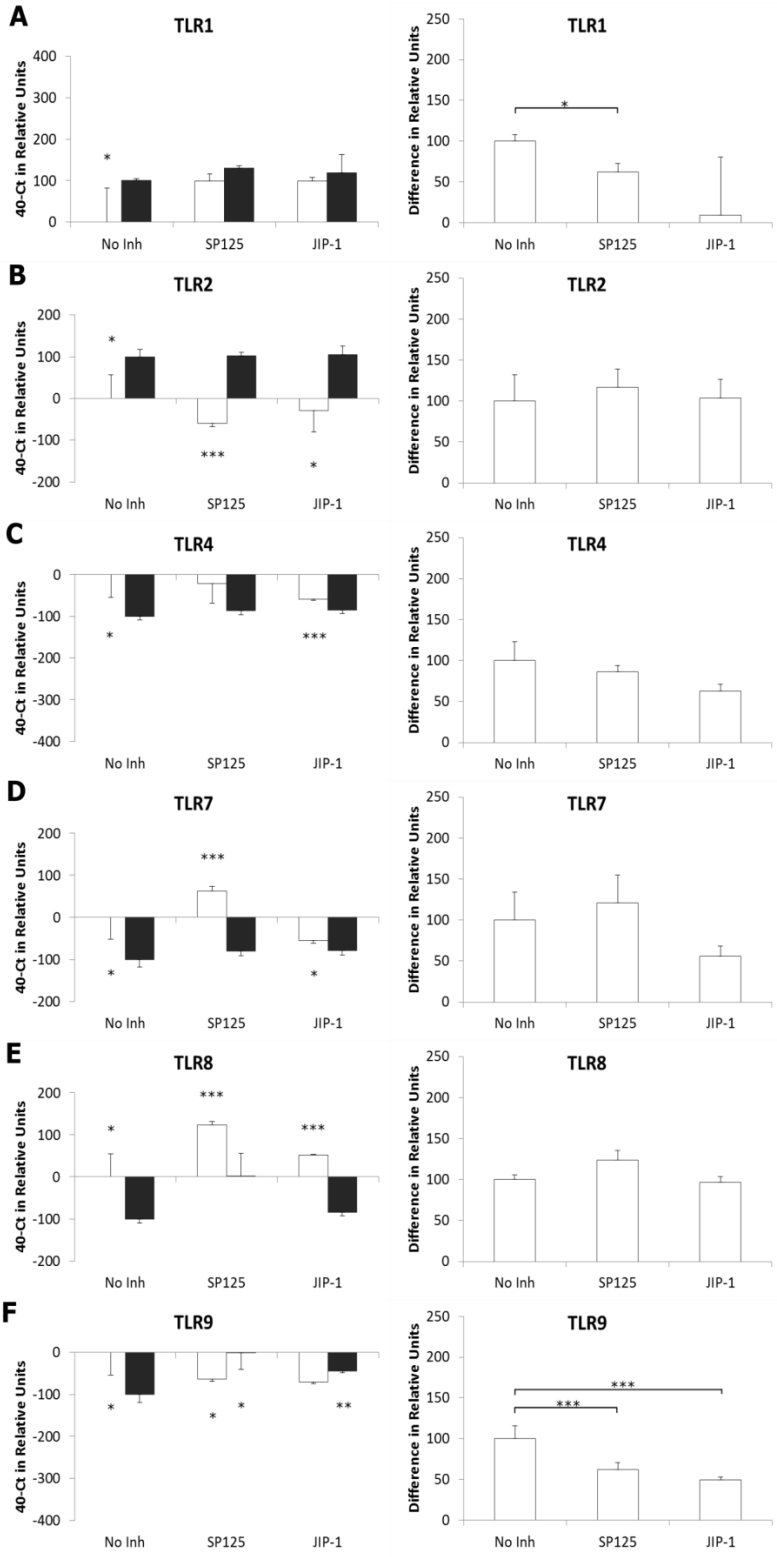


Figure 5.4: Effect of inhibiting JNK signalling on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In relative unit of 40-Ct graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

TLR1 up-regulation due to a large variation between samples (Figure 5.4A). For TLR2 mRNA expression, treatment with either SP125 or JIP-1 did not affect its basal level in inhibitor-only stimulated cells. In PAM treated cells, TLR2 transcript level was not affected by treatment with either inhibitor. Overall, the magnitude of PAM-induced TLR2 up-regulation was not affected by SP125 or JIP-1 treatment (Figure 5.4B).

TLR4 underwent down-regulation following PAM stimulation. Treatment with SP125 or JIP-1 inhibitors did not affect TLR4 basal expression in inhibitor-only treated cells, nor did they alter PAM-induced TLR4 down-regulation in PAM stimulated cells (Figure 5.4C).

PAM stimulation reduced expression of TLR7, 8 and 9 mRNA levels in RAW cells. In inhibitor-only treated cells, TLR7 basal expression level was not affected by treatment of SP125 or JIP-1, although a slight but not significant increase in its mRNA level was observed with SP125. In PAM stimulated cells, SP125 or JIP-1 pre-treatment did not rescue the down-regulation of TLR7. Overall, inhibition of JNK by SP125 or JIP-1 stimulation did not affect the magnitude of PAM-induced TLR7 down-regulation (Figure 5.4D). TLR8 transcript level was significantly up-regulated following SP125 treatment in inhibitor-only treated cells ($p<0.05$) however JIP-1 treatment did not induce the same TLR8 mRNA change. In PAM stimulated cells, treatment of neither inhibitor modulated TLR8 transcriptional regulation (Figure 5.4E). The basal level of TLR9 mRNA in inhibitor-only treated cells was not affected by either SP125 or JIP-1 stimulation. In PAM treated cells, treatment with SP125 ($p<0.05$) and JIP-1 ($p<0.01$) substantially rescued the down-regulation of TLR9. Overall, inhibition of JNK signalling through SP125 and JIP-1 stimulation significantly reduced the magnitude of PAM induced TLR9 down-regulation in RAW cells ($p<0.001$) (Figure 5.4F).

5.2.3.3 Pharmacological inhibition of P38 pathway

The inhibitor SB580 was used to investigate the effect of P38 signalling inhibition on PAM induced TLR regulation. SB580 directly inhibits the P38 MAPK protein through

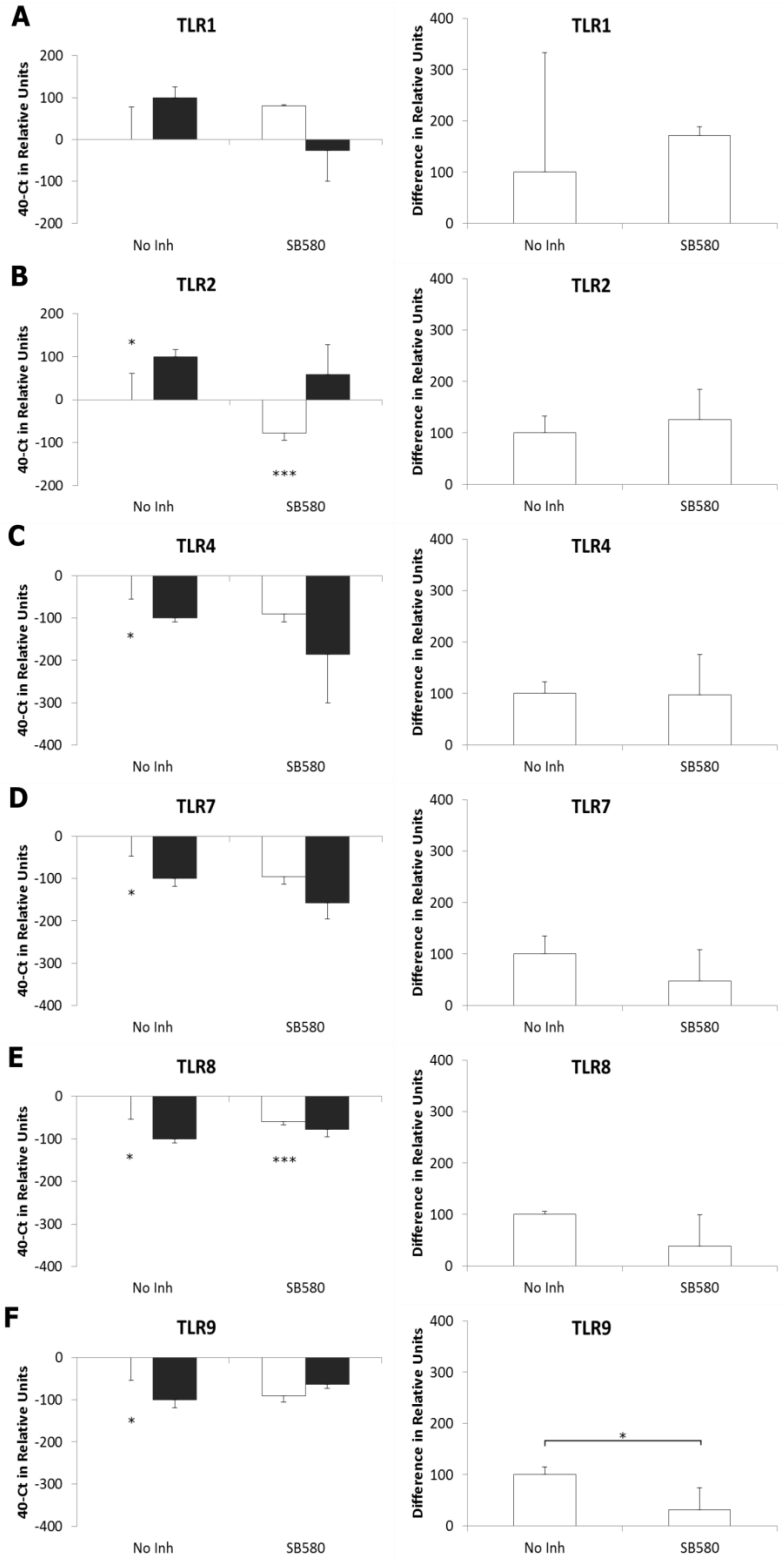


Figure 5.5: Effect of inhibiting P38 signalling on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In relative unit of 40-Ct graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

competitive binding to its ATP-binding pocket. The raw 40-Ct results for the effect of P38 inhibition on PAM induced TLR mRNA changes are indicated in Appendix Figure 5.

PAM stimulation normally increases the transcriptional expression of TLR1 and TLR2 in RAW cells however only TLR2 mRNA level was up-regulated ($p<0.05$) following PAM stimulation in this experiment. Although exhibiting a small increase, the TLR1 up-regulation was not significant due to the large variation between samples ($p=0.72$) (Figure 5.5A). Inhibition of P38 by SB580 did not affect the basal or PAM induced expression of TLR1 or TLR2 mRNA in inhibitor-only treated cells or in PAM and inhibitor co-incubated cells (Figure 5.5A-B). Similar to TLR1/2, neither the TLR4 basal mRNA expression nor the PAM induced mRNA expression was modulated following treatment of SB580 (Figure 5.5C).

The TLR7, 8 and 9 transcript levels were down-regulated following PAM stimulation. Treatment with SB580 did not affect the mRNA levels of TLR7 family members in inhibitor-only treated cells or in PAM stimulated cells (Figure 5.5 D-E). However, a trend of rescued TLR9 mRNA down-regulation was observed in cells co-incubated with both PAM and SB580 compared to cells stimulated with only PAM agonist ($p=0.06$). Taking this background change into account, the magnitude of PAM induced TLR9 down-regulation was reduced by treatment with SB580 ($p<0.05$) (Figure 5.5F).

5.2.3.4 Pharmacological inhibition of NFκB pathway

Celastrol was used to investigate the effect of NFκB signalling in PAM induced TLR regulation. The 40-Ct raw data are presented in Appendix Figure 6.

In the TLR1/2 family, both TLRs were up-regulated following PAM stimulation. Inhibition of NFκB signalling pathway by celastrol substantially increased TLR1 mRNA expression in inhibitor-only treated cells ($p<0.001$). Upon PAM stimulation, celastrol treatment did not alter the PAM induced TLR1 up-regulation. Taking into account of both basal and PAM induced regulation, the magnitude of PAM induced TLR1 up-

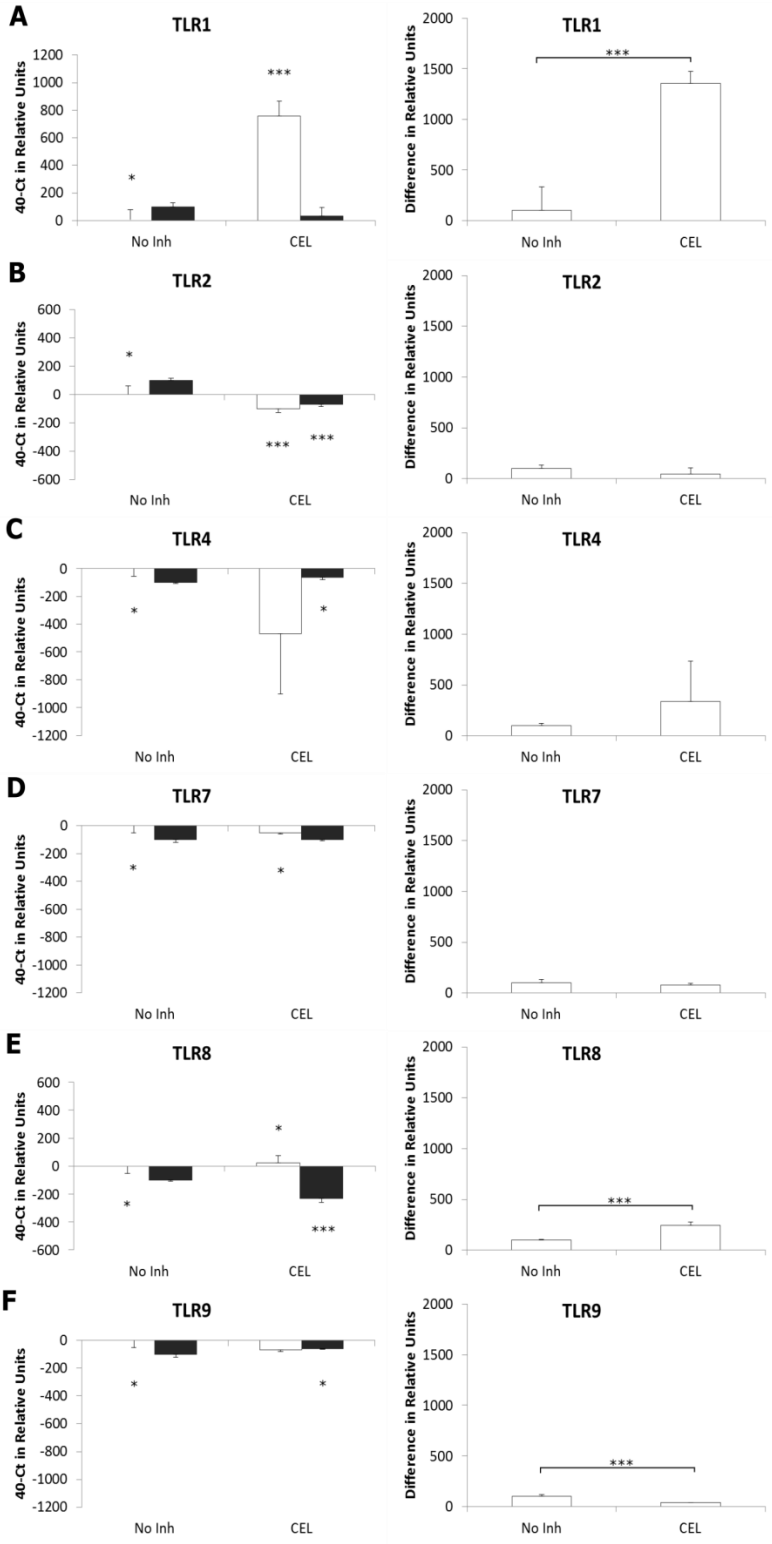


Figure 5.6: Effect of inhibiting NFκB signalling on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In relative unit of 40-Ct graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

regulation was substantially modulated ($p<0.001$) due to the huge increase of TLR1 basal mRNA level in celastrol-only treated cells (Figure 5.6A). In contrast, celastrol treatment induced a non-significant trend of down-regulation in the basal TLR2 mRNA level compared to the resting cells ($p=0.14$). In PAM stimulated cells, celastrol treatment completely prevented TLR2 up-regulation ($p<0.001$). Therefore the magnitude of PAM induced TLR2 up-regulation was not substantially affected by celastrol treatment. This is because celastrol treatment altered both basal and PAM induced regulation of TLR2 to the same level (Figure 5.6B).

TLR4 was down-regulated following PAM stimulation. In inhibitor-only treated cells, celastrol treatment did not alter the TLR4 basal expression although large variation between samples was observed. This large variation also masked out the effect of celastrol stimulation in altering PAM induced TLR4 transcriptional changes. In PAM treated cells, the down-regulation of TLR4 was partially rescued by celastrol stimulation ($p<0.05$). However when considering the effects of celastrol treatment with or without PAM, the inhibitor did not alter the magnitude of PAM induced TLR4 down-regulation due to the large variation in the basal expression of TLR4 mRNA levels in celastrol-only treated cells (Figure 5.6C).

PAM stimulation reduced TLR7, 8 and 9 mRNA levels in RAW cells. In inhibitor-only treated cells, celastrol treatment did not change the basal expression of TLR7, 8 or 9. In PAM stimulated cells, celastrol treatment induced completely different changes in the transcriptional regulation for each of the TLR7 family members. The PAM induced down-regulation of TLR7 was unaffected by celastrol treatment in RAW cells (Figure 5.6D). On the contrary, regulation of the other two members of the TLR7 family, TLR8 and TLR9, were both altered by treatment with celastrol. The Inhibitor stimulation substantially reduced TLR8 mRNA expression below PAM induced down-regulation level in agonist-treated cells ($p<0.001$) (Figure 5.6E). Whereas for TLR9, the inhibitor stimulation partially rescued the PAM induced transcriptional down-regulation ($p<0.05$)

(Figure 5.6F). Overall, treatment with celastrol increased the magnitude of PAM induced TLR8 down-regulation ($p<0.001$) (Figure 5.6E) while reduced the magnitude of PAM induced TLR9 down-regulation ($p<0.001$) (Figure 5.6F). However, celastrol stimulation did not affect the PAM induced TLR7 down-regulation (Figure 5.6D).

5.3 Discussion

PAM induced activation of various signalling pathways have been documented in previous research. However most studies focused on signalling cascades responsible for the regulation of inflammatory mediators, whereas consideration of signalling pathways in the transcriptional regulation of TLRs was lacking. Therefore this study aimed to systematically address the signalling pathways responsible for TLR regulation following PAM stimulation in the selected cell line RAW264.7.

PAM stimulus induced phosphorylation of all three MAPK terminal components ERK, JNK and P38 was identified by western blotting. The highest phosphorylation levels of all these components were detected at 30 mins following agonist stimulation, consistent with previous findings in BMDC, BPL and pulmonary epithelial cells (Peng, Zhang 2014, Leng, Chen et al. 2010, Berube, Bourdon et al. 2009). In non-stimulated RAW cells, phosphorylation of ERK but not JNK or P38 was also observed, which may be due to other roles that ERK1/2 play, including regulation of cellular growth and proliferation (Roux, Blenis 2004). In addition, ERK phosphorylation is known for maintaining mitochondrial function and ATP production in human alveolar macrophages, which in turn, prolong the survival of alveolar macrophages during stress (Monick, Powers et al. 2008). It is possible that the activation of ERK in non-stimulated RAW cells is responsible for regulating cellular mechanism for coping with changing environment during the experiments.

A range of pharmacological inhibitors were used to investigate the signalling components that maintain the basal levels and regulate the PAM induced TLR mRNA levels in non-stimulated and PAM treated cells. This study uncovered the signalling pathways responsible for the maintenance of the basal TLR mRNA expression which have been neglected in previous studies. In non-stimulated cells, inhibition of NFκB increased basal TLR1 mRNA level and reduced basal TLR2 mRNA level. Inhibition of ERK1/2 down-regulated the transcript level of TLR4 in non-stimulated cells. While

A

Inhibitor Effect on Basal TLR mRNA levels				
Inhibitor	**PD98059**	**SP600125**	**SB203580**	**Celastrol**
Target	**ERK**	**JNK**	**P38**	**NFκB**
TLR1	80.98	99.67	79.55	759.53
TLR2	28.54	-59.69	-77.39	-100.40
TLR4	-61.92	-21.15	-90.61	-438.49
TLR7	-23.34	62.03	-95.24	-50.04
TLR8	74.87	123.93	-59.66	25.29
TLR9	-45.38	-63.87	-90.36	-70.19

B

Inhibitor Effect on PAM-induced TLR mRNA levels				
Inhibitor	**PD98059**	**SP600125**	**SB203580**	**Celastrol**
Target	**ERK**	**JNK**	**P38**	**NFκB**
TLR1 (100)	208.70	129.87	-26.85	33.15
TLR2 (100)	220.31	102.31	59.13	-66.82
TLR4 (-100)	-85.59	-87.09	-185.92	-65.24
TLR7 (-100)	-93.03	-80.95	-158.03	-99.83
TLR8 (-100)	-23.42	0.12	-78.24	-231.10
TLR9 (-100)	-3.76	-1.12	-62.84	-59.82

C

Differences of Inhibitor Effect on TLR levels				
Inhibitor	**PD98059**	**SP600125**	**SB203580**	**Celastrol**
Target	**ERK**	**JNK**	**P38**	**NFκB**
TLR1 (100)	108.05	62.31	171.90	1354.10
TLR2 (100)	176.11	116.64	126.47	42.62
TLR4 (100)	51.56	86.61	97.54	338.76
TLR7 (100)	115.97	120.78	47.64	78.59
TLR8 (100)	82.97	123.74	38.08	244.78
TLR9 (100)	35.37	61.76	31.12	36.99

Table 5.7: Signalling pathways mediating PAM induced TLR responses in RAW cells. Values in the tables depict the mean relative units of TLR mRNA levels. Red highlight colour indicates statistically significant ($p<0.05$) up-regulation; blue highlight colour indicates statistically significant down-regulation in inhibitor-treated cells compared to (A) non-stimulated cells; (B) PAM treated cells. Green highlight colour indicates statistic significant magnitude differences between PAM induced and basal TLR mRNA levels.

inhibition of either MAPK or NFκB signalling pathway did not affect the basal transcript level of TLR7, inhibition of ERK or JNK substantially increased the basal TLR8 mRNA level and inhibition of JNK reduced the basal TLR9 mRNA level (Figure 5.7A). These results suggest that different signalling pathways are responsible for the basal mRNA expression of different TLRs although some similarities for TLRs within the same family were detected.

In PAM stimulated cells, both TLR1 and 2 were up-regulated. Inhibition of ERK increased the TLR1 transcript level above the PAM induced level, while inhibition of JNK, P38 and NFκB did not significantly affect the PAM induced TLR1 up-regulation (Figure 5.7B). When comparing the PAM induced mRNA level to the basal level, only inhibition of JNK reduced PAM induced TLR1 up-regulation, suggesting JNK signalling is responsible for the elevated TLR1 mRNA level following PAM stimulation (Figure 5.7C). This result is consistent with previous finding where stimulation of PAM resulted in JNK1 mediated TLR1 mRNA up-regulation (Izadi, Motameni et al. 2007). Overall taking into account the basal and PAM induced TLR1 transcript levels, the interaction between ERK, JNK and NFκB signalling cascades was responsible for the TLR1 transcriptional regulation. Indeed, inhibition of ERK also up-regulated TLR2 above PAM induced level (Figure 5.7B). Similar to this result, ERK has been shown to down-regulate TLR2 mRNA level following LPS stimulation (Matsuguchi, Musikacharoen et al. 2000). Inhibition of NFκB fully rescued PAM induced TLR2 up-regulation (Figure 5.7B). Hence both ERK and NFκB signalling pathways were responsible for the TLR2 basal and PAM induced transcriptional regulation.

Signalling pathways responsible for PAM induced TLR cross-regulation have not been addressed in previous studies. The current study provides the first comprehensive signalling analysis for the cross-regulating TLRs in RAW cells. In PAM treated cells, inhibition of NFκB partially rescued TLR4 down-regulation (Figure 5.7B), indicating the role of NFκB signalling pathway in mediating the PAM induced TLR4 transcriptional

regulation. Similar to TLR4, NFκB signalling pathway mediated PAM induced transcriptional regulation of TLR8 and TLR9. Inhibition of NFκB reduced TLR8 mRNA level below the PAM induced level while partially rescued the down-regulation of TLR9 mRNA level. In addition, inhibition of JNK also rescued the TLR9 down-regulation induced by PAM (Figure 5.7B). When comparing the PAM induced mRNA levels to the basal levels, these results indicate the antagonistic interaction between ERK, JNK and NFκB for the transcriptional regulation of TLR8 in order to neutralise the effect of ERK and JNK in TLR8 basal regulation. Similarly, the transcriptional regulation of TLR9 is also mediated by a combination of MAPK and NFκB signalling pathways. Both the basal level and PAM induced mRNA level of TLR7 were not affected by inhibition of MAPK or NFκB signalling pathways, suggesting that the transcriptional regulation of TLR7 induced by PAM may be independent of both pathways but dependent on a mystery pathway.

Signalling control for the transcriptional regulation of TLR9 was the most complex in TLR7 family. TLR9 mRNA regulation required the presence of all MAPK terminal cascades ERK, JNK and P38 as well as NFκB signalling. Signalling required for TLR8 transcriptional regulation was simpler than TLR9 but more complex than TLR7. A combination of ERK, JNK and NFκB, but not P38 contributed to the TLR8 mRNA regulation in RAW cells. TLR7 transcriptional regulation was the least complex among TLR7, 8 and 9. Neither MAPK nor NFκB signalling pathways were responsible for the PAM induced TLR7 transcriptional changes. Interestingly, this is coherent with the evolutionary history of TLR7, 8 and 9. Structural phylogenetic analysis revealed TLR7 and 8 are recent gene duplications prior to the origin of mammals. Both TLRs are closely located on the X chromosome in placental mammals (Hughes, Piontkivska 2008, Dunne, O'Neill 2005). Although located within the same TLR family cluster, TLR9 is associated more distantly to TLR7 and 8. In addition, phylogenetic analyses of the TIR domain of the adaptor molecules revealed the same ancestral characteristics of

TLR7, 8 and 9 (Roach, Racioppi et al. 2013, Song, Jin et al. 2012, Roach, Glusman et al. 2005). The TLR signalling cascades also evolved. Recent changes to the TLR signalling pathways include addition of TRIF molecules and duplication of *NFκB* and *IκB* genes in vertebrates (Song, Jin et al. 2012). It is possible that signalling pathways recruited by the ancient TLR9 is more straight forward and with less negative regulators whereas the signalling pathways recruited by the TLR7 and TLR8 are more tightly regulated with adaptive molecules and more negative regulators. Hence the levels of signalling complexity for TLR7, 8 and 9 may be reflective of the evolutionary characteristics of the TLR7 family.

In summary, this chapter identified signalling pathways responsible for the basal expression and the PAM induced expression changes of the cognate- and cross-regulating TLRs. Transcriptional regulation of all TLRs requires a combination of MAPK and NFκB signalling pathways. The question remains of how these signalling pathways coordinate to regulate TLRs transcription. The next chapter will investigate the effect of multiple signalling pathways on TLR transcriptional regulation.

Chapter 6 The effects of signalling synergy on the transcriptional regulation of TLRs

6.1 Introduction

The integration of signalling in innate immunity may occur at different stages: when multiple receptors bind to the same ligand, when shared signalling transduction is induced or common down-stream immune regulators are activated (Thaiss, Levy et al. 2016). Upon activation through agonist binding, only a small number of signalling pathways are activated by large groups of immune receptors. Hence interactions between signalling pathways by facilitating modulators and permitting cross-pathway signal amplification or inhibition is important for the fine tuning of the host immune response.

Global analysis of signalling networks with a combination of microarray and clustering methods revealed TLR mediated cross-interactions among several signalling pathways, mainly between the direct MAPK and NFκB pathways and the indirect PI3K/Akt and JAK-STAT pathways (Natarajan, Lin et al. 2006). A number of studies have indicated the effects of PI3K pathway on TLR induced cytokine production. In human dendritic cells, inhibition of PI3K signalling abolished production of IL12 following dual agonist stimulation with both TLR3 or 4 agonists and TLR8 agonist (Makela, Strengell et al. 2009). Sly et al. (2004) observed that inhibition of the PI3K pathway with its pharmacological inhibitor, Wortmannin, down-regulated LPS induced production of TNFα, IL1β and IL6 in bone marrow derived macrophages and mast cells. Whereas Hochdorfer et al. (2011) found that inhibition of PI3K pathway with the same inhibitor reduced amount of TNFα and IL6 while increased IL1β production in BMMC. The differential effect of PI3K signalling on the production of two cytokines was also observed in human monocytes. The LPS induced IL12 production was up-regulated by treatment with PI3K inhibitor whereas IL10 production was down-regulated (Martin, Schifferle et al. 2003). This may be due to the interactions between PI3K and ERK

signalling. ERK signalling was shown to induce IL10 production and supress IL12 production in murine macrophage cells (Yi, Yoon et al. 2002). Moreover, the inhibition of PI3K pathway prevents ERK activation (Sandig, Bulfone-Paus 2012), which in turn, lead to the differential regulation of IL10 and IL12. The PI3K pathway also acts as a negative regulator for MAPK and NFκB pathways in some cell types (Troutman, Bazan et al. 2012). PI3K mutant mice produced greater amounts of IL12 through increased P38 activity following TLR activation in dendritic cells (Fukao, Tanabe et al. 2002). In thioglycollate induced macrophages, PI3K mutant mice also expressed increased levels of TNFα and IL6 through up-regulated ERK and JNK phosphorylation (Luyendyk, Schabbauer et al. 2008). PDPK1, a crucial regulator of Akt phosphorylation in PI3K signalling, suppressed TLR2 or TLR4 induced TNFα and IL6 production in macrophages through inhibition of phosphorylated IκBα (Chaurasia, Mauer et al. 2010). In addition, Akt signalling downstream of PI3K was shown to regulate miRNA species, such as the miRNA-155 which targeted the negative regulator of PI3K, SHIP1, and subsequently affected TLR mediated cytokine production through the TLR-PI3K signalling (Bauerfeld, Rastogi et al. 2012, Androulidaki, Iliopoulos et al. 2009, O'Connell, Chaudhuri et al. 2009).

The JAK-STAT signalling pathway is downstream of IFNγ, one of the most potent macrophage activating factors (Schroder, Sweet et al. 2006). Activation of IFNγ induced JAK-STAT pathway dependent increased levels of mRNA encoding TLR2, 4, 6 and 9 receptors as well as protein expression of TLR signalling molecules, such as CD14, MD-2, MyD88 and IRAK1(Mochizuki, Kobayashi et al. 2004, Tamai, Sugawara et al. 2003, Dalpke, Eckerle et al. 2003, Bosisio, Polentarutti et al. 2002, Ahmad-Nejad, Hacker et al. 2002, Adib-Conquy, Cavaillon 2002, Mita, Dobashi et al. 2001). IFNγ also affected the regulation of transcription factors downstream of TLR signalling. Pre-treatment of murine macrophages with IFNγ increased LPS induced activation of transcriptional factor NFκB (Held, Weihua et al. 1999). In human monocytes, exposure

to IL10 reduced production of inflammatory cytokines such as IL6 and TNFα. IFNγ stimulation supressed the negative regulatory effect of IL10 for TLR2 and TLR4 mediated cytokine production through down-regulation of c-JUN transcript and reduced the DNA binding ability of the transcriptional factor AP-1 (Hu, Chen et al. 2007, de Wit, Hoogstraten et al. 1996). In addition, IFNγ supressed the protein expression of negative regulators for TLR4 signalling such as SOCS1, SOCS3 and ST2L (Brint, Xu et al. 2004, Crespo, Filla et al. 2002, Stoiber, Kovarik et al. 1999).

TLR signalling pathways affect the IFNγ signalling by regulating the transcription factors, such as STAT1 downstream of JAK-STAT pathway. Activation of transcription factor STAT1 requires phosphorylation at 2 sites: tyrosine 701 (Y701) and serine 727 (S727) (Varinou, Ramsauer et al. 2003, Nair, DaFonseca et al. 2002, Wen, Zhong et al. 1995). TLR signalling pathways are directly involved in S-phosphorylation of STAT1, with the activation of all TLRs inducing phosphorylation of STAT1 at S727 residue via P38 signalling, which is dependent on direct MyD88 and TRIF pathways rather than the indirect IRF pathways (Luu, Greenhill et al. 2014, Schroder, Spille et al. 2007, Schroder, Sweet et al. 2006, Zhao, Kong et al. 2006, Dalpke, Eckerle et al. 2003, Rhee, Jones et al. 2003). Furthermore, TRAF6, downstream of MyD88 and TRIF, also interacts with STAT1 directly (Luu, Greenhill et al. 2014, Nguyen, Chatterjee-Kishore et al. 2003).

While cross-talk between some direct and indirect TLR signalling pathways have been characterised, only a few studies have addressed signalling synergy between MAPK and NFκB pathways which are both directly downstream of TLR activation. Co-incubation of TLR2 and TLR4 agonists induced hyporesponsiveness in macrophages with reduced TNFα production, which is dependent on impaired JNK and NFκB signalling (Sato, Nomura et al. 2000). Co-incubation of TLR2 and TLR4 agonists, TLR2 and TLR7 agonists or TLR4 and TLR7 agonists induced secretion of IL6 and IL12 in

BMDC. Inhibition of P38 and JNK largely impaired IL6 secretion and inhibition of ERK and P38 substantially reduced production of IL12P40 (Mitchell, Yong et al. 2010).

Results from the previous chapter indicated complex signalling involvement of individual MAPK and NFκB pathways following stimulation with PAM. However it is unclear whether synergy between multiple pathways may affect the transcriptional regulation of TLRs. This chapter hereby aimed to address the effect of multiple MAPK signalling pathways and interactions between MAPK and NFκB pathways on PAM induced TLR mRNA productions.

6.2 Results

6.2.1 Multiple MAPK signalling cascades mediated immune responses

The effects of multiple MAPK signalling pathways were elucidated using combinations of signalling inhibitors. By inhibiting 2 MAPK signalling cascades with dual pharmacological inhibitors, it could be determined whether the effects of individual MAPK pathways were additive, antagonistic or independent. Complete MAPK inhibition was achieved with a combination of ERK, JNK and P38 inhibitors: PD98059, SP600125 and SB203580. Inhibition of individual MAPK or NFκB signalling pathways was also performed as replicates to previous experiments and controls in this experiment, although these results were excluded from this chapter as they were discussed in the previous chapter. A figure indicating the 40-Ct value of the TLRs is shown in Appendix Figure 7.

6.2.1.1 Multiple MAPK signalling cascades mediated IL1β mRNA changes

Inhibition of multiple MAPK signalling pathways affected IL1β transcript level in non-agonist stimulated cells. Increased basal IL1β mRNA level was detected following inhibition of double MAPK signalling pathways with PD059 and SP125 ($p<0.001$), PD059 and SB580 ($p<0.001$), SP125 and SB580 ($p<0.01$) as well as inhibition of complete MAPK signalling with PD059, SP125 and SB580 ($p<0.001$). In agonist stimulated cells, PAM treatment up-regulated IL1β transcriptional expression. This elevated mRNA level was not affected by inhibition of multiple MAPK signalling pathways (Figure 6.1A). However, the magnitude of PAM induced IL1β up-regulation was reduced following double inhibitor treatment with PD059 with SB580 as well as SP125 with SB580 when taking into account the inhibitory effect of multiple MAPK signalling pathways on non-agonist stimulated cells. Complete inhibition of MAPK signalling pathways with 3 inhibitors did not significantly reduce the magnitude of PAM induced IL1β up-regulation due to large variations between samples (Figure 6.1B).

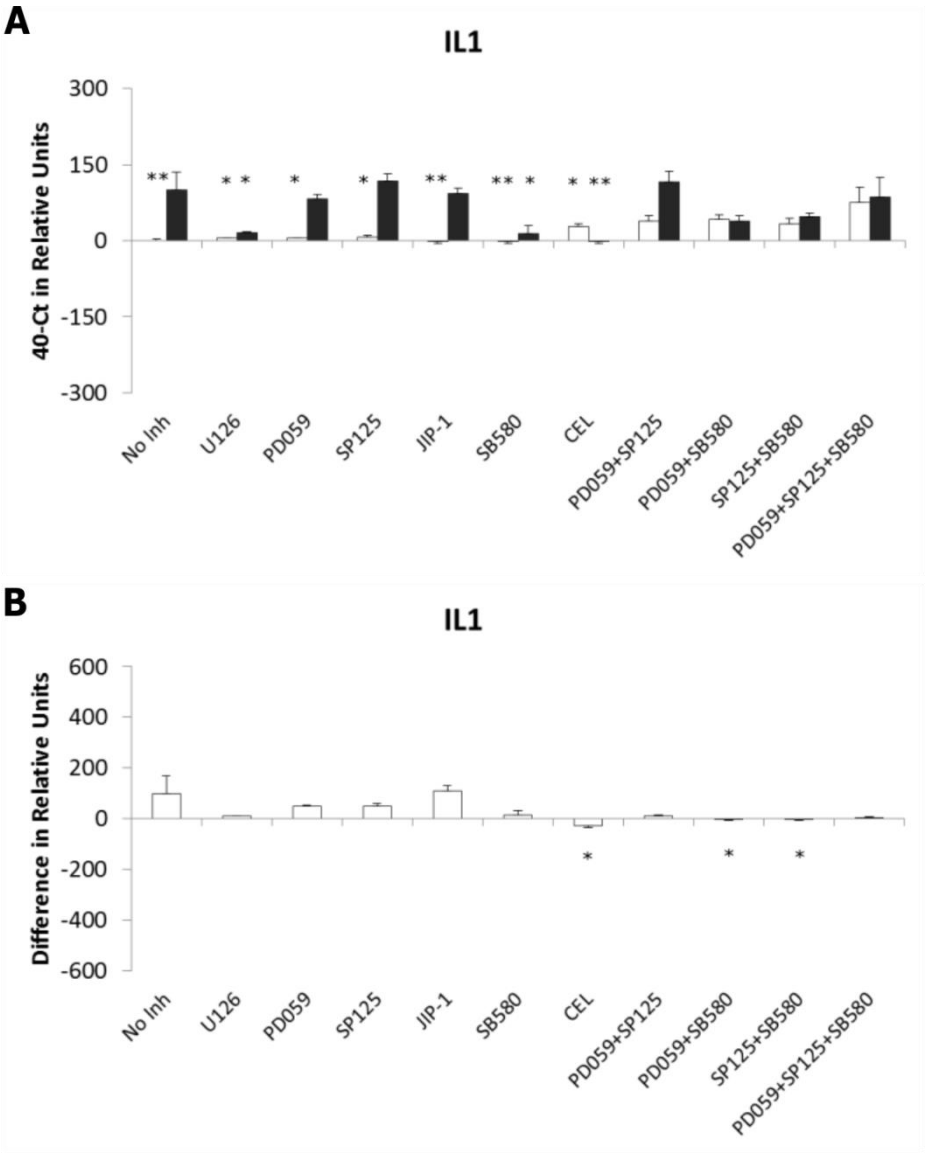


Figure 6.1: Effect of individual and multiple MAPK signalling inhibition on PAM-induced IL1 transcriptional expression in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A) or magnitude of change in relative quantity (B). In 40-Ct in relative units graphs, the clear bars represent IL1 expression in non-stimulated or inhibitor-only treated cells; the black bars represent IL1 expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels of the differences between the bar over which they are placed and PAM treated cells: *p< 0.05, **p< 0.01, ***p< 0.001.

6.2.1.2 Multiple MAPK signalling cascades mediated TLR mRNA changes

Inhibition of double and triple MAPK signalling pathways up-regulated TLR1 mRNA production in inhibitor-only treated cells, with some inducing larger changes than others. Dual inhibition with PD059 and SP125 as well as SP125 and SB580 both increased TLR1 mRNA level compared to non-stimulated cells ($p<0.05$). Treatment with other inhibitor combinations, PD059 and SB580 ($p<0.01$) as well as PD059, SP125 and SB580 ($p<0.05$) also substantially increased TLR1 transcriptional expression compared to agonist induced up-regulation level. In PAM stimulated cells, treatment with dual or triple inhibitors also increased TLR1 mRNA expression above the PAM up-regulated level (Figure 6.2A). However, the magnitude of PAM induced TLR1 up-regulation was not altered by inhibition of multiple MAPK signalling considering TLR transcriptional changes in both inhibitor-only and PAM with inhibitor co-incubated cells (Figure 6.2B).

The transcript level of TLR2 was not affected by inhibition of 2 or 3 MAPK signalling pathways in inhibitor-only treated cells, although a trend of TLR2 down-regulation was observed following treatment of PD059 with SB580 and SP125 with SB580. In PAM stimulated cells, pre-treatment with SP125 and SB580 ($p<0.001$) fully prevented PAM induced TLR2 up-regulation. However, co-incubation of PAM with the other combinations of dual inhibitors PD059 and SP125 or PD059 and SB580 did not alter PAM induced TLR2 up-regulation. In a similar fashion, complete inhibition of MAPK signalling pathways with a combination of 3 inhibitors PD059, SP125 and SB580 did not change the TLR2 up-regulation in PAM treated cells (Figure 6.2C). Although the combination of SP125 and SB580 fully rescued TLR2 mRNA expression in PAM treated cells, it also reduced basal TLR2 transcript level in some samples, causing the large variations. Hence the magnitude of PAM induced TLR2 up-regulation was not altered by inhibition of multiple MAPK signalling cascades (Figure 6.2D).

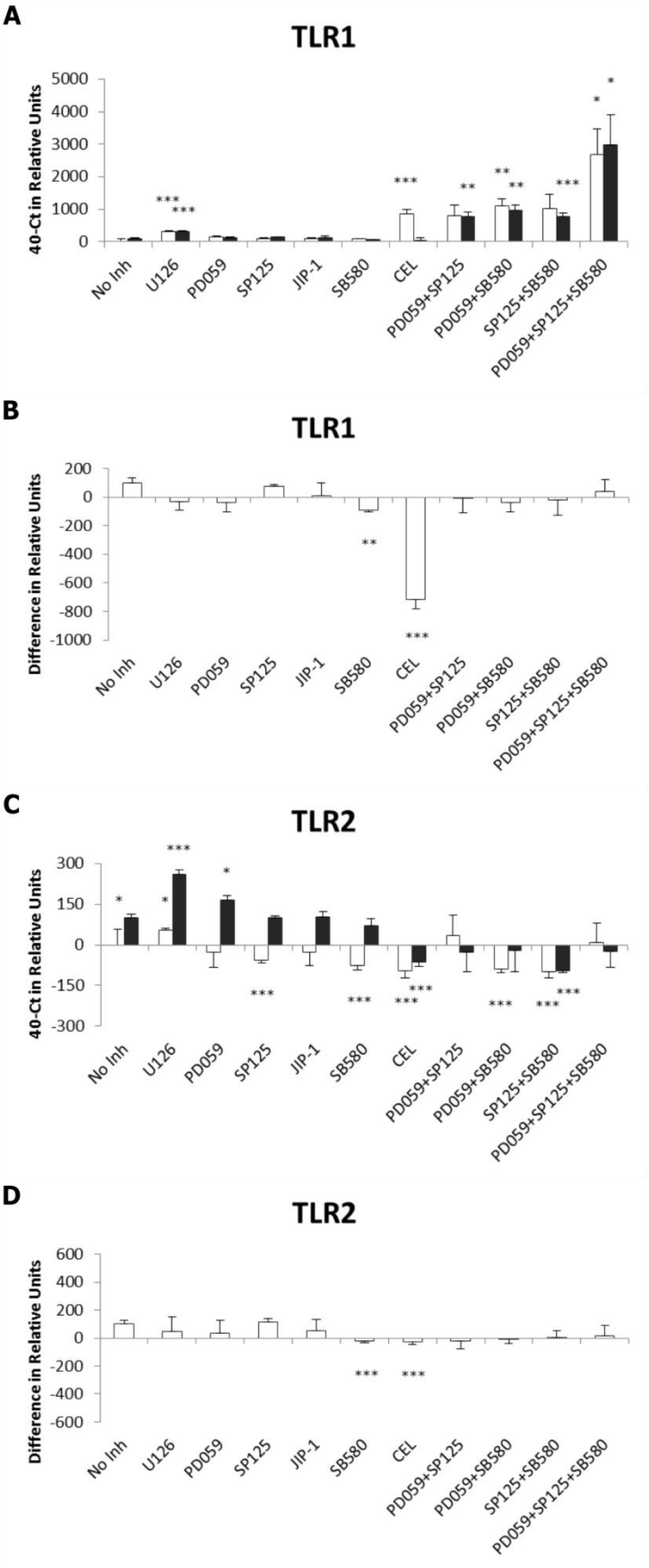


Figure 6.2: Effect of MAPK signalling inhibition on PAM-induced TLR1 family mRNA changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A, C) or magnitude of change in relative quantity (B, D). In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-only treated cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

Inhibition of multiple MAPK signalling cascades substantially reduced TLR4 mRNA level in inhibitor-only treated cells ($p<0.01$) except for dual inhibitor treatment with PD059 and SP125, which did not change the TLR4 basal expression. In PAM stimulated cells, pre-treatment with dual inhibitors PD059 and SP125 did not affect the TLR4 down-regulation induced by PAM. In contrast, co-incubation of PAM with dual inhibitors PD059 and SB580 ($p<0.001$), or SP125 and SB580 ($p<0.001$) substantially further reduced TLR4 mRNA levels compared to the PAM induced down-regulation level. Similarly, inhibition of all three MAPK signalling pathways also further down-regulated TLR4 transcript level in PAM treated cells ($p<0.001$) (Figure 6.3A). Overall, due to inhibition of the MAPK pathways down-regulation of TLR4 mRNA levels in both inhibitor-only and agonist treated cells, inhibition of multiple MAPK signalling pathways did not affect the magnitude of PAM induced TLR4 down-regulation (Figure 6.3B).

The TLR7 transcript level was not affected by inhibition of dual or triple MAPK signalling cascades in inhibitor-only treated cells or in PAM and inhibitor co-incubated stimulated cells (Figure 6.4A-B). The TLR8 mRNA level was increased following stimulation with dual inhibitors PD059 and SP125 ($p<0.05$) in inhibitor-only treated cells compared to the resting cells. Treatment with the dual inhibitors PD059 and SB580, SP125 and SB580 or the triple inhibitors did not affect the basal expression of TLR8 although a trend of increased TLR8 mRNA was observed following stimulation with dual inhibitors SP125 and SB580 ($p=0.14$) as well as the triple inhibitors ($p=0.09$). In PAM treated cells, stimulation with dual inhibitors and triple inhibitors largely prevented TLR8 down-regulation induced by PAM except for treatment with dual inhibitors PD059 and SB580 (Figure 6.4C). However, co-incubation of PAM and inhibitors did not up-regulate TLR8 transcript level to match the inhibitor-only induced level. Hence inhibition of dual and triple MAPK signalling cascades did not affect the magnitude of TLR8 down-regulation induced by PAM (Figure 6.4D). Similar to TLR7, inhibition of

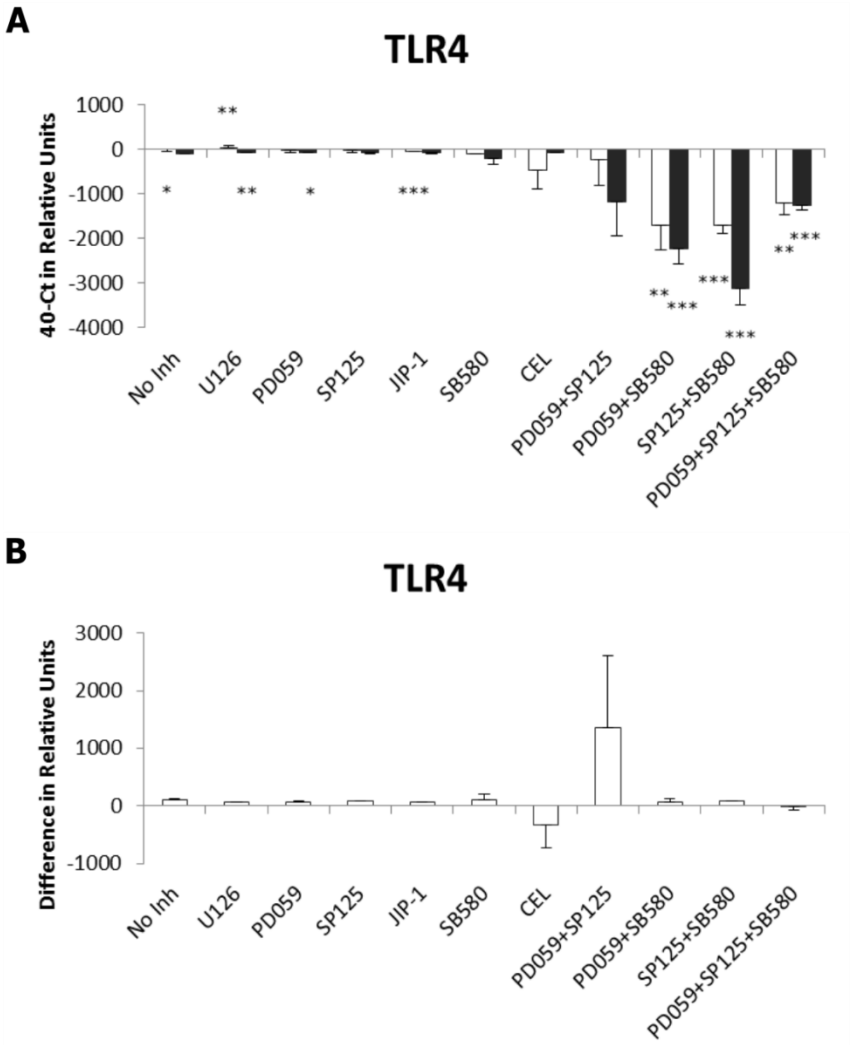


Figure 6.3: Effect of individual and multiple MAPK signalling inhibition on PAM-induced TLR4 transcriptional regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A) or magnitude of change in relative quantity (B). In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-only treated cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

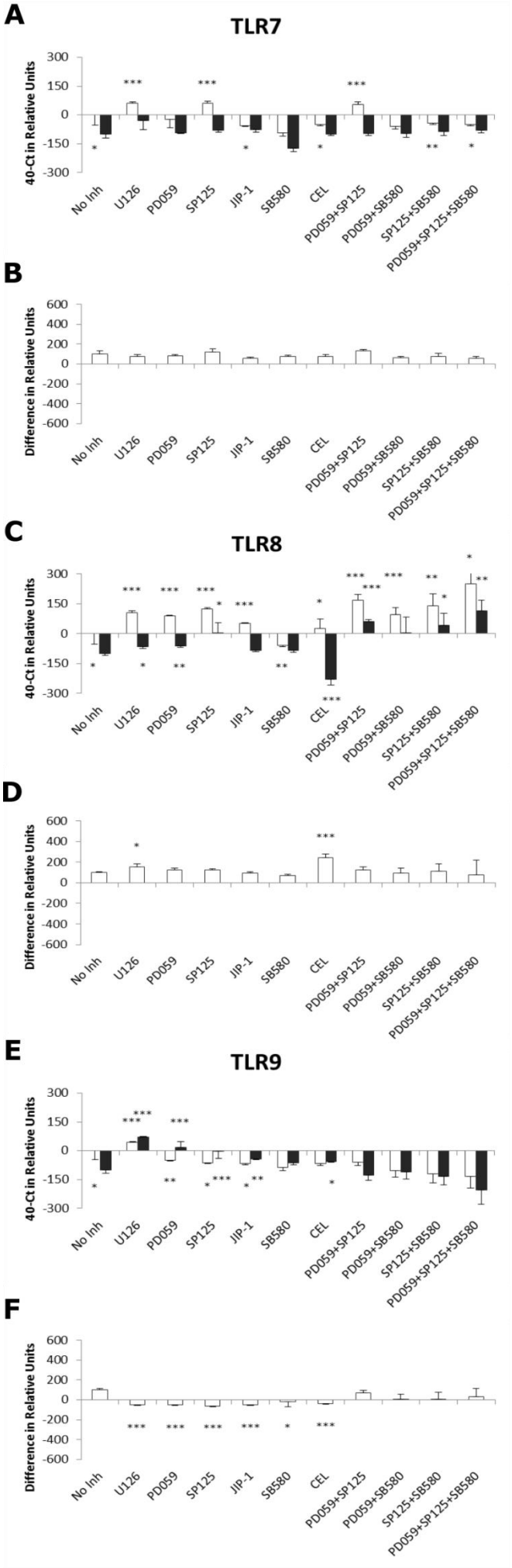


Figure 6.4: Effect of MAPK signalling inhibition on PAM-induced TLR7 family mRNA changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A, C, E) or magnitude of change in relative quantity (B, D, F). In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-only treated cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

multiple MAPK signalling pathways did not substantially affect the expression of TLR9 in inhibitor-only treated cells or in PAM and inhibitors co-incubated cells in this particular experiment (Figure 6.4E-F).

6.2.2 Synergy between MAPK and NFκB pathways for the transcriptional regulation of TLRs

As both MAPK and NFκB signalling pathways are involved in the regulation of TLRs, it is important to understand the synergy between the two pathways. The effects of signalling synergy between individual MAPK signalling cascades and NFκB were investigated using combinations of inhibitors targeting ERK with NFκB, JNK with NFκB or P38 with NFκB. The effect of synergy between the complete MAPK signalling pathway and NFκB pathway was studied using a combination of 4 chemical inhibitors, PD059, SP125, SB580 and celastrol. Cells incubated with both PAM and MAPK signalling inhibitors were used as one of the control conditions in this experiment, additional to PAM stimulated or non-stimulated cells.

6.2.2.1 Inhibition of individual MAPK signalling terminal cascades with NFκB

Stimulation with PAM increased the transcript levels of both TLR1 and 2 in RAW cells. Inhibition of individual MAPK signalling cascades did not affect PAM induced transcriptional regulation of cognate TLRs although a trend of increase in mRNA level was observed following treatment with PD059 inhibitor. On the contrary, when individual MAPK signalling terminal cascades and NFκB signalling were both inhibited, PAM induced up-regulation of the cognate TLRs were fully prevented ($p<0.001$) (Figure 6.5A-B).

The cross-regulating TLR mRNA levels were all reduced following PAM stimulation. The down-regulation of TLR4 or TLR7 was not affected by treatment with individual MAPK signalling inhibitors with or without NFκB inhibitor (Figure 6.5C-D). Treatment with PD059 partially rescued the down-regulation of TLR8 ($p<0.01$) whereas treatment

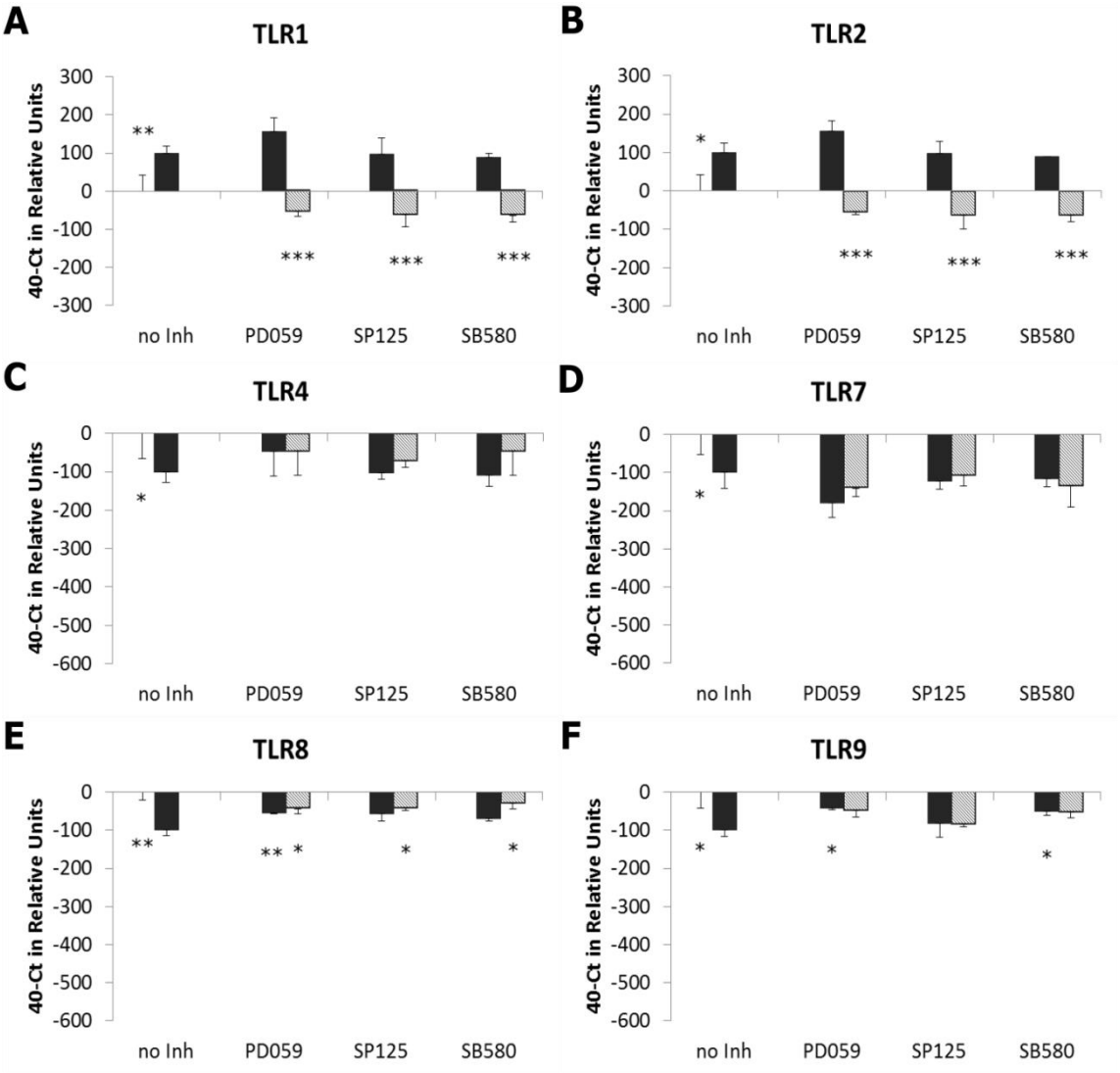


Figure 6.5: Effect of inhibiting individual MAPK signalling cascade with/without NFκB signalling on PAM-induced TLR transcriptional changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity. The clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in PAM-stimulated or PAM and MAPK inhibitor co-incubated cells; the grey bars represent TLR expression in cells treated with both MAPK and NFκB inhibitors, as well as PAM agonist. Error bars and significance levels denoted by asterisks are the same as previous figures.

with SP125 or SB580 alone did not affect the TLR8 mRNA level in PAM stimulated cells. When treated with both MAPK and NFκB inhibitors, all three treatments: PD059 with celastrol ($p<0.05$), SP125 with celastrol ($p<0.05$) and SB580 with celastrol ($p<0.05$) partially rescued PAM induced TLR8 down-regulation (Figure 6.5E). The down-regulation of TLR9 was partially rescued following treatment with PD059 ($p<0.05$) or SB580 ($p<0.05$). Although indicating a trend of increase in TLR9 transcript level, treatment with SP125 did not alter the reduced TLR9 mRNA level due to large variation between samples. Similarly, although a trend of rescuing TLR9 down-regulation was observed, treatment with individual MAPK signalling inhibitors with NFκB signalling inhibitor did not affect the TLR9 mRNA level in PAM stimulated cells (Figure 6.5F).

The effect of inhibiting individual MAPK signalling cascades with or without NFκB signalling on TLR transcriptional regulation is also presented in 40-Ct value in Appendix Figure 8.

6.2.2.2 Inhibition of the three MAPK signalling cascades with NFκB pathway

The effect of inhibiting the three MAPK signalling cascades with or without NFκB pathway inhibition was similar for both cognate TLRs. PAM induced transcriptional changes of the cognate TLR1 or 2 were not affected by suppressing MAPK signalling pathway in this experiment. However, PAM induced up-regulation of TLR1 ($p<0.01$) and 2 ($p<0.001$) were fully prevented when both MAPK and NFκB signalling pathways were suppressed (Figure 6.6A-B).

Inhibition of the three MAPK signalling cascades with or without NFκB signalling inhibition did not alter the down-regulation of TLR4 or TLR7 in PAM stimulated cells (Figure 6.6C-D). On the other hand, while inhibition of complete MAPK signalling pathways did not affect transcriptional regulation of TLR8, inhibition of both MAPK with NFκB pathway substantially rescued TLR8 down-regulation (Figure 6.6E). Down-regulation of TLR9 was fully rescued by inhibition of complete MAPK signalling

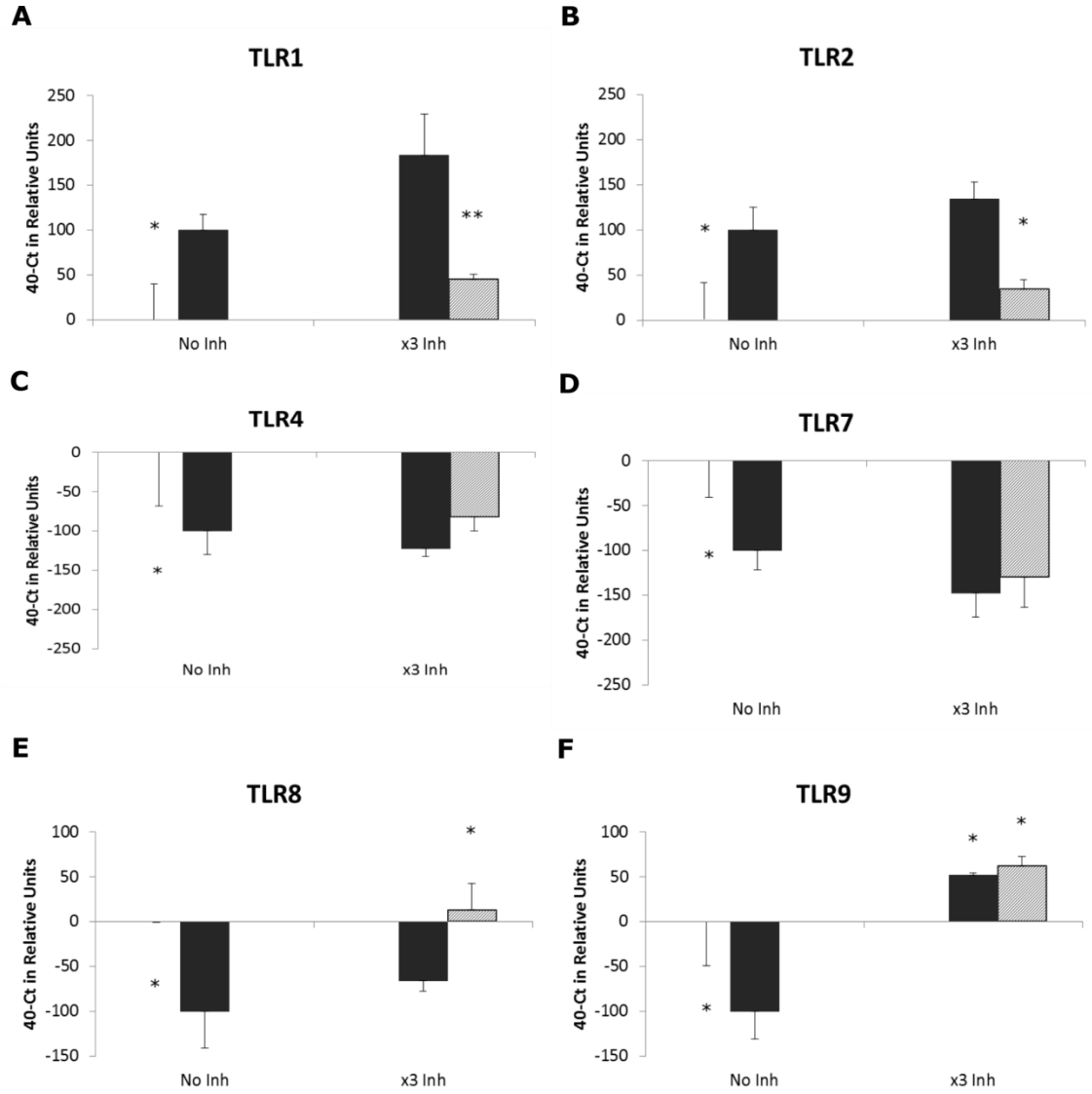


Figure 6.6: Effect of complete inhibition of MAPK signalling pathway with/without NFκB signalling on PAM-induced TLR transcriptional changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity. The clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in PAM-stimulated or PAM and MAPK inhibitors co-incubated cells; the grey bars represent TLR expression in cells treated with both MAPK and NFκB inhibitors, as well as PAM agonist. Error bars and significance levels denoted by asterisks are the same as previous figures.

pathways ($p<0.05$) and inhibition of NFκB pathway in addition to the MAPK pathways did not affect this result ($p<0.05$) (Figure 6.6F).

The effect of complete MAPK and NFκB signalling inhibition on PAM induced TLR transcriptional changes is also presented as raw 40-Ct values in Appendix Figure 9.

6.2.3 Pharmacological inhibition altered phosphorylation status of MAPK terminal cascades

PAM induced phosphorylation of MAPK signalling terminal components ERK, JNK and P38 was discussed in Chapter 5 (see section 5.2.1). In brief, all three signalling components were activated following PAM stimulation. PAM induced ERK1/2 phosphorylation was detected from 10 mins post stimulation and it only started reducing from 40 mins post agonist stimulation. Phosphorylation of JNK1/2 and P38 was detected between 10 mins and 50 mins following PAM treatment.

Results in Section 5.2.3 and 6.2.1 indicated a complex association between signalling pathways hence it was important to understand how pharmacological inhibition affects the activation of individual signalling cascades. The effect of pharmacological inhibition on the phosphorylation status of individual MAPK and NFκB signalling terminal components was assessed. In one experiment, inhibitor treatments PD059, U126, SP125, SB580 and celastrol targeting the respective signalling components ERK1/2, MEK1/2, JNK1/2, P38 and NFκB were performed in parallel. In a separate experiment, an additional inhibitor of JNK signalling, JIP-1 was treated alongside SP125. Results from the two experiments are both presented in this section.

6.2.3.1 Phosphorylation status of ERK1/2 following inhibition of MAPK or NFκB

ERK1/2 was phosphorylated at residues Thr202/Tyr204 and Thr185/Tyr187 on p44 and p42 following PAM stimulation with detectable basal phosphorylation in non-stimulated cells. Inhibition of ERK1/2 and MEK1/2 with the respective inhibitors, PD059 and U126, eliminated the phosphorylation of ERK1/2 in both inhibitor-only and PAM

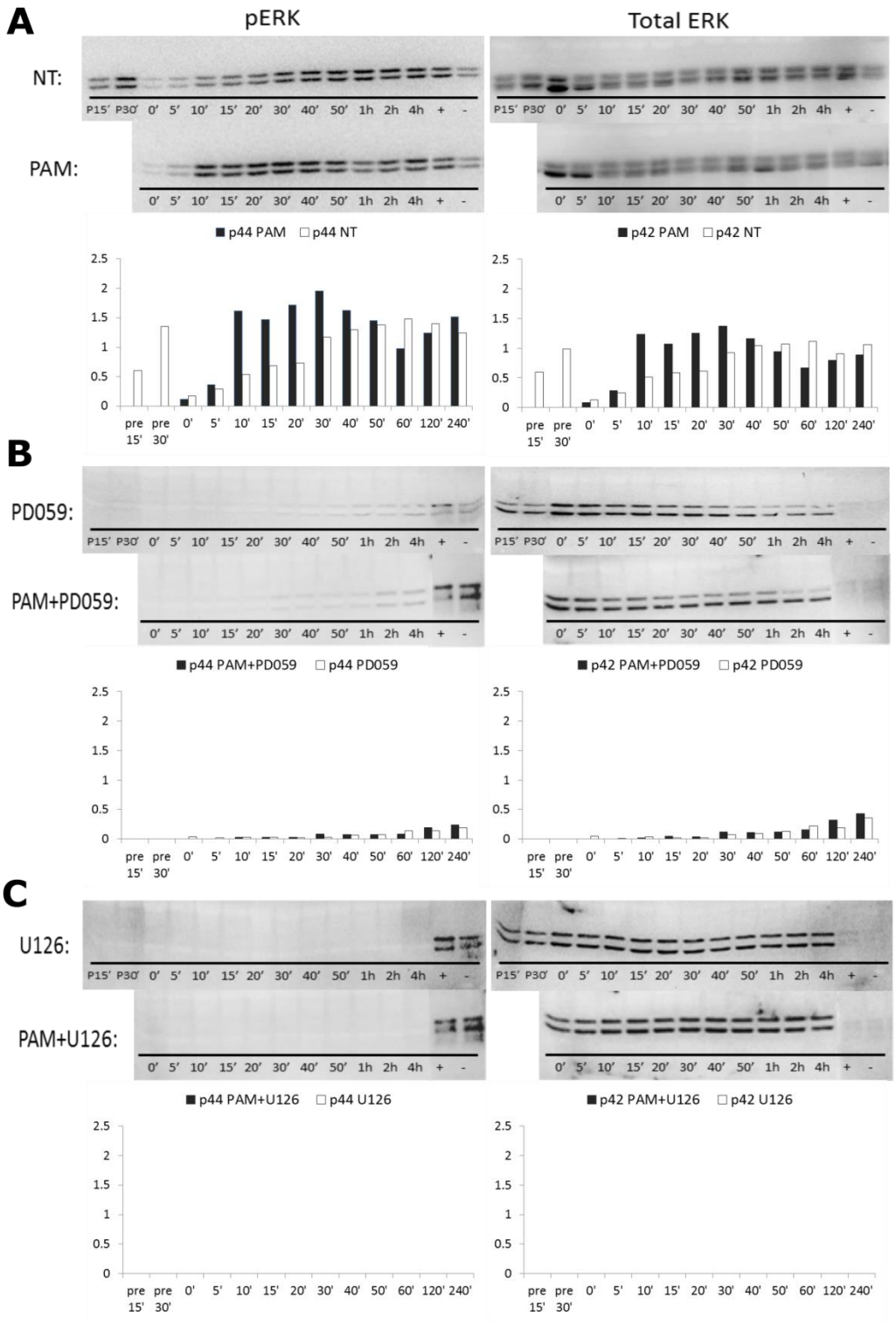


Figure 6.7: Effect of ERK and MEK inhibition on PAM-induced ERK1/2 signalling activation. Images represent the phosphor- or total- status of ERK1/2 in PAM treated (A), PAM and PD059 co-incubated (B) and PAM and U126 co-incubated cells (C). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

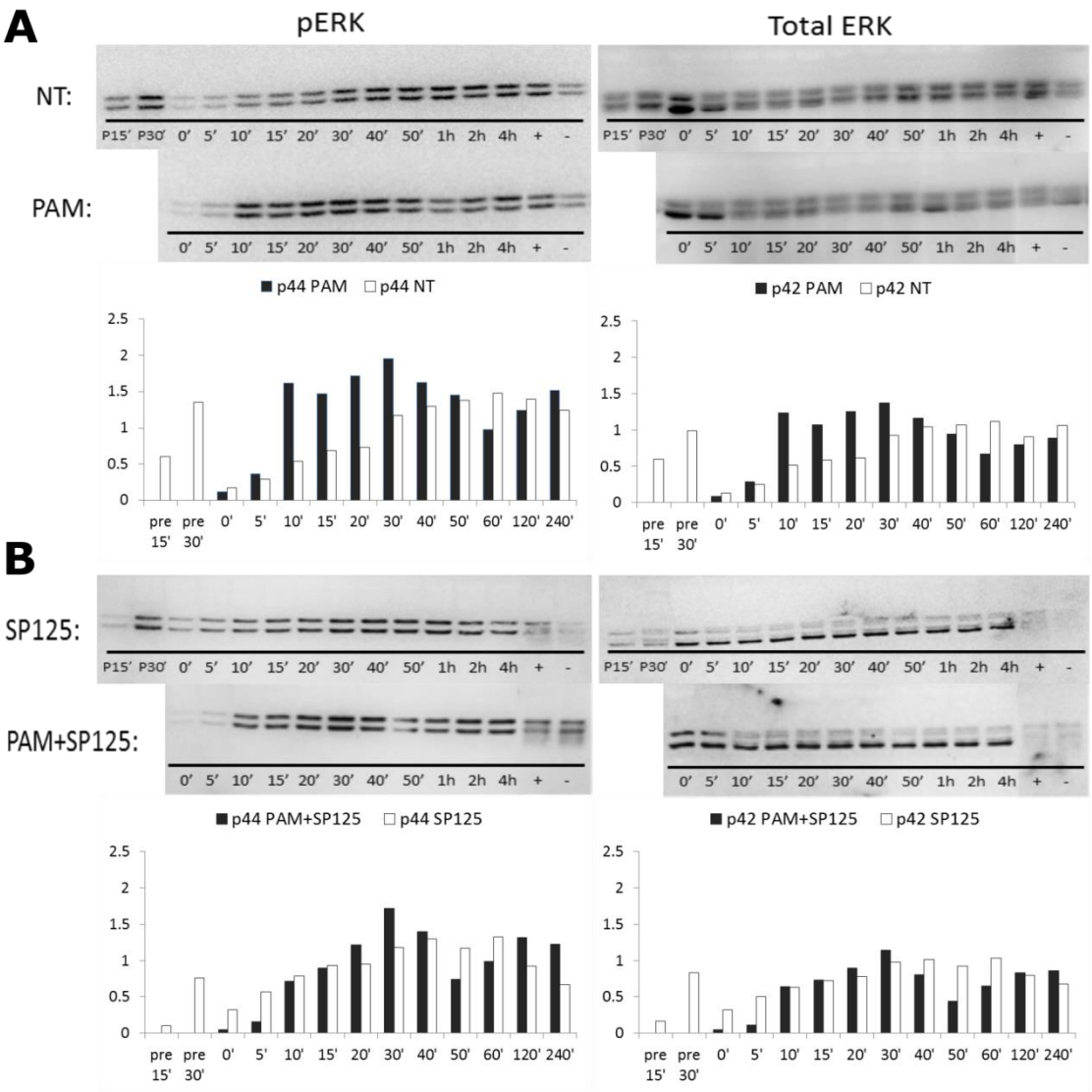


Figure 6.8: Effect of JNK inhibition on PAM-induced ERK1/2 signalling activation. Images represent the phosphor- or total- status of ERK1/2 in PAM treated (A), PAM and SP125 co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

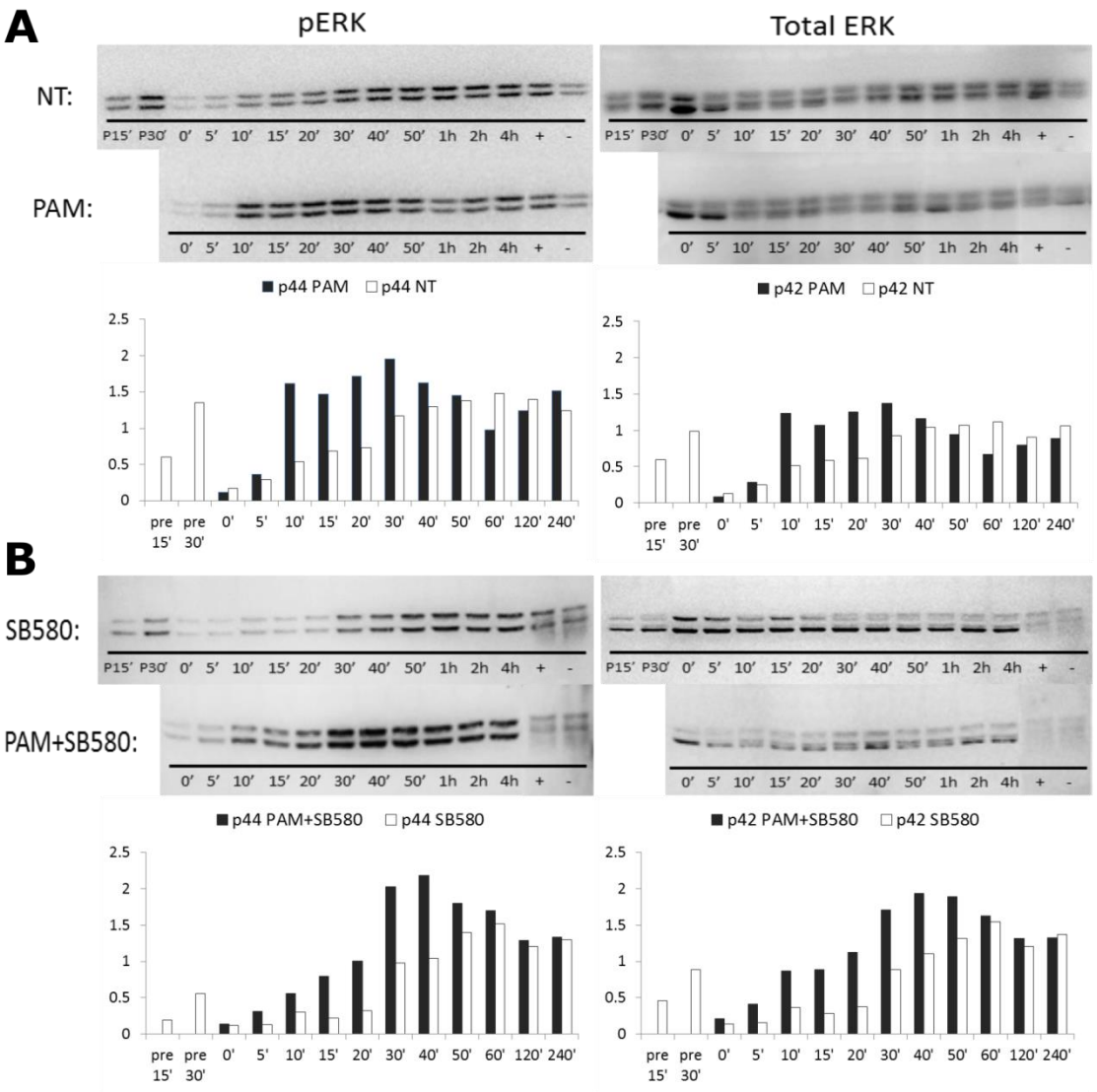


Figure 6.9: Effect of P38 inhibition on PAM-induced ERK1/2 signalling activation. Images represent the phosphor- or total- status of ERK1/2 in PAM treated (A), PAM and SB580 co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

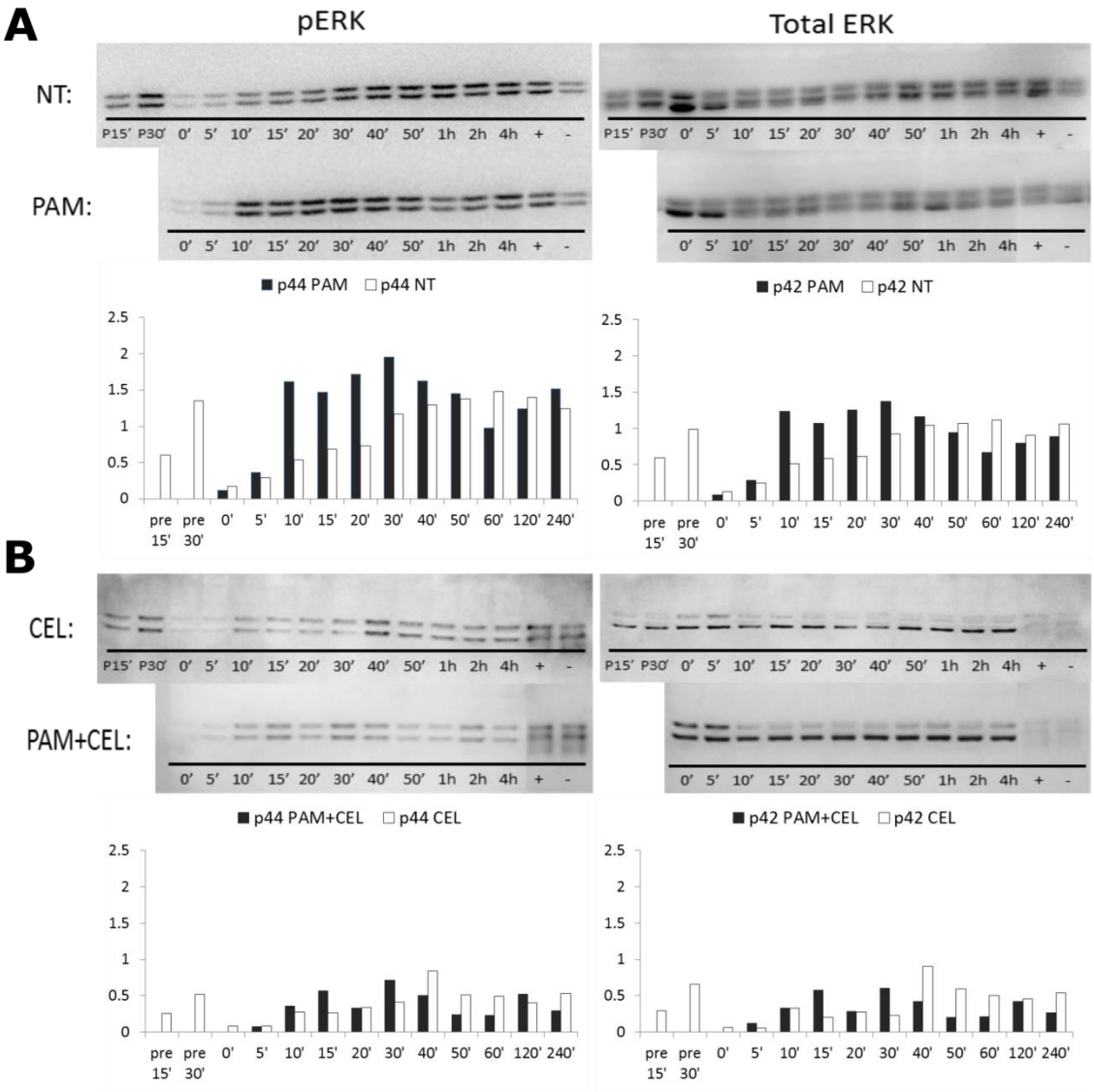


Figure 6.10: Effect of NFκB inhibition on PAM-induced ERK1/2 signalling activation. Images represent the phosphor- or total- status of ERK1/2 in PAM treated (A), PAM and celastrol co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

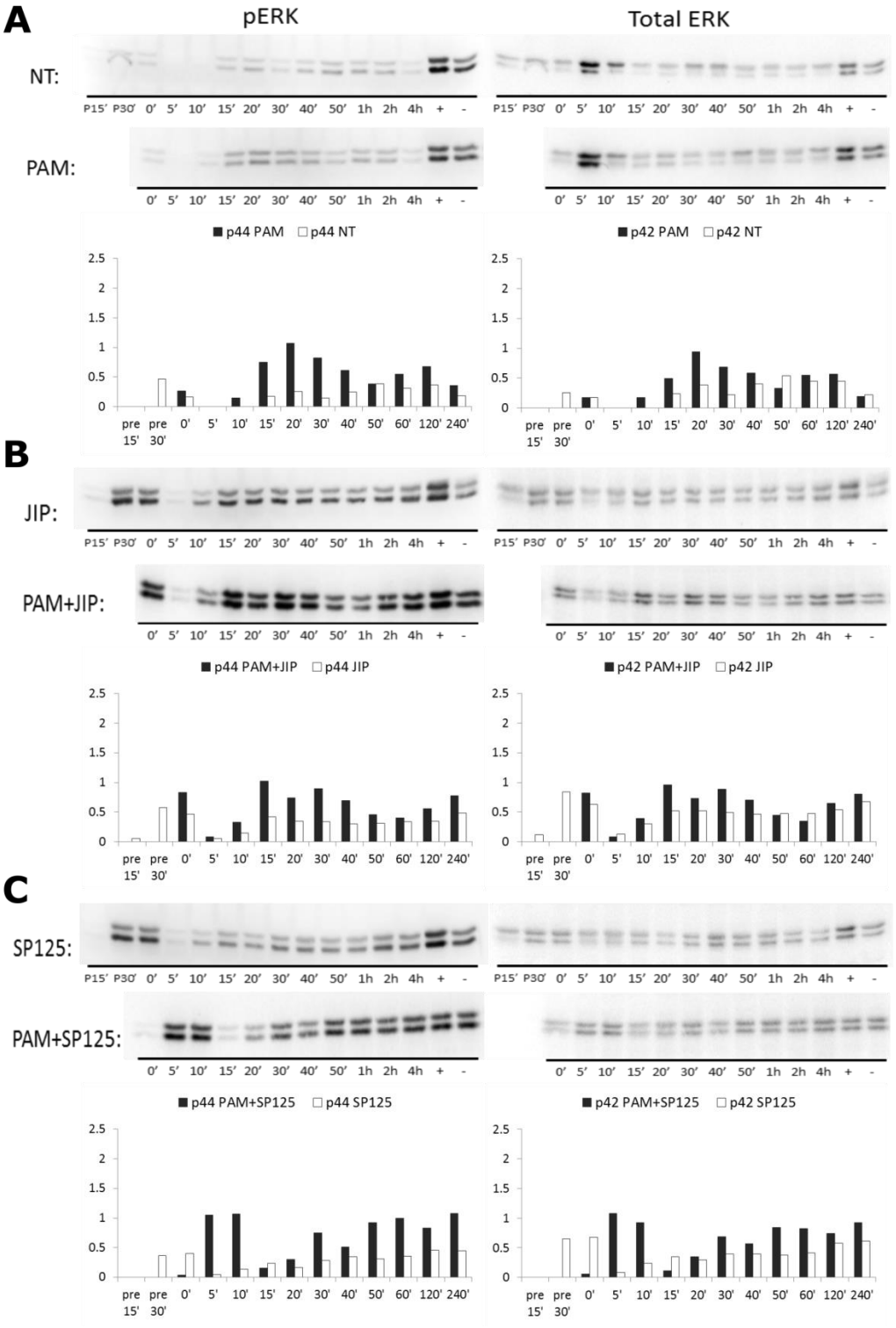


Figure 6.11: Effect of JNK inhibition on PAM-induced ERK1/2 signalling activation. Images represent the phosphor- or total- status of ERK1/2 in PAM treated (A), PAM and JIP-1 co-incubated (B) and PAM and SP125 co-incubated cells (C). Graphs represent densitometry analysis of band intensity in relation to the positive control band normalised to total ERK. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

stimulated cells (Figure 6.7B-C). Treatment with SP125 reduced the magnitude of ERK1/2 phosphorylation at early time points between 10 mins and 20 mins in PAM stimulated cells. However it did not affect the phosphorylation status of ERK1/2 in inhibitor-only treated cells at these time points. The phosphorylation level of ERK1/2 from 30 mins after PAM stimulation was also not affected by SP125 treatment (Figure 6.8B). Treatment with SB580 delayed the phosphorylation of ERK1/2 in both inhibitor-only and PAM stimulated cells. The same inhibitor treatment also increased the quantity of phosphorylated p44 and p42 protein induced by PAM stimulation at time points from 30 mins onwards. Interestingly, SB580 inhibitor treatment also affected the amount of total ERK protein present in the cells. Co-incubation with PAM and SB580 reduced the quantity of ERK2 protein whereas SB580 alone did not induce the same effect (Figure 6.9B). Celastrol treatment reduced phosphorylation of ERK1/2 in both inhibitor-only treated cells and PAM treated cells. PAM induced activation of ERK1/2 was only observed at 15 mins and 30 mins time points in PAM and celastrol co-incubated cells (Figure 6.10B).

To investigate the interactions between MAPK pathways, the effect of two JNK inhibitors on ERK1/2 phosphorylation status was compared. Activation of ERK1/2 protein was detected from 10 mins to 40 mins following PAM stimulation. Treatment with JIP-1 inhibitor did not alter phosphorylation status of ERK1/2 (Figure 6.11A). Treatment with SP125 reduced phosphorylation of ERK between 15 mins to 20 mins but increased ERK activation at later time points. Strong phosphorylation signal was also recorded in PAM and SP125 co-incubated cells at 5 mins and 10 mins time points. However, as this observation was not made in previous experiments, it is possible that the strong phosphorylation signals at these time points may be due to contamination during cell harvest (Figure 6.11B).

6.2.3.2 Phosphorylation status of JNK1/2 following inhibition of MAPK or NFκB

JNK1/2 was activated at p54 and p46 from 10 mins after PAM stimulation. Treatment with PD059 and U126 affected JNK phosphorylation pattern differently. Co-incubation of ERK inhibitor PD059 and PAM activated JNK at 0 mins and 5 mins but did not change the quantity of phospho-JNK protein induced by PAM (Figure 6.12B). On the contrary, co-incubation of the MEK inhibitor U126 with PAM reduced phosphorylation of JNK1/2 but did not alter the time points at which JNK was activated (Figure 6.12C). Treatment with SP125 reduced the quantity of phosphorylated p54 and p46 protein across all time points in PAM treated cells (Figure 6.13B). Treatment with SB580, on the other hand, increased the quantity of phosphorylated p54 and p46 protein from 10 mins time point following PAM stimulation. The same inhibitor treatment also up-regulated basal JNK phosphorylation at early time points between 0 min and 30 mins in inhibitor-only stimulated cells (Figure 6.14B). Celastrol treatment did not affect the phosphorylation status of JNK1/2 (Figure 6.15B).

When comparing the effect of different JNK inhibitors, different pattern of JNK activation was detected following stimulation of JIP-1 or SP125. Treatment with SP125 inhibited PAM induced phosphorylation of JNK1/2, which was similar to previous experiments (Figure 6.16C). Treatment with JIP-1 reduced the quantity of phosphorylated p54 and p46 protein from 20 mins after PAM stimulation but did not affect JNK phosphorylation at early time points from 0 min to 15 mins (Figure 6.16B).

6.2.3.3 Phosphorylation status of P38 following inhibition of MAPK or NFκB

MAPK signalling cascade P38 was activated from 10 mins following PAM stimulation. Treatment with U126 did not affect the quantity of phospho-P38 protein (Figure 6.17C) whereas treatment with PD059 down-regulated the phosphorylation of P38 protein in PAM treated cells (Figure 6.17B). Similar to PD059, treatment with SP125 also reduced P38 activation across all time points (Figure 6.18B). Pre-incubation of inhibitor SB580 down-regulated PAM induced P38 activation between 0 min and 40 mins but did not affect P38 activation from 50 mins onwards (Figure 6.19B). Celastrol treatment

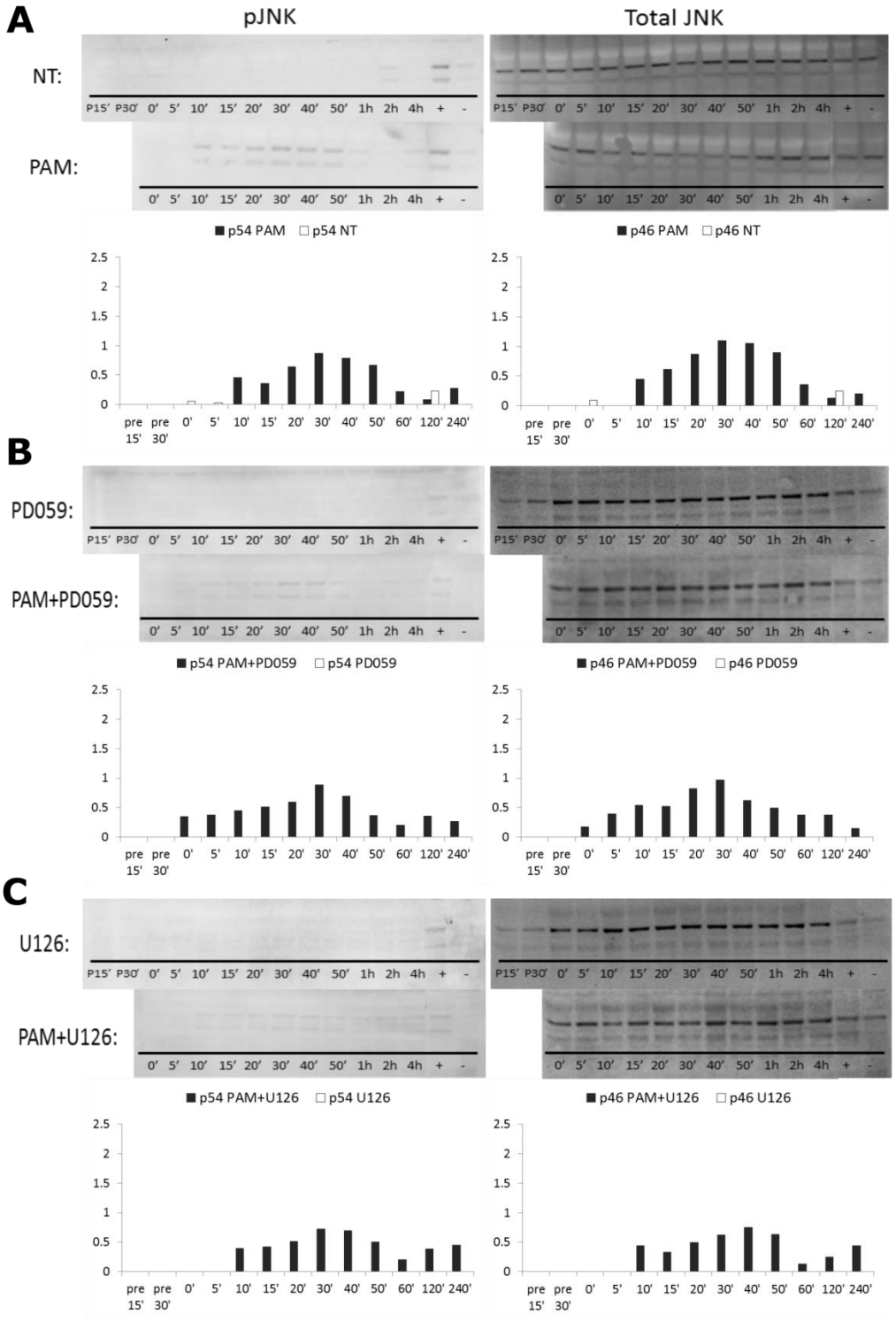


Figure 6.12: Effect of ERK and MEK inhibition on PAM-induced JNK1/2 signalling activation. Images represent the phosphor- or total- status of JNK1/2 in PAM treated (A), PAM and PD059 co-incubated (B) and PAM and U126 co-incubated cells (C). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

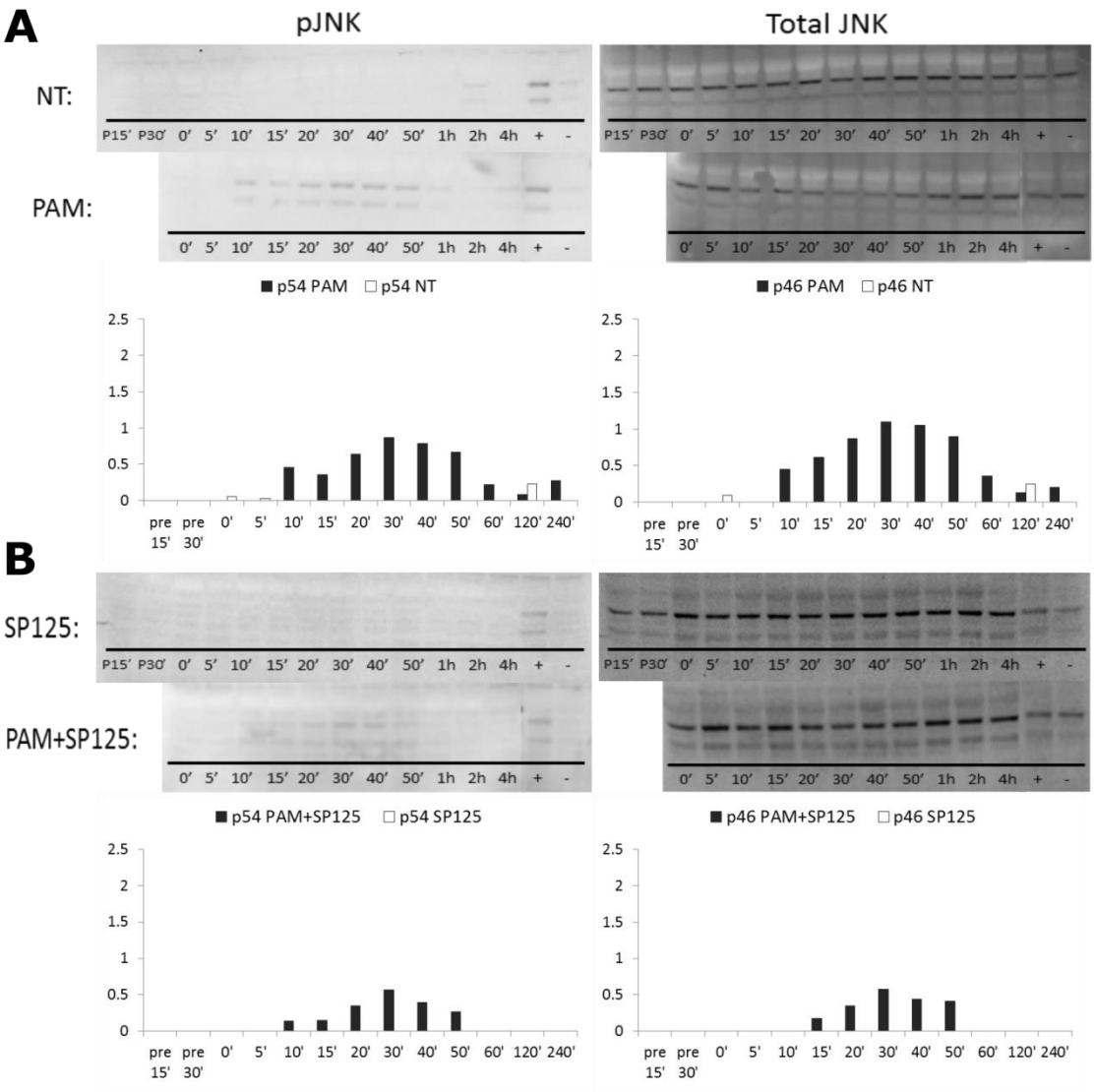


Figure 6.13: Effect of JNK inhibition on PAM-induced JNK1/2 signalling activation. Images represent the phosphor- or total- status of JNK1/2 in PAM treated (A), PAM and SP125 co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

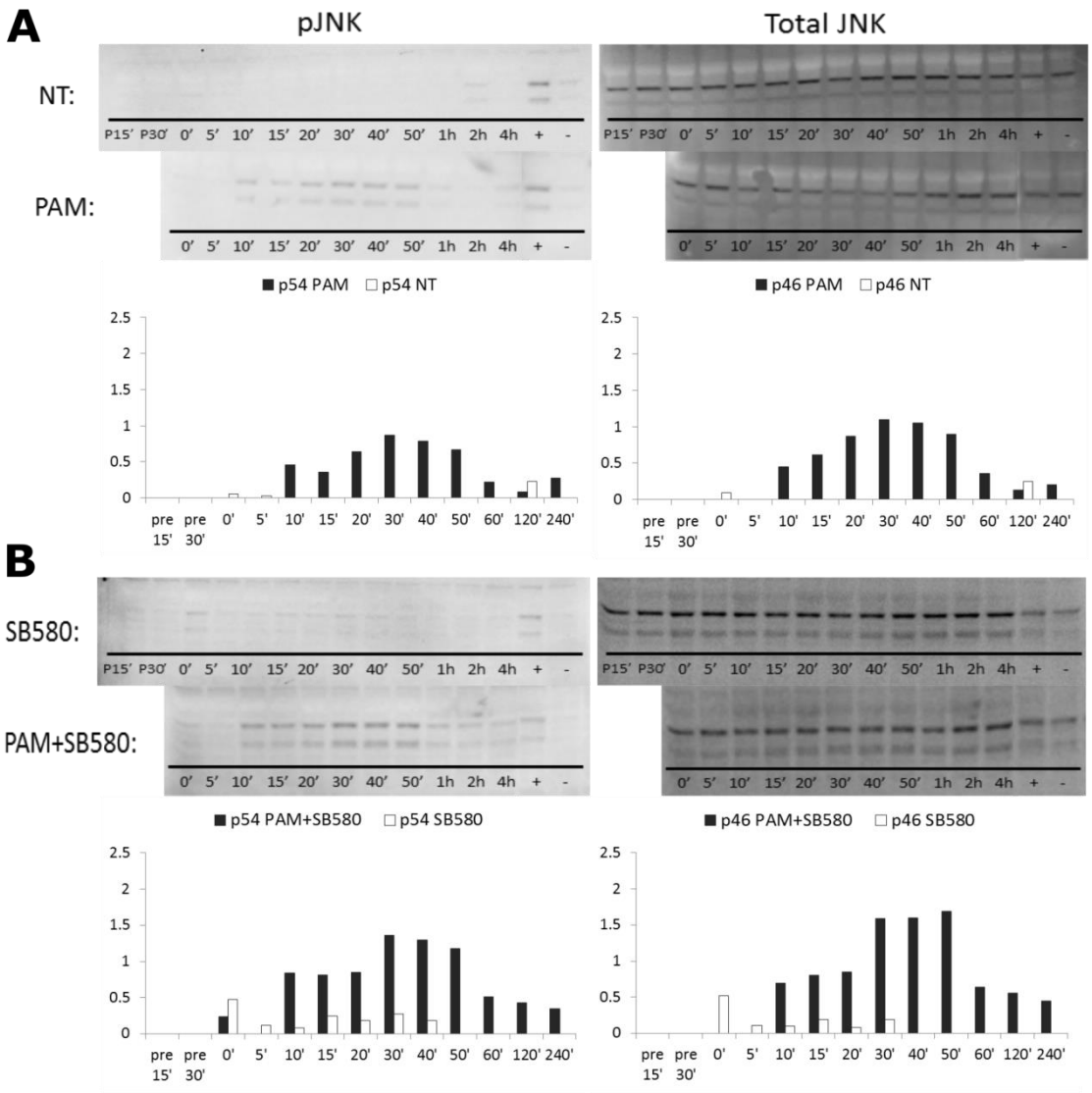


Figure 6.14: Effect of P38 inhibition on PAM-induced JNK1/2 signalling activation. Images represent the phosphor- or total- status of JNK1/2 in PAM treated (A), PAM and SB580 co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

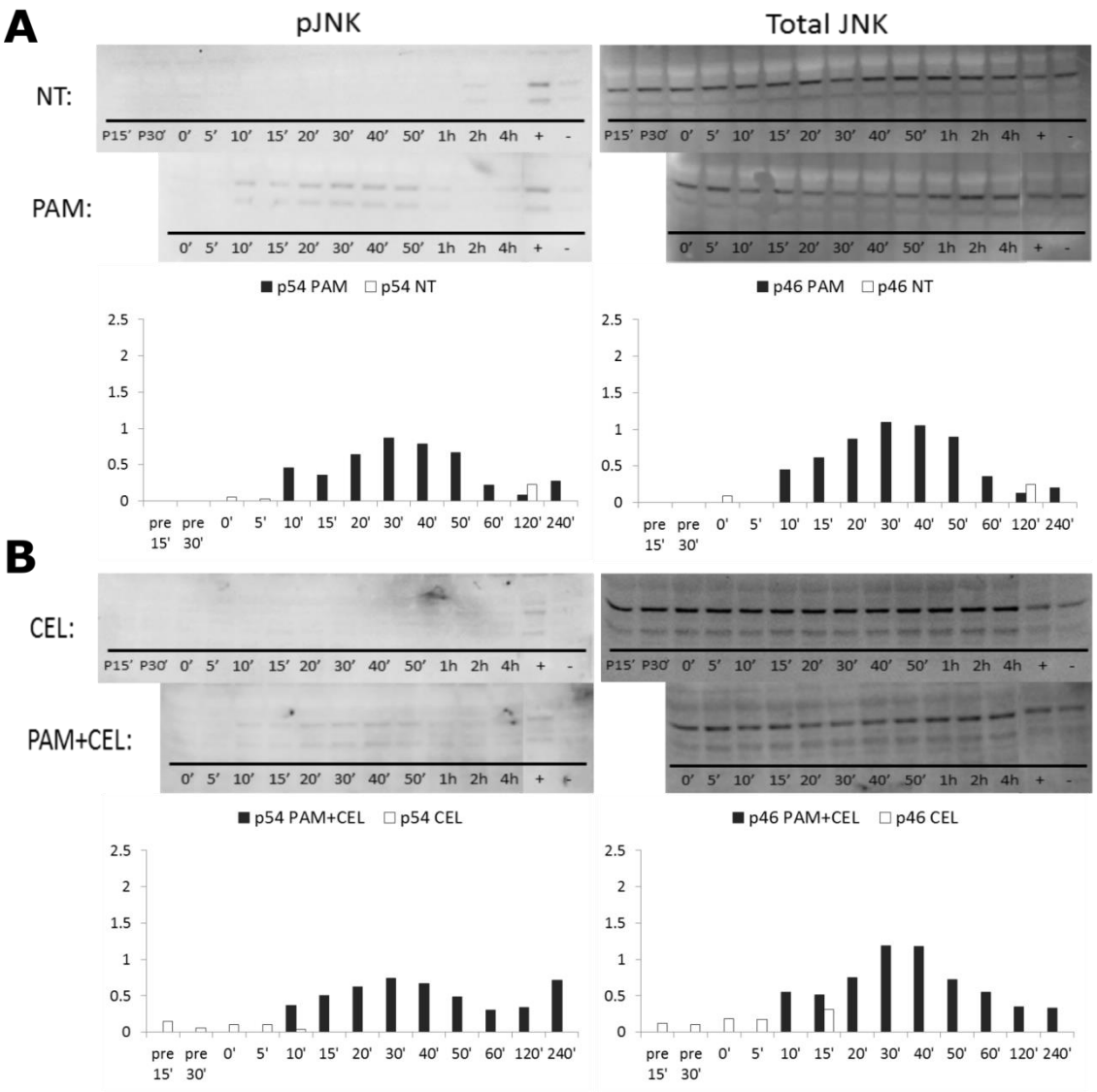


Figure 6.15: Effect of NFκB inhibition on PAM-induced JNK1/2 signalling activation. Images represent the phosphor- or total- status of JNK1/2 in PAM treated (A), PAM and celastrol co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

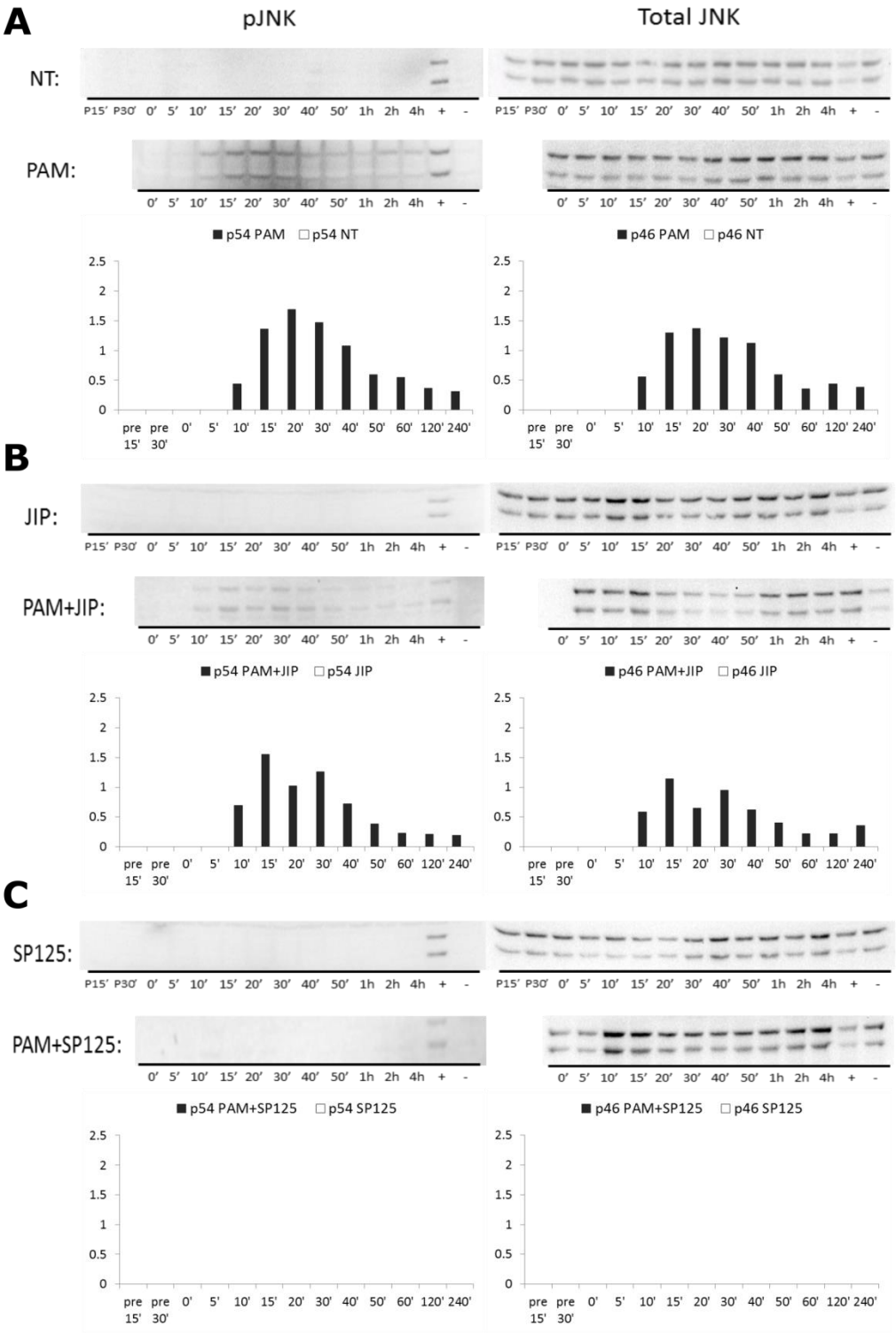


Figure 6.16: Effect of JNK inhibition on PAM-induced JNK1/2 signalling activation. Images represent the phosphor- or total- status of JNK1/2 in PAM treated (A), PAM and JIP-1 co-incubated (B) and PAM and SP125 co-incubated cells (C). Graphs represent densitometry analysis of band intensity in relation to the positive control band normalised to total ERK. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

did not alter the phosphorylation status of P38 in inhibitor-only or PAM stimulated cells (Figure 6.20B).

In a separate experiment, PAM induced P38 activation was observed from 15 mins post agonist stimulation, with the quantity of phospho-P38 peaked between 15 mins and 30 min time points (Figure 6.21A). SP125 inhibitor treatment reduced phosphorylation of P38 protein in PAM stimulated cells, especially between 15 mins and 30 min time points (Figure 6.21C). This observation was consistent with the previous experiment (Figure 6.18B). In contrast, JIP-1 inhibitor treatment increased the quantity of phospho-P38 protein following PAM stimulation. In addition, JIP-1 treatment extended PAM induced P38 activation from 15 mins to 30 mins to 10 mins to 40 mins post agonist stimulation (Figure 6.21B).

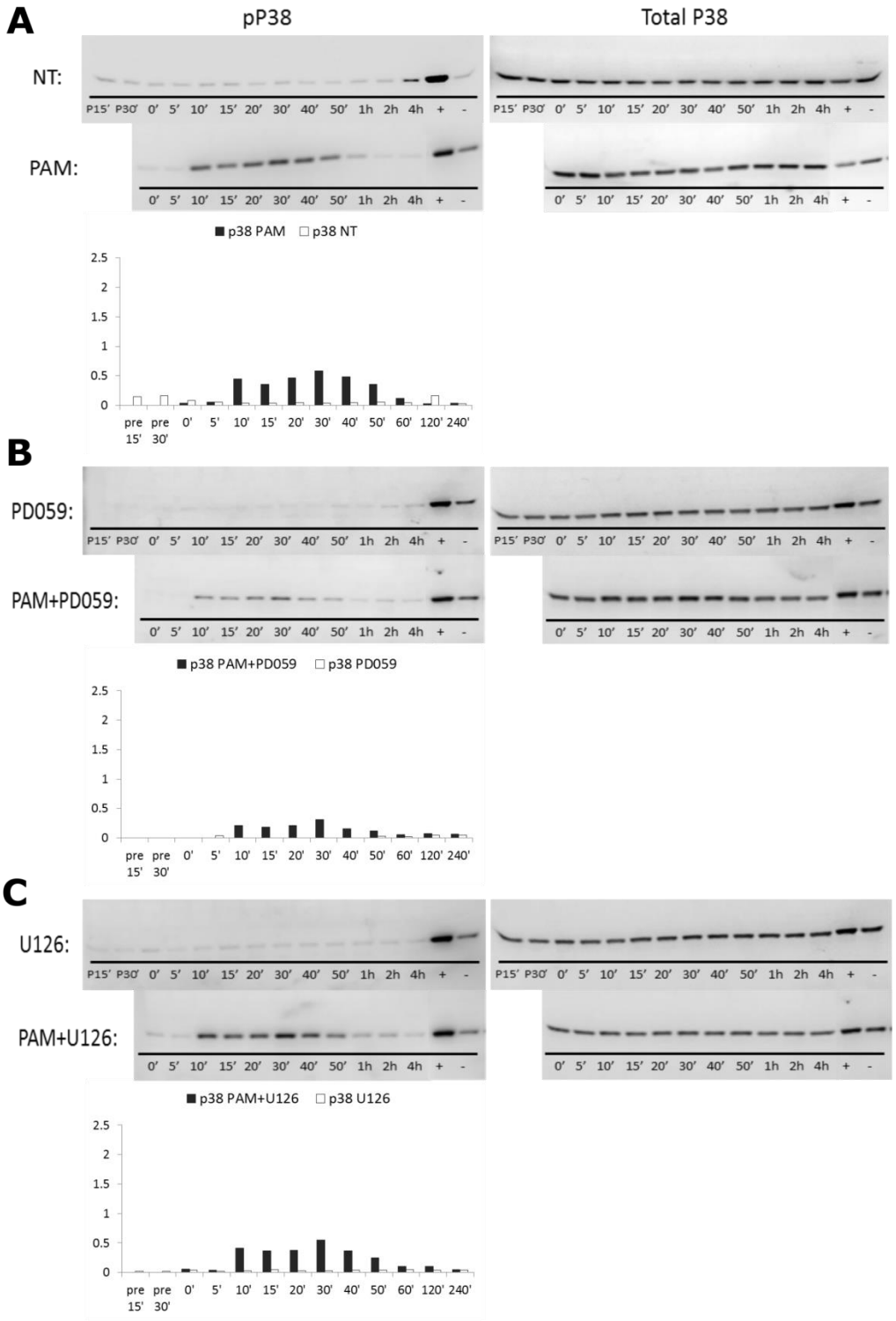


Figure 6.17: Effect of ERK and MEK inhibition on PAM-induced P38 signalling activation. Images represent the phosphor- or total- status of P38 in PAM treated (A), PAM and PD059 co-incubated (B) and PAM and U126 co-incubated cells (C). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

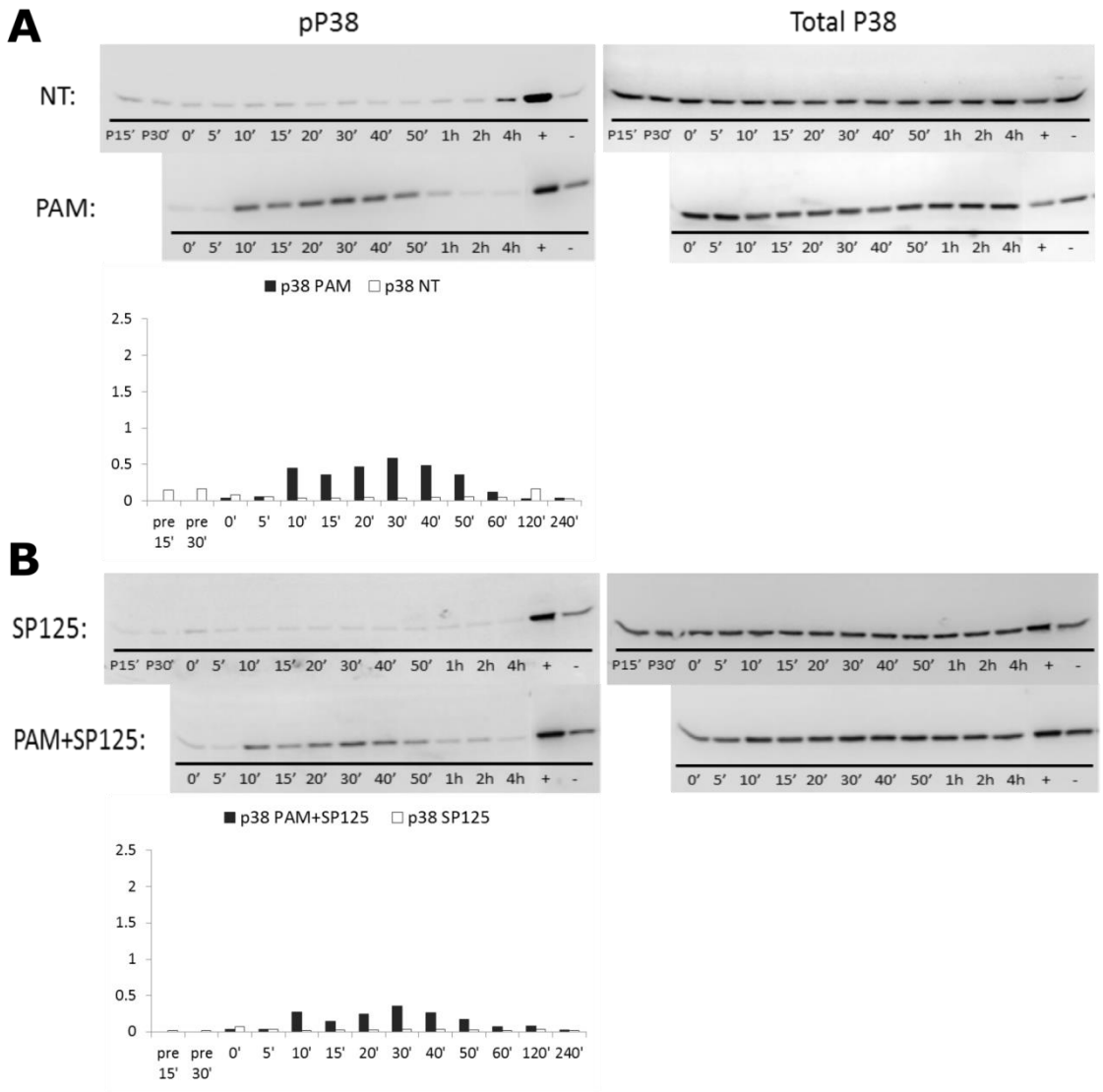


Figure 6.18: Effect of JNK inhibition on PAM-induced P38 signalling activation. Images represent the phosphor- or total- status of P38 in PAM treated (A), PAM and SP125 co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

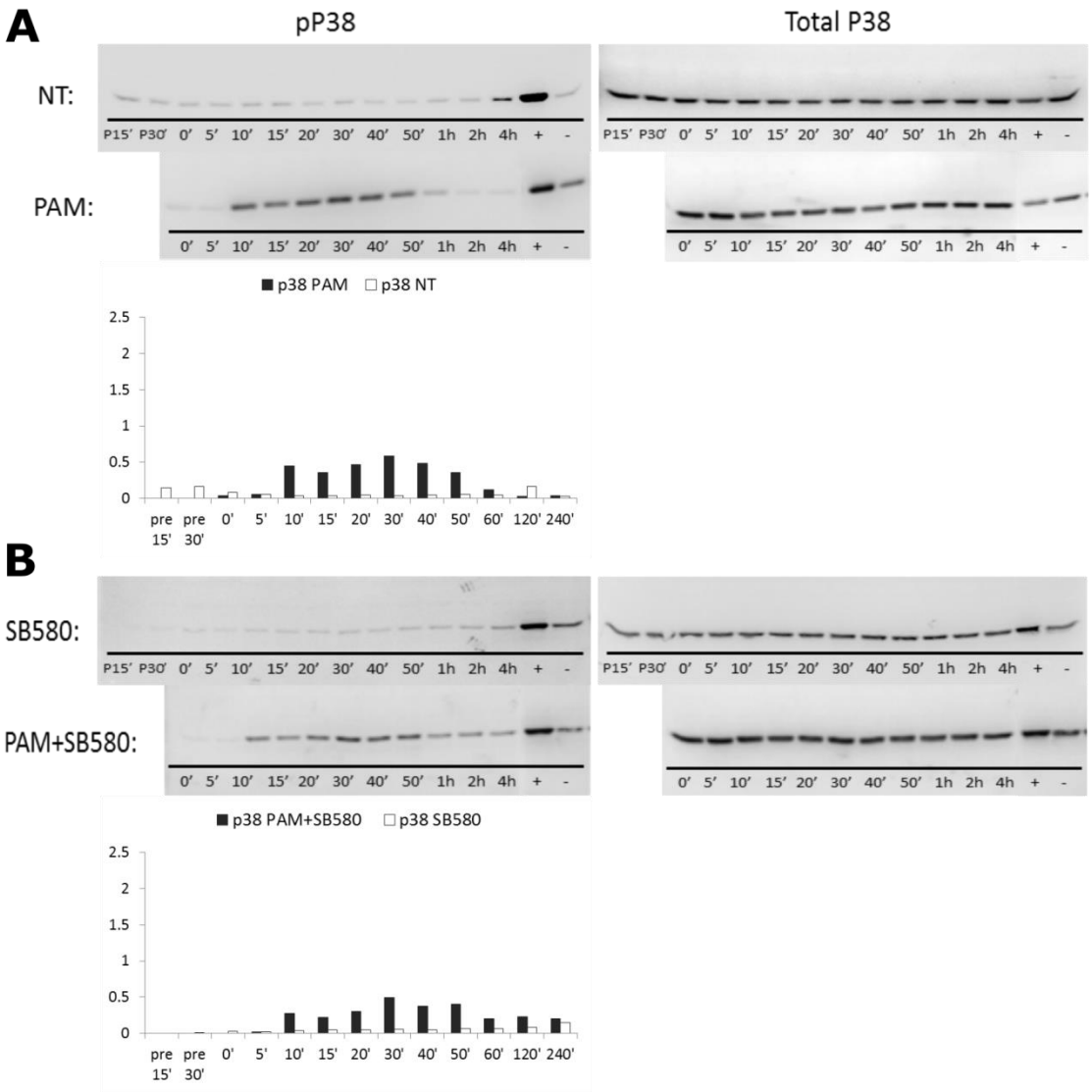


Figure 6.19: Effect of P38 inhibition on PAM-induced P38 signalling activation. Images represent the phosphor- or total- status of P38 in PAM treated (A), PAM and SB580 co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

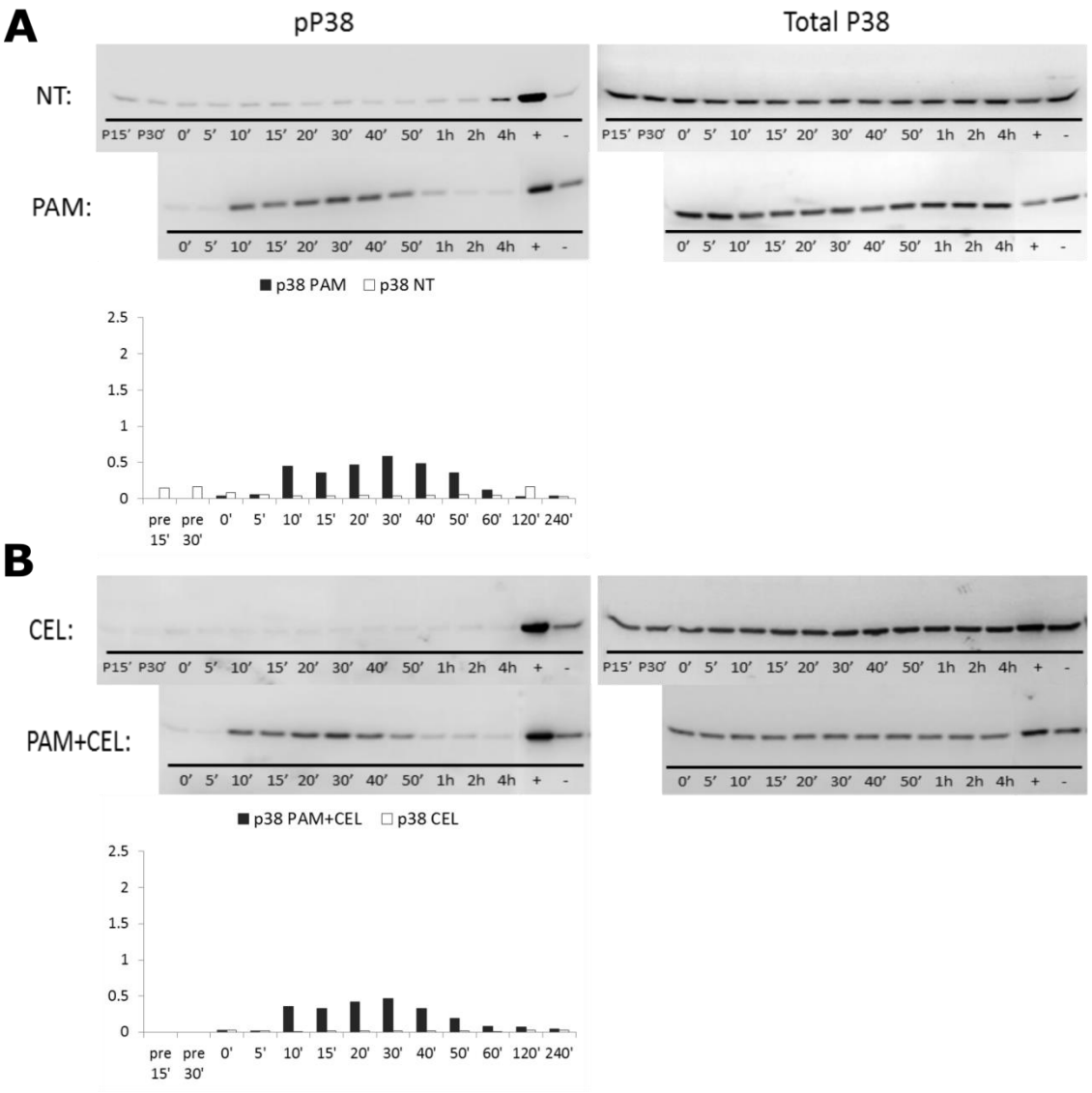


Figure 6.20: Effect of NFκB inhibition on PAM-induced P38 signalling activation. Images represent the phosphor- or total- status of P38 in PAM treated (A), PAM and celastrol co-incubated cells (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

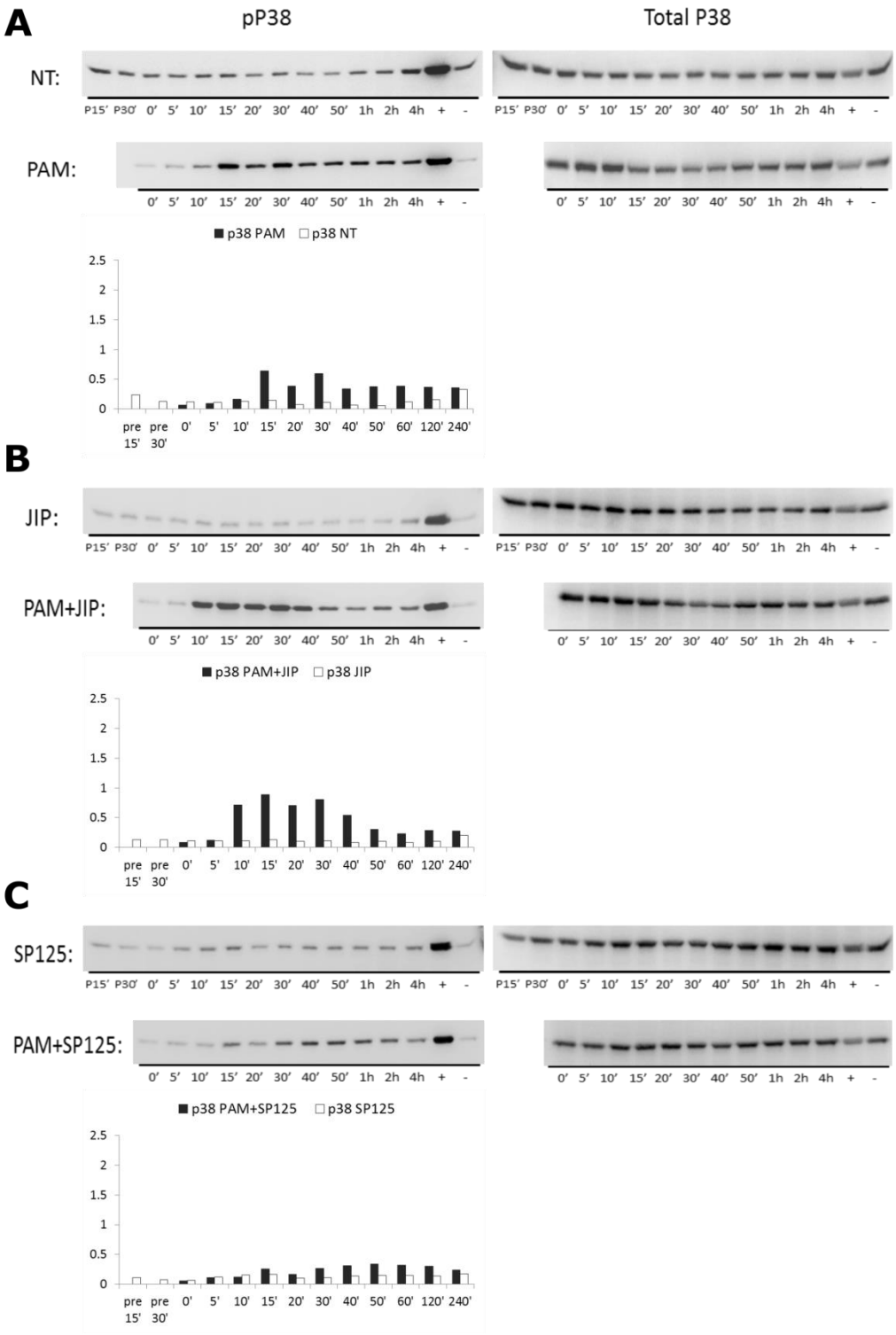


Figure 6.21: Effect of JNK inhibition on PAM-induced P38 signalling activation. Images represent the phosphor- or total- status of P38 in PAM treated (A), PAM and JIP-1 co-incubated (B) and PAM and SP125 co-incubated cells (C). Graphs represent densitometry analysis of band intensity in relation to the positive control band normalised to total ERK. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphorylation status in non-stimulated cells or inhibitor-only treated cells; the black bars represent phosphorylation status in PAM-stimulated cells or PAM and inhibitor co-incubated cells.

6.3 Discussion

Activation of TLRs results in the recruitment of downstream signalling pathways. The main signalling pathways involve terminal signalling elements, ERK, JNK, P38 and NFκB. Some studies have identified synergy between these signalling cascades with other signalling pathways, for example, the PI3K/Akt pathway and the JAK/STAT pathway. Interactive pathways may have positive or inhibitory effects to one another. However, signalling interactions between MAPK and NFκB pathways directly downstream TLRs have been largely neglected. Experiments in this chapter systemically addressed the synergy between TLR signalling pathways and the contribution of pathway interactions to PAM induced transcriptional regulation of TLRs. To explore these interactions, single, double, triple and quadruple inhibitor combinations were performed on untreated and PAM treated macrophages.

Previous studies have supported the central role of MAPK and NFκB in mediating immune responses (O'Neill, Golenbock et al. 2013, Peroval, Boyd et al. 2013, Huang, Shi et al. 2009, O'Neill, Bowie 2007, Roux, Blenis 2004). Results from my experiments not only confirmed this finding, but also advanced the understanding of signalling interactions between the MAPK and NFκB pathways. In this discussion, I take into account both the individual signalling effects (chapter 5) and the synergetic signalling effects (chapter 6) to analyse the signalling networks used for the transcriptional regulation for each TLRs.

Inhibition of dual MAPK signalling terminal components and complete MAPK pathway up-regulated TLR1 mRNA levels in cells treated with and without PAM, suggesting combinations of MAPK signalling cascades are responsible for maintaining the basal expression of TLR1 (Table 6.22A). Although a trend of up-regulation was detected, inhibition of individual MAPK pathway components did not affect TLR1 basal mRNA level (Figure 5.7A), suggesting an additive effect of multiple MAPK signalling cascades on TLR1. Inhibition of both MAPK and NFκB pathways suppressed PAM induced TLR1

A

Multiple Inhibitor Effect on Basal TLR mRNA levels (compared with to resting level)								
Inhibitor	Multiple MAPK Inhibitors				MAPK and NFκB Inhibitors			
	PD059+SP125	PD059+SB580	SP125+SB580	x3 Inhs	PD059+Cel	SP125+Cel	SB580+Cel	x4 Inhs
Target	ERK+JNK	ERK+P38	JNK+P38	MAPK	ERK+NFκB	JNK+NFκB	P38+NFκB	MAPK+NFκB
TLR1	↑	↑	↑	↑	ND	ND	ND	ND
TLR2	—	—	—	—	ND	ND	ND	ND
TLR4	—	↓	↓	↓	ND	ND	ND	ND
TLR7	—	—	—	—	ND	ND	ND	ND
TLR8	↑	—	—	—	ND	ND	ND	ND
TLR9	—	—	—	—	ND	ND	ND	ND

B

Multiple Inhibitor Effect on PAM-induced TLR mRNA levels (compared to PAM-induced level)								
Inhibitor	Multiple MAPK Inhibitors				MAPK and NFκB Inhibitors			
	PD059+SP125	PD059+SB580	SP125+SB580	x3 Inhs	PD059+Cel	SP125+Cel	SB580+Cel	x4 Inhs
Target	ERK+JNK	ERK+P38	JNK+P38	MAPK	ERK+NFκB	JNK+NFκB	P38+NFκB	MAPK+NFκB
TLR1	↑	↑	↑	↑	↓	↓	↓	↓
TLR2	—	—	↓	—	↓	↓	↓	↓
TLR4	—	↓	↓	↓	—	—	—	—
TLR7	—	—	—	—	—	—	—	—
TLR8	↑	—	↑	↑	↑	↑	↑	↑
TLR9	—	—	—	—	—	—	—	↑

C

Magnitude Differences of Inhibitor Effect on PAM-induced TLR mRNA levels								
Inhibitor	Multiple MAPK Inhibitors				MAPK and NFκB Inhibitors			
	PD059+SP125	PD059+SB580	SP125+SB580	x3 Inhs	PD059+Cel	SP125+Cel	SB580+Cel	x4 Inhs
Target	ERK+JNK	ERK+P38	JNK+P38	MAPK	ERK+NFκB	JNK+NFκB	P38+NFκB	MAPK+NFκB
TLR1	—	—	—	—	ND	ND	ND	ND
TLR2	—	—	—	—	ND	ND	ND	ND
TLR4	—	—	—	—	ND	ND	ND	ND
TLR7	—	—	—	—	ND	ND	ND	ND
TLR8	—	—	—	—	ND	ND	ND	ND
TLR9	—	—	—	—	ND	ND	ND	ND

Table 6.22: Synergy between multiple signalling pathways which mediates PAM induced TLRs responses in RAW cells. Red arrow indicates statistically significant up-regulation ($p<0.05$); blue arrow indicates significant down-regulation ($p<0.05$); yellow lines indicates no significant changes of TLR mRNA levels compared to (A) non-stimulated cells; (B) PAM treated cells; (C) Magnitude differences between PAM induced and basal mRNA levels.

up-regulation (Table 6.22B). The effect of inhibiting individual or triple MAPK signalling cascades was overpowered by effect of inhibiting the NFκB pathway, suggesting that PAM induced TLR1 transcriptional levels are balanced by the MAPK and NFκB signalling.

Inhibition of multiple MAPK signalling cascades did not affect the basal expression of TLR2 in inhibitor-only treated cells. PAM induced TLR2 up-regulation was substantially reduced by inhibition of JNK and P38 (Table 6.22B), suggesting the effect of JNK and P38 in mediating PAM induced TLR transcriptional regulation. In addition, inhibition of both MAPK and NFκB pathways also supressed PAM induced TLR2 up-regulation (Table 6.22B), representing the effect of inhibiting NFκB alone (Figure 5.7B). On the contrary, inhibition of individual MAPK signalling cascade, ERK, up-regulated TLR2 transcript level in PAM treated cells (Figure 5.7B). Altogether these results suggest that JNK, P38 and NFκB pathways are responsible for mediating PAM induced TLR2 transcriptional regulation while ERK signalling is responsible for maintaining basal expression of TLR2 in PAM treated cells.

Inhibition of dual MAPK signalling cascades, ERK and P38, JNK and P38 as well as inhibition of all MAPK signalling components, reduced TLR4 mRNA level in cells treated with and without PAM (Table 6.22A-B). This result indicated the additive effect of inhibiting individual MAPK signalling cascades (Figure 5.7B), suggesting that the MAPK pathway is important for the maintenance of TLR4 basal expression. Inhibition of NFκB signalling rescued PAM induced TLR4 regulation (Figure 5.7B), suggesting that the NFκB pathway is responsible for mediating TLR4 down-regulation in PAM treated cells. Simultaneous inhibition of MAPK and NFκB pathways did not affect TLR4 mRNA level (Table 6.22B) due to the balancing effect between the two pathways.

Results in chapter 5 and chapter 6 both indicated that transcriptional regulation of TLR7 in RAW cells was not affected by inhibition of individual or combinations of MAPK and NFκB pathways (Figure 5.7, Table 6.22). Cross-talk between TLR2 and

TLR7 has previously been observed however direct signalling responsible for this cross-regulation was not addressed (Liu, Simmons et al. 2012, Wenink, Santegoets et al. 2009). Inhibitors for upstream signalling components, such as MEK1/2, MEK4/7 and MEK3/6, may be used to identify the signalling pathway responsible for regulating TLR7 mRNA level in RAW cells.

Inhibition of ERK and JNK up-regulated TLR8 transcript level in inhibitor-only treated cells and PAM treated cells (Table 6.22A-B). Similar to this result, inhibition of JNK and P38 as well as inhibition of all MAPK components also increased TLR8 mRNA level in PAM treated cells (Table 6.22B). These results indicated the additive effects of MAPK pathway when compared with results for inhibiting each MAPK signalling cascades and suggested the combination of MAPK signalling components, JNK and P38 are responsible for down-regulating TLR8 mRNA level in PAM treated cells whereas P38 alone does not contribute to the regulation of TLR8 transcription. Inhibition of NFκB alone further down-regulated TLR8 mRNA level in PAM treated cells (Figure 5.7). However the effect of NFκB on TLR8 transcriptional regulation was overpowered by the effect of MAPK signalling when both MAPK and NFκB pathways were inhibited.

Inhibition of multiple MAPK pathways or inhibition of individual MAPK signalling cascades with NFκB signalling both did not affect the transcriptional regulation of TLR9 (Table 6.22A-B). This may be due to differential effects of MAPK and NFκB pathways for TLR9 regulation. Both pathways indicated trends for influencing the basal expression of TLR9 in inhibitor-only treated cells, at the same time, contributed to the down-regulation of TLR9 in PAM treated cells (Figure 5.7A-B). Complete inhibition of MAPK with NFκB pathways rescued PAM induced TLR9 down-regulation (Table 6.22B), which is similar to the effect of inhibiting NFκB pathway alone.

Inhibition of dual MAPK signalling terminal components, ERK and JNK, ERK and P38, JNK and P38 or inhibition of all three MAPK pathways did not affect the magnitude of PAM induced TLR transcriptional changes (Table 6.22C). This was due to the effect of

inhibiting multiple MAPK signalling cascades on TLR mRNA levels in non-agonist treated cells.

Signalling cross-talk between MAPK and NFκB pathways is complex, with a unique signalling pattern mediating transcriptional regulation for each TLR. This may be due to differential effects of individual pathway inhibition on the signalling network (Wagner, Nebreda 2009, Trinchieri, Sher 2007, Shen, Godlewski et al. 2003, Zhang, Liu 2002). Signal flow from a supressed pathway may be redirected through a different pathway via common upstream mediators. For instance, treatment with SB203580 increased phosphorylation of ERK and treatment with PD98059 increased phosphorylation of P38 during differentiation of human osteosarcoma cells (Shimo, Matsumura et al. 2007). This redirected phospho signal may be mediated via the common upstream cascade MEKK3 (Kyriakis, Avruch 2001). In addition, inhibition of one pathway may cause suppression of negative regulators in a different pathway. For instance, inhibition of ERK reduced the production of DUSP which negatively regulates JNK. Similarly, inhibition of P38 reduced the production of PP1 or PP2A phosphatases which inhibits ERK activity (Fey, Croucher et al. 2012). Furthermore, inhibition of ERK signalling with U126 in LPS stimulated cells reduced the secretion of NFκB inhibitory protein IκBα (Aga, Watters et al. 2004). However, these signalling interactions may be cell type specific or agonist specific. Hence, the phosphorylation status of all MAPK signalling terminal cascades were assessed to determine the effect of pharmacological inhibition on PAM induced phosphorylation status of signalling components other than their supposed target (Table 6.23).

All inhibitor treatments substantially down-regulated the phosphorylation level of their respective target signalling cascades. However, inhibition of ERK and MEK affected phospho status of JNK and P38 different. MEK inhibition reduced phosphorylation of JNK protein but did not affect P38 protein. Inhibition of ERK reduced phosphorylation

Inhibitor Effect on Phosphor Status of MAPK Signalling Cascades						
Inhibitor	U126	PD98059	SP600125	JIP-1	SB203580	Celastrol
Target	MEK	ERK	JNK		P38	NFκB
pERK				-	>	
pJNK		<				-
pP38	-			<->		-

Table 6.23: Effect of MAPK and NFκB signalling inhibition on phosphorylation status of each MAPK signalling cascades. Red highlight colour indicates increased phosphor signal; blue highlight colour indicates reduced phosphor signal; the forward arrow (>) indicates a delaying effect; the backward arrow (<) indicates an advancing effect; two sided arrows (<->) indicates prolonged effect at both earlier and later time points; single line (-) indicates no effect.

level of P38 protein as well as accelerated PAM induced JNK phosphorylation to an earlier time point.

The two JNK inhibitors, SP125 and JIP-1 affected MAPK signalling cascades differently. SP125 stimulation reduced phosphorylation level of ERK and P38. Treatment with JIP1 increased the phosphorylation level of P38 as well as prolonged its phosphorylation but did not affect phospho ERK protein. The differential effect between the two JNK inhibitors is likely due to their binding characteristics. SP125 is a reversible inhibitor for competitive ATP-binding whereas JIP-1 inhibitor directly targets at the JNK-binding domain (JBD) which is more specific to JNK protein (Tanemura, Momose et al. 2009, Uchida, Gee et al. 2008, Stebbins, De et al. 2008, Tanoue, Adachi et al. 2000). In addition, although both MKK4 and MKK7 are upstream of JNK, JNK interacting protein (JIP) only binds to MKK7 and JNK, but does not interact with MKK4(Coffey, Hongisto et al. 2000, Whitmarsh, Davis 1998, Dickens, Rogers et al. 1997). Together, these findings suggest that JNK activity may be channelled towards alternative kinases that interact with JNK via motif other than JBD/MKK7 when JNK is inhibited at JBD site by JIP-1 inhibitor.

Inhibition of P38 increased phosphorylation of ERK and JNK protein, as well as extended the phosphorylation period of ERK protein. The observation of P38 signalling supress phospho ERK activation was also reported by Shimo (2007) in human bone cancer cells. In addition, inhibition of P38 increased phospho-c-Jun expression was detected following LPS treatment in RAW cells (Supplemental Data Figure S5) (Xu, Jagannath et al. 2007). Inhibition of NFκB pathway reduced phosphorylation of ERK but did not affect phospho JNK or phospho P38 protein in PAM stimulated RAW cells.

The effect of pharmacological inhibitions on the phosphorylation status of the non-targeting MAPK signalling cascades may reflect the complex interactions among these pathways. Apart from redirection of signal flow and suppression of signalling negative regulators, pharmacological inhibition may also induce non-specific knock down of a

variety of other kinase proteins, known as the “off-target” effect. These “off-target” effects are caused by binding of a competitive inhibitor to a common structure on kinase proteins, such as the ATP-binding sites, which lacks structural specificity. The “off-target” side effects may be reduced by replacing the competitive inhibitors with chemical inhibitors targeting the specific allosteric domains of the proteins (Tanemura, Momose et al. 2009). As almost all pharmacological inhibitors used in the studies presented in chapter 5 and 6 were competitive inhibitors (except JIP-1), results from these chapters must be validated with alternative means of signalling inhibition, for instance, siRNA gene silencing. This is discussed in detail in the next chapter.

Chapter 7 Inhibition of MAPK signalling pathways with small interfering RNAs (siRNAs)

7.1 Introduction

Three of the terminal MAPK signalling cascades are responsible for regulating TLR transcription: ERK, JNK and P38. The ERK family consists mainly of ERK1/2 and ERK5. ERK1/2 but not ERK5 was recognised as the classical signalling regulator for TLR mediated immune responses since the 1990s even though ERK5 was thought to activate transcription factors involved in immune functioning, such as AP-1 and c-JUN (Morrison 2012, Roux, Blenis 2004, Chen, Gibson et al. 2001, Kyriakis, Avruch 2001). However, it was recently discovered that ERK5 is responsible for regulating TLR2- and TLR4-dependent cytokine production in human and mouse monocytes and endothelial cells (Wilhelmsen, Xu et al. 2015, Wilhelmsen, Mesa et al. 2012). The JNK family consists of JNK1, JNK2 and JNK3 in 10 different isoforms. As JNK3 is primarily expressed in the brain, only JNK1 and JNK2 are involved in the regulation of cytokine production (Kyriakis, Avruch 2001, Kyriakis, Avruch 1990). The P38 family consists of 4 family members: P38α, P38β, P38γ and P38δ. P38α and P38β are involved in regulating immune responses whereas P38γ and P38δ are involved in cell differentiation and development in some cell types (Eckert, Efimova et al. 2003, Court, dos Remedios et al. 2002).

Cell-permeable pharmacological inhibitors have been extensively used for transient inhibition of signalling pathway components due to their rapid on- and off-rates, which cannot be achieved with gene knockdown approach (Cohen 2009). However most chemical inhibitors lack specificity due to the fact that these inhibitors target the ATP-binding site which has structural similarities among a range of proteins. The MAPK family, which shares conserved docking (CD) motif for their activation and regulation, suffer badly from the chemical off-target effects (Wauson, Guerra et al. 2013, Miyazawa 2011, Tanoue, Adachi et al. 2000, Davies, Reddy et al. 2000).

Both miRNAs and siRNAs are small (~15-30 nucleotides) RNA sequences which act as critical negative regulators in a wide range of organisms (Lam, Chow et al. 2015, Carthew, Sontheimer 2009, Mack 2007, Rana 2007). The first miRNA, lin-4 was discovered in *C. elegans* for its ability to negatively regulate the LIN-14 protein in development (Lee, Feinbaum et al. 1993). To date, more than 30,000 miRNAs have been described in over 200 species (He, Jing et al. 2014). In human, miRNA was predicted to make up 3% of the whole human genome with each targeting hundreds of mRNA sequences. Some miRNAs contribute as key 'fine-tuners' for immune functioning through inhibiting the translation of mRNAs (He, Jing et al. 2014, O'Neill, Sheedy et al. 2011, Quinn, O'Neill 2011). Some miRNAs target TLR signalling pathway components, for instance, miR-155 targets P38 in LPS-induced human monocyte-derived dendritic cells (Ceppi, Pereira et al. 2009); miR-9 and miR-210 both target NFκB in murine and human monocytes (Qi, Qiao et al. 2012, Bazzoni, Rossato et al. 2009). SiRNAs share structural and functional similarities to miRNAs. The first siRNA was identified in plants as a defence mechanism against viral invasion (Mello, Conte 2004). Unlike miRNAs which target multiple mRNA sequences, siRNA only binds to sequences at full complementariness hence only inducing specific gene silencing (Lam, Chow et al. 2015).

In this chapter, synthetic siRNAs which target specific TLR signalling components were used to validate pharmacological inhibition results from chapter 5.

7.2 Results

Treatment with siRNAs was used as an alternative inhibition method to the pharmacological inhibition of MAPK signalling. The synthetic siRNAs used in these experiments were 19 mer RNA sequences which target the conserved protein-coding transcripts for all isoforms of each MAPK components (ERK1, ERK2, JNK1, JNK2 and P38α) that is involved in TLR regulation. On the other hand, the siRNAs were designed to avoid the conserved amino acid sequences in the common docking (CD) domains between ERK, JNK, P38 (Figure 7.1A) and among other MAPK family members to avoid potential off-target effects caused by imperfect pairing (Jackson, Linsley 2010, Tanoue, Adachi et al. 2000). Two siRNA targeting each gene was used as advised by the manufacturer, to ensure the knockdown efficiency (>70%). Scrambled siRNA (SC siRNA) which consists of 19 mer RNA sequence with no gene target was used as negative control in all siRNA experiments. In addition, siRNA targeting GAPDH gene was used as additional negative control in experiments designed to characterise the effect of ERK1 inhibition in PAM treated cells.

7.2.1 Targeted mRNA knockdown of ERK, JNK, P38 and off-target effect of siRNAs

The targeted mRNA knockdown and off-target effects on the transcript levels of ERK, JNK and P38 gene by siRNA treatments were measured by qRT-PCR. Results were adjusted against scrambled siRNA treatment, normalised to GAPDH gene and represented in fold changes (Figure 7.2).

Treatment with siRNAs targeting ERK1 and ERK2 induced high level of ERK1 but not ERK2 knockdown. The ERK1 transcript level reduced 86% compared to cells treated with SC siRNA whereas ERK2 transcript level only reduced 32%. However, treatment with the ERK1/2-targeting siRNA also somewhat affected the transcript level of JNK and P38. Expression of JNK1 and P38α increased 27% and 60% respectively whereas expression of JNK2 decreased 52% (Figure 7.2A). Treatment with siRNAs targeting

A

Gene	Bioinformatic Description	CD site sequence
MAPK3 (ERK1)	4 protein-coding transcripts	GATCCGACAGAT
MAPK1 (ERK2)	3 protein-coding transcripts	GACCCAAGTGAT
MAPK8 (JNK1)	6 protein-coding transcripts	GATCCTTCAGAAGCAGAA
MAPK9 (JNK2)	7 protein-coding transcripts	GACCCCGCTGAAGCAGAA
MAPK14 (P38α)	6 protein-coding transcripts	GACCCATCTCAT

B

```
NM_011952.2|    ATCCCAGGAGGACCTTAATTGCATCATTAACATGAAGGCCCGAAACTACCTGCAGTCTCT
siRNA1          -----------------attgcatcattaacatgaa-----------------------
                                  ******************

NM_011952.2|    TGACCTGCTGGACCGGATGTTAACCTTCAACCCAAACAAGCGCATCACAGTAGAGGAAGC
siRNA2          -----------------gttaaccttcaacccaaac-----------------------
                                  ******************
```

C

```
NM_011949.3|    AGAACATCATTGGCATCAATGACATCATCCGGGCACCAACCATTGAGCAAATGAAAGATG
siRNA1          ----------------------------------------cattgagcaaatgaaagat-
                                                         ******************

NM_011949.3|    TGGGTATTCTTGGATCTCCATCACAGGAAGATCTGAATTGTATAATAAATTTAAAAGCTA
siRNA2          --------------------------------tgaattgtataataaattt--------
                                                 ******************
```

D

```
NM_016700.4     TCCCGGACAAGCAGTTAGATGAGAGGGAGCACACAATAGAGGAGTGGAAAGAACTGATAT
siRNA1          --ccggacaagcagttagatg---------------------------------------
                  ******************

NM_016700.4     AAGGAATAGTGTGTGCAGCTTATGATGCCATTCTTGAAAGAAATGTTGCAATCAAGAAGC
siRNA2          -aggaatagtgtgtgcagct----------------------------------------
                 ******************
```

E

```
XM_006533379.1    CAGCTAGGAACACCATCCGCAGAGTTCATGAAGAAACTTCAGCCAACTGTAAGGAATTAT
siRNA1            -------------------agagttcatgaagaaactt--------------------
                                     ******************

XM_006533379.1    ATTCAGCTGGTATCATTCATAGAGATTTGAAGCCTAGCAACATTGTAGTAAAATCAGACT
siRNA2            ----------------------------------------attgtagtaaaatcagact
                                                          ******************
```

F

```
NM_011951.3|    ACAGGGACCTAAAGCCCAGCAACCTAGCTGTGAACGAAGACTGTGAGCTCAAGATTCTGG
siRNA1          ----------------------------gtgaacgaagactgtgagc------------
                                            ******************

NM_011951.3|    CCTACCGAGAGTTGCGTCTGCTGAAGCACATGAAACACGAAAATGTGATTGGTCTGTTGG
siRNA2          -------------------------------tgaaacacgaaaatgtgat-----------
                                               ******************
```

Figure 7.1: Bioinformatics analysis of siRNA targeting ERK1/2, JNK1/2 and P38α. The conserved transcript sequences between isotypes and the sequences of common docking (CD) domains are summarised in table (A). SiRNAs were designed to target the conserved transcript sequences between isotypes and to avoid the CD domains of ERK1 (B), ERK2 (C), JNK1 (D), JNK2 (E) and P38α (F).

JNK1 and JNK2 substantially reduced JNK2 mRNA level compared to SC siRNA treated cells. A 77% reduction in JNK2 transcript level was observed whereas JNK1 transcript level only reduced 22%. Treatment with JNK1/2-targeting siRNAs did not induce substantial off-target transcriptional changes to ERK or P38. Expression of ERK1 decreased 39% whereas expression of ERK2 and P38 increased 30% and 34% respectively in cells incubated with JNK1/2-targeting siRNAs (Figure 7.2B). Treatment with siRNAs targeting P38 exhibited little reduction in the transcript levels of all MAPK signalling components including P38. The mRNA level of P38 only decreased 50% compared to cells treated with SC siRNA (Figure 7.2C).

Due to the lack of knockdown efficiency on the P38 transcript, no changes in P38 protein expression was observed following siRNA treatments (data not shown). Similarly, no changes in JNK protein expression was observed from 16 hrs to 120 hrs post siRNA treatment (data not shown) although substantial mRNA knockdown of JNK2 was detected. On the contrary, reduced protein expression of ERK1 and ERK2 was observed from 24 hrs after treatment with siRNAs targeting ERK1 and ERK2 mRNAs. Densitometry analysis revealed that the ERK1 protein level reduced over 80% in cells incubation with siRNA targeting ERK1/2 for 72 hrs compared with cells treated with SC siRNA (Figure 7.3A).

In experiments with PAM agonist stimulation, treatment with ERK1-targeting siRNAs reduced protein expression of ERK1 in cells regardless of agonist stimulation. This reduction in the ERK1 protein level led to the decreased phosphorylation of ERK1 when incubated with PAM agonist (Figure 7.3B).

7.2.2 The effect of ERK1 knockdown on PAM induced transcriptional regulation of IL1β and TLRs

The effect of ERK1 knockdown induced by treatment with ERK1-targeting siRNA was compared to 2 negative controls: SC siRNA treatment and GAPDH-targeting siRNA treatment. Both negative control treatments did not induce detectable off-target effects.

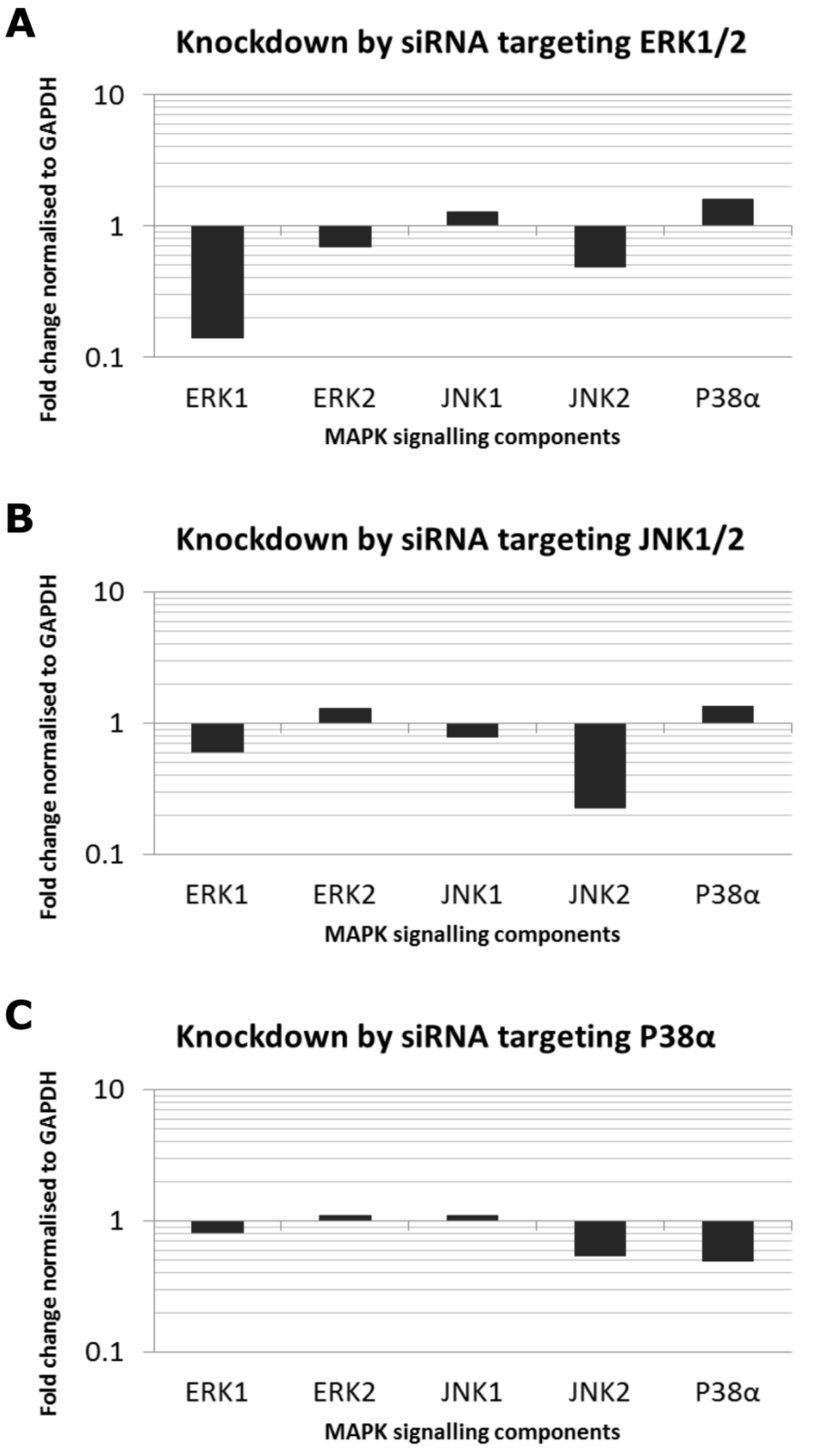


Figure 7.2: On-target and off-target knockdown induced by siRNA targeting ERK1 and 2 (A), JNK 1 and 2 (B) and P38α (C). The x axis indicates the genes tested by qPCR; the y axis indicates fold changes values presented on a logarithmic scale (log base=10). Results were adjusted against scrambled siRNA treatment and normalised to GAPDH.

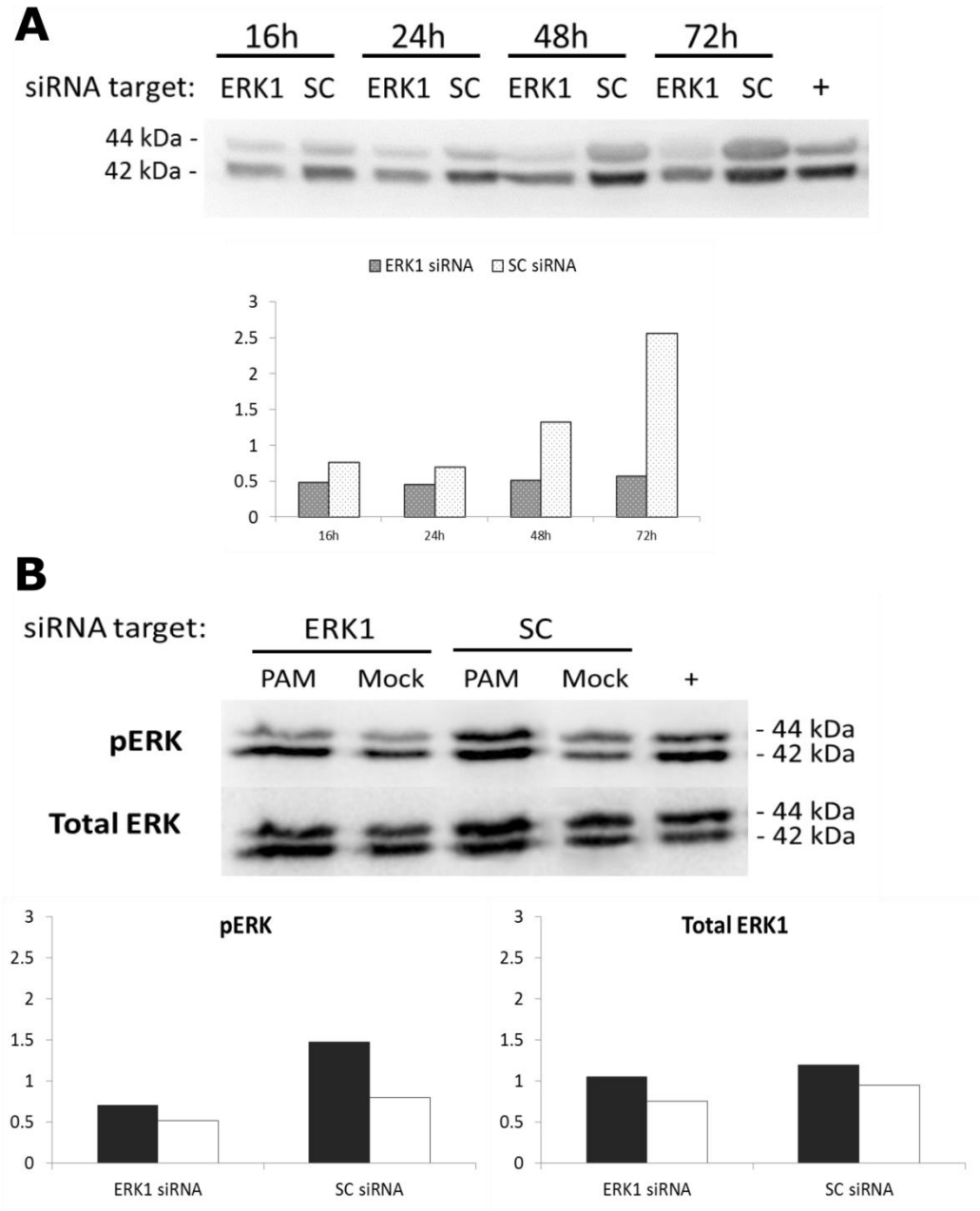


Figure 7.3: ERK1-targeting siRNA induced knockdown of ERK protein in time course (A), when treated with/without PAM agonist at 48hr post siRNA transfection (B). Graphs represent densitometry analysis of band intensity in relation to the positive control band. In panel A, the x axis indicates time post siRNA transfection; the y axis indicates percentage intensity of the positive control. The light grey bars represent ERK signal in SC siRNA treated cells; the dark grey bars represent ERK signal in ERK1 siRNA treated cells. In panel B, the x axis indicates the siRNA treatments; the y axis indicates percentage intensity of the positive control. The clear bars represent phosphor- or total- ERK signals in mock treated cells; the black bars represent phosphor or total- ERK signals in PAM treated cells.

In non-stimulated cells, treatment with siRNAs targeting ERK1, GAPDH or scrambled sequence all did not affect the basal expression of IL1β. In PAM-stimulated cells, the PAM induced transcriptional up-regulation of IL1β was reduced by knockdown of ERK1 ($p<0.05$) but not with knockdown of GAPDH (Figure 7.4).

The transcript levels of cognate- and cross-regulating TLRs were measured following treatment with siRNAs targeting ERK1, GAPDH and scrambled sequence. Knockdown of the ERK1 protein did not affect the mRNA levels of TLR1, 2, 4 or 7 in both non-stimulated and PAM stimulated cells (Figure 7.5A-D). In contrast, inhibition of the ERK1 protein expression substantially increased the basal transcript level of TLR8 in non-stimulated cells ($p<0.05$) while further reduced its transcript level below PAM induced down-regulation level in PAM treated cells ($p<0.05$). Overall, the magnitude of PAM induced TLR8 down-regulation remained un-altered by treatment with ERK1-targeting siRNA due to large variation between samples (Figure 7.5E). Inhibition of ERK1 protein expression did not affect the basal transcript level of TLR9 in non-stimulated cells. On the other hand, the same treatment fully prevented TLR9 down-regulation in PAM stimulated cells ($p<0.05$). Hence inhibition of ERK1 protein substantially reduced the magnitude of PAM induced TLR9 transcriptional changes (Figure 7.5F).

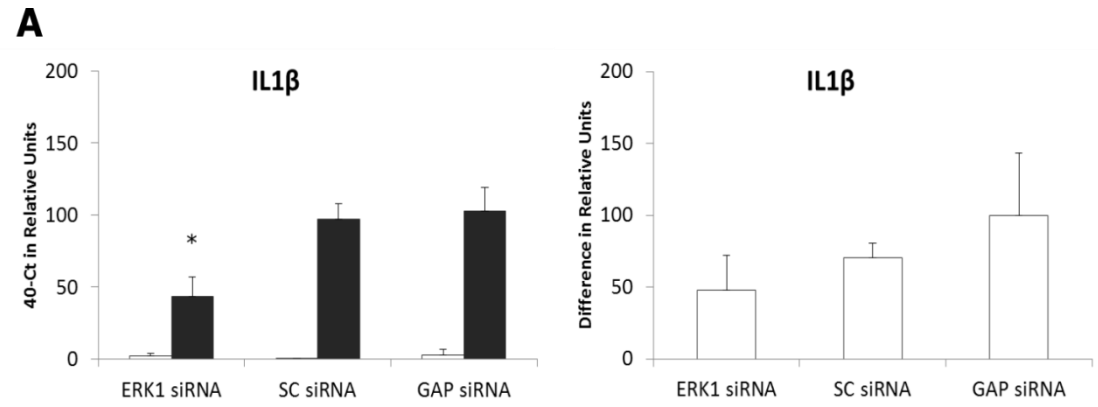


Figure 7.4: Effect of inhibiting ERK1 with siRNA on PAM-induced IL1β transcriptional expression in RAW cells. The x axis indicates siRNA treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative units graphs, the clear bars represent IL1β expression in non-stimulated cells; the black bars represent IL1β expression in PAM-stimulated cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels of the differences between the bar over which they are placed and GAP siRNA treated control cells: *p< 0.05, **p< 0.01, ***p< 0.001.

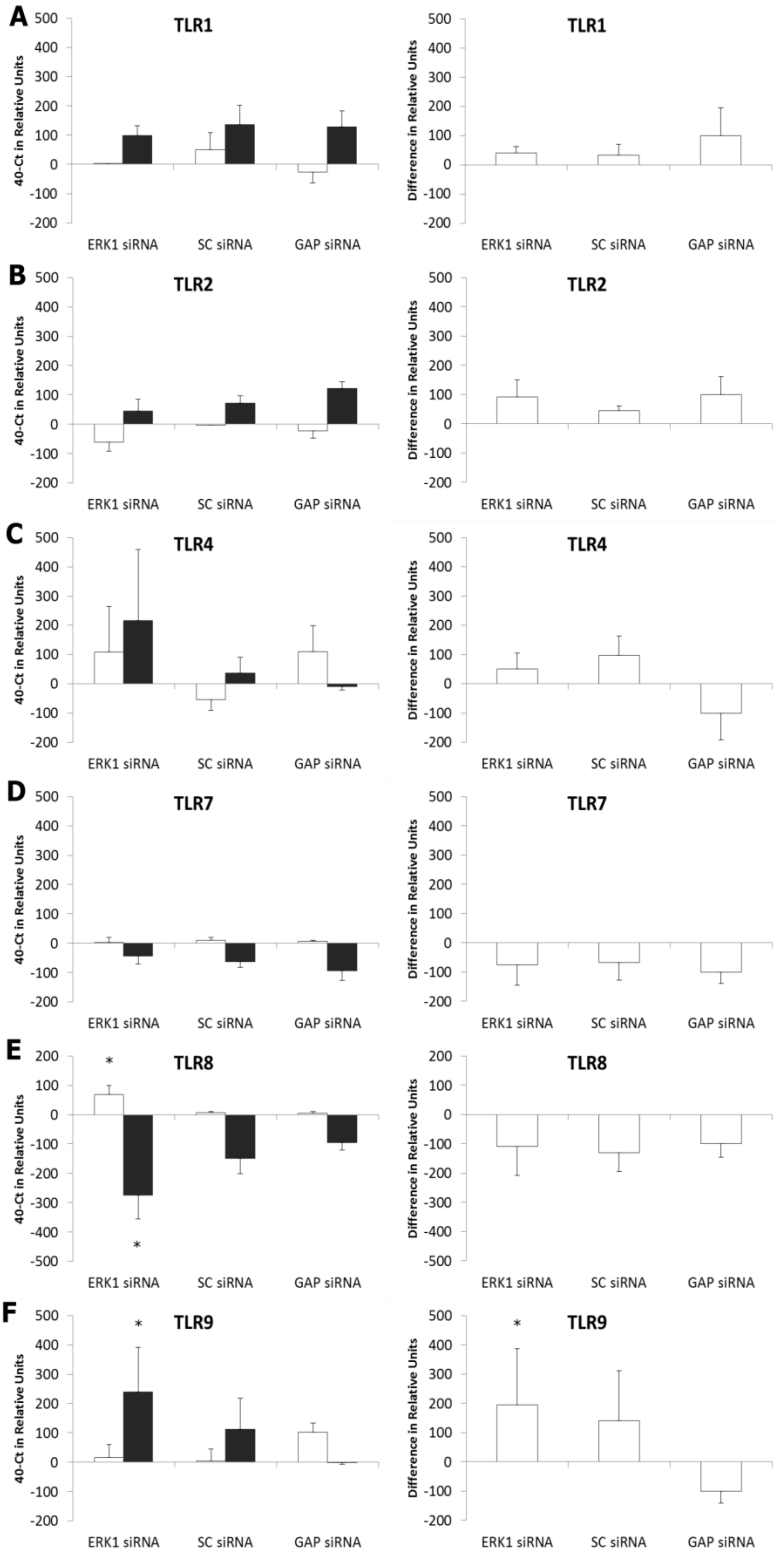


Figure 7.5: Effect of inhibiting ERK1 with siRNA on PAM-induced TLR transcriptional expression in RAW cells. The x axis indicates siRNA treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in PAM-stimulated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

7.3 Discussion

Gene silencing with siRNAs have been widely used over the past few years as an alternative to the pharmacological inhibitors due to their specificity. In this chapter, I first explored the siRNA induced protein knockdown for the MAPK terminal signalling cascades. Then I investigated the effect of ERK1 protein knockdown on PAM induced transcriptional changes of IL1β and TLRs.

To ensure the knockdown efficiency and target specificity, siRNAs were designed to bind to conserved transcript sequences of all protein-coding isoforms while avoid the specific conserved docking motifs of the MAPK family. Over 70% target gene knockdown were achieved following 48 hr incubation with ERK1-targeting siRNA or JNK2-targeting siRNA. While inhibition of the ERK1 gene expression induced ERK1 protein knockdown after 24 hrs, inhibition of JNK2 gene expression did not change the JNK2 protein level at 16 hr, 24 hr, 48 hr, 72 hr, 96 hr or 120 hr after treatment even though both ERK and JNK protein have a half-life at +/- 24 hrs (Boutros, Chevet et al. 2008, Satoh, Parent et al. 2002). These results suggest that the target gene knockdown does not guarantee the target protein knockdown hence expression of the target proteins must be checked for all siRNA experiments.

Inhibition of the ERK1 protein expression by siRNA treatment increased basal mRNA level of TLR8 and fully rescued the PAM induced TLR9 down-regulation whereas it did not affect TLR7 mRNA level. These results are consistent with the effects of chemical inhibition through treatment with U126 and PD059 (see chapter 5.2.3.1), suggesting that ERK1 is the main contributing ERK signalling cascade for the transcriptional regulation of TLR8 and TLR9 in RAW cells. On the other hand, while chemical inhibition of ERK1/2 increased mRNA levels of TLR1 and TLR2 in both non-treated and PAM treated cells, siRNA inhibition of ERK1 protein did not affect the mRNA levels of TLR1 or 2. In a similar manner, chemical inhibition of ERK1/2 reduced the basal transcript level of TLR4 whereas siRNA inhibition of ERK1 protein did not affect the

basal mRNA level of TLR4. These differential effects between chemical inhibition and gene knockdown may be caused by a wider range of target, more complete inhibition or the “off-target” inhibition by chemical inhibitors. The chemical inhibitors U126 and PD98059 both inhibit the phosphorylation of ERK1/2 protein as well as ERK5 (Chen, Gibson et al. 2001, Kamakura, Moriguchi et al. 1999). Hence ERK2 and/or ERK5, but not ERK1 may be responsible for PAM induced transcriptional regulation of TLR1, 2 and 4. Western blot analysis revealed differences in the inhibition of phospho-ERK between chemical inhibitors and siRNAs. Treatment with U126 or PD98059 obstructed the phosphorylation of ERK completely whereas siRNA treatment only reduced phosphorylation of ERK1 by half. This result suggests that reduced phosphorylation of ERK1 protein may be sufficient to induce the TLR transcriptional changes following PAM stimulation. In addition, both chemical inhibitors U126 and PD98059 are known to cause off-target effects. Treatment with U126 at 10 μM inhibits MKK4 by 29%, MKK7 by 21% (both directly upstream of JNK1/2) and P38α by 25%. Treatment with PD98059 at 50 μM increases JNK1 by 11% and inhibits P38 by 15% (Wauson, Guerra et al. 2013). Hence it is possible that the differences between transcriptional changes of TLR1, 2 and 4 induced by chemical inhibition and siRNA knockdown may be due to the off-target effects.

Both the ERK1-targeting and the JNK2-targeting siRNAs affected the transcriptional expression of other MAPK genes. Treatment with the ERK1-targeting siRNA reduced mRNA levels of ERK2 and JNK2 while increased transcript levels of JNK1 and P38α. Similarly, Treatment with the JNK2-targeting siRNA reduced mRNA levels of ERK1 and JNK1 while increased transcript levels of JNK2 and P38α. The “off-target” knockdowns may be caused by imperfect matching sequence at the 3’ UTR region of cellular mRNAs to the siRNA strand (Jackson, Linsley 2010) whereas the increased mRNA expression of MAPK signalling cascades may be caused by TLR7/8

recognising the siRNA sequences as PAMPs (Chen, Liang et al. 2013, Fabbri 2012, Jackson, Linsley 2010).

Chemical modifications on siRNAs could increase their specificity and prevent TLR mediated immune responses. For instance, the locked nucleic acid (LNA) modification increases the thermostability of siRNAs which result in the reduced immunogenicity by making the ssRNA sequences less targetable for TLR7/8 (Broering, Real et al. 2014, Bramsen, Kjems 2012). All siRNAs used in this chapter was modified with LNA and a dTdT overhang.

In summary, treatment with ERK1-targeting siRNAs confirmed results from previous chapter that ERK1 contributed to the transcriptional regulation of TLR8 and 9 but not for TLR7. However, function of other MAPK signalling cascades on the cognate and cross-regulating TLR transcriptional changes require future work.

Chapter 8 Indirect regulation of PAM induced TLR mRNA expression

8.1 Introduction

The coordination between signalling pathways for mediating the transcriptional regulation of TLRs can be complex. Integration of direct TLR signalling pathways involves cross-activation or cross-inhibition (Thaiss, Levy et al. 2016). These types of signalling interaction have been described in Chapter 6. In addition, the transcriptional regulation of TLRs may be mediated through the positive/negative feedback loops induced by the production of immune regulators. This type of signalling modification is described as the indirect regulation of TLR transcription.

A number of studies have described the inhibitory effects of suppressor of cytokine signalling (SOCS) proteins on TLR signalling, SOCS1 and SOCS3 in particular (Kondo, Kawai et al. 2012, Yoshimura, Yasukawa 2012, Yoshimura, Naka et al. 2007, Liew, Xu et al. 2005). Kinjyo et al. (2002) and Nakagawa et al. (2002) both reported the increased production of nitric oxide, TNFα and IL12 in response to TLR4 or TLR9 agonists in macrophages lack SOCS1. Mice with SOCS1 deficiency were more susceptible to sepsis than the wild-types. Furthermore, the production of SOCS1 was induced by stimulation with TLR4 or TLR9 agonists (Naka, Fujimoto et al. 2005). This negative feedback loop between SOCS1 and TLR4/TLR9 is either through direct inhibition of the MyD88-dependent pathway or by indirectly supressing the IFNβ signalling without affecting the MAPK or NFκB signalling pathways (Kawasaki, Kawai 2014, Baetz, Frey et al. 2004).

The integrin family are proteins which facilitates adhesion of leukocytes for cell migration. Some integrins also influences cell functions (Means, Luster 2010). Yee et al. (2013) indicated that the β2 integrin family supressed the production of IL12 and IL6 in response to TLR2 or TLR9 agonists. Wang et al. (2010) demonstrated that the β2 integrin supressed the protein secretion of IL6 and TNF in response to TLR4 activation. Some integrins positively regulate TLR mediated innate responses, for instance,

activation of αvβ3 integrin increased the production of IFN, IL10 and IL2 induced through TLR2 and TLR3 signalling in response to the herpes simplex virus 1 (HSV) (Casiraghi, Gianni et al. 2016, Gianni, Campadelli-Fiume 2014, Gianni, Leoni et al. 2012).

The other key protein regulators for TLR signalling include, SARM and TAG which target TRIF, SOCS3 which targets TRAF3, SHP and A20 which target TRAF6, Bcl-3 and ABIN-3 which target NFκB. A detailed description of the negative regulators for TLR signalling pathways can be found in chapter 1 (section 1.3.3). Most of these listed signalling regulators are mediated though the tyrosine-based activation motif (ITAM)-coupled receptor associated signalling pathways. Downstream of the ITAM-coupled receptors and β2 integrins, the high-avidity calcium dependent DAP12-Syk-Pyk2 pathway induces the production of IL10, STAT3, SOCS3, ABIN-3, Hes1 and A20, regulators which inhibit TLR signalling or cytokine transcription. In addition, the low-avidity/tonic engagement through the ITAM-coupled receptors can also attenuate TLR signalling through activation of the tyrosine phosphatase SHP-1 (Wang, Gordon et al. 2010, Ivashkiv 2008, Pasquier, Launay et al. 2005).

Previous studies have identified the effects and mechanisms by which the indirect feedbacks affect TLR induced immune responses, however little is known about the effects and mechanisms of indirect feedbacks on the transcriptional regulation of TLRs. This chapter aimed to investigate the effect of protein production on mediating PAM induced TLR transcriptional changes with two methods. Firstly, supernatant which contained secreted proteins from PAM incubated cells was collected and treated on freshly cultured cells to examine the effect of secreted protein on regulating TLR transcription. Secondly, chemical inhibitors for protein secretion (brefeldin A), protein transportation (monensin) and translation (cycloheximide), which obstruct TLR mediated protein production therefore dampen the secondary feedback on TLR regulation, were used to study the effect of protein production on TLR transcription.

8.2 Results

8.2.1 Effect of secreted protein on PAM induced IL1β and TLR mRNA levels

Murine macrophage cell line RAW264.7 was incubated with PAM for 4 hrs, washed 5 times with complete medium followed by further incubation with medium for another 4 hrs. Both basal ($p<0.01$) and PAM induced transcript levels ($p<0.001$) of IL1β were higher in washed cells than in cells incubated with PAM for straight 8 hrs. However no substantial differences between the magnitudes of PAM induced IL1β up-regulation was observed in cells incubated with PAM for 4 hr or 8 hr (Figure 8.1). In a similar manner, shorter incubation time with PAM agonist did not affect the magnitudes of TLR transcriptional changes induced by PAM (Figure 8.2). Although the 4 hr incubation with PAM increased the TLR2 mRNA level above the 8 hr PAM up-regulated level ($p<0.05$) (Figure 8.2B) and reduced the TLR4 mRNA level below the 8 hr PAM down-regulated level ($p<0.05$) (Figure 8.2C).

The last medium wash (LW) and supernatant from the 4 hr medium incubation (SN) was collected and treated on freshly cultured cells for 8 hrs. Both LW and SN treatments induced up-regulation of IL1β mRNA levels higher than the 8 hr PAM induced level ($p<0.01$). However the magnitude of IL1β up-regulation induced by LW or SN treatment was not substantially different to that induced by PAM treatment when taking into account of both basal and treatments induced levels (Figure 8.3A). Treatment with LW and SN induced transcriptional regulation of TLR2 and TLR4 in a similar manner to the 8 hr PAM incubation. TLR2 mRNA level was up-regulated whereas TLR4 mRNA level was down-regulated following incubation with LW or SN (Figure 8.3B-C).

Next, I investigated the shortest incubation time with PAM for cells to induce the same transcriptional changes as induced by 8 hr PAM stimulation. RAW cells were incubated with PAM for 15 mins, washed 5 times with complete medium followed by medium incubation for the rest of 8 hrs. Both basal expression and PAM induced expression of

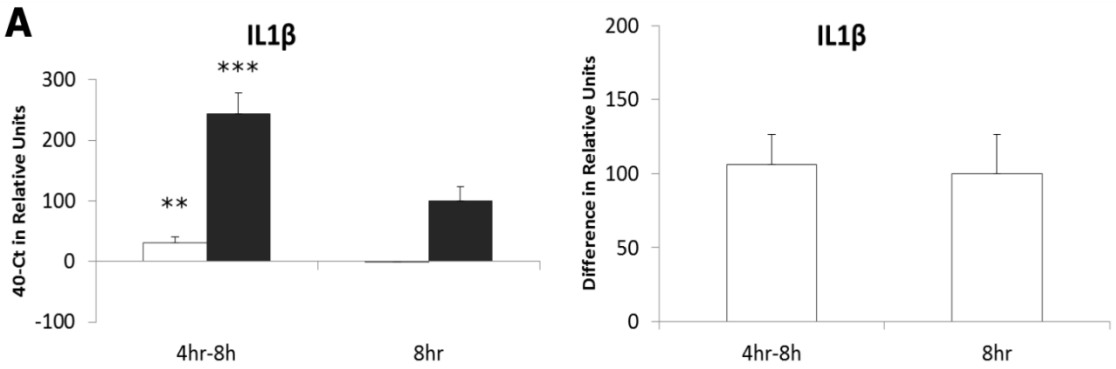


Figure 8.1: Transcriptional expression of IL1β induced by 4hr PAM incubation followed by 4hr media incubation in RAW cells. The x axis indicates treatments (4hr-8h: 4hr PAM incubation, washed 5 times followed by further 4hr media incubation; 8hr: 8hr PAM incubation straight); the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative units graphs, the clear bars represent IL1β expression in non-stimulated cells; the black bars represent IL1β expression in PAM-stimulated cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels of the differences between the bar over which they are placed and Mock or PAM treated for 8hr incubation control cells: *p< 0.05, **p< 0.01, ***p< 0.001.

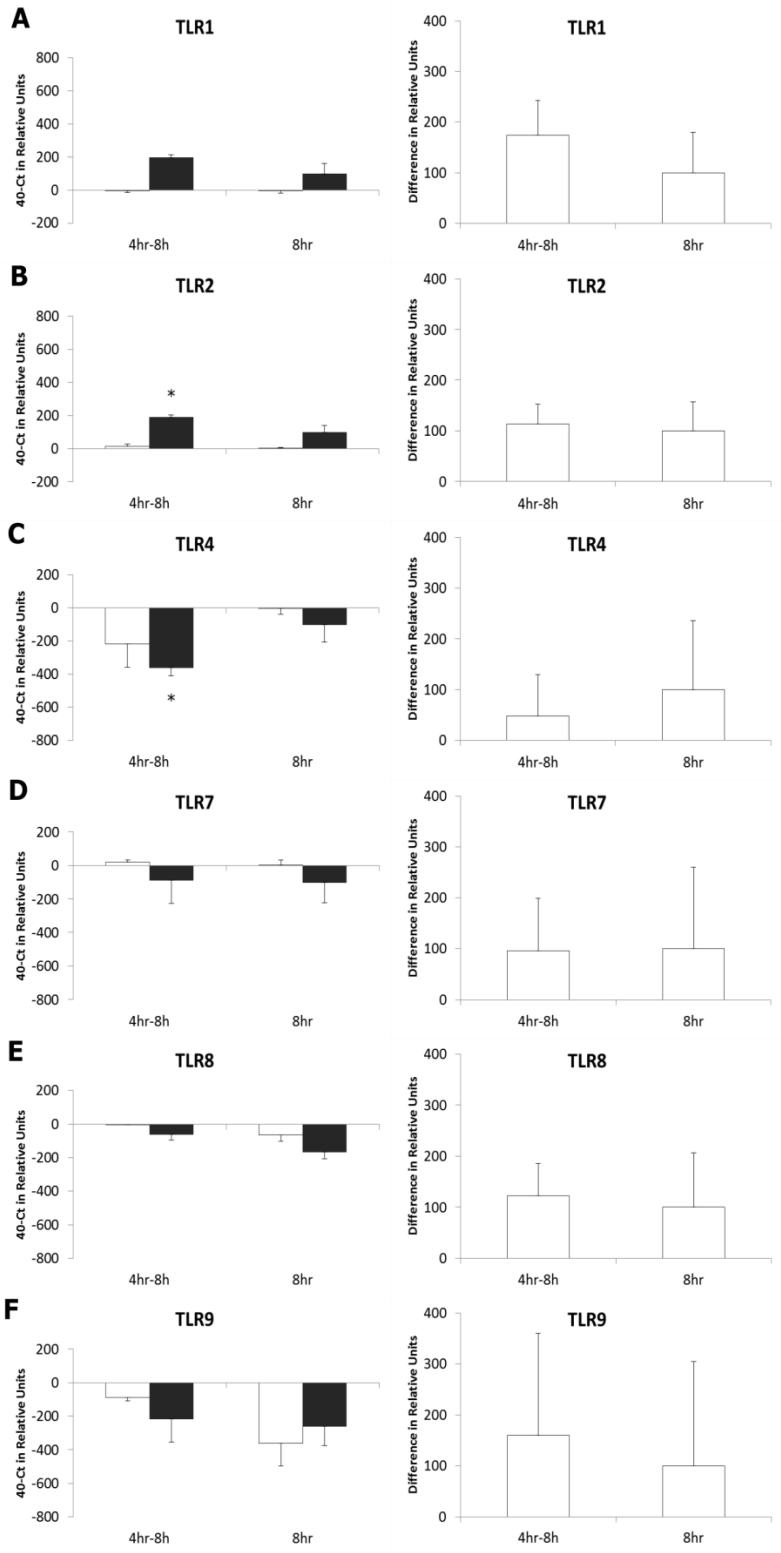


Figure 8.2: Transcriptional expression of TLRs induced by 4hr PAM incubation followed by 4hr media incubation in RAW cells. The x axis indicates treatment times; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in PAM-stimulated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

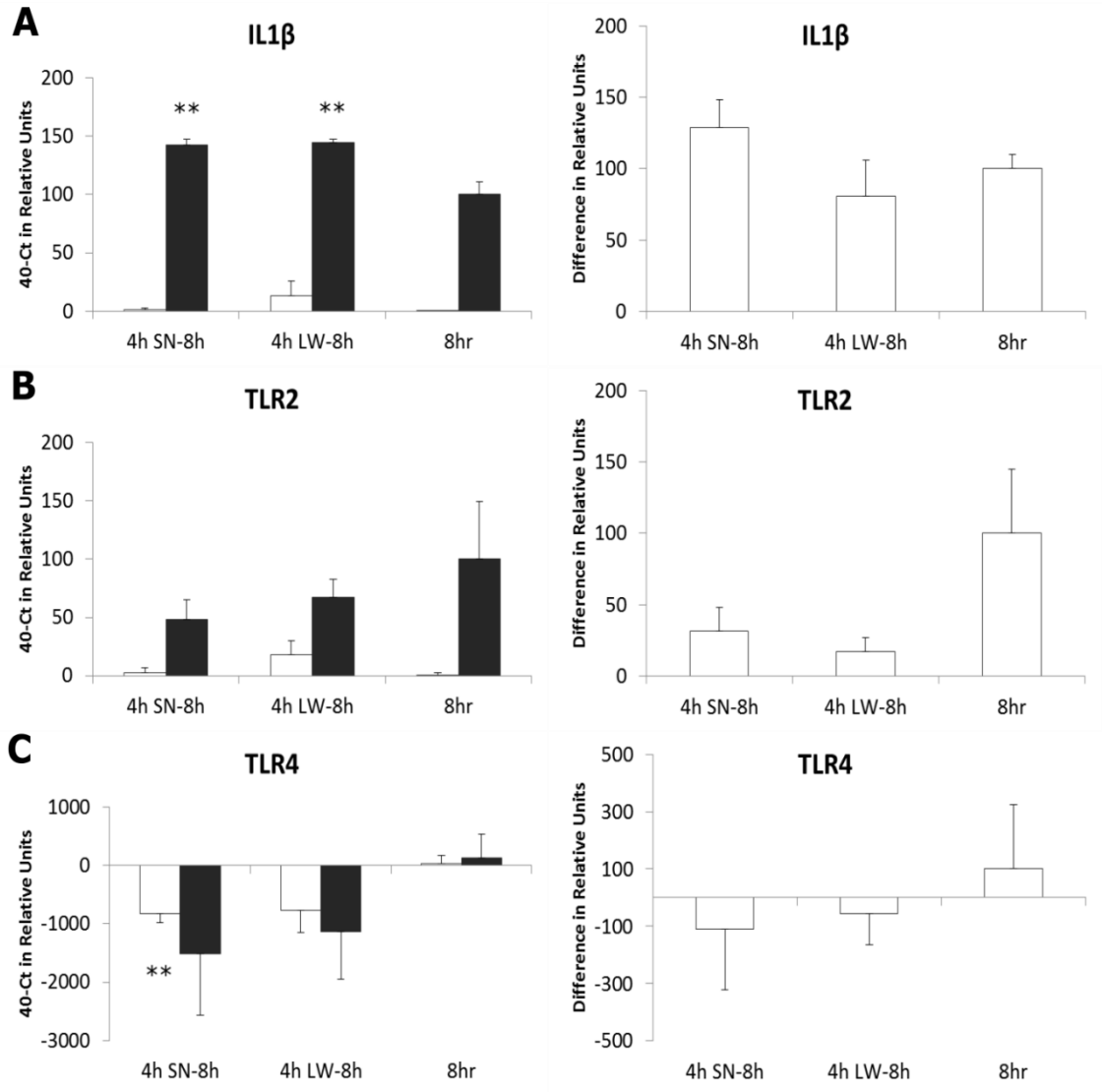


Figure 8.3: Transcriptional expression of IL1β and TLRs induced by 4hr supernatant (SN) or last wash (LW) treatment in RAW cells. The x axis indicates treatment types; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative units graphs, the clear bars represent gene expression in non-stimulated cells; the black bars represent gene expression in SN or LW stimulated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

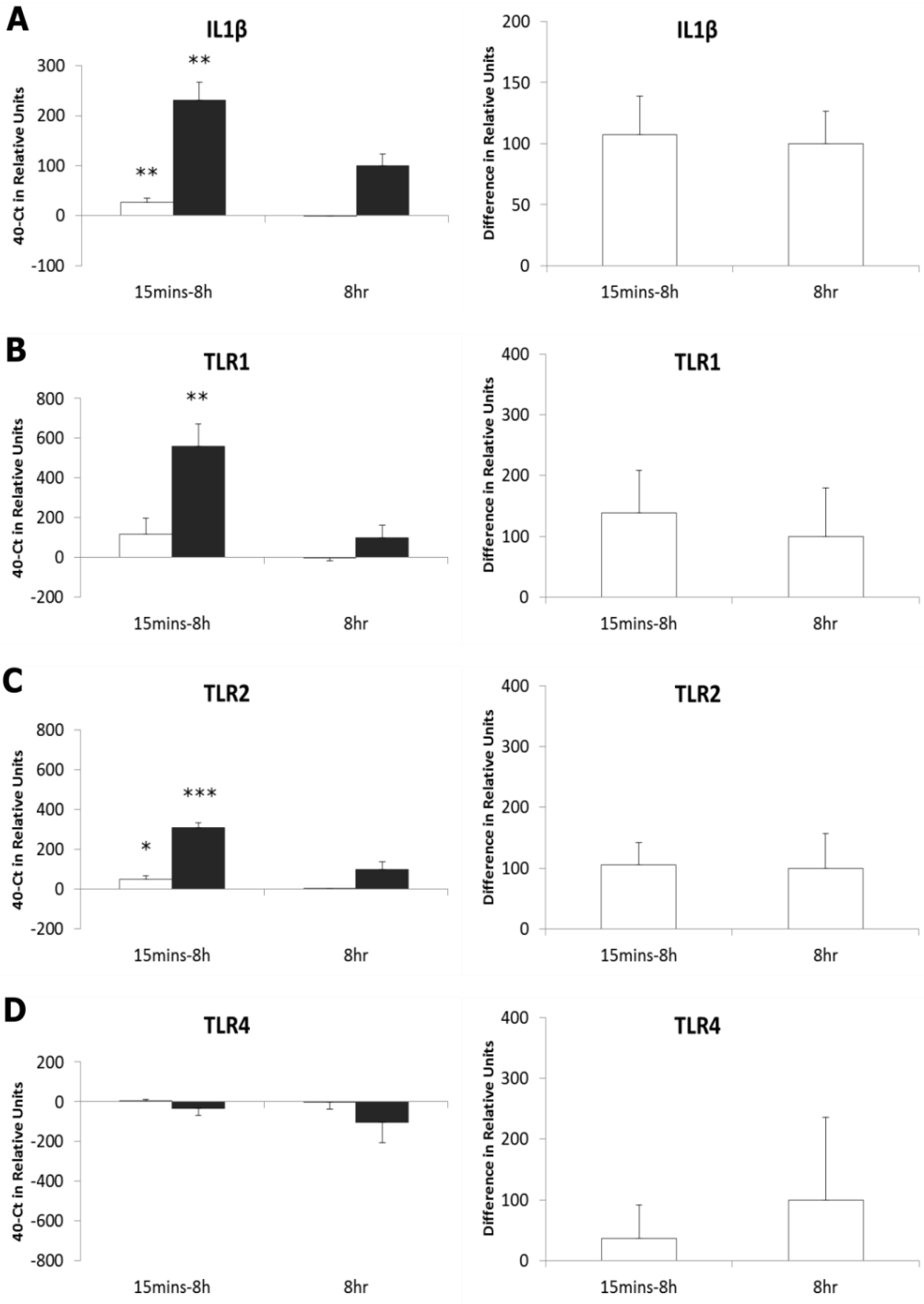


Figure 8.4: Transcriptional expression of IL1β and TLRs induced by 15mins PAM incubation followed by 7hr and 45mins media incubation in RAW cells. The x axis indicates treatment times; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative units graphs, the clear bars represent gene expression in non-stimulated cells; the black bars represent gene expression in PAM-stimulated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

IL1β were higher in washed cells ($p<0.01$) than in cells incubated for 8 hrs straight. However the magnitudes of IL1β up-regulation induced by 8 hr PAM incubation was the same as 15 mins PAM incubation when taking into account of the basal transcript level change in the washed cells (Figure 8.4A). Likewise, shorter PAM incubation time did not alter the magnitudes of transcriptional changes of TLR1, 2 and 4 induced by 8 hr PAM incubation (Figure 8.4B-D). Although the 15 mins PAM incubation increased mRNA levels of TLR1 ($p<0.01$) and TLR2 ($p<0.001$) above the 8 hr PAM induced levels (Figure 8.4B-C).

8.2.2 High concentration of PAM is required for inducing the transcriptional changes of IL1β and TLRs in RAW cells

Substantial up-regulation of IL1β was induced by PAM stimulation at 1.6 μg/ ml ($p<0.01$), although a small but non-significant trend of IL1β up-regulation was induced by PAM stimulation at 150 ng/ ml (Figure 8.5A). Substantial up-regulation of TLR2 ($p<0.05$) and down-regulation of TLR8 ($p<0.05$) were only induced by PAM stimulation at 1.6 μg/ml but not at concentrations at or below 150 ng/ ml (Figure 8.5B-C).

8.2.3 Effect of inhibiting translation, protein transportation and secretion on PAM induced IL1β mRNA level

Inhibitors for protein transportation, secretion and translation are known for their toxicity to cells (Nylander, Kalies 1999, Mollenhauer, Morre et al. 1990). Cells were tested with a range of inhibitor concentrations and were treated with inhibitors at concentrations that did not affect the cell viability in experiments presented in this chapter (section 2.6 for inhibitor concentrations; cell viability data not shown). In addition, cells were either treated with inhibitors for 1 hr prior to the 8 hr agonist incubation (described hereafter as “pre-incubation”) or treated with inhibitors and agonist at the same time for 8 hrs (described hereafter as “co-incubation”). Solvent EtOH and DMSO were used as control treatments for inhibitor monensin and brefeldin

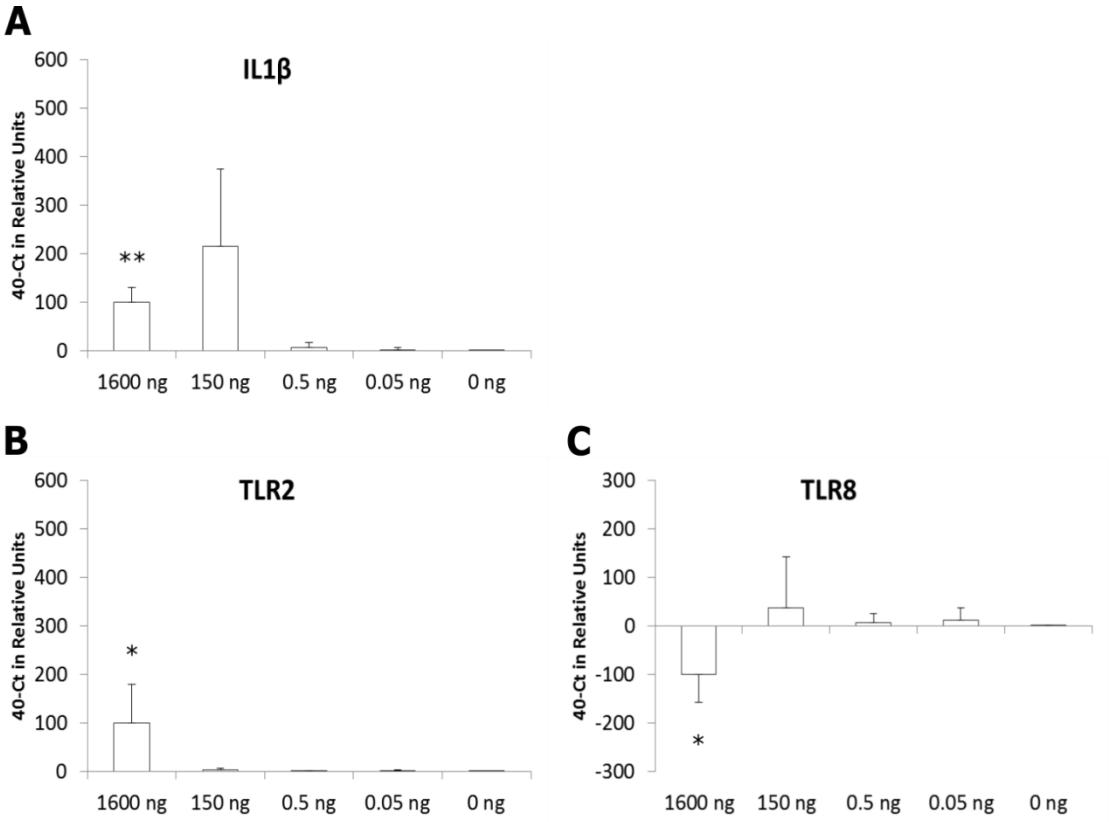


Figure 8.5: Transcriptional expression of IL1β and TLR following PAM titration in RAW cells. The x axis indicates PAM treatment concentration; the y axis indicates mean 40 – Ct values in relative quantity. Error bars presented are at 95% confidence intervals and asterisks signify significance levels of the differences between the bar over which they are placed and the 0 ng mock treated samples: *p< 0.05, **p< 0.01, ***p< 0.001.

A respectively. A figure indicating the raw 40-Ct values of the TLRs is shown in Appendix Figure 11.

Inhibition of translation by treatment with cycloheximide (CHX) increased the basal mRNA expression of IL1β ($p<0.01$) but did not alter the PAM induced up-regulation of IL1β mRNA level (Figure 8.6A). Hence the magnitude of PAM induced up-regulation of IL1β was reduced by treatment with CHX, taking into account of the basal expression changes ($p<0.05$) (Figure 8.6B). Inhibition of protein transportation by treatment with monensin reduced the basal mRNA expression of IL1β in cells pre-incubated with monensin ($p<0.01$) for 9 hrs but not in cells incubated with monensin for 8 hrs due to larger variation. In PAM stimulated cells, both pre-incubation and co-incubation with monensin rescued the up-regulation of IL1β mRNA levels ($p<0.01$) (Figure 8.6A). When comparing the effect of monensin treatment to its appropriate control, EtOH, pre-incubation with monensin reduced the magnitude of PAM induced IL1β transcriptional up-regulation ($p<0.05$) (Figure 8.6B). Inhibition of protein secretion by treatment with brefeldin reduced PAM induced up-regulation of IL1β mRNA levels in cells both pre-incubated with brefeldin and co-incubated with both PAM and brefeldin ($p<0.01$) (Figure 8.6A). When comparing the effect of brefeldin to its control, DMSO, co-incubation of brefeldin and PAM reduced the magnitude of PAM induced IL1β transcriptional up-regulation ($p<0.05$) (Figure 8.6B).

8.2.4 Effect of inhibiting translation, protein transportation and secretion on PAM induced TLR mRNA levels

The basal mRNA levels of TLR1 and TLR2 were altered by different inhibitors and their control treatments. Reduced TLR1 basal mRNA level was measured following treatment with monensin ($p<0.01$), EtOH ($p<0.05$), brefeldin ($p<0.05$) or DMSO ($p<0.01$) whereas the basal mRNA level of TLR2 was not affected by any inhibitors or control treatments (Figure 8.7A, C). In the presence of agonist PAM, pre-incubation with monensin ($p<0.01$), brefeldin ($p<0.05$) or DMSO ($p<0.001$) as well as co-incubation

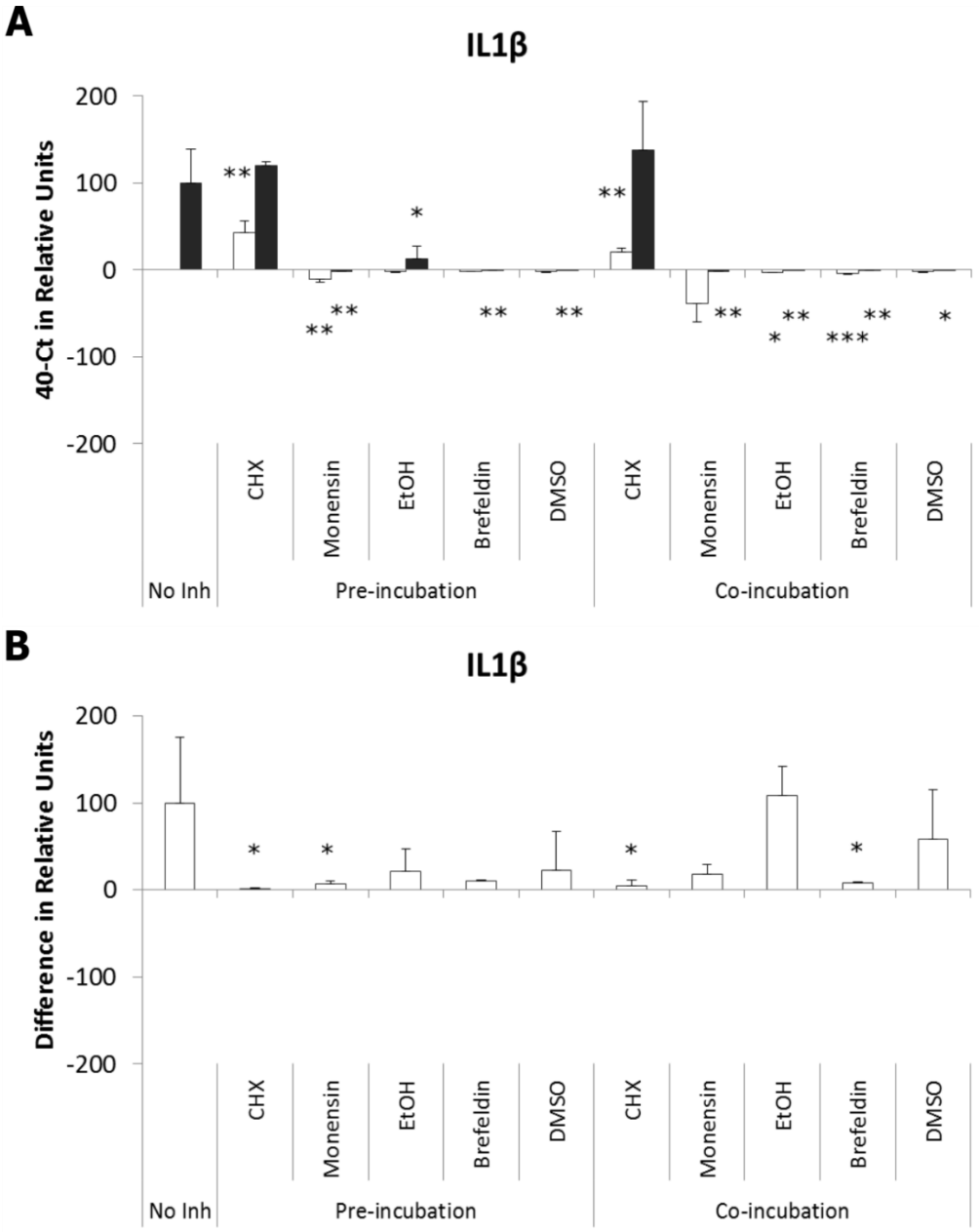


Figure 8.6: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced IL1 transcriptional expression in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A) or magnitude of change in relative quantity (B). In 40-Ct in relative units graphs, the clear bars represent IL1 expression in non-stimulated or inhibitor-only treated cells; the black bars represent IL1 expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels of the differences between the bar over which they are placed and Mock or PAM-treated control cell groups: *p< 0.05, **p< 0.01, ***p< 0.001.

with monensin ($p<0.01$), EtOH ($p<0.05$) or brefeldin ($p<0.01$) rescued the TLR1 up-regulation induced by PAM (Figure 8.7A). When compared with their appropriate controls, both pre- and co-incubation with monensin ($p<0.001$) and pre-incubation with brefeldin ($p<0.05$) substantially reduced the magnitude of TLR1 up-regulation induced by PAM stimulation (Figure 8.7B). The PAM induced TLR2 mRNA up-regulation was reduced by pre-incubation with EtOH ($p<0.01$) as well as co-incubation with monensin ($p<0.01$) or brefeldin ($p<0.01$) (Figure 8.7C). However none of these inhibitor treatments significantly reduced the magnitude of PAM induced TLR2 transcriptional up-regulation (Figure 8.7D).

In inhibitor-only treated cells, the basal mRNA expression of TLR4 was reduced following incubation with monensin ($p<0.05$). At the presence of PAM, treatment with monensin ($p<0.001$), EtOH ($p<0.05$) or DMSO ($p<0.05$) reduced the mRNA expression of TLR4 below the PAM down-regulated level (Figure 8.8A). However, none of the inhibitor treatments affected the magnitude of PAM induced TLR4 down-regulation when comparing the inhibitor treatments to their appropriate controls (Figure 8.8B).

The transcriptional regulations of TLR7/8/9 were altered by different treatments. The basal mRNA expression of TLR7 was not altered by any treatments except EtOH ($p<0.05$). In PAM stimulated cells, pre-incubation with monensin ($p<0.05$), brefeldin ($p<0.05$) or DMSO ($p<0.05$) as well as co-incubation with monensin ($p<0.01$), EtOH ($p<0.05$), brefeldin ($p<0.01$) or DMSO ($p<0.001$) reduced TLR7 mRNA levels below the PAM down-regulated level (Figure 8.9A). However, the effects of the inhibitors were not significantly different to their respective controls, hence inhibition of protein production did not alter the magnitude of PAM induced TLR7 transcriptional down-regulation (Figure 8.9B). The basal mRNA expression of TLR8 was reduced by treatment with CHX ($p<0.001$) or monensin ($p<0.01$) while increased by EtOH ($p<0.01$). In PAM stimulated cells, treatment with monensin reduced TLR8 transcript level below the PAM down-regulated level ($p<0.05$). Pre-incubation with EtOH prior to PAM

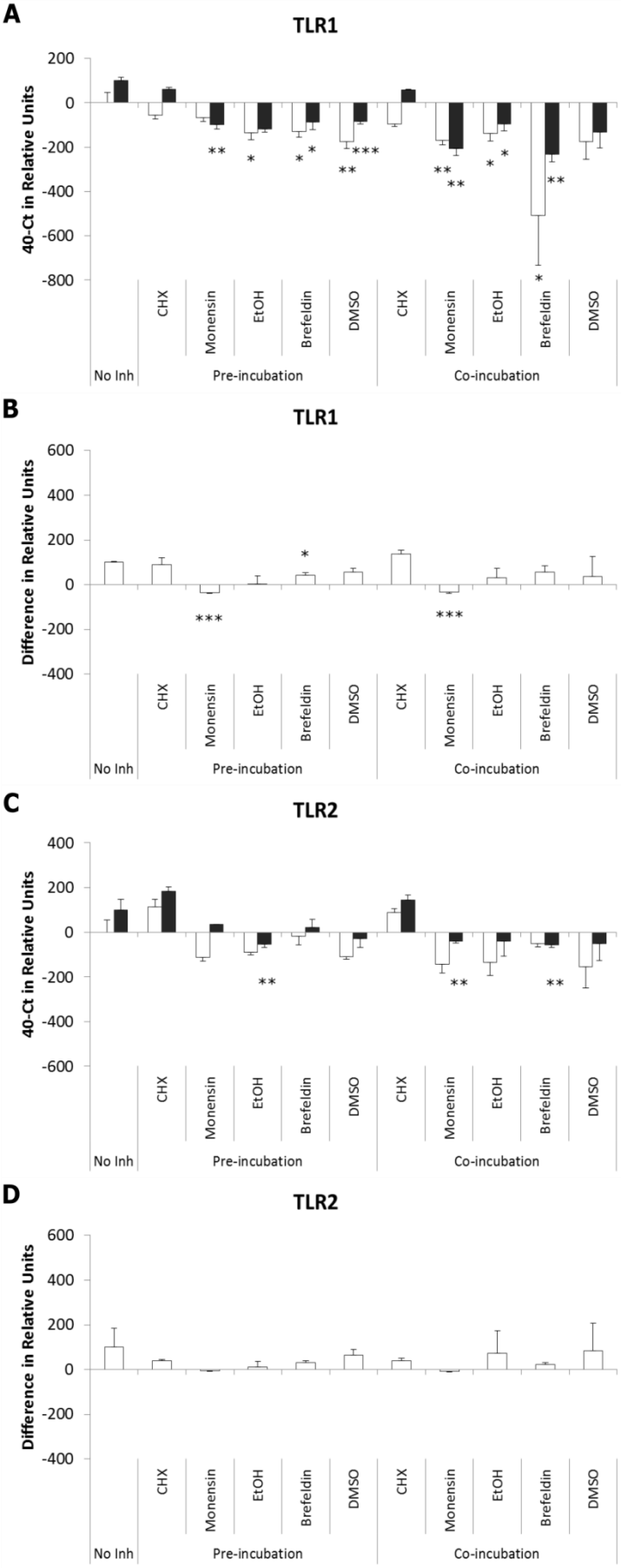


Figure 8.7: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced TLR1 family mRNA changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A, C) or magnitude of change in relative quantity (B, D). In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-only treated cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

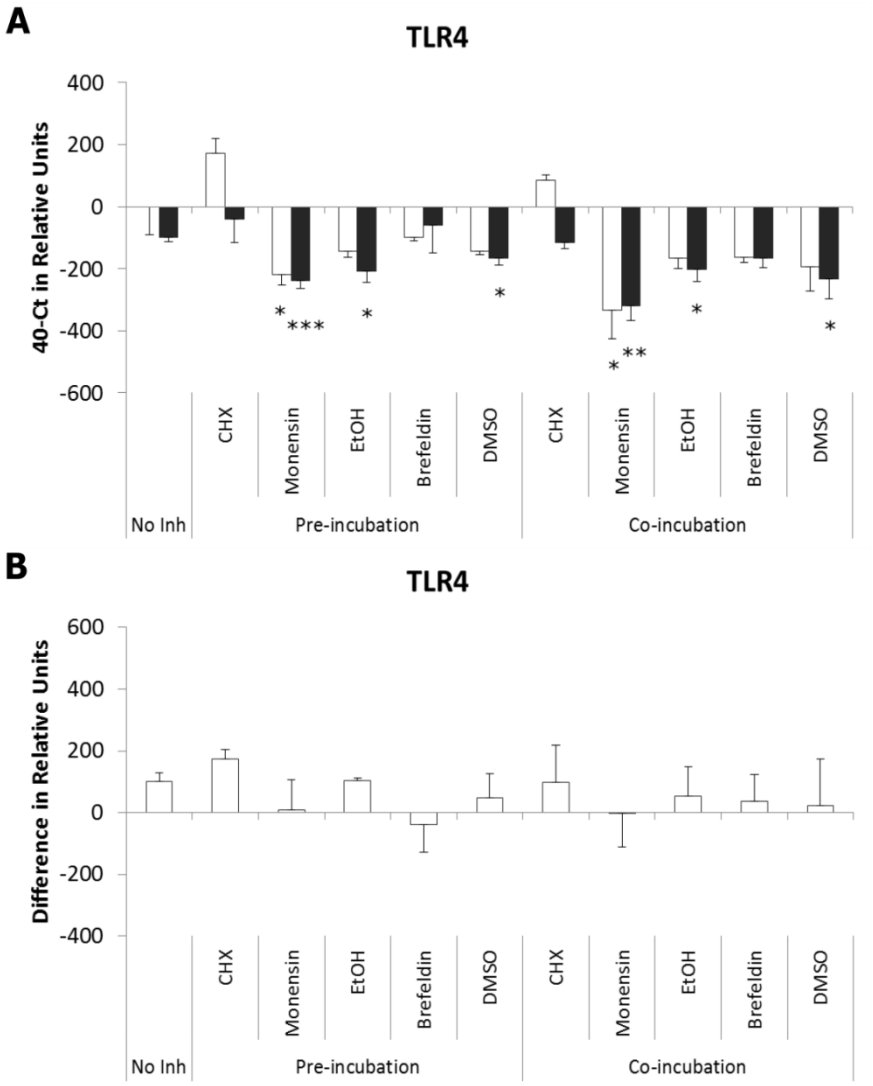


Figure 8.8: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced TLR4 family mRNA changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A) or magnitude of change in relative quantity (B). In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-only treated cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

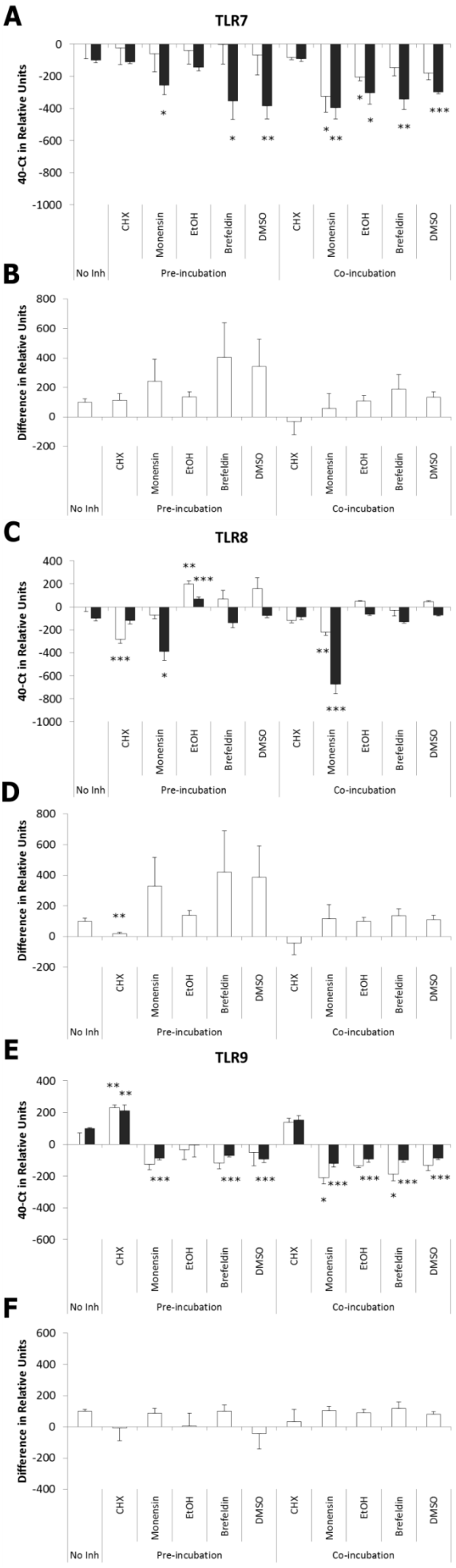


Figure 8.9: Effect of inhibiting protein transportation, protein secretion and translation on PAM-induced TLR7 family mRNA changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity (A, C, E) or magnitude of change in relative quantity (B, D, F). In 40-Ct in relative units graphs, the clear bars represent TLR expression in non-stimulated or inhibitor-only treated cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

stimulation rescued the TLR8 transcriptional down-regulation ($p<0.001$) whereas similar effect was not observed when EtOH was co-incubated with PAM (Figure 8.9C). Overall, the magnitude of PAM induced TLR8 down-regulation was only reduced by pre-treatment with CHX, when taking into account of the basal transcript levels (Figure 8.9D). The basal TLR9 transcript level was reduced by treatment with monensin ($p<0.05$) or brefeldin ($p<0.05$) while increased by CHX ($p<0.01$). In PAM stimulated cells, treatment with monensin ($p<0.001$), brefeldin ($p<0.001$) or DMSO ($p<0.001$) reduced TLR9 mRNA levels compared to its basal level whereas co-incubation with EtOH prevented the PAM induced TLR9 up-regulation ($p<0.001$) (Figure 8.9E). Overall, the magnitude of PAM induced TLR9 transcriptional changes was not altered by inhibition of protein production when compared to the control treatments (Figure 8.9F).

8.3 Discussion

To investigate the effect of secreted proteins from cell culture supernatant, macrophages were incubated with PAM for shorter time period instead of continuously for 8 hrs. Supernatant from the 4 hr culture was collected and treated on fresh cells. The shorter incubation time with PAM at 15 mins or 4 hrs both did not alter the magnitudes of IL1β or TLR transcriptional changes induced by the agonist. This result suggests that a short stimulus of PAM is sufficient to generate the TLR transcription patterns measured at 8 hrs later. Interestingly, both the basal mRNA level and the PAM induced mRNA level of IL1β were increased in cells washed with medium. This increase in the production of IL1β transcript may be the result of stress induced DAMP recognition due to repetitive washing.

Treatment with the after-washing culture supernatant (SN) on freshly cultured cells induced the same transcriptional changes of IL1β and TLR2/4 compared to PAM induced levels. This result suggests that the transcriptional regulation of IL1β and TLR2/4 is due to the feedback of secreted proteins in culture supernatant. However, as macrophages engulf foreign materials and release waste debris constantly, it is possible that macrophages may expel a small amount of PAM residue as waste material without digesting it with lysosome. Similar macrophage behaviour was reported in previous research where the engulfed living cell with PtdSer signal was released from the macrophage before transferred to lysosomes (Supplementary materials Movie S3) (Segawa, Kurata et al. 2014). To determine the minimum working concentration of PAM for inducing the transcriptional regulation of IL1β and TLRs, a titration was then conducted. Results indicated that substantial regulation of IL1β and TLR mRNAs were only induced by PAM at 1.6 μg/ml but not by concentration at 150 ng/ml or under. Therefore the remaining PAM residue left in the cell culture after 5 washes should not be sufficient to induce the IL1β and TLR transcriptional regulation.

I then investigated the effect of protein production on PAM induced IL1β and TLR transcriptional regulation. The magnitude of PAM induced IL1β up-regulation was rescued by inhibition of translation, protein transportation and secretion, suggesting that PAM induce IL1β transcriptional up-regulation can be mediated by indirect feedback mechanisms. This finding is consistent with previous research where signalling through cytokine receptors may activate a positive feedback loop for the regulation of IL1β (Beutler 2009). The magnitude of PAM induced up-regulation of TLR1 mRNA level was partially prevented by inhibiting protein transportation and secretion, suggesting that the TLR1 mRNA increase induced by PAM is partially regulated by feedback through secreted proteins. The magnitude of PAM induced down-regulation of TLR8 mRNA level was rescued by inhibition of translation but not by protein transportation or secretion. This result suggests that this TLR8 mRNA decrease is regulated by feedback through intracellular proteins.

Together, results from the SN treatment experiments and the protein inhibition experiments suggest that SN induced mRNA changes of TLRs may be regulated by the combination of the low concentration of PAM residue in the culture supernatant and the protein production mediated indirect feedback. However, when the last medium wash (LW) was collected and used to stimulate cells alongside SN treatment as a control, the LW control treatment also induced the same transcriptional changes of IL1β and TLR2/4. This is inconsistent with results from the PAM agonist titration experiment and may require further investigation.

Overall, data from this chapter provided important evidence indicating that PAM induced transcriptional regulation of the cognate TLR1 was partially mediated by indirect feedback loops through the production of intracellular or secreted proteins. This result confirmed pervious research where a number of cytokines, i.e.: downstream feedback of IL5, could act as signalling regulators for the transcriptional regulation of cognate TLRs (Yoshimura, Naka et al. 2007, Heeg, Dalpke 2003). Furthermore, this

chapter was the first to identify the indirect feedback mechanism for PAM induced cross-regulation of TLR8.

Chapter 9 Comparative studies of TLR transcriptional regulation in different species or induced by different agonists

9.1 Introduction

Transcriptional regulation of TLRs mediated by the same agonist may vary due to cell type differences (See Chapter 4.1) or species differences. A number of studies explored TLR regulations in species other than *Mus musculus*. Peroval et al. (2013) investigated agonist induced transcriptional profile of cognate and cross-regulating TLRs in chicken macrophage cell line HD11. PAM stimulation reduced mRNA expression of all TLRs in chicken except TLR15. Similarly, LPS stimulation reduced transcriptional expression of all TLRs except TLR1.2 and TLR15. Zarember et al. (2002) examined LPS induced transcriptional changes of cognate and cross-regulating TLRs in human monocyte/macrophage cell line THP1. Treatment with LPS increased transcript levels of TLR1, 2, 3, 4, 5, 7 and 8 whereas mRNA levels of TLR6, 9 and 10 remained unchanged. In murine macrophage cell line RAW264.7, PAM stimulation up-regulated mRNA levels of TLR1 and 2 while down-regulated transcript levels of TLR4, 5, 7, 8 and 9; LPS stimulation increased transcript levels of TLR1, 2 and 3 while reduced mRNA levels of TLR5 (see Chapter 3). This differential regulation of TLRs among various species or induced by different agonists may be due to the activation and balancing effect between different signalling pathways. In addition, physiological differences between species may be a contributing factor to the transcriptional regulation of TLRs. For instance, circadian clock oscillations may affect the TLR regulations in the same way in animals with opposite circadian rhythm. In mice, TLR9 mRNA level increased, which mediated more potent immune responses during the active phase (at night) (Silver, Arjona et al. 2012) (see Chapter 4.2.4). It is possible that circadian rhythm may also increase the transcript level of TLR9 and in turn, induce stronger immune responses during the active phase (at day) in chicken.

Therefore, this chapter aimed firstly, to address the signalling activation responsible for PAM mediated TLR responses in chicken and to compare the signalling differences between chicken and mice; secondly, to explore the effect of day/night oscillation on TLR mRNA levels and immune responses in chicken and lastly, to compare the signalling pathways used for PAM and LPS mediated TLR transcriptional changes in murine macrophages.

9.2 Results

9.2.1 PAM induced IL1β and TLR transcriptional changes in HD11 cell line

Substantial increase in IL1β mRNA level was measured following PAM stimulation from 1.6 ng/ml upwards. The magnitude of IL1β transcriptional up-regulation increased according to the increasing PAM concentration (Figure 9.1A). In a similar manner, low concentration of PAM stimulus at 8 ng/ml reduced transcript level of cognate TLR2.1 and TLR2.2 and cross-regulating TLR7. The magnitude of TLR2.1, TLR2.2 and TLR7 down-regulation increased according to the increasing concentration of PAM (Figure 9.1B-D).

Phosphorylation status of MAPK signalling terminal components, ERK, JNK and P38 was measured. Phosphorylation of ERK1 at p44 was observed in both non-stimulated cells and PAM treated cells whereas phosphorylation of ERK2 was not detected in HD11 cells. PAM treatment induced elevated phospho level of ERK1 which exceeded the basal level between 15 mins and 60 mins post stimulation. PAM induced phosphorylation of ERK1 peaked at 30mins and stayed constant until basal phosphorylation slowly caught up (Figure 9.2A). The basal phosphorylation of JNK1/2 and P38 remained stable at low levels (Figure 9.2B-C). PAM induced phosphorylation of JNK1/2 was measured between 15 mins and 60 mins following agonist treatment, with phospho levels peaked at 30mins (Figure 9.2B). Phosphorylation of P38 was measured from 5 mins to 120 mins following PAM stimulation. However its phospho level was slow to peak, with the maximum phospho intensity documented between 40 mins to 60 mins post agonist stimulation (Figure 9.2C).

9.2.2 Influence of day/night oscillation on agonist induced IL1β and TLR mRNA expression in chicken splenic macrophages

To compare the differences of IL1β and TLR transcriptional regulation between species, day/night oscillation experiment was conducted in primary chicken splenic

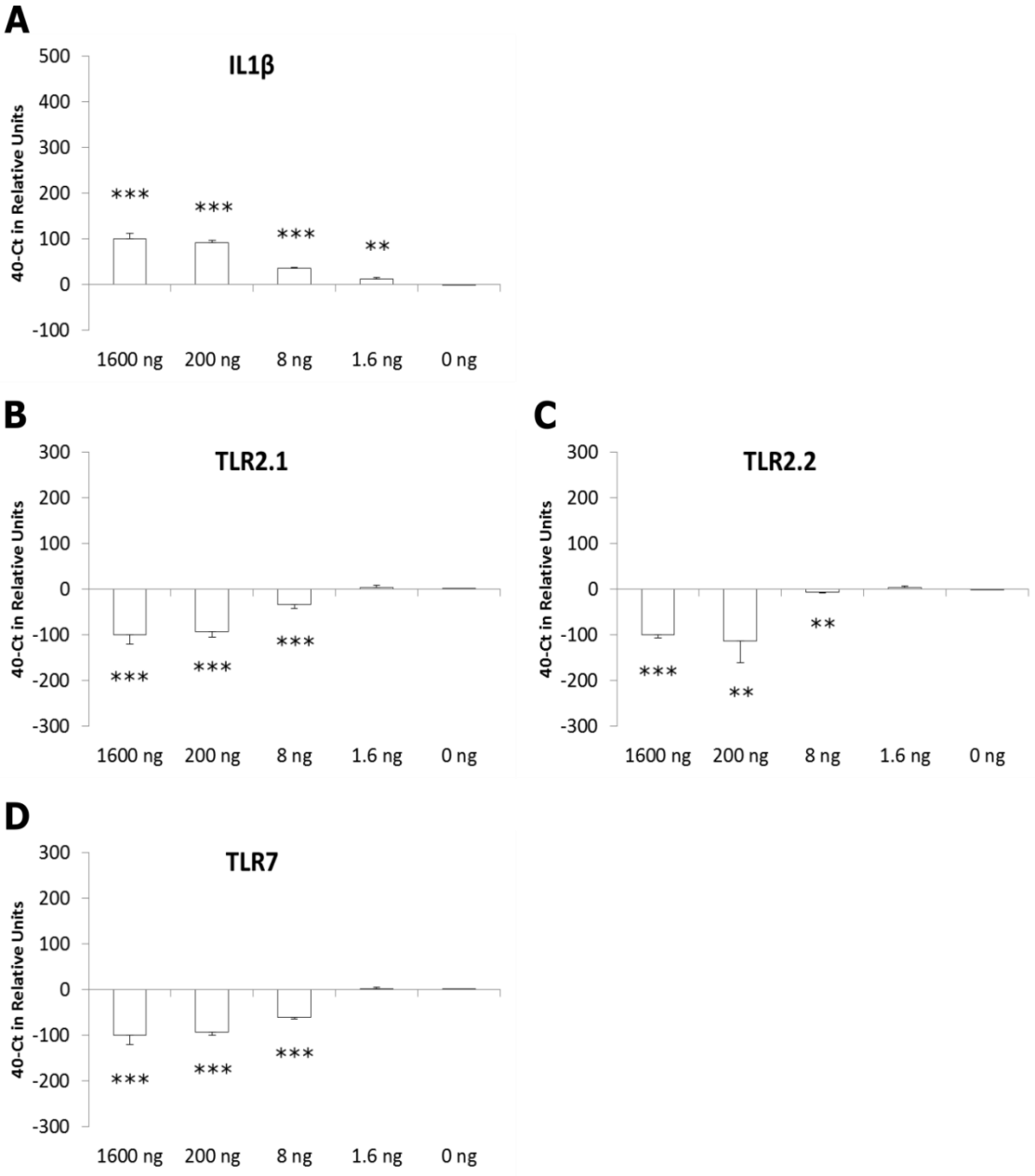


Figure 9.1: Transcriptional expression of IL1β and TLR following PAM titration in HD11 cells. The x axis indicates PAM treatment concentration; the y axis indicates mean 40 – Ct values in relative quantity based on the assumption that mock treatment (water) induced TLR cycle value is defined at 0 units, while PAM induced TLR cycle value is defined at 100 units. Error bars and significance levels denoted by asterisks are the same as previous figures.

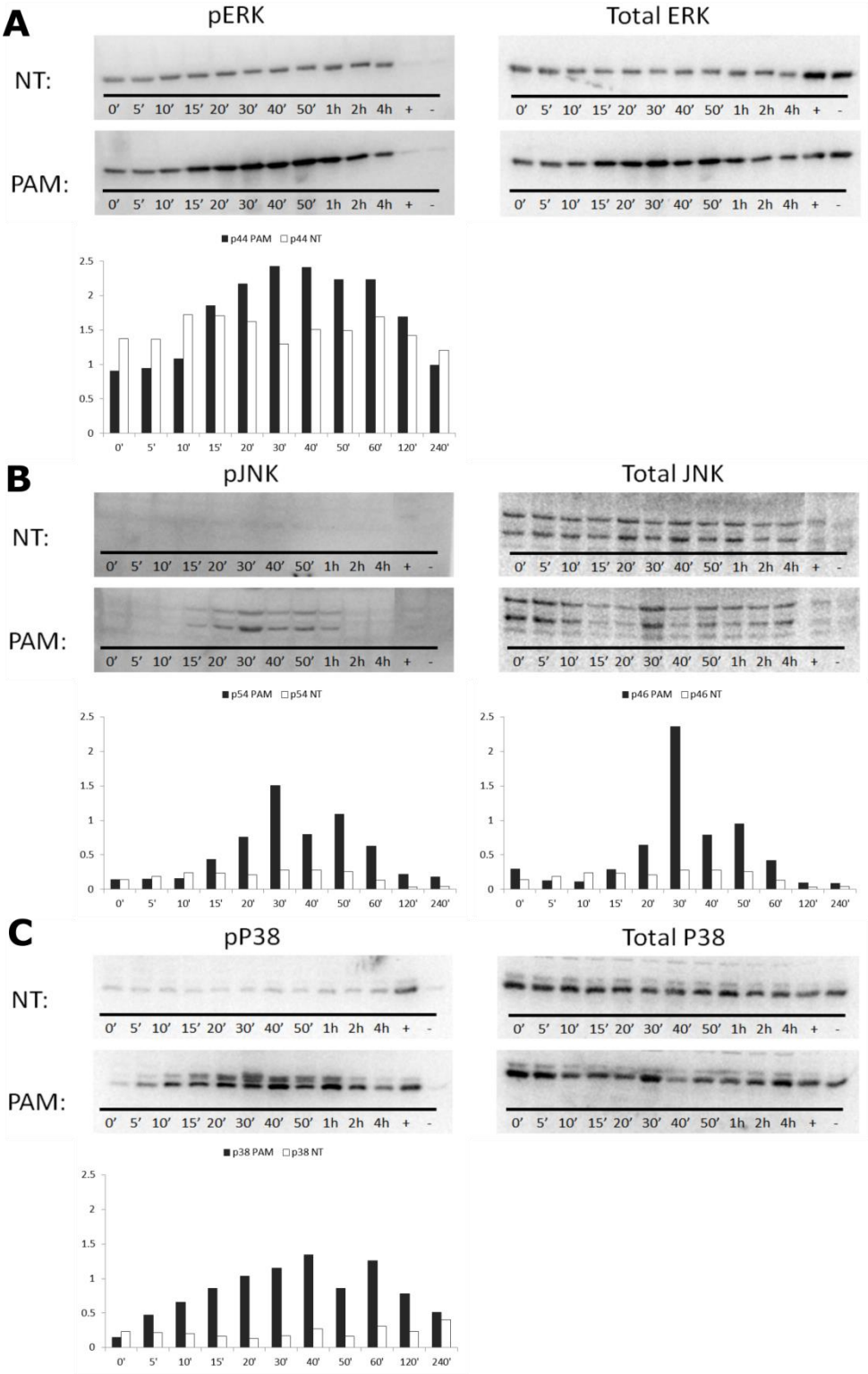


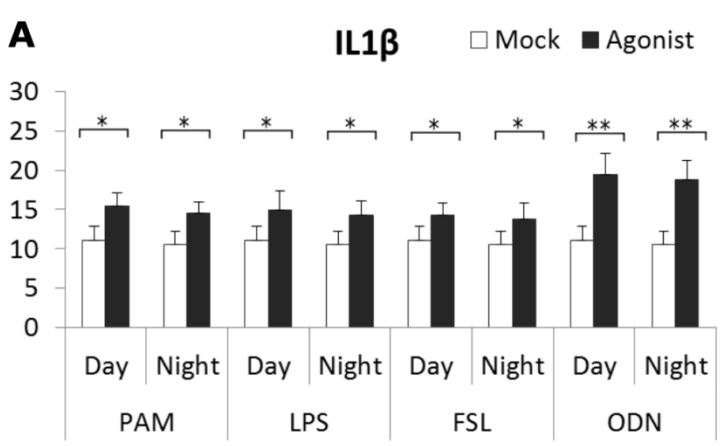
Figure 9.2: PAM-induced MAPK signalling activation in HD11 cells. Images represent the phosphor- or total- status of protein ERK1/2 (A), JNK1/2 (B) or P38 (C) in PAM treated and non-treated cells. Graphs represent densitometry analysis of band intensity in relation to the positive control band. The x axis indicates incubation time; the y axis indicates percentage intensity of the positive control. The clear bars represent protein phosphorylation status in non-stimulated cells; the black bars represent protein phosphorylation status in PAM-stimulated cells.

macrophages. Agonists were treated at the same time points as in murine day/night oscillation experiment and transcript levels of IL1β and TLRs were measured 8 hrs after agonist stimulation (see section 4.2.3). The effect of day/night oscillation on agonist induced transcriptional regulation of IL1β was measured in outbred red jungle fowl chicken and inbred line 6 chicken. Basal expression of IL1β remained constant at day and at night in both outbred and inbred chicken (Figure 9.3). Treatment with agonists PAM, LPS, FSL or ODN all increased transcript levels of IL1β. The magnitude of agonists induced IL1β up-regulation was not altered by treatment time in outbred chicken due to larger variations between different chicken samples (Figure 9.3A). In inbred line 6 chicken, the magnitude of LPS induced IL1β up-regulation was substantially higher at day than at night whereas PAM, FSL or ODN did not induce differential regulation of IL1β (Figure 9.3B).

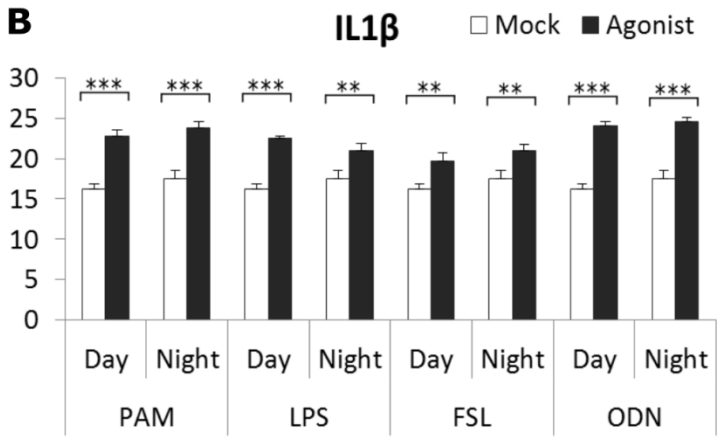
The effect of day/night oscillation on TLR4 in inbred line 6 chicken was then examined as TLR4 is cognate to LPS stimulation. The basal transcript level of TLR4 remained constant at day and at night. LPS, FSL and ODN down-regulated TLR4 mRNA levels at night ($p<0.05$) whereas none of these agonists altered TLR4 transcript levels at day. However the magnitude of TLR4 transcriptional changes was not different between day and night following LPS, FSL or ODN stimulation due to variation among samples. PAM treatment did not affect TLR4 transcriptional regulation at any time (Figure 9.4).

9.2.3 Signalling pathways required for LPS induced TLR transcriptional changes in murine RAW264.7 cell line

LPS treatment up-regulated mRNA levels of TLR1, TLR2 and TLR3 while down-regulated mRNA level of TLR5 in RAW cells. Pharmacological inhibitors for MAPK and NFκB signalling terminal components were used on cells treated with LPS to compare the signalling pathways responsible for mediating LPS and PAM induced transcriptional regulation of TLR1 and TLR2. Treatment with ERK inhibitor (PD059),

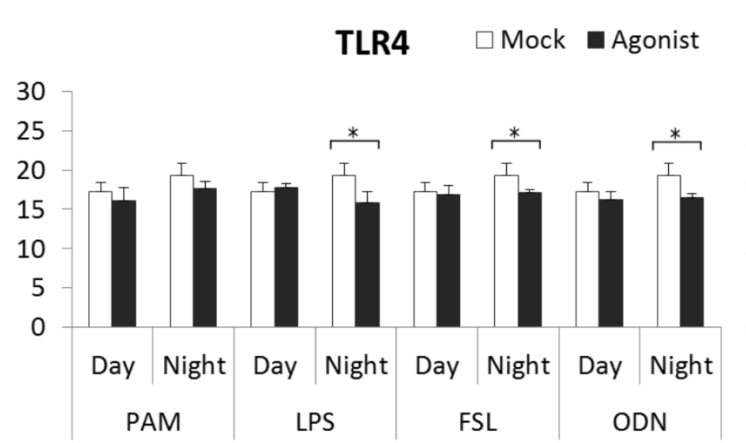


IL1β	Day	Night
PAM	15.27	13.15
LPS	12.32	12.03
FSL	9.02	9.51
ODN	50.32	49.91



IL1β	Day	Night
PAM	33.06	30.49
LPS	30.07	11.72
FSL	11.25	11.57
ODN	46.55	37.94

Figure 9.3: Effect of Day/Night oscillation on agonist-induced IL1β responses in splenic macrophages in outbred red jungle fowl chicken (A) or inbred chicken line 6 (B). The x axis indicates the treatment time (Day vs Night) and the agonist used: PAM, LPS, FSL or ODN; the y axis indicates mean 40 – Ct values adjusted for 28S. The clear bars represent TLR expression in non-stimulated splenic macrophages; the black bars represent TLR expression in agonist-stimulated splenic macrophages. Error bars and significance levels denoted by asterisks are the same as previous figures. Values in the tables depict average fold increase of mRNA levels in agonist-stimulated cells compared with non-stimulated cells. Green highlight colour indicates statistically significant differences of the fold changes between samples measured at day and night.



TLR4	Day	Night
PAM	-3.42	-9.56
LPS	1.18	-61.78
FSL	-0.38	-22.61
ODN	-1.88	-35.31

Figure 9.4: Effect of Day/Night oscillation on agonist-induced TLR4 transcriptional regulations in inbred chicken line 6 splenic macrophages. The x axis indicates the treatment time (Day vs Night) and the agonist used: PAM, LPS, FSL or ODN; the y axis indicates mean 40 – Ct values adjusted for 28S. The clear bars represent TLR expression in non-stimulated macrophages; the black bars represent TLR expression in agonist-stimulated macrophages. Error bars and significance levels denoted by asterisks are the same as previous figures. Values in the tables depict average fold increase (positive value) or fold decrease (negative value) of mRNA levels in agonist-stimulated cells compared with non-stimulated cells.

JNK inhibitor (SP125) or P38 inhibitor (SB580) did not affect the basal expression of TLR1 or TLR2 in inhibitor-only treated cells, although a trend of TLR1 down-regulation was observed following treatment with SP125 (Figure 9.5A). Similarly, inhibition of individual MAPK signalling terminal components or inhibition of individual MAPK signalling components with NFκB pathways did not alter the elevated levels of TLR1 or TLR2 in LPS treated cells (Figure 9.5A-B). However, magnitude of LPS induced up-regulation of TLR1 was further increased by treatment with SP125 due to the trend of reduced TLR1 basal level (Figure 9.5A).

Inhibition of complete MAPK signalling with a combination of three inhibitors, PD059, SP125 and SB580 affected transcriptional regulation of TLR1 and TLR2 differently. The basal expression of TLR1 was reduced whereas basal expression of TLR2 was unaffected by inhibiting complete MAPK signalling in inhibitor-only treated cells (Figure 9.6). In LPS treated cells, the up-regulation of TLR2 was prevented by inhibiting MAPK pathway with ($p<0.05$) or without ($p<0.01$) NFκB signalling inhibitors whereas the up-regulation of TLR1 was not affected by either treatment. Overall, the magnitude of LPS induced up-regulation of TLR1 and TLR2 was not affected by inhibition of MAPK pathway with or without NFκB signalling (Figure 9.6A-B).

Inhibition of NFκB pathway with CEL prevented up-regulation of TLR1 ($p<0.05$) and TLR2 ($p<0.05$) induced by LPS. However, the effect of CEL on basal expression of TLR1 and TLR2 in inhibitor-only treated cells remained unknown (Figure 9.7).

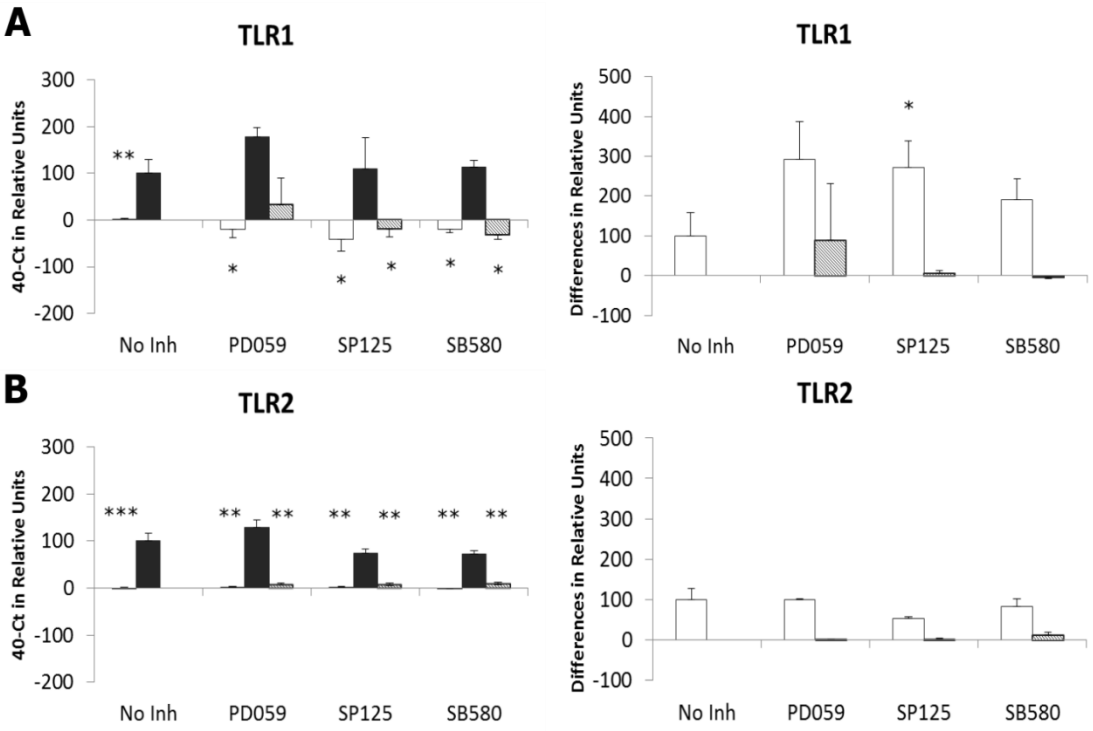


Figure 9.5: Effect of inhibiting individual MAPK signalling cascade with/without NFκB signalling on LPS-induced TLR1 and TLR2 transcriptional changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative quantity graphs, the clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in LPS-stimulated or LPS and MAPK inhibitor co-incubated cells; the grey bars represent TLR expression in cells treated with both MAPK and NFκB inhibitors, as well as LPS agonist. Error bars presented are at 95% confidence intervals and asterisks signify significance levels of the differences between the bar over which they are placed and LPS stimulated cells: *p< 0.05, **p< 0.01, ***p< 0.001.

.

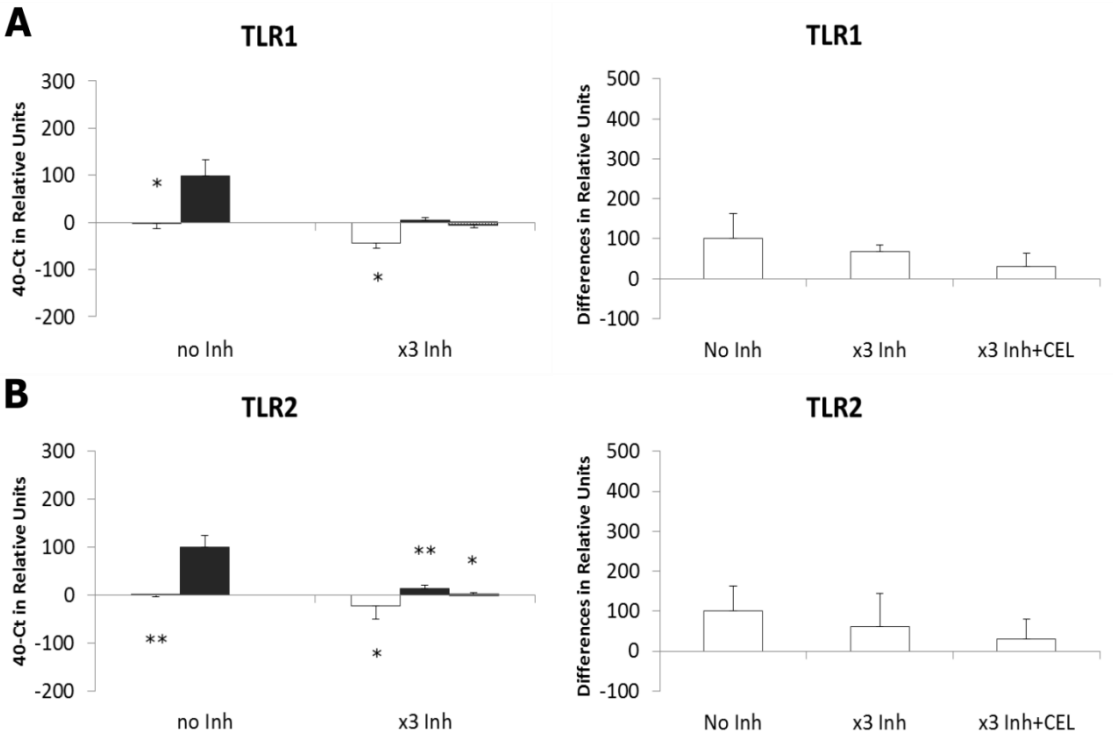


Figure 9.6: Effect of complete inhibition of MAPK signalling cascade with/without NFκB signalling on LPS-induced TLR1 and TLR2 transcriptional changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity or magnitude of change in relative quantity. In 40-Ct in relative unit graphs, the clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in LPS-stimulated or LPS and MAPK inhibitor co-incubated cells; the grey bars represent TLR expression in cells treated with both MAPK and NFκB inhibitors, as well as LPS agonist. Error bars and significance levels denoted by asterisks are the same as previous figures.

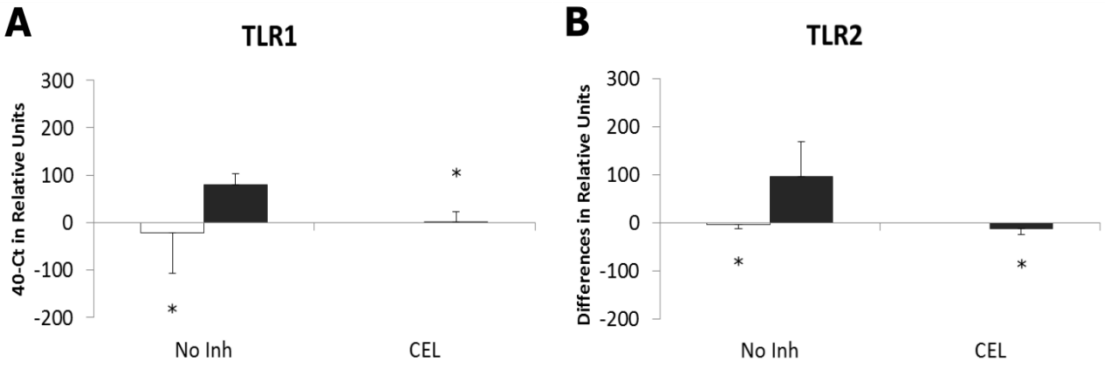


Figure 9.7: Effect of inhibiting NFκB signalling on LPS-induced TLR1 and TLR2 transcriptional changes in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values in relative quantity. The clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in LPS-stimulated or LPS and NFκB inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.

9.3 Discussion

Toll-like receptor families and their signalling molecules are highly conserved across species, with great similarity in their functionalities. Some TLRs are associated with similar health traits in different species. Polymorphism of *TLR1* and *TLR6* can induce susceptibility to bacterial infections, causing diseases such as mastitis in cattle, listeriosis in mice and tuberculosis human (Jann, King et al. 2009). On the other hand, agonist induced regulation of TLRs and TLR mediated immune responses can differ between species (Peroval, Boyd et al. 2013, Zarember, Godowski 2002). Hence studies of TLR signalling and regulation in different species is of great importance for the discovery of vaccine adjuvants and the application of animal models for the study of human diseases.

PAM induced transcriptional regulation of cognate- and cross-regulating TLRs as well as TLR mediated cytokine production at a much lower concentration in chicken than in mice. This is consistent with the fact that same concentration of PAM induced longer phosphorylation time periods and higher magnitudes of phosphorylation levels of MAPK signalling terminal components in chicken than in mouse (Figure 9.8). Chicken share majorities of its TLRs with mammals, but also share TLR15 and TLR21 with reptiles (Roach, Glusman et al. 2005). The transcriptional regulations of PAM induced cognate TLRs were different in these two species. TLR1/2 was up-regulated in mouse while down-regulated in chicken. Signalling pathways responsible for mediating the TLR transcriptional changes in these two species were then compared. Inhibition of ERK signalling increased mRNA level of TLR1 in mouse and TLR1.1 in chicken, suggesting that ERK signalling is important for the regulation of PAM induced TLR1 in mouse and TLR1.1 in chicken. Furthermore, inhibition of ERK reduced transcript level of TLR2.2 in chicken below the PAM induced level as well as increased transcript level of TLR2 in mouse above the PAM induced level, suggesting that ERK signalling is also important for the fine tuning of TLR2 in mouse and TLR2.2 in chicken. On the other

Mice	pERK	pJNK	pP38
Time	10' - 30'	10' - 50'	10' - 50'
Magnitude			
Chicken	pERK	pJNK	pP38
Time	15' - 60'	15' - 120'	5' - 120'
Magnitude			

Table 9.8: PAM-induced MAPK signalling activation in murine RAW264.7 cell line vs chicken HD11 cell line. Values in the tables depict the time period during which PAM-induced phosphor-ERK, phosphor-JNK or phosphor-P38 was measured. Darker colour highlights indicate longer activation time period or larger magnitude of phosphor levels compared to the positive control bands.

Species		Chicken				Mice			
TLR\Inhibitors		PD059	SP125	SB580	CEL	PD059	SP125	SB580	CEL
TLR1	TLR1.1		-	-	-		-	-	-
	TLR1.2	-		-					
TLR2	TLR2.1	-	-		-		-	-	
	TLR2.2		-		-				

Table 9.9: Signalling pathways mediating PAM induced TLR1/2 transcriptional regulation in HD11 cells vs RAW cells. Red highlight colour indicates statistically significant ($p<0.05$) up-regulation; blue highlight colour indicates statistically significant down-regulation. Lighter shade is in comparison to the basal expression of TLR1/2; darker shade is in comparison to PAM induced up- or down-regulation levels.

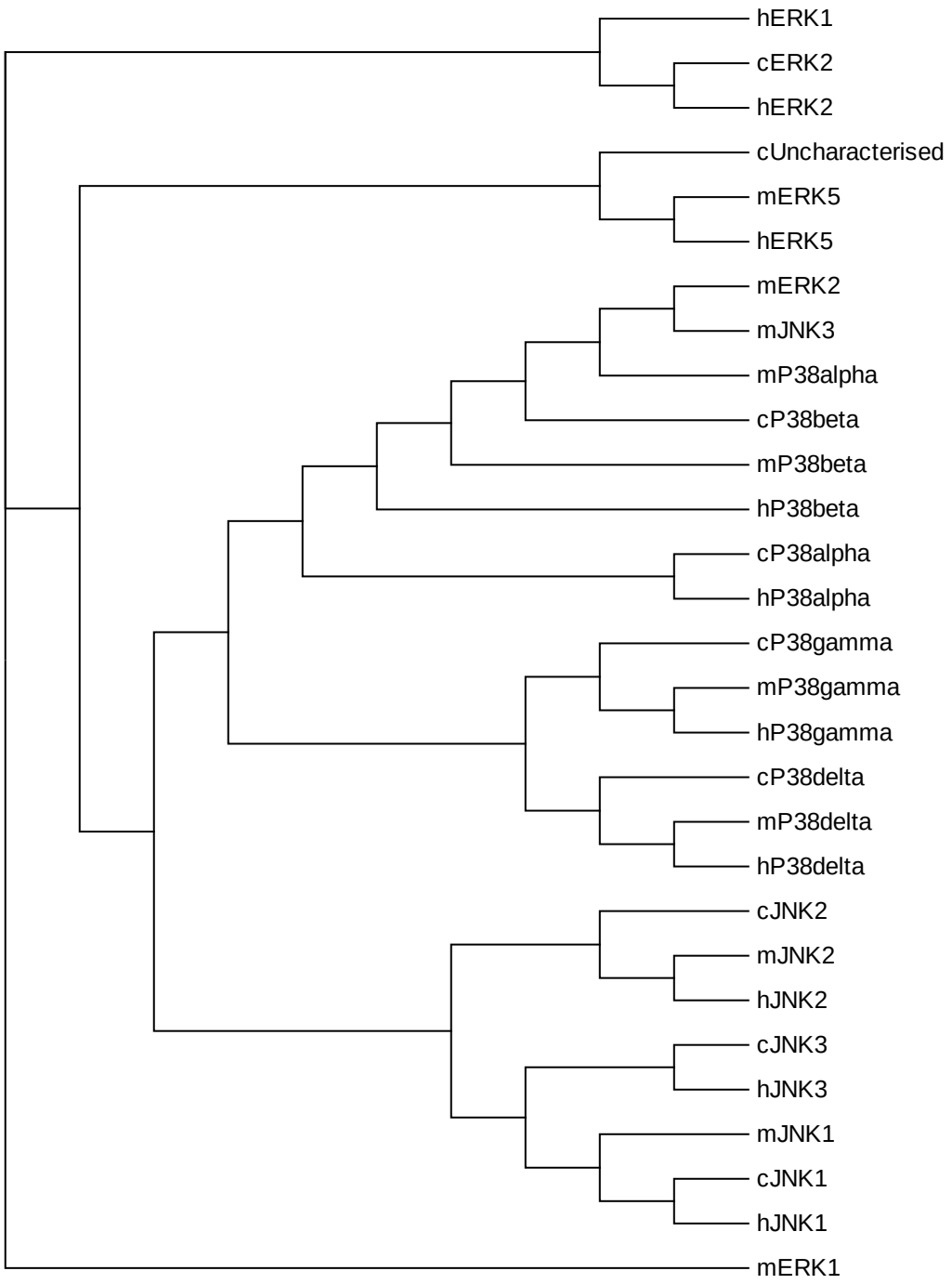


Figure 9.10: An uncharacterised chicken amino acid sequence is closer related to ERK5 in mouse and human than ERK1 or ERK2.

A

Inhibitor Effect on Basal TLR mRNA levels				
TLR	TLR1		TLR2	
Agonist	PAM	LPS	PAM	LPS
PD059 (ERK)	80.98	-18.91	28.54	2.12
SP125 (JNK)	99.67	-41.42	-59.69	1.80
SB580 (P38)	79.55	-18.94	-77.39	-0.70
CEL (NFκB)	759.53	ND	-100.40	ND

B

Inhibitor Effect on PAM-induced TLR mRNA levels				
TLR	TLR1 (100)		TLR2 (100)	
Agonist	PAM	LPS	PAM	LPS
PD059 (ERK)	208.70	178.91	220.31	128.31
SP125 (JNK)	129.87	109.29	102.31	73.59
SB580 (P38)	-26.85	114.08	59.13	71.85
CEL (NFκB)	33.15	1.01	-66.82	-11.87

C

Differences of Inhibitor Effect on TLR levels				
TLR	TLR1 (100)		TLR2 (100)	
Agonist	PAM	LPS	PAM	LPS
PD059 (ERK)	108.05	291.47	176.11	100.21
SP125 (JNK)	62.31	271.08	116.64	53.84
SB580 (P38)	171.90	190.34	126.47	83.17
CEL (NFκB)	1354.10	ND	42.62	ND

Table 9.11: Signalling pathways mediating PAM and LPS induced TLR1/2 transcriptional regulation in RAW cells. Values in the tables depict the mean relative units of TLR mRNA levels. Red highlight colour indicates statistically significant ($p<0.05$) up-regulation; blue highlight colour indicates statistically significant down-regulation in inhibitor-treated cells compared to (A) non-stimulated cells; (B) PAM treated cells. Green highlight colour indicates statistic significant magnitude differences between PAM induced and basal TLR mRNA levels.

hand, transcriptional regulation of TLR1.2 and 2.1 in chicken were mediated by different signalling pathways compared to TLR1/2 in mouse (Table 9.9). Bioinformatics analysis revealed that chicken lack ERK1 gene which is prominent in regulating TLR transcription in mice. The annotated chicken ERK1 gene on Ensembl ('cUncharacterised' on the figure) is, in fact, most closely related to ERK5 in human and mouse (Figure 9.10). This difference in the available signalling cascades in chicken and in mouse may be one of the reasons why TLR transcription was regulated differently between these two species.

Circadian rhythm is known to affect TLR mediated immune responses (Zhou, Wang et al. 2015, Curtis, Fagundes et al. 2015, Nakao 2014, Mukherji, Kobiita et al. 2013, Silver, Arjona et al. 2012, Gibbs, Blaikley et al. 2012, Keller, Mazuch et al. 2009). Murine cells immunised with ligand-adjuvanted vaccines at the highest expression time point of TLR9 induced substantially more IFNγ in 4 weeks' time than cells immunised at the lowest TLR9 expression time point (Silver, Arjona et al. 2012). This finding may help improve the efficiency of ligand-adjuvanted vaccines by selecting the appropriate time for immunisation. Chicken vaccination has great importance due to the large number of consumption worldwide. In this chapter, the effect of circadian rhythm on agonist induced TLR regulations in chicken was explored. Although LPS induced higher magnitude of IL1β during the active phase of chicken (at day than at night), no difference in the magnitude of TLR4 regulation was detected between day and night.

At last, signalling pathways responsible for PAM and LPS induced TLR1/2 transcriptional regulation in murine macrophage was compared. Inhibition of NFκB reduced TLR2 up-regulation in both agonists treated cells, suggesting that NFκB signalling is important for mediating PAM and LPS induced TLR2 responses. Inhibition of JNK signalling reduced TLR1 transcript level in PAM treated cells while increased TLR1 mRNA level in LPS treated cells, suggesting that JNK signalling is also contributing for the fine-tuning of TLR1 regulation (Table 9.11).

Overall, these data present complex signalling patterns for mediating TLR responses. Similarities and differences of the signalling pathways responsible for TLR transcriptional regulations by different agonist or in different species were both observed, suggesting that the fine tuning of TLR regulation requires the co-ordination of all signalling pathways.

Chapter 10 Conclusion

The production of innate immune responses is essential for host defence. However, too much inflammation can be dangerous to the host body, causing diseases such as sepsis and autoimmune disorders. Hence, several complex mechanisms have been employed for the tight control of innate responses by supressing TLR sensitivity during prolonged or repeated exposure to their agonists. One of these mechanisms is the transcriptional regulation of toll-like receptors (TLRs).

10.1 Agonist induced TLR transcription pattern in murine cell lines and splenic macrophages

Data presented in chapter 3 demonstrated the TLR transcriptional changes induced by agonists derived from bacterial surface structures: PAM, LPS and FLA. Different agonists induced similar transcriptional changes for some TLRs. Transcript levels of the TLR1/2 family were up-regulated and transcript levels of TLR4 were down-regulated by both PAM and LPS. For other TLRs, different transcription patterns were induced by different agonists. Treatment with PAM agonist induced down-regulation of the non-cognate TLR5 and TLR7/8/9 mRNA levels, treatment with LPS agonist induced up-regulation of the non-cognate TLR3, TLR5 and TLR13 mRNA levels whereas treatment with FLA did not induce any TLR transcriptional changes in RAW cells. This agonist-dependent TLR transcription patterns were also observed in previous study where the mRNA levels of the TLR1/2 family were up-regulated by PAM but down-regulated by LTA in RAW cells (Dalpke, Lehner et al. 2005).

Both PAM and LPS induced similar transcriptional changes of the cognate TLRs in P388D1 cells compared to RAW cells. Transcript levels of the TLR2 and TLR6 was up-regulated by PAM and transcript levels of TLR4 were down-regulated by LPS in P388D1 cells. However transcriptional regulation of the cross-regulating TLRs was not observed in P388D1 cells. As P388D1 cells is less sensitive to dual stimulation of LPS and IFNγ than RAW cells (Walloschke, Fuhrmann et al. 2010), it is likely that this

hyposensitivity of P388D1 cells may be due to the lack of transcriptional regulation of the cross-regulating TLRs. In addition, TLR9 is present on RAW cells but not on P388D1 cells (Shibata, Takemura et al. 2012), this difference in the TLR repertoires present on the cells may be a contributing factor for mediating the different TLR transcription patterns between cell lines.

Studies presented in chapter 4 identified the TLR transcription pattern in splenic macrophages from two murine strains and compared this to the TLR transcription patterns in cell lines. PAM induced the same transcriptional up-regulation of the cognate TLR1/2 between the two mouse strains and between primary macrophages vs. cell lines. However, although LPS induced the same transcriptional down-regulation of the cognate TLR4 between cell lines and splenic macrophages from BALB/c mice, TLR4 mRNA level increased following treatment with LPS in macrophages from C57Bl/6 mouse. In addition, treatment with PAM down-regulated TLR4 mRNA level in murine intestinal cells while up-regulated TLR4 transcript level in B cells (Jagannathan, Hasturk et al. 2009, van Aubel, Keestra et al. 2007). Together, these results suggest that transcriptional regulation of TLR4 may be dependent on cell types.

Studies presented in chapter 4 were the first to comprehensively characterise thr transcriptional regulation of the non-cognate TLRs induced by PAM, FLA, CL097 and ODN in murine splenic macrophages. Similar studies were conducted previously in chicken HD11 cells and in human THP1 cells (LPS treatment only) but not in mice (Peroval, Boyd et al. 2013a, Zarember, Godowski 2002). As mice have been widely used as a popular animal model for the studies of human diseases, my data filled in the gaps for species-species comparison (discussed in chapter 9) and provided important insights for the understanding of TLR mediated immune responses. Similar to that in murine macrophage cell lines, TLR transcription patterns differ according to agonists but with some TLRs consistently up- or down-regulated by various agonists. For instance, the mRNA levels of TLR1 and TLR2 increased following treatment with

PAM, LPS, FLA, CL097 and ODN; the mRNA levels of TLR8 reduced following treatment with PAM, LPS, FLA and ODN, suggesting that the transcriptional regulation of TLR1/2 and TLR8 induced by these agonists may be mediated through the same signalling pathways. This was then explored in chapter 5, 6 and 9.

10.2 Direct and indirect regulation of TLR transcriptional changes

Production of TLR mediated immune responses requires activation of both MAPK and NFκB signalling pathways, directly downstream of TLR activation. In murine cross-regulation model, both ERK and NFκB signalling pathways contributed to LPS induced up-regulation of TLR2 mRNA level (Matsuguchi, Musikacharoen et al. 2000), indicating that the transcriptional regulation of TLRs may also require activation of both MAPK and NFκB signalling pathways. Results presented in chapter 5 and chapter 6 were the first to systemically analyse functions of the direct MAPK and NFκB signalling pathways in regulating PAM induced TLR transcription in murine model, with chapter 5 focused on the effects of individual signalling cascades, ERK, JNK, P38 and NFκB and chapter 6 focused on the synergetic effects (i.e.: additive, antagonistic or non-interfering) between two or more signalling cascades.

Transcriptional regulation of TLRs mediates innate immune responses by modifying the availability of TLR molecules present in the cells, for instance, TLR hypersensitivity may be caused by over-expression of TLRs. This over-expression of TLR molecules can be due to the sudden increase in TLR transcription induced by agonists or the accumulated basal TLR expression. Few studies have addressed signalling controls for agonist induced TLR regulation (Izadi, Motameni et al. 2007, Matsuguchi, Musikacharoen et al. 2000) while signalling controls for the basal TLR regulation have been completely neglected. Hence, one of the important findings presented in chapter 5 is the signalling contribution for the basal mRNA expression of TLRs in resting macrophages. Pharmacological inhibition revealed that NFκB pathway contributed to the basal mRNA expression of TLR1 and TLR2, ERK pathway contributed to the basal

mRNA expression of TLR4 and TLR8 and JNK pathway contributed to the basal mRNA expression of TLR8 and TLR9, indicating that the basal transcription of TLRs from the same family may be regulated by similar signalling pathways.

In case of agonist induced TLR transcriptional regulation, both individual and multiple pathways contributing to the transcriptional regulation of PAM induced TLRs were observed. NFκB pathway contributed to down-regulation of TLR4 and TLR8 mRNA levels and JNK pathway contributed to the up-regulation of TLR1 mRNA levels induced by PAM. In addition, both ERK and NFκB pathways mediated the up-regulation of TLR2 mRNA level and both JNK and NFκB signalling pathways mediated the down-regulation of TLR9 mRNA level.

Taking into account the signalling pathways for the regulation of both the basal and PAM induced TLR transcription, these results indicated complex and distinct signalling pattern for each TLR, with multiple signalling pathways collaborating to achieve the fine tuning of TLR regulation. Detailed interactions between ERK, JNK, P38 and NFκB signalling pathways were systematically analysed in chapter 6.

By using combinations of double, triple or quadruple pharmacological signalling inhibitors, I was able to identify whether the interactions between signalling pathways were additive, antagonistic or non-interfering. Transcription of TLR1 was regulated by NFκB pathway which mediated PAM induced up-regulation and the combination of ERK, JNK and P38 signalling cascades which mediated the basal expression. Similar to TLR1, transcription of TLR4 was also mediated by both NFκB and MAPK signalling pathways in the same way. Transcription of TLR2 was regulated by the combination of JNK, P38 and NFκB pathways which mediated PAM induced up-regulation and ERK signalling which mediated the basal expression. Transcription of TLR9 was regulated by the combination of MAPK and NFκB pathways for both PAM induced down-regulation and basal expression. The transcriptional regulation of TLR8 is interesting: PAM induced TLR8 down-regulation was regulated by the balancing effect between

the combination of JNK and P38 signalling cascades and the NFκB pathway. Furthermore, the basal expression of TLR8 was regulated by the combination of ERK and JNK signalling cascades. Overall, this study was the first to comprehensively identify the collaborative effects between MAPK and NFκB signalling pathways, with data indicating that PAM induced TLR transcription were regulated by the additive and balancing effects between ERK, JNK, P38 and NFκB signalling cascades except for TLR7.

Although cross-talk between TLR4, 5, 7 and 9 have previously been reported (Palazzo, Gariboldi et al. 2008), none have studied the signalling pathways recruited for TLR2 and TLR7 cross-talk. As PAM induced TLR7 down-regulation was not altered by inhibition of MAPK or NFκB pathways, it is possible that TLR7 transcription is regulated by the feedback effect of immune regulators on signalling pathways. Hence this indirect signalling regulation was discussed in chapter 8. Results demonstrated that PAM induced transcriptional regulation of TLR1 and TLR8 were partially mediated by indirect feedback via production of intracellular or secreted proteins. However TLR7 transcription was not mediated by indirect feedback. Hence PAM induced transcriptional regulation of TLR7 may be dependent on alternative pathways other than the MAPK or NFκB pathways.

10.3 Side effects of pharmacological inhibition and alternative signalling inhibition with siRNAs

Pharmacological inhibition of a single signalling pathway may affect the signal flow through other pathways by three mechanisms: supressing the phosphorylation of other signal pathways by targeting a conserved protein structure, increasing the phosphorylation of other signal pathways by reducing the production of signalling negative regulators or redirecting the signal flow from the supressed pathway to a different pathway through a common upstream signal cascades (Fey, Croucher et al. 2012, Tanemura, Momose et al. 2009, Shimo, Matsumura et al. 2007). In chapter 6, I

investigated the side effects of pharmacological inhibition on MAPK and NFκB signalling in PAM treated cells. Results indicated that pharmacological inhibition not only changed phosphorylation status of non-targeted signalling cascades but also altered the timing of signal flow in non-targeted pathways by accelerating, delaying or prolonging phosphorylation. The effect of signalling inhibitors on phosphorylation timing have not been previously addressed in murine macrophages treated with PAM agonist. Together with the study of circadian effect on TLR regulation (chapter 4), these results provide important insights which may help discovering the most efficient stimulation time point for disease treatment.

Studies presented in chapter 7 discussed inhibition of ERK signalling with synthetic siRNAs, a signalling inhibition method more precise in targeting signalling cascades than pharmacological inhibition. Results confirmed the contribution of ERK1 signalling for the transcriptional regulation of TLR8 and TLR9 but not for the maintenance of TLR2 basal expression, suggesting that basal mRNA expression of TLR2 may be regulated by ERK2 or ERK5 rather than ERK1.

10.4 Species-specific TLR transcriptional regulation

Transcriptional regulation of TLRs in mice was compared to other model species, chicken and human (Peroval, Boyd et al. 2013b, Zarember, Godowski 2002) in chapter 9. Similarities and differences in agonist induced TLR transcriptional changes were observed. In both human THP1 cells and murine RAW cells, treatment with LPS increased transcript levels of TLR1, TLR2 TLR3 and TLR5. On the other hand, transcript level of TLR4 was down-regulated in RAW cells whereas it was increased in THP1 cells. In addition, transcript levels of TLR7 and TLR8 were increased in THP1 cells whereas they remained unchanged in RAW cells. These differences may be due to the differential signalling pathways occupied in these two species. Recent study has found that the IRAK proteins function differently between mouse and human, with TLR signalling in mouse macrophages mainly dependent on IRAK2 and IRAK4 while

human macrophages mainly dependent on IRAK1 and IRAK4 (Sun, Li et al. 2016). It is possible that the different functions of IRAK proteins may affect TLR transcription induced by LPS agonist. In chicken HD11 cells and RAW cells, although PAM up-regulated TLR1/2 mRNA levels in mouse but down-regulated TLR1/2 mRNA levels in chicken, ERK signalling was shown to be responsible for PAM induced transcriptional changes of both TLR1/2 in mouse and TLR1.1 and TLR1.2 in chicken, indicating the signalling conservancy between species.

In addition, circadian oscillation studies presented in chapter 4 and chapter 9 revealed TLR induced immune responses in a time-dependent manner despite of the natural active/resting phase of the species. The magnitude of transcriptional up-regulation of IL1β induced by FLA and ODN was more during the day than during the night in murine macrophages. Similarly, the magnitude of transcriptional up-regulation of IL1β induced by LPS was more during the day than during the night in chicken macrophages. However this result contradicts with previous finding where the production of IL1β protein induced by ODN treatment during the night was more than that during the day in murine macrophages (Silver, Arjona et al. 2012). Hence further studies are required to confirm the finding.

10.5 Future work

This thesis has presented the extent of the TLR signalling complexity which is one of the vital control mechanisms for the transcriptional regulation of the cognate and the non-cognate TLRs. Signalling synergy between MAPK and NFκB pathways was address for the first time in this thesis. It was discovered that a group of signalling components may have a combined additive or antagonistic effect for maintaining the basal expression of each TLR while a different combination of signalling components may work together for regulating the agonist induced transcriptional changes of the same TLR. Among the signalling synergies, the balancing effect between one or more MAPK signalling components and NFκB signalling pathway is the most intriguing. So

far, it is still unclear how exactly the signalling pathways affect each other and how much do they contribute to the transcriptional regulation of the TLRs directly. These questions may be explored using petri-net bioinformatics tools where each signal impulse is translated into a token of energy passing through the signalling network (Gogolinska, Nowak, 2012). The petri-net approach will be able to generate simulated TLR transcription results based on existing experimental assumptions under shorter time period. The results from petri-net simulation can then be confirmed with a few key experiments. As understanding signal flow and outcome is important for pharmacological studies, this research has the potential to benefit future vaccine and disease treatments.

A second method to investigate the effect of specific signalling components is knock-out studies by using the Clustered regularly interspaced short palindromic repeats (CRISPR) technique. CRISPR, originally discovered in prokaryotic cells, has been developed into a genome editing tool for both cell lines and primary cells (Rath, Amlinger, et al., 2015; Bhaya, Davison, et al., 2011). Signalling component knock-out study on primary cells may reveal TLR transcriptional changes more relevant to the physiology of mouse/human. On the other hand, altering signalling regulatory genes may cause severe physiological side effects. For instance, the *ERK2*-deficient mice have low survival rate due to impaired embryonic development (Chan, Gu, et al., 2013). It is possible that CRISPR may also cause off-target effects that could be difficult to interpret.

Signalling pathways responsible for PAM induced TLR7 transcriptional down-regulation remains a mystery. As the direct TLR signalling pathways cross-talk with indirect pathways such as the PI3K-Akt pathway, it would be interesting to investigate whether such indirect pathway is responsible for mediating the PAM induced TLR7 transcriptional changes.

Signalling pathways are largely conserved between species, however some species-species variation in signalling cascades have been identified, for instance, chicken lack ERK5 and TRAM which are present in mammals and functions of IRAK protein differ between mouse and human (Sun, Li et al. 2016, Gillespie, Shamovsky et al. 2011, Keestra, van Putten 2008). It would be interesting to investigate the roles of these particular signalling cascades in regulating agonist induced TLR transcription.

Agonist induced TLR transcription patterns vary in different cell subsets due to the unique TLR repertoires present on different cells. Analysis of agonist induced TLR transcriptional changes in increased cell subsets would permit the development of more detailed hypotheses regarding to the functionalities of TLR transcriptional regulation.

The study about the effect of cytokine feedback on TLR transcription (indirect signalling) is rather incomplete due to the unexpected effects induced by treatment with last wash medium. It may be necessary to use microarray to determine the concentration of cytokines present in cell culture supernatant and to use synthetic cytokines instead of cell culture supernatant to study the effect of feedback loops.

Following the production of TLR mRNAs, several regulatory mechanisms occurring at the level of post-transcription and protein translation may play important roles in the fine tuning of TLRs (Carpenter, Ricci et al. 2014). It would be interesting to investigate the role of alternative splicing/alternative polyadenylation, mRNA stability control and translation initiation control in regulating agonist induced TLR transcription.

Most importantly, dual TLR agonist stimulation and TLR tolerance studies revealed differential regulation of cytokine production through complex signalling synergy (Murphy, Xiong et al. 2015, Mitchell, Yong et al. 2010, Biswas, Lopez-Collazo 2009, Ghosh, Mickelson et al. 2007, Dalpke, Lehner et al. 2005, Otte, Cario et al. 2004, Sato, Nomura et al. 2000). To fully understand the role of TLR transcriptional regulation in mediating innate immune responses, it will be necessary to investigate how the TLR

transcriptional changes would affect TLR protein production and the functions of agonist induced TLR protein production.

References

ABDERRAZAK, A., SYROVETS, T., COUCHIE, D., EL HADRI, K., FRIGUET, B., SIMMET, T. and ROUIS, M., 2015. NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases. *Redox biology,* **4**, pp. 296-307.

ADAMOVICH, Y., LADEUIX, B., GOLIK, M., KOENERS, M.P., ASHER, G., 2016. Rhythmic oxygen levels reset circadian clocks through HIF1α. *Cell Metabolism*. **25**, pp. 1-9.

ADIB-CONQUY, M. and CAVAILLON, J.M., 2002. Gamma interferon and granulocyte/monocyte colony-stimulating factor prevent endotoxin tolerance in human monocytes by promoting interleukin-1 receptor-associated kinase expression and its association to MyD88 and not by modulating TLR4 expression. *The Journal of biological chemistry,* **277**(31), pp. 27927-27934.

AGA, M., WATTERS, J.J., PFEIFFER, Z.A., WIEPZ, G.J., SOMMER, J.A. and BERTICS, P.J., 2004. Evidence for nucleotide receptor modulation of cross talk between MAP kinase and NF-kappa B signaling pathways in murine RAW 264.7 macrophages. *American journal of physiology.Cell physiology,* **286**(4), pp. C923-30.

AHMAD-NEJAD, P., HACKER, H., RUTZ, M., BAUER, S., VABULAS, R.M. and WAGNER, H., 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *European journal of immunology,* **32**(7), pp. 1958-1968.

AJIBADE, A.A., WANG, H.Y. and WANG, R.F., 2013. Cell type-specific function of TAK1 in innate immune signaling. *Trends in immunology,* **34**(7), pp. 307-316.

AKIRA, S. and TAKEDA, K., 2004. Toll-like receptor signalling. *Nature reviews.Immunology,* **4**(7), pp. 499-511.

AKIRA, S., UEMATSU, S. and TAKEUCHI, O., 2006a. Pathogen recognition and innate immunity. *Cell,* **124**(4), pp. 783-801.

AKIRA, S., YAMAMOTO, M. and TAKEDA, K., 2003. Role of adapters in Toll-like receptor signalling. *Biochemical Society transactions,* **31**(Pt 3), pp. 637-642.

ALEXOPOULOU, L., HOLT, A.C., MEDZHITOV, R. and FLAVELL, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* **413**(6857), pp. 732-738.

AMIT, I., WINTER, D.R. and JUNG, S., 2015. The role of the local environment and epigenetics in shaping macrophage identity and their effect on tissue homeostasis. *Nature immunology,* **17**(1), pp. 18-25.

AN, H., ZHAO, W., HOU, J., ZHANG, Y., XIE, Y., ZHENG, Y., XU, H., QIAN, C., ZHOU, J., YU, Y., LIU, S., FENG, G. and CAO, X., 2006. SHP-2 phosphatase negatively regulates the TRIF adaptor protein-dependent type I interferon and proinflammatory cytokine production. *Immunity,* **25**(6), pp. 919-928.

ANDROULIDAKI, A., ILIOPOULOS, D., ARRANZ, A., DOXAKI, C., SCHWORER, S., ZACHARIOUDAKI, V., MARGIORIS, A.N., TSICHLIS, P.N. and TSATSANIS, C., 2009. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. *Immunity,* **31**(2), pp. 220-231.

APPLEQUIST, S.E., WILLIN, P.R., LJUNGGREN, H.G., 2002. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int.Immunol.* **14**(9), pp. 1065-1074

AREAL, H., ABRANTES, J. and ESTEVES, P.J., 2011. Signatures of positive selection in Toll-like receptor (TLR) genes in mammals. *BMC evolutionary biology,* **11**, pp. 368-2148-11-368.

ASEA, A., REHLI, M., KABINGU, E., BOCH, J.A., BARE, O., AURON, P.E., STEVENSON, M.A. and CALDERWOOD, S.K., 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *The Journal of biological chemistry,* **277**(17), pp. 15028-15034.

BAETZ, A., FREY, M., HEEG, K. and DALPKE, A.H., 2004. Suppressor of cytokine signaling (SOCS) proteins indirectly regulate toll-like receptor signaling in innate immune cells. *The Journal of biological chemistry,* **279**(52), pp. 54708-54715.

BARRENSCHEE, M., LEX, D. and UHLIG, S., 2010. Effects of the TLR2 agonists MALP-2 and Pam3Cys in isolated mouse lungs. *PloS one,* **5**(11), pp. e13889.

BAUERFELD, C.P., RASTOGI, R., PIROCKINAITE, G., LEE, I., HUTTEMANN, M., MONKS, B., BIRNBAUM, M.J., FRANCHI, L., NUNEZ, G. and SAMAVATI, L., 2012. TLR4-mediated AKT activation is MyD88/TRIF dependent and critical for induction of oxidative phosphorylation and mitochondrial transcription factor A in murine macrophages. *Journal of immunology (Baltimore, Md.: 1950),* **188**(6), pp. 2847-2857.

BAZZONI, F., ROSSATO, M., FABBRI, M., GAUDIOSI, D., MIROLO, M., MORI, L., TAMASSIA, N., MANTOVANI, A., CASSATELLA, M.A. and LOCATI, M., 2009. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(13), pp. 5282-5287.

BEESON, P.B., 1946. Development of tolerance to typhoid bacterial pyrogen and its abolition by reticulo-endothelial blockade. *Proceedings of the Society for Experimental Biology and Medicine.Society for Experimental Biology and Medicine (New York, N.Y.),* **61**, pp. 248-250.

BELL, J.K., MULLEN, G.E., LEIFER, C.A., MAZZONI, A., DAVIES, D.R. and SEGAL, D.M., 2003. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends in immunology,* **24**(10), pp. 528-533.

BELL-PEDERSEN, D., CASSONE, V.M., EARNEST, D.J., GOLDEN, S.S., HARDIN, P.E., THOMAS, T.L., ZORAN, M.J., 2005. Carcadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat. Rev. Gene.* **6**, pp. 544-556

BERUBE, J., BOURDON, C., YAO, Y. and ROUSSEAU, S., 2009. Distinct intracellular signaling pathways control the synthesis of IL-8 and RANTES in TLR1/TLR2, TLR3 or NOD1 activated human airway epithelial cells. *Cellular signalling,* **21**(3), pp. 448-456.

BEUG, H., VON KIRCHBACH, A., DÖDERLEIN, G., CONSCIENCE, J. and GRAF, T., 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell,* **18**(2), pp. 375-390.

BEUTLER, B., 2009a. Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. *Immunological reviews,* **227**(1), pp. 248-263.

BEUTLER, B.A., 2009b. TLRs and innate immunity. *Blood,* **113**(7), pp. 1399-1407.

BHAYA, D., DAVISON, M., BARRANGOU, R., 2011. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Ann. Rev. Gene*, **45**. pp. 273-297

BIRKY, T.L., BRAY, M.S., 2014. Understanding circadian gene function: animal models of tissue-specific circadian disruption. *IUBMB Life*, **66**(1), pp. 34-41

BISWAS, A., WILMANSKI, J., FORSMAN, H., HRNCIR, T., HAO, L., TLASKALOVA-HOGENOVA, H. and KOBAYASHI, K.S., 2011. Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine. *European journal of immunology,* **41**(1), pp. 182-194.

BISWAS, S.K. and LOPEZ-COLLAZO, E., 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in immunology,* **30**(10), pp. 475-487.

BOONE, D.L., TURER, E.E., LEE, E.G., AHMAD, R.C., WHEELER, M.T., TSUI, C., HURLEY, P., CHIEN, M., CHAI, S., HITOTSUMATSU, O., MCNALLY, E., PICKART, C. and MA, A., 2004. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nature immunology,* **5**(10), pp. 1052-1060.

BOSISIO, D., POLENTARUTTI, N., SIRONI, M., BERNASCONI, S., MIYAKE, K., WEBB, G.R., MARTIN, M.U., MANTOVANI, A. and MUZIO, M., 2002. Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. *Blood,* **99**(9), pp. 3427-3431.

BOTOS, I., SEGAL, D.M. and DAVIES, D.R., 2011. The structural biology of Toll-like receptors. *Structure (London, England : 1993),* **19**(4), pp. 447-459.

BOUTROS, T., CHEVET, E. and METRAKOS, P., 2008. Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. *Pharmacological reviews,* **60**(3), pp. 261-310.

BOYD, A.C., PEROVAL, M.Y., HAMMOND, J.A., PRICKETT, M.D., YOUNG, J.R. and SMITH, A.L., 2012. TLR15 is unique to avian and reptilian lineages and recognizes a yeast-derived agonist. *Journal of immunology (Baltimore, Md.: 1950),* **189**(10), pp. 4930-4938.

BRAMSEN, J.B. and KJEMS, J., 2012. Development of Therapeutic-Grade Small Interfering RNAs by Chemical Engineering. *Frontiers in genetics,* **3**, pp. 154.

BRETZ, J.D., WILLIAMS, S.C., BAER, M., JOHNSON, P.F. and SCHWARTZ, R.C., 1994. C/EBP-related protein 2 confers lipopolysaccharide-inducible expression of interleukin 6 and monocyte chemoattractant protein 1 to a lymphoblastic cell line. *Proceedings of the National Academy of Sciences of the United States of America,* **91**(15), pp. 7306-7310.

BRINT, E.K., XU, D., LIU, H., DUNNE, A., MCKENZIE, A.N., O'NEILL, L.A. and LIEW, F.Y., 2004a. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nature immunology,* **5**(4), pp. 373-379.

BROERING, R., REAL, C.I., JOHN, M.J., JAHN-HOFMANN, K., ICKENSTEIN, L.M., KLEINEHR, K., PAUL, A., GIBBERT, K., DITTMER, U., GERKEN, G. and SCHLAAK, J.F., 2014. Chemical modifications on siRNAs avoid Toll-like-receptor-mediated activation of the hepatic immune system in vivo and in vitro. *International immunology,* **26**(1), pp. 35-46.

BROWNLIE, R., ZHU, J., ALLAN, B., MUTWIRI, G.K., BABIUK, L.A., POTTER, A. and GRIEBEL, P., 2009. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Molecular immunology,* **46**(15), pp. 3163-3170.

BROZ, P. and MONACK, D.M., 2013. Newly described pattern recognition receptors team up against intracellular pathogens. *Nature reviews.Immunology,* **13**(8), pp. 551-565.

BRUBAKER, S.W., BONHAM, K.S., ZANONI, I. and KAGAN, J.C., 2015. Innate immune pattern recognition: a cell biological perspective. *Annual Review of Immunology,* **33**, pp. 257-290.

BRYANT, C.E., SPRING, D.R., GANGLOFF, M. and GAY, N.J., 2010. The molecular basis of the host response to lipopolysaccharide. *Nature reviews.Microbiology,* **8**(1), pp. 8-14.

BULUT, Y., FAURE, E., THOMAS, L., EQUILS, O. and ARDITI, M., 2001. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *Journal of immunology (Baltimore, Md.: 1950),* **167**(2), pp. 987-994.

BURCH-SMITH, T.M. and DINESH-KUMAR, S.P., 2007. The functions of plant TIR domains. *Science's STKE : signal transduction knowledge environment,* **2007**(401), pp. pe46.

BURNS, K., JANSSENS, S., BRISSONI, B., OLIVOS, N., BEYAERT, R. and TSCHOPP, J., 2003. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. *The Journal of experimental medicine,* **197**(2), pp. 263-268.

BURNS, K., MARTINON, F., ESSLINGER, C., PAHL, H., SCHNEIDER, P., BODMER, J.L., DI MARCO, F., FRENCH, L. and TSCHOPP, J., 1998. MyD88, an adapter protein involved in interleukin-1 signaling. *The Journal of biological chemistry,* **273**(20), pp. 12203-12209.

CABRAL, E.S., GELDERBLOM, H., HORNUNG, R.L., MUNSON, P.J., MARTIN, R. and MARQUES, A.R., 2006. Borrelia burgdorferi lipoprotein-mediated TLR2 stimulation causes the down-regulation of TLR5 in human monocytes. *The Journal of infectious diseases,* **193**(6), pp. 849-859.

CAMPOS, M.A., ALMEIDA, I.C., TAKEUCHI, O., AKIRA, S., VALENTE, E.P., PROCOPIO, D.O., TRAVASSOS, L.R., SMITH, J.A., GOLENBOCK, D.T. and GAZZINELLI, R.T., 2001. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *Journal of immunology (Baltimore, Md.: 1950),* **167**(1), pp. 416-423.

CARL, V.S., BROWN-STEINKE, K., NICKLIN, M.J.H. and SMITH, M.F., 2002. Toll-like Receptor 2 and 4 (TLR2 and TLR4) Agonists Differentially Regulate Secretory Interleukin-1 Receptor Antagonist Gene Expression in Macrophages. *Journal of Biological Chemistry,* **277**(20), pp. 17448-17456.

CARPENTER, S., RICCI, E.P., MERCIER, B.C., MOORE, M.J. and FITZGERALD, K.A., 2014. Post-transcriptional regulation of gene expression in innate immunity. *Nature reviews.Immunology,* **14**(6), pp. 361-376.

CARTHEW, R.W. and SONTHEIMER, E.J., 2009. Origins and Mechanisms of miRNAs and siRNAs. *Cell,* **136**(4), pp. 642-655.

CARTY, M., GOODBODY, R., SCHRODER, M., STACK, J., MOYNAGH, P.N. and BOWIE, A.G., 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nature immunology,* **7**(10), pp. 1074-1081.

CASIRAGHI, C., GIANNI, T. and CAMPADELLI-FIUME, G., 2016. alphavbeta3 Integrin Boosts the Innate Immune Response Elicited in Epithelial Cells through Plasma Membrane and Endosomal Toll-Like Receptors. *Journal of virology,* **90**(8), pp. 4243-4248.

CEPPI, M., PEREIRA, P.M., DUNAND-SAUTHIER, I., BARRAS, E., REITH, W., SANTOS, M.A. and PIERRE, P., 2009. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(8), pp. 2735-2740.

CHAMAILLARD, M., HASHIMOTO, M., HORIE, Y., MASUMOTO, J., QIU, S., SAAB, L., OGURA, Y., KAWASAKI, A., FUKASE, K., KUSUMOTO, S., VALVANO, M.A., FOSTER, S.J., MAK, T.W., NUNEZ, G. and INOHARA, N., 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nature immunology,* **4**(7), pp. 702-707.

CHAMBERS, J.M. 1992. Linear models. Statistical Models in S, *Wadsworth & Brooks/Cole, Pacific Grove, California*.

CHAN, G., GU, S.Q., NEEL, B.G., 2013. *ERK1* and *ERK2* are required for maintenance of hematopoietic stem cells and adult hematopoiesis. *Blood*, **121**, pp. 3594-3598.

CHANG, M., JIN, W. and SUN, S.C., 2009. Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. *Nature immunology,* **10**(10), pp. 1089-1095.

CHASE, M.A., WHEELER, D.S., LIERL, K.M., HUGHES, V.S., WONG, H.R. and PAGE, K., 2007. Hsp72 induces inflammation and regulates cytokine production in airway epithelium through a TLR4- and NF-kappaB-dependent mechanism. *Journal of immunology (Baltimore, Md.: 1950),* **179**(9), pp. 6318-6324.

CHAURASIA, B., MAUER, J., KOCH, L., GOLDAU, J., KOCK, A.S. and BRUNING, J.C., 2010. Phosphoinositide-dependent kinase 1 provides negative feedback inhibition to Toll-like receptor-mediated NF-kappaB activation in macrophages. *Molecular and cellular biology,* **30**(17), pp. 4354-4366.

CHEN, N.J., CHIO, I.I., LIN, W.J., DUNCAN, G., CHAU, H., KATZ, D., HUANG, H.L., PIKE, K.A., HAO, Z., SU, Y.W., YAMAMOTO, K., DE POOTER, R.F., ZUNIGA-PFLUCKER, J.C., WAKEHAM, A., YEH, W.C. and MAK, T.W., 2008. Beyond tumor necrosis factor receptor: TRADD signaling in toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America,* **105**(34), pp. 12429-12434.

CHEN, X., LIANG, H., ZHANG, J., ZEN, K. and ZHANG, C.Y., 2013. microRNAs are ligands of Toll-like receptors. *RNA (New York, N.Y.),* **19**(6), pp. 737-739.

CHEN, Z., GIBSON, T.B., ROBINSON, F., SILVESTRO, L., PEARSON, G., XU, B., WRIGHT, A., VANDERBILT, C. and COBB, M.H., 2001. MAP kinases. *Chemical reviews,* **101**(8), pp. 2449-2476.

CHEN, Z.J., 2005. Ubiquitin signalling in the NF-kappaB pathway. *Nature cell biology,* **7**(8), pp. 758-765.

CHI, H., BARRY, S.P., ROTH, R.J., WU, J.J., JONES, E.A., BENNETT, A.M. and FLAVELL, R.A., 2006. Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proceedings of the National Academy of Sciences of the United States of America,* **103**(7), pp. 2274-2279.

CHOW, A., HUGGINS, M., AHMED, J., HASHIMOTO, D., LUCAS, D., KUNISAKI, Y., PINHO, S., LEBOEUF, M., NOIZAT, C., VAN ROOIJEN, N., TANAKA, M., ZHAO, Z.J., BERGMAN, A., MERAD, M. and FRENETTE, P.S., 2013. CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress. *Nature medicine,* **19**(4), pp. 429-436.

COFFEY, E.T., HONGISTO, V., DICKENS, M., DAVIS, R.J. and COURTNEY, M.J., 2000. Dual roles for c-Jun N-terminal kinase in developmental and stress responses in cerebellar granule neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **20**(20), pp. 7602-7613.

COHEN, P., 2009. Guidelines for the effective use of chemical inhibitors of protein function to understand their roles in cell regulation. *The Biochemical journal,* **425**(1), pp. 53-54.

COLEMAN, A.E., FOREST, S.T., MCNEIL, N., KOVALCHUK, A.L., RIED, T. and JANZ, S., 1999. Cytogenetic analysis of the bipotential murine pre-B cell lymphoma, P388,

and its derivative macrophage-like tumor, P388D1, using SKY and CGH. *Leukemia,* **13**(10), pp. 1592-1600.

COURT, N.W., DOS REMEDIOS, C.G., CORDELL, J. and BOGOYEVITCH, M.A., 2002. Cardiac expression and subcellular localization of the p38 mitogen-activated protein kinase member, stress-activated protein kinase-3 (SAPK3). *Journal of Molecular and Cellular Cardiology,* **34**(4), pp. 413-426.

CRESPO, A., FILLA, M.B. and MURPHY, W.J., 2002. Low responsiveness to IFN-gamma, after pretreatment of mouse macrophages with lipopolysaccharides, develops via diverse regulatory pathways. *European journal of immunology,* **32**(3), pp. 710-719.

CURTIS, A.M., FAGUNDES, C.T., YANG, G., PALSSON-MCDERMOTT, E.M., WOCHAL, P., MCGETTRICK, A.F., FOLEY, N.H., EARLY, J.O., CHEN, L., ZHANG, H., XUE, C., GEIGER, S.S., HOKAMP, K., REILLY, M.P., COOGAN, A.N., VIGORITO, E., FITZGERALD, G.A. and O'NEILL, L.A., 2015. Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1. *Proceedings of the National Academy of Sciences of the United States of America,* .

CUSSON-HERMANCE, N., KHURANA, S., LEE, T.H., FITZGERALD, K.A. and KELLIHER, M.A., 2005. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. *The Journal of biological chemistry,* **280**(44), pp. 36560-36566.

DALPKE, A.H., ECKERLE, S., FREY, M. and HEEG, K., 2003. Triggering of Toll-like receptors modulates IFN-gamma signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signaling. *European journal of immunology,* **33**(7), pp. 1776-1787.

DALPKE, A.H., LEHNER, M.D., HARTUNG, T. and HEEG, K., 2005. Differential effects of CpG-DNA in Toll-like receptor-2/-4/-9 tolerance and cross-tolerance. *Immunology,* **116**(2), pp. 203-212.

DAMBUZA, I.M. and BROWN, G.D., 2015. C-type lectins in immunity: recent developments. *Current opinion in immunology,* **32**, pp. 21-27.

DANN, S., 2000. Collin Pittendrigh, Jurgen Aschoff, and the natural entrainment of circadian systems. *J Biol Rhythms*, **15**, pp. 195-207.

DAVIES, L.C., JENKINS, S.J., ALLEN, J.E. and TAYLOR, P.R., 2013. Tissue-resident macrophages. *Nature immunology,* **14**(10), pp. 986-995.

DAVIES, L.C., ROSAS, M., SMITH, P.J., FRASER, D.J., JONES, S.A. and TAYLOR, P.R., 2011. A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. *European journal of immunology,* **41**(8), pp. 2155-2164.

DAVIES, L.C. and TAYLOR, P.R., 2015. Tissue-resident macrophages: then and now. *Immunology,* **144**(4), pp. 541-548.

DAVIES, S.P., REDDY, H., CAIVANO, M. and COHEN, P., 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *The Biochemical journal,* **351**(Pt 1), pp. 95-105.

DE NARDO, D., DE NARDO, C.M., NGUYEN, T., HAMILTON, J.A. and SCHOLZ, G.M., 2009a. Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages. *Journal of immunology (Baltimore, Md.: 1950),* **183**(12), pp. 8110-8118.

DE WIT, H., HOOGSTRATEN, D., HALIE, R.M. and VELLENGA, E., 1996. Interferon-gamma modulates the lipopolysaccharide-induced expression of AP-1 and NF-kappa B at the mRNA and protein level in human monocytes. *Experimental hematology,* **24**(2), pp. 228-235.

DEBIERRE-GROCKIEGO, F., CAMPOS, M.A., AZZOUZ, N., SCHMIDT, J., BIEKER, U., RESENDE, M.G., MANSUR, D.S., WEINGART, R., SCHMIDT, R.R., GOLENBOCK, D.T., GAZZINELLI, R.T. and SCHWARZ, R.T., 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii. *Journal of immunology (Baltimore, Md.: 1950),* **179**(2), pp. 1129-1137.

DEN HAAN, J.M. and KRAAL, G., 2012. Innate immune functions of macrophage subpopulations in the spleen. *Journal of innate immunity,* **4**(5-6), pp. 437-445.

DICKENS, M., ROGERS, J.S., CAVANAGH, J., RAITANO, A., XIA, Z., HALPERN, J.R., GREENBERG, M.E., SAWYERS, C.L. and DAVIS, R.J., 1997. A cytoplasmic inhibitor of the JNK signal transduction pathway. *Science (New York, N.Y.),* **277**(5326), pp. 693-696.

DIEBOLD, S.S., KAISHO, T., HEMMI, H., AKIRA, S. and REIS E SOUSA, C., 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science (New York, N.Y.),* **303**(5663), pp. 1529-1531.

DIEHL, G.E., YUE, H.H., HSIEH, K., KUANG, A.A., HO, M., MORICI, L.A., LENZ, L.L., CADO, D., RILEY, L.W. and WINOTO, A., 2004. TRAIL-R as a negative regulator of innate immune cell responses. *Immunity,* **21**(6), pp. 877-889.

DUNNE, A. and O'NEILL, L.A., 2005. Adaptor usage and Toll-like receptor signaling specificity. *FEBS letters,* **579**(15), pp. 3330-3335.

ECKERT, R.L., EFIMOVA, T., BALASUBRAMANIAN, S., CRISH, J.F., BONE, F. and DASHTI, S., 2003. p38 Mitogen-activated protein kinases on the body surface--a function for p38 delta. *The Journal of investigative dermatology,* **120**(5), pp. 823-828.

ERMOLAEVA, M.A., MICHALLET, M.C., PAPADOPOULOU, N., UTERMOHLEN, O., KRANIDIOTI, K., KOLLIAS, G., TSCHOPP, J. and PASPARAKIS, M., 2008. Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. *Nature immunology,* **9**(9), pp. 1037-1046.

FABBRI, M., 2012. TLRs as miRNA receptors. *Cancer research,* **72**(24), pp. 6333-6337.

FARHAT, K., RIEKENBERG, S., HEINE, H., DEBARRY, J., LANG, R., MAGES, J., BUWITT-BECKMANN, U., ROSCHMANN, K., JUNG, G., WIESMULLER, K.H. and ULMER, A.J., 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *Journal of leukocyte biology,* **83**(3), pp. 692-701.

FAUSTIN, B., LARTIGUE, L., BRUEY, J.M., LUCIANO, F., SERGIENKO, E., BAILLY-MAITRE, B., VOLKMANN, N., HANEIN, D., ROUILLER, I. and REED, J.C., 2007. Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Molecular cell,* **25**(5), pp. 713-724.

FEY, D., CROUCHER, D.R., KOLCH, W. and KHOLODENKO, B.N., 2012. Crosstalk and signaling switches in mitogen-activated protein kinase cascades. *Frontiers in physiology,* **3**, pp. 355.

FISCHER, M. and EHLERS, M., 2008. Toll-like receptors in autoimmunity. *Annals of the New York Academy of Sciences,* **1143**, pp. 21-34.

FITZGERALD, K.A., PALSSON-MCDERMOTT, E.M., BOWIE, A.G., JEFFERIES, C.A., MANSELL, A.S., BRADY, G., BRINT, E., DUNNE, A., GRAY, P., HARTE, M.T., MCMURRAY, D., SMITH, D.E., SIMS, J.E., BIRD, T.A. and O'NEILL, L.A., 2001. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature,* **413**(6851), pp. 78-83.

FITZGERALD, K.A., ROWE, D.C., BARNES, B.J., CAFFREY, D.R., VISINTIN, A., LATZ, E., MONKS, B., PITHA, P.M. and GOLENBOCK, D.T., 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *The Journal of experimental medicine,* **198**(7), pp. 1043-1055.

FOSTER, S.L., HARGREAVES, D.C. and MEDZHITOV, R., 2007. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature,* **447**(7147), pp. 972-978.

FREUDENBERG, M.A. and GALANOS, C., 1988. Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. *Infection and immunity,* **56**(5), pp. 1352-1357.

FUKAO, T., TANABE, M., TERAUCHI, Y., OTA, T., MATSUDA, S., ASANO, T., KADOWAKI, T., TAKEUCHI, T. and KOYASU, S., 2002. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nature immunology,* **3**(9), pp. 875-881.

GALLUCCI, S., LOLKEMA, M. and MATZINGER, P., 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nature medicine,* **5**(11), pp. 1249-1255.

GANZ, T., 2012. Macrophages and systemic iron homeostasis. *Journal of innate immunity,* **4**(5-6), pp. 446-453.

GAUTIER, E.L., IVANOV, S., WILLIAMS, J.W., HUANG, S.C., MARCELIN, G., FAIRFAX, K., WANG, P.L., FRANCIS, J.S., LEONE, P., WILSON, D.B., ARTYOMOV, M.N., PEARCE, E.J. and RANDOLPH, G.J., 2014. Gata6 regulates aspartoacylase expression in resident peritoneal macrophages and controls their survival. *The Journal of experimental medicine,* **211**(8), pp. 1525-1531.

GAY, N.J. and KEITH, F.J., 1991. Drosophila Toll and IL-1 receptor. *Nature,* **351**(6325), pp. 355-356.

GHOSH, A.K., SINHA, D., MUKHERJEE, S., BISWAS, R. and BISWAS, T., 2015. LPS stimulates and Hsp70 down-regulates TLR4 to orchestrate differential cytokine

response of culture-differentiated innate memory CD8+ T cells. *Cytokine,* **73**(1), pp. 44-52.

GHOSH, T.K., MICKELSON, D.J., SOLBERG, J.C., LIPSON, K.E., INGLEFIELD, J.R. and ALKAN, S.S., 2007. TLR–TLR cross talk in human PBMC resulting in synergistic and antagonistic regulation of type-1 and 2 interferons, IL-12 and TNF-α. *International immunopharmacology,* **7**(8), pp. 1111-1121.

GIANNI, T. and CAMPADELLI-FIUME, G., 2014. The epithelial alphavbeta3-integrin boosts the MYD88-dependent TLR2 signaling in response to viral and bacterial components. *PLoS pathogens,* **10**(11), pp. e1004477.

GIANNI, T., LEONI, V., CHESNOKOVA, L.S., HUTT-FLETCHER, L.M. and CAMPADELLI-FIUME, G., 2012. Alphavbeta3-Integrin is a Major Sensor and Activator of Innate Immunity to Herpes Simplex Virus-1. *Proceedings of the National Academy of Sciences of the United States of America,* **109**(48), pp. 19792-19797.

GIBBS, J.E., BLAIKLEY, J., BEESLEY, S., MATTHEWS, L., SIMPSON, K.D., BOYCE, S.H., FARROW, S.N., ELSE, K.J., SINGH, D., RAY, D.W. and LOUDON, A.S., 2012. The nuclear receptor REV-ERBalpha mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *Proceedings of the National Academy of Sciences of the United States of America,* **109**(2), pp. 582-587.

GILCHRIST, M., THORSSON, V., LI, B., RUST, A.G., KORB, M., ROACH, J.C., KENNEDY, K., HAI, T., BOLOURI, H. and ADEREM, A., 2006. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature,* **441**(7090), pp. 173-178.

GILLESPIE, M., SHAMOVSKY, V. and D'EUSTACHIO, P., 2011. Human and chicken TLR pathways: manual curation and computer-based orthology analysis. *Mammalian genome : official journal of the International Mammalian Genome Society,* **22**(1-2), pp. 130-138.

GINHOUX, F., SCHULTZE, J.L., MURRAY, P.J., OCHANDO, J. and BISWAS, S.K., 2015. New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nature immunology,* **17**(1), pp. 34-40.

GIRARDIN, S.E., BONECA, I.G., CARNEIRO, L.A., ANTIGNAC, A., JEHANNO, M., VIALA, J., TEDIN, K., TAHA, M.K., LABIGNE, A., ZAHRINGER, U., COYLE, A.J., DISTEFANO, P.S., BERTIN, J., SANSONETTI, P.J. and PHILPOTT, D.J., 2003. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science (New York, N.Y.),* **300**(5625), pp. 1584-1587.

GLASS, C.K. and NATOLI, G., 2015. Molecular control of activation and priming in macrophages. *Nature immunology,* **17**(1), pp. 26-33.

GOGOLINSKA, A., NOWAK, W., 2012. Petri nets approach to modelling of immune system and autism. *Artificial Imm. Syst. Lecture notes in computer science*. **7597**, pp. 86-99

GOHDA, J., MATSUMURA, T. and INOUE, J., 2004. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor

domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. *Journal of immunology (Baltimore, Md.: 1950),* **173**(5), pp. 2913-2917.

GRAHAM, L.M. and BROWN, G.D., 2009. The Dectin-2 family of C-type lectins in immunity and homeostasis. *Cytokine,* **48**(1-2), pp. 148-155.

GREAVES, D.R. and GORDON, S., 2009. The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. *Journal of lipid research,* **50 Suppl**, pp. S282-6.

GRIBAR, S.C., ANAND, R.J., SODHI, C.P. and HACKAM, D.J., 2008. The role of epithelial Toll-like receptor signaling in the pathogenesis of intestinal inflammation. *Journal of leukocyte biology,* **83**(3), pp. 493-498.

GUO, H., CALLAWAY, J.B. and TING, J.P., 2015. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nature medicine,* **21**(7), pp. 677-687.

HACKER, H., REDECKE, V., BLAGOEV, B., KRATCHMAROVA, I., HSU, L.C., WANG, G.G., KAMPS, M.P., RAZ, E., WAGNER, H., HACKER, G., MANN, M. and KARIN, M., 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature,* **439**(7073), pp. 204-207.

HACKER, H., TSENG, P.H. and KARIN, M., 2011. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. *Nature reviews.Immunology,* **11**(7), pp. 457-468.

HAEHNEL, V., SCHWARZFISCHER, L., FENTON, M.J. and REHLI, M., 2002. Transcriptional regulation of the human toll-like receptor 2 gene in monocytes and macrophages. *Journal of immunology (Baltimore, Md.: 1950),* **168**(11), pp. 5629-5637.

HASHIMOTO, C., HUDSON, K.L. and ANDERSON, K.V., 1988. The Toll gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell,* **52**(2), pp. 269-279.

HAYASHI, F., SMITH, K.D., OZINSKY, A., HAWN, T.R., YI, E.C., GOODLETT, D.R., ENG, J.K., AKIRA, S., UNDERHILL, D.M. and ADEREM, A., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature,* **410**(6832), pp. 1099-1103.

HE, X., JING, Z. and CHENG, G., 2014. MicroRNAs: new regulators of Toll-like receptor signalling pathways. *BioMed research international,* **2014**, pp. 945169.

HEEG, K. and DALPKE, A., 2003. TLR-induced negative regulatory circuits: role of suppressor of cytokine signaling (SOCS) proteins in innate immunity. *Vaccine,* **21 Suppl 2**, pp. S61-7.

HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H. and BAUER, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science (New York, N.Y.),* **303**(5663), pp. 1526-1529.

HEINZ, S., HAEHNEL, V., KARAGHIOSOFF, M., SCHWARZFISCHER, L., MULLER, M., KRAUSE, S.W. and REHLI, M., 2003. Species-specific regulation of Toll-like

receptor 3 genes in men and mice. *The Journal of biological chemistry,* **278**(24), pp. 21502-21509.

HELD, T.K., WEIHUA, X., YUAN, L., KALVAKOLANU, D.V. and CROSS, A.S., 1999. Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. *Infection and immunity,* **67**(1), pp. 206-212.

HEMMI, H., TAKEUCHI, O., KAWAI, T., KAISHO, T., SATO, S., SANJO, H., MATSUMOTO, M., HOSHINO, K., WAGNER, H., TAKEDA, K. and AKIRA, S., 2000. A Toll-like receptor recognizes bacterial DNA. *Nature,* **408**(6813), pp. 740-745.

HIBINO, T., LOZA-COLL, M., MESSIER, C., MAJESKE, A.J., COHEN, A.H., TERWILLIGER, D.P., BUCKLEY, K.M., BROCKTON, V., NAIR, S.V., BERNEY, K., FUGMANN, S.D., ANDERSON, M.K., PANCER, Z., CAMERON, R.A., SMITH, L.C. and RAST, J.P., 2006. The immune gene repertoire encoded in the purple sea urchin genome. *Developmental biology,* **300**(1), pp. 349-365.

HIDMARK, A., VON SAINT PAUL, A. and DALPKE, A.H., 2012. Cutting edge: TLR13 is a receptor for bacterial RNA. *Journal of immunology (Baltimore, Md.: 1950),* **189**(6), pp. 2717-2721.

HITOTSUMATSU, O., AHMAD, R.C., TAVARES, R., WANG, M., PHILPOTT, D., TURER, E.E., LEE, B.L., SHIFFIN, N., ADVINCULA, R., MALYNN, B.A., WERTS, C. and MA, A., 2008. The ubiquitin-editing enzyme A20 restricts nucleotide-binding oligomerization domain containing 2-triggered signals. *Immunity,* **28**(3), pp. 381-390.

HOCHDORFER, T., KUHNY, M., ZORN, C.N., HENDRIKS, R.W., VANHAESEBROECK, B., BOHNACKER, T., KRYSTAL, G. and HUBER, M., 2011. Activation of the PI3K pathway increases TLR-induced TNF-alpha and IL-6 but reduces IL-1beta production in mast cells. *Cellular signalling,* **23**(5), pp. 866-875.

HOFFMANN, A., LEVCHENKO, A., SCOTT, M.L., BALTIMORE, D., 2002. The NFκB signalling molecule: temporal control and selective gene activation. *Science*, **298**(5596), pp.1241-5

HORNG, T., BARTON, G.M., FLAVELL, R.A. and MEDZHITOV, R., 2002. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature,* **420**(6913), pp. 329-333.

HORNG, T., BARTON, G.M. and MEDZHITOV, R., 2001. TIRAP: an adapter molecule in the Toll signaling pathway. *Nature immunology,* **2**(9), pp. 835-841.

HORNUNG, V., ABLASSER, A., CHARREL-DENNIS, M., BAUERNFEIND, F., HORVATH, G., CAFFREY, D.R., LATZ, E. and FITZGERALD, K.A., 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature,* **458**(7237), pp. 514-518.

HORNUNG, V., ELLEGAST, J., KIM, S., BRZOZKA, K., JUNG, A., KATO, H., POECK, H., AKIRA, S., CONZELMANN, K.K., SCHLEE, M., ENDRES, S. and HARTMANN, G., 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science (New York, N.Y.),* **314**(5801), pp. 994-997.

HOSHINO, K., TAKEUCHI, O., KAWAI, T., SANJO, H., OGAWA, T., TAKEDA, Y., TAKEDA, K. and AKIRA, S., 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *Journal of immunology (Baltimore, Md.: 1950),* **162**(7), pp. 3749-3752.

HOTHORN, T., BRETZ, F. and WESTFALL, P., 2008. Simultaneous inference in general parametric models. *Biometrical journal.Biometrische Zeitschrift,* **50**(3), pp. 346-363.

HU, X., CHEN, J., WANG, L. and IVASHKIV, L.B., 2007. Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. *Journal of leukocyte biology,* **82**(2), pp. 237-243.

HUANG, G., SHI, L.Z. and CHI, H., 2009. Regulation of JNK and p38 MAPK in the immune system: signal integration, propagation and termination. *Cytokine,* **48**(3), pp. 161-169.

HUANG, Y., TEMPERLEY, N.D., REN, L., SMITH, J., LI, N. and BURT, D.W., 2011. Molecular evolution of the vertebrate TLR1 gene family--a complex history of gene duplication, gene conversion, positive selection and co-evolution. *BMC evolutionary biology,* **11**, pp. 149-2148-11-149.

HUGHES, A.L. and PIONTKIVSKA, H., 2008. Functional diversification of the toll-like receptor gene family. *Immunogenetics,* **60**(5), pp. 249-256.

HYAKUSHIMA, N., MITSUZAWA, H., NISHITANI, C., SANO, H., KURONUMA, K., KONISHI, M., HIMI, T., MIYAKE, K. and KUROKI, Y., 2004. Interaction of soluble form of recombinant extracellular TLR4 domain with MD-2 enables lipopolysaccharide binding and attenuates TLR4-mediated signaling. *Journal of immunology (Baltimore, Md.: 1950),* **173**(11), pp. 6949-6954.

IHEKWABA, A.E.C., BROOMHEAD, D.S., GRIMLEY, R.L., BENSON, N., KELL, D.B., 2004. Sensitivity analysis of parameters controlling oscillatory signalling in the NFκB pathway: the role of IKK and IκBα. *Systems Bio.*, **1**(1), pp. 93-103

IP, Y.T., REACH, M., ENGSTROM, Y., KADALAYIL, L., CAI, H., GONZALEZ-CRESPO, S., TATEI, K. and LEVINE, M., 1993. Dif, a dorsal-related gene that mediates an immune response in Drosophila. *Cell,* **75**(4), pp. 753-763.

ISHIDA, A., AKITA, K., MORI, Y., TANIDA, S., TODA, M., INOUE, M. and NAKADA, H., 2014. Negative regulation of Toll-like receptor-4 signaling through the binding of glycosylphosphatidylinositol-anchored glycoprotein, CD14, with the sialic acid-binding lectin, CD33. *The Journal of biological chemistry,* **289**(36), pp. 25341-25350.

ISRAEL, A., 2010. The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harbor perspectives in biology,* **2**(3), pp. a000158.

IVASHKIV, L.B., 2008. A signal-switch hypothesis for cross-regulation of cytokine and TLR signalling pathways. *Nature reviews.Immunology,* **8**(10), pp. 816-822.

IWAMI, K.I., MATSUGUCHI, T., MASUDA, A., KIKUCHI, T., MUSIKACHAROEN, T. and YOSHIKAI, Y., 2000. Cutting edge: naturally occurring soluble form of mouse Toll-

like receptor 4 inhibits lipopolysaccharide signaling. *Journal of immunology (Baltimore, Md.: 1950),* **165**(12), pp. 6682-6686.

IZADI, H., MOTAMENI, A.T., BATES, T.C., OLIVERA, E.R., VILLAR-SUAREZ, V., JOSHI, I., GARG, R., OSBORNE, B.A., DAVIS, R.J., RINCON, M. and ANGUITA, J., 2007. c-Jun N-terminal kinase 1 is required for Toll-like receptor 1 gene expression in macrophages. *Infection and immunity,* **75**(10), pp. 5027-5034.

JACKSON, A.L. and LINSLEY, P.S., 2010. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nature reviews.Drug discovery,* **9**(1), pp. 57-67.

JAGANNATHAN, M., HASTURK, H., LIANG, Y., SHIN, H., HETZEL, J.T., KANTARCI, A., RUBIN, D., MCDONNELL, M.E., VAN DYKE, T.E., GANLEY-LEAL, L.M. and NIKOLAJCZYK, B.S., 2009. TLR cross-talk specifically regulates cytokine production by B cells from chronic inflammatory disease patients. *Journal of immunology (Baltimore, Md.: 1950),* **183**(11), pp. 7461-7470.

JANEWAY, C.A., TRAVERS, J.P., WALPORT, M. and SHLOMCHIK, M.J., 2005. Immunobiology. *Garland Science Publishing,* .

JANEWAY, C.A.,Jr, 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor symposia on quantitative biology,* **54 Pt 1**, pp. 1-13.

JANN, O.C., KING, A., CORRALES, N.L., ANDERSON, S.I., JENSEN, K., AIT-ALI, T., TANG, H., WU, C., COCKETT, N.E., ARCHIBALD, A.L. and GLASS, E.J., 2009. Comparative genomics of Toll-like receptor signalling in five species. *BMC genomics,* **10**, pp. 216-2164-10-216.

JANN, O.C., WERLING, D., CHANG, J.S., HAIG, D. and GLASS, E.J., 2008. Molecular evolution of bovine Toll-like receptor 2 suggests substitutions of functional relevance. *BMC evolutionary biology,* **8**, pp. 288-2148-8-288.

JANSSENS, S., BURNS, K., TSCHOPP, J. and BEYAERT, R., 2002. Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. *Current biology : CB,* **12**(6), pp. 467-471.

JIANG, X. and CHEN, Z.J., 2011. The role of ubiquitylation in immune defence and pathogen evasion. *Nature reviews.Immunology,* **12**(1), pp. 35-48.

JIANG, Z., MAK, T.W., SEN, G. and LI, X., 2004. Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. *Proceedings of the National Academy of Sciences of the United States of America,* **101**(10), pp. 3533-3538.

JIANG, Z., NINOMIYA-TSUJI, J., QIAN, Y., MATSUMOTO, K. and LI, X., 2002. Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. *Molecular and cellular biology,* **22**(20), pp. 7158-7167.

JIN, J., SAMUVEL, D.J., ZHANG, X., LI, Y., LU, Z., LOPES-VIRELLA, M.F. and HUANG, Y., 2011. Coactivation of TLR4 and TLR2/6 coordinates an additive

augmentation on IL-6 gene transcription via p38MAPK pathway in U937 mononuclear cells. *Molecular immunology,* **49**(3), pp. 423-432.

JIN, M.S. and LEE, J.O., 2008. Structures of the toll-like receptor family and its ligand complexes. *Immunity,* **29**(2), pp. 182-191.

JONES, B.W., MEANS, T.K., HELDWEIN, K.A., KEEN, M.A., HILL, P.J., BELISLE, J.T. and FENTON, M.J., 2001a. Different Toll-like receptor agonists induce distinct macrophage responses. *Journal of leukocyte biology,* **69**(6), pp. 1036-1044.

KAMAKURA, S., MORIGUCHI, T. and NISHIDA, E., 1999. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. *The Journal of biological chemistry,* **274**(37), pp. 26563-26571.

KANG, J.Y. and LEE, J.O., 2011. Structural biology of the Toll-like receptor family. *Annual Review of Biochemistry,* **80**, pp. 917-941.

KANG, K., REILLY, S.M., KARABACAK, V., GANGL, M.R., FITZGERALD, K., HATANO, B. and LEE, C.H., 2008. Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell metabolism,* **7**(6), pp. 485-495.

KANNEGANTI, T.D., LAMKANFI, M. and NUNEZ, G., 2007. Intracellular NOD-like receptors in host defense and disease. *Immunity,* **27**(4), pp. 549-559.

KARIMA, R., MATSUMOTO, S., HIGASHI, H. and MATSUSHIMA, K., 1999. The molecular pathogenesis of endotoxic shock and organ failure. *Molecular medicine today,* **5**(3), pp. 123-132.

KATO, H., SATO, S., YONEYAMA, M., YAMAMOTO, M., UEMATSU, S., MATSUI, K., TSUJIMURA, T., TAKEDA, K., FUJITA, T., TAKEUCHI, O. and AKIRA, S., 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity,* **23**(1), pp. 19-28.

KATO, H., TAKAHASI, K. and FUJITA, T., 2011. RIG-I-like receptors: cytoplasmic sensors for non-self RNA. *Immunological reviews,* **243**(1), pp. 91-98.

KATO, H., TAKEUCHI, O., SATO, S., YONEYAMA, M., YAMAMOTO, M., MATSUI, K., UEMATSU, S., JUNG, A., KAWAI, T., ISHII, K.J., YAMAGUCHI, O., OTSU, K., TSUJIMURA, T., KOH, C.S., REIS E SOUSA, C., MATSUURA, Y., FUJITA, T. and AKIRA, S., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature,* **441**(7089), pp. 101-105.

KAWAI, T., ADACHI, O., OGAWA, T., TAKEDA, K. and AKIRA, S., 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity,* **11**(1), pp. 115-122.

KAWAI, T. and AKIRA, S., 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity,* **34**(5), pp. 637-650.

KAWAI, T. and AKIRA, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology,* **11**(5), pp. 373-384.

KAWAI, T. and AKIRA, S., 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *International immunology,* **21**(4), pp. 317-337.

KAWAI, T. and AKIRA, S., 2006. TLR signaling. *Cell death and differentiation,* **13**(5), pp. 816-825.

KAWAI, T., TAKAHASHI, K., SATO, S., COBAN, C., KUMAR, H., KATO, H., ISHII, K.J., TAKEUCHI, O. and AKIRA, S., 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature immunology,* **6**(10), pp. 981-988.

KAWASAKI, T. and KAWAI, T., 2014. Toll-like receptor signaling pathways. *Frontiers in immunology,* **5**, pp. 461.

KAWASAKI, T., TAKEMURA, N., STANDLEY, D.M., AKIRA, S. and KAWAI, T., 2013. The second messenger phosphatidylinositol-5-phosphate facilitates antiviral innate immune signaling. *Cell host & microbe,* **14**(2), pp. 148-158.

KEESTRA, A.M., DE ZOETE, M.R., BOUWMAN, L.I. and VAN PUTTEN, J.P., 2010. Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. *Journal of immunology (Baltimore, Md.: 1950),* **185**(1), pp. 460-467.

KEESTRA, A.M. and VAN PUTTEN, J.P., 2008. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. *Journal of immunology (Baltimore, Md.: 1950),* **181**(6), pp. 4354-4362.

KELLER, M., MAZUCH, J., ABRAHAM, U., EOM, G.D., HERZOG, E.D., VOLK, H.D., KRAMER, A. and MAIER, B., 2009. A circadian clock in macrophages controls inflammatory immune responses. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(50), pp. 21407-21412.

KENNY, E.F. and O'NEILL, L.A., 2008. Signalling adaptors used by Toll-like receptors: an update. *Cytokine,* **43**(3), pp. 342-349.

KERSCHER, B., WILLMENT, J.A. and BROWN, G.D., 2013. The Dectin-2 family of C-type lectin-like receptors: an update. *International immunology,* **25**(5), pp. 271-277.

KIMURA, A., NAKA, T., NAKAHAMA, T., CHINEN, I., MASUDA, K., NOHARA, K., FUJII-KURIYAMA, Y. and KISHIMOTO, T., 2009. Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *The Journal of experimental medicine,* **206**(9), pp. 2027-2035.

KINJYO, I., HANADA, T., INAGAKI-OHARA, K., MORI, H., AKI, D., OHISHI, M., YOSHIDA, H., KUBO, M. and YOSHIMURA, A., 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity,* **17**(5), pp. 583-591.

KOBAYASHI, K., HERNANDEZ, L.D., GALAN, J.E., JANEWAY, C.A.,Jr, MEDZHITOV, R. and FLAVELL, R.A., 2002. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell,* **110**(2), pp. 191-202.

KOBAYASHI, K.S., CHAMAILLARD, M., OGURA, Y., HENEGARIU, O., INOHARA, N., NUNEZ, G. and FLAVELL, R.A., 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science (New York, N.Y.),* **307**(5710), pp. 731-734.

KOHYAMA, M., ISE, W., EDELSON, B.T., WILKER, P.R., HILDNER, K., MEJIA, C., FRAZIER, W.A., MURPHY, T.L. and MURPHY, K.M., 2009. Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. *Nature,* **457**(7227), pp. 318-321.

KOLLEWE, C., MACKENSEN, A.C., NEUMANN, D., KNOP, J., CAO, P., LI, S., WESCHE, H. and MARTIN, M.U., 2004. Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. *The Journal of biological chemistry,* **279**(7), pp. 5227-5236.

KONDO, T., KAWAI, T. and AKIRA, S., 2012a. Dissecting negative regulation of Toll-like receptor signaling. *Trends in immunology,* **33**(9), pp. 449-458.

KOPP, A., BUECHLER, C., BALA, M., NEUMEIER, M., SCHOLMERICH, J. and SCHAFFLER, A., 2010. Toll-like receptor ligands cause proinflammatory and prodiabetic activation of adipocytes via phosphorylation of extracellular signal-regulated kinase and c-Jun N-terminal kinase but not interferon regulatory factor-3. *Endocrinology,* **151**(3), pp. 1097-1108.

KOPP, E.B. and MEDZHITOV, R., 1999. The Toll-receptor family and control of innate immunity. *Current opinion in immunology,* **11**(1), pp. 13-18.

KORESSAAR, T. and REMM, M., 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics (Oxford, England),* **23**(10), pp. 1289-1291.

KRUG, A., FRENCH, A.R., BARCHET, W., FISCHER, J.A., DZIONEK, A., PINGEL, J.T., ORIHUELA, M.M., AKIRA, S., YOKOYAMA, W.M. and COLONNA, M., 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity,* **21**(1), pp. 107-119.

KRUG, A., LUKER, G.D., BARCHET, W., LEIB, D.A., AKIRA, S. and COLONNA, M., 2004. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood,* **103**(4), pp. 1433-1437.

KRUISBEEK, A.M., 2001. Isolation of mouse mononuclear cells. *Current protocols in immunology / edited by John E.Coligan ...[et al.],* **Chapter 3**, pp. Unit 3.1.

KUMAR, H., KAWAI, T. and AKIRA, S., 2011. Pathogen recognition by the innate immune system. *International reviews of immunology,* **30**(1), pp. 16-34.

KUMAR, H., KAWAI, T. and AKIRA, S., 2009. Toll-like receptors and innate immunity. *Biochemical and biophysical research communications,* **388**(4), pp. 621-625.

KYRIAKIS, J.M. and AVRUCH, J., 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiological Reviews,* **81**(2), pp. 807-869.

KYRIAKIS, J.M. and AVRUCH, J., 1990. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *The Journal of biological chemistry,* **265**(28), pp. 17355-17363.

LABRECQUE, N., CERMAKIAN, N., 2015. Circadian clocks in the immune systems. *J. Biol. Rhythms*, **30**(4), pp. 277-90

LAM, J.K., CHOW, M.Y., ZHANG, Y. and LEUNG, S.W., 2015. siRNA Versus miRNA as Therapeutics for Gene Silencing. *Molecular therapy.Nucleic acids,* **4**, pp. e252.

LANG, T. and MANSELL, A., 2007. The negative regulation of Toll-like receptor and associated pathways. *Immunology and cell biology,* **85**(6), pp. 425-434.

LARKIN, M.A., BLACKSHIELDS, G., BROWN, N.P., CHENNA, R., MCGETTIGAN, P.A., MCWILLIAM, H., VALENTIN, F., WALLACE, I.M., WILM, A., LOPEZ, R., THOMPSON, J.D., GIBSON, T.J. and HIGGINS, D.G., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics,* **23**(21), pp. 2947-2948.

LEBOUDER, E., REY-NORES, J.E., RUSHMERE, N.K., GRIGOROV, M., LAWN, S.D., AFFOLTER, M., GRIFFIN, G.E., FERRARA, P., SCHIFFRIN, E.J., MORGAN, B.P. and LABETA, M.O., 2003. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. *Journal of immunology (Baltimore, Md.: 1950),* **171**(12), pp. 6680-6689.

LEE, R.C., FEINBAUM, R.L. and AMBROS, V., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell,* **75**(5), pp. 843-854.

LEE, S.M., KOK, K.H., JAUME, M., CHEUNG, T.K., YIP, T.F., LAI, J.C., GUAN, Y., WEBSTER, R.G., JIN, D.Y. and PEIRIS, J.S., 2014. Toll-like receptor 10 is involved in induction of innate immune responses to influenza virus infection. *Proceedings of the National Academy of Sciences of the United States of America,* **111**(10), pp. 3793-3798.

LEMAITRE, B., MEISTER, M., GOVIND, S., GEORGEL, P., STEWARD, R., REICHHART, J.M. and HOFFMANN, J.A., 1995. Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila. *The EMBO journal,* **14**(3), pp. 536-545.

LEMAITRE, B., NICOLAS, E., MICHAUT, L., REICHHART, J.M. and HOFFMANN, J.A., 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell,* **86**(6), pp. 973-983.

LENG, C.H., CHEN, H.W., CHANG, L.S., LIU, H.H., LIU, H.Y., SHER, Y.P., CHANG, Y.W., LIEN, S.P., HUANG, T.Y., CHEN, M.Y., CHOU, A.H., CHONG, P. and LIU, S.J., 2010. A recombinant lipoprotein containing an unsaturated fatty acid activates NF-kappaB through the TLR2 signaling pathway and induces a differential gene profile from a synthetic lipopeptide. *Molecular immunology,* **47**(11-12), pp. 2015-2021.

LEWIS, S.H. and OBBARD, D.J., 2014. Recent insights into the evolution of innate viral sensing in animals. *Current opinion in microbiology,* **20**, pp. 170-175.

LI, X.D. and CHEN, Z.J., 2012. Sequence specific detection of bacterial 23S ribosomal RNA by TLR13. *eLife,* **1**, pp. e00102.

LIEW, F.Y., XU, D., BRINT, E.K. and O'NEILL, L.A., 2005a. Negative regulation of toll-like receptor-mediated immune responses. *Nature reviews.Immunology,* **5**(6), pp. 446-458.

LIM, K.H. and STAUDT, L.M., 2013. Toll-like receptor signaling. *Cold Spring Harbor perspectives in biology,* **5**(1), pp. a011247.

LIN, Y., LEE, H., BERG, A.H., LISANTI, M.P., SHAPIRO, L. and SCHERER, P.E., 2000. The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. *The Journal of biological chemistry,* **275**(32), pp. 24255-24263.

LINDSTROM, M.L. and BATES, D.M., 1990. Nonlinear mixed effects models for repeated measures data. *Biometrics,* **46**(3), pp. 673-687.

LIU, H., CAO, H., WU, M., WANG, H., SUN, W. and XU, R., 2004. **Differential Expression of PKC Isoforms and Their Tumoricidal Activity in Two Macrophage Cell Lines: Involvement of Nitric Oxide-dependent Mechanisms**. *The Chinese-German Journal of Clinical Oncology,* **3**(2), pp. 101-105.

LIU, X., CHEN, W., WANG, Q., LI, L. and WANG, C., 2013. Negative regulation of TLR inflammatory signaling by the SUMO-deconjugating enzyme SENP6. *PLoS pathogens,* **9**(6), pp. e1003480.

LIU, Y., SHEPHERD, E.G. and NELIN, L.D., 2007. MAPK phosphatases--regulating the immune response. *Nature reviews.Immunology,* **7**(3), pp. 202-212.

LIU, Y.C., SIMMONS, D.P., LI, X., ABBOTT, D.W., BOOM, W.H. and HARDING, C.V., 2012. TLR2 signaling depletes IRAK1 and inhibits induction of type I IFN by TLR7/9. *Journal of immunology (Baltimore, Md.: 1950),* **188**(3), pp. 1019-1026.

LOWREY, P.L., TAKAHASHI, J.S., 2011. Genetics of circadian rhythms in mammalian model organisms. *Adv Genet.* **74**, pp. 175-230.

LUND, J., SATO, A., AKIRA, S., MEDZHITOV, R. and IWASAKI, A., 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *The Journal of experimental medicine,* **198**(3), pp. 513-520.

LUND, J.M., ALEXOPOULOU, L., SATO, A., KAROW, M., ADAMS, N.C., GALE, N.W., IWASAKI, A. and FLAVELL, R.A., 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proceedings of the National Academy of Sciences of the United States of America,* **101**(15), pp. 5598-5603.

LUU, K., GREENHILL, C.J., MAJOROS, A., DECKER, T., JENKINS, B.J. and MANSELL, A., 2014. STAT1 plays a role in TLR signal transduction and inflammatory responses. *Immunology and cell biology,* **92**(9), pp. 761-769.

LUYENDYK, J.P., SCHABBAUER, G.A., TENCATI, M., HOLSCHER, T., PAWLINSKI, R. and MACKMAN, N., 2008. Genetic analysis of the role of the PI3K-Akt pathway in lipopolysaccharide-induced cytokine and tissue factor gene expression in monocytes/macrophages. *Journal of immunology (Baltimore, Md.: 1950),* **180**(6), pp. 4218-4226.

MACK, G.S., 2007. MicroRNA gets down to business. *Nature biotechnology,* **25**(6), pp. 631-638.

MAKELA, S.M., STRENGELL, M., PIETILA, T.E., OSTERLUND, P. and JULKUNEN, I., 2009. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *Journal of leukocyte biology,* **85**(4), pp. 664-672.

MALMGAARD, L., MELCHJORSEN, J., BOWIE, A.G., MOGENSEN, S.C. and PALUDAN, S.R., 2004. Viral activation of macrophages through TLR-dependent and -independent pathways. *Journal of immunology (Baltimore, Md.: 1950),* **173**(11), pp. 6890-6898.

MARCHANT, E.G., MISTLBERGER, R.E., 1996. Entrainment and phase shifting of circadian rhythms in mice by forced treadmill running. *Physiol Behav.* **60**(2), pp. 657-63.

MARION, J., 2014. Toll-Like Receptors: Evolution and Structure. *Springer New York,* , pp. 1-7.

MARSHAK-ROTHSTEIN, A., 2006. Toll-like receptors in systemic autoimmune disease. *Nature reviews.Immunology,* **6**(11), pp. 823-835.

MARSHALL, J.D., HEEKE, D.S., GESNER, M.L., LIVINGSTON, B. and VAN NEST, G., 2007. Negative regulation of TLR9-mediated IFN-alpha induction by a small-molecule, synthetic TLR7 ligand. *Journal of leukocyte biology,* **82**(3), pp. 497-508.

MARTIN, C.A. and DORF, M.E., 1990. Interleukin-6 production by murine macrophage cell lines P388D1 and J774A.1: stimulation requirements and kinetics. *Cellular immunology,* **128**(2), pp. 555-568.

MARTIN, M., SCHIFFERLE, R.E., CUESTA, N., VOGEL, S.N., KATZ, J. and MICHALEK, S.M., 2003. Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. *Journal of immunology (Baltimore, Md.: 1950),* **171**(2), pp. 717-725.

MATHUR, R., OH, H., ZHANG, D., PARK, S.G., SEO, J., KOBLANSKY, A., HAYDEN, M.S. and GHOSH, S., 2012. A mouse model of Salmonella typhi infection. *Cell,* **151**(3), pp. 590-602.

MATSUGUCHI, T., MUSIKACHAROEN, T., OGAWA, T. and YOSHIKAI, Y., 2000. Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. *Journal of immunology (Baltimore, Md.: 1950),* **165**(10), pp. 5767-5772.

MATSUSHIMA, N., MIYASHITA, H., ENKHBAYAR, P. and KRETSINGER, R.H., 2015. Comparative Geometrical Analysis of Leucine-Rich Repeat Structures in the Nod-Like and Toll-Like Receptors in Vertebrate Innate Immunity. *Biomolecules,* **5**(3), pp. 1955-1978.

MATSUSHIMA, N., TANAKA, T., ENKHBAYAR, P., MIKAMI, T., TAGA, M., YAMADA, K. and KUROKI, Y., 2007. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. *BMC genomics,* **8**, pp. 124.

MAURYA, M.R., BENNER, C., PRADERVAND, S., GLASS, C. and SUBRAMANIAM, S., 2007. Systems biology of macrophages. *Advances in Experimental Medicine and Biology,* **598**, pp. 62-79.

MCCORMACK, J.E., WADE, T., MORALES, H., KAPPLER, J. and MARRACK, P., 1991. Analysis of class II MHC structure in thymic nurse cells. *Cellular immunology,* **138**(2), pp. 413-422.

MCCOY, S.L., KURTZ, S.E., HAUSMAN, F.A., TRUNE, D.R., BENNETT, R.M. and HEFENEIDER, S.H., 2004. Activation of RAW264.7 macrophages by bacterial DNA and lipopolysaccharide increases cell surface DNA binding and internalization. *The Journal of biological chemistry,* **279**(17), pp. 17217-17223.

MEANS, T.K. and LUSTER, A.D., 2010. Integrins limit the Toll. *Nature immunology,* **11**(8), pp. 691-693.

MEDZHITOV, R. and HORNG, T., 2009. Transcriptional control of the inflammatory response. *Nature reviews.Immunology,* **9**(10), pp. 692-703.

MEDZHITOV, R. and JANEWAY, C.A.,Jr, 1997. Innate immunity: impact on the adaptive immune response. *Current opinion in immunology,* **9**(1), pp. 4-9.

MEDZHITOV, R., PRESTON-HURLBURT, P. and JANEWAY, C.A.,Jr, 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature,* **388**(6640), pp. 394-397.

MELLO, C.C. and CONTE, D.,Jr, 2004. Revealing the world of RNA interference. *Nature,* **431**(7006), pp. 338-342.

MEYLAN, E., BURNS, K., HOFMANN, K., BLANCHETEAU, V., MARTINON, F., KELLIHER, M. and TSCHOPP, J., 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nature immunology,* **5**(5), pp. 503-507.

MIAO, E.A., ALPUCHE-ARANDA, C.M., DORS, M., CLARK, A.E., BADER, M.W., MILLER, S.I. and ADEREM, A., 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nature immunology,* **7**(6), pp. 569-575.

MICHALLET, M.C., MEYLAN, E., ERMOLAEVA, M.A., VAZQUEZ, J., REBSAMEN, M., CURRAN, J., POECK, H., BSCHEIDER, M., HARTMANN, G., KONIG, M., KALINKE, U., PASPARAKIS, M. and TSCHOPP, J., 2008. TRADD protein is an essential component of the RIG-like helicase antiviral pathway. *Immunity,* **28**(5), pp. 651-661.

MIGGIN, S.M. and O'NEILL, L.A., 2006. New insights into the regulation of TLR signaling. *Journal of leukocyte biology,* **80**(2), pp. 220-226.

MITA, Y., DOBASHI, K., SHIMIZU, Y., NAKAZAWA, T. and MORI, M., 2001. Toll-like receptor 2 and 4 surface expressions on human monocytes are modulated by interferon-gamma and macrophage colony-stimulating factor. *Immunology letters,* **78**(2), pp. 97-101.

MITCHELL, D., YONG, M., SCHRODER, W., BLACK, M., TIRRELL, M. and OLIVE, C., 2010. Dual stimulation of MyD88-dependent Toll-like receptors induces synergistically enhanced production of inflammatory cytokines in murine bone marrow-derived dendritic cells. *The Journal of infectious diseases,* **202**(2), pp. 318-329.

MIYAZAWA, K., 2011. Encountering unpredicted off-target effects of pharmacological inhibitors. *Journal of Biochemistry,* **150**(1), pp. 1-3.

MOCHIZUKI, S., KOBAYASHI, M., SUZUKI, T., OIKAWA, A., KOSEKI, T., NISHIHARA, T. and HASEGAWA, K., 2004. Gamma-interferon enhances expression of CD14/MyD88 and subsequent responsiveness to lipopolysaccharide from Actinobacillus actinomycetemcomitans in human gingival fibroblasts. *Journal of periodontal research,* **39**(5), pp. 333-343.

MOLLENHAUER, H.H., MORRE, D.J. and ROWE, L.D., 1990. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochimica et biophysica acta,* **1031**(2), pp. 225-246.

MONICK, M.M., POWERS, L.S., BARRETT, C.W., HINDE, S., ASHARE, A., GROSKREUTZ, D.J., NYUNOYA, T., COLEMAN, M., SPITZ, D.R. and HUNNINGHAKE, G.W., 2008. Constitutive ERK MAPK activity regulates macrophage ATP production and mitochondrial integrity. *Journal of immunology (Baltimore, Md.: 1950),* **180**(11), pp. 7485-7496.

MORRISON, D.K., 2012. MAP kinase pathways. *Cold Spring Harbor perspectives in biology,* **4**(11), pp. 10.1101/cshperspect.a011254.

MUKHERJI, A., KOBIITA, A., YE, T. and CHAMBON, P., 2013. Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. *Cell,* **153**(4), pp. 812-827.

MURPHY, M., XIONG, Y., PATTABIRAMAN, G., QIU, F. and MEDVEDEV, A.E., 2015. Pellino-1 positively regulates Toll-like Receptor (TLR) 2 and TLR4 signaling and is suppressed upon induction of endotoxin tolerance. *The Journal of biological chemistry,* .

MUZIO, M., NI, J., FENG, P. and DIXIT, V.M., 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science (New York, N.Y.),* **278**(5343), pp. 1612-1615.

NAGATA, S., 2007. Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors. *Immunological reviews,* **220**, pp. 237-250.

NAIR, J.S., DAFONSECA, C.J., TJERNBERG, A., SUN, W., DARNELL, J.E.,Jr, CHAIT, B.T. and ZHANG, J.J., 2002. Requirement of Ca2+ and CaMKII for Stat1 Ser-727 phosphorylation in response to IFN-gamma. *Proceedings of the National Academy of Sciences of the United States of America,* **99**(9), pp. 5971-5976.

NAKA, T., FUJIMOTO, M., TSUTSUI, H. and YOSHIMURA, A., 2005. Negative regulation of cytokine and TLR signalings by SOCS and others. *Advances in Immunology,* **87**, pp. 61-122.

NAKAGAWA, R., NAKA, T., TSUTSUI, H., FUJIMOTO, M., KIMURA, A., ABE, T., SEKI, E., SATO, S., TAKEUCHI, O., TAKEDA, K., AKIRA, S., YAMANISHI, K., KAWASE, I., NAKANISHI, K. and KISHIMOTO, T., 2002. SOCS-1 participates in negative regulation of LPS responses. *Immunity,* **17**(5), pp. 677-687.

NAKAO, A., 2014. Temporal regulation of cytokines by the circadian clock. *Journal of immunology research,* **2014**, pp. 614529.

NATARAJAN, M., LIN, K.M., HSUEH, R.C., STERNWEIS, P.C. and RANGANATHAN, R., 2006. A global analysis of cross-talk in a mammalian cellular signalling network. *Nature cell biology,* **8**(6), pp. 571-580.

NEGISHI, H., OHBA, Y., YANAI, H., TAKAOKA, A., HONMA, K., YUI, K., MATSUYAMA, T., TANIGUCHI, T. and HONDA, K., 2005. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proceedings of the National Academy of Sciences of the United States of America,* **102**(44), pp. 15989-15994.

NELSON, D.E., IHEKWABA, A.E.C., ELLIOTT, M., JOHNSEN, J.R., GIBNEY, C.A., FOREMAN, B.E., NELSON, G., SEE, V., HORTON, C.A., SPILLER D.G., EDWARDS S.W., MCDOWELL, H.P., UNITT, J.F., SULLIVAN, E., GRIMLEY, R., BENSON, N., BROOMHEAD, D., KELL, D.B., WHITE, M.R.H., 2004. Oscillations in NFkB signalling control the dynamics of gene expression. *Science*, **306**(5696), pp. 704-708

NEWTON, K. and DIXIT, V.M., 2012. Signaling in innate immunity and inflammation. *Cold Spring Harbor perspectives in biology,* **4**(3), pp. 10.1101/cshperspect.a006049.

NGUYEN, H., CHATTERJEE-KISHORE, M., JIANG, Z., QING, Y., RAMANA, C.V., BAYES, J., COMMANE, M., LI, X. and STARK, G.R., 2003. IRAK-dependent phosphorylation of Stat1 on serine 727 in response to interleukin-1 and effects on gene expression. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research,* **23**(4), pp. 183-192.

NI, M., MACFARLANE, A.W.,4th, TOFT, M., LOWELL, C.A., CAMPBELL, K.S. and HAMERMAN, J.A., 2012. B-cell adaptor for PI3K (BCAP) negatively regulates Toll-like receptor signaling through activation of PI3K. *Proceedings of the National Academy of Sciences of the United States of America,* **109**(1), pp. 267-272.

NILSEN, N.J., VLADIMER, G.I., STENVIK, J., ORNING, M.P., ZEID-KILANI, M.V., BUGGE, M., BERGSTROEM, B., CONLON, J., HUSEBYE, H., HISE, A.G., FITZGERALD, K.A., ESPEVIK, T. and LIEN, E., 2015. A role for the adaptor proteins TRAM and TRIF in toll-like receptor 2 signaling. *The Journal of biological chemistry,* **290**(6), pp. 3209-3222.

NISHIHARA, T., KOGA, T. and HAMADA, S., 1988. Production of an interleukin-1 inhibitor by cell line P388D1 murine macrophages stimulated with Haemophilus actinomycetemcomitans lipopolysaccharide. *Infection and immunity,* **56**(11), pp. 2801-2807.

NOUR, A.M., YEUNG, Y.G., SANTAMBROGIO, L., BOYDEN, E.D., STANLEY, E.R. and BROJATSCH, J., 2009. Anthrax lethal toxin triggers the formation of a membrane-associated inflammasome complex in murine macrophages. *Infection and immunity,* **77**(3), pp. 1262-1271.

NYLANDER, S. and KALIES, I., 1999. Brefeldin A, but not monensin, completely blocks CD69 expression on mouse lymphocytes: efficacy of inhibitors of protein secretion in protocols for intracellular cytokine staining by flow cytometry. *Journal of immunological methods,* **224**(1-2), pp. 69-76.

O'BRIEN, J., WILSON, I., ORTON, T. and POGNAN, F., 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European journal of biochemistry / FEBS,* **267**(17), pp. 5421-5426.

O'CONNELL, R.M., CHAUDHURI, A.A., RAO, D.S. and BALTIMORE, D., 2009. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(17), pp. 7113-7118.

ODA K., KITANO H., 2006. A Comprehensive map of the toll-like receptor signalling network. *Mol Syst Biol*., 2:2006.0015.

O'DEA, E.L., BARKEN, D., PERALTA, R.Q., TRAN, K.T., WERNER, S.L., KEARNS, J.D., LEVCHENKO, A., HOFFMANN, A., 2007. A homeostatic model of IκB metabolism to control constitutive NFκB activity. *Mol Syst Biol.*, **3**(1)

ODEGAARD, J.I., RICARDO-GONZALEZ, R.R., GOFORTH, M.H., MOREL, C.R., SUBRAMANIAN, V., MUKUNDAN, L., RED EAGLE, A., VATS, D., BROMBACHER, F., FERRANTE, A.W. and CHAWLA, A., 2007. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature,* **447**(7148), pp. 1116-1120.

OGANESYAN, G., SAHA, S.K., GUO, B., HE, J.Q., SHAHANGIAN, A., ZARNEGAR, B., PERRY, A. and CHENG, G., 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature,* **439**(7073), pp. 208-211.

OHASHI, K., BURKART, V., FLOHE, S. and KOLB, H., 2000. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *Journal of immunology (Baltimore, Md.: 1950),* **164**(2), pp. 558-561.

OHTO, U., SHIBATA, T., TANJI, H., ISHIDA, H., KRAYUKHINA, E., UCHIYAMA, S., MIYAKE, K. and SHIMIZU, T., 2015. Structural basis of CpG and inhibitory DNA recognition by Toll-like receptor 9. *Nature,* .

OKABE, Y. and MEDZHITOV, R., 2015. Tissue biology perspective on macrophages. *Nature immunology,* **17**(1), pp. 9-17.

OKABE, Y. and MEDZHITOV, R., 2014. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell,* **157**(4), pp. 832-844.

OLDENBURG, M., KRUGER, A., FERSTL, R., KAUFMANN, A., NEES, G., SIGMUND, A., BATHKE, B., LAUTERBACH, H., SUTER, M., DREHER, S., KOEDEL, U., AKIRA, S., KAWAI, T., BUER, J., WAGNER, H., BAUER, S., HOCHREIN, H. and KIRSCHNING, C.J., 2012. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science (New York, N.Y.),* **337**(6098), pp. 1111-1115.

O'NEILL, L.A. and BOWIE, A.G., 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature reviews.Immunology,* **7**(5), pp. 353-364.

O'NEILL, L.A., GOLENBOCK, D. and BOWIE, A.G., 2013. The history of Toll-like receptors - redefining innate immunity. *Nature reviews.Immunology,* **13**(6), pp. 453-460.

O'NEILL, L.A., SHEEDY, F.J. and MCCOY, C.E., 2011. MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nature reviews.Immunology,* **11**(3), pp. 163-175.

OTTE, J.M., CARIO, E. and PODOLSKY, D.K., 2004. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology,* **126**(4), pp. 1054-1070.

PALAZZO, M., GARIBOLDI, S., ZANOBBIO, L., DUSIO, G.F., SELLERI, S., BEDONI, M., BALSARI, A. and RUMIO, C., 2008. Cross-talk among Toll-like receptors and their ligands. *International immunology,* **20**(5), pp. 709-718.

PARK, B.S. and LEE, J.O., 2013a. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental & molecular medicine,* **45**, pp. e66.

PARK, B.S. and LEE, J.O., 2013b. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental & molecular medicine,* **45**, pp. e66.

PASQUIER, B., LAUNAY, P., KANAMARU, Y., MOURA, I.C., PFIRSCH, S., RUFFIE, C., HENIN, D., BENHAMOU, M., PRETOLANI, M., BLANK, U. and MONTEIRO, R.C., 2005. Identification of FcalphaRI as an inhibitory receptor that controls inflammation: dual role of FcRgamma ITAM. *Immunity,* **22**(1), pp. 31-42.

PENG, Y. and ZHANG, L., 2014. Activation of the TLR1/2 pathway induces the shaping of the immune response status of peripheral blood leukocytes. *Experimental and therapeutic medicine,* **7**(6), pp. 1708-1712.

PERDIGUERO, E.G. and GEISSMANN, F., 2015. The development and maintenance of resident macrophages. *Nature immunology,* **17**(1), pp. 2-8.

PERKINS, D.J., POLUMURI, S.K., PENNINI, M.E., LAI, W., XIE, P. and VOGEL, S.N., 2013. Reprogramming of murine macrophages through TLR2 confers viral resistance via TRAF3-mediated, enhanced interferon production. *PLoS pathogens,* **9**(7), pp. e1003479.

PEROVAL, M.Y., BOYD, A.C., YOUNG, J.R. and SMITH, A.L., 2013a. A critical role for MAPK signalling pathways in the transcriptional regulation of toll like receptors. *PloS one,* **8**(2), pp. e51243.

PETNICKI-OCWIEJA, T., CHUNG, E., ACOSTA, D.I., RAMOS, L.T., SHIN, O.S., GHOSH, S., KOBZIK, L., LI, X. and HU, L.T., 2013. TRIF mediates Toll-like receptor 2-dependent inflammatory responses to Borrelia burgdorferi. *Infection and immunity,* **81**(2), pp. 402-410.

PFAFFL, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research,* **29**(9), pp. e45.

PIAO, W., SONG, C., CHEN, H., DIAZ, M.A., WAHL, L.M., FITZGERALD, K.A., LI, L. and MEDVEDEV, A.E., 2009. Endotoxin tolerance dysregulates MyD88- and Toll/IL-1R domain-containing adapter inducing IFN-beta-dependent pathways and increases expression of negative regulators of TLR signaling. *Journal of leukocyte biology,* **86**(4), pp. 863-875.

PICHLMAIR, A., SCHULZ, O., TAN, C.P., NASLUND, T.I., LILJESTROM, P., WEBER, F. and REIS E SOUSA, C., 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science (New York, N.Y.),* **314**(5801), pp. 997-1001.

POBEZINSKAYA, Y.L., KIM, Y.S., CHOKSI, S., MORGAN, M.J., LI, T., LIU, C. and LIU, Z., 2008. The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. *Nature immunology,* **9**(9), pp. 1047-1054.

POLTORAK, A., HE, X., SMIRNOVA, I., LIU, M.Y., VAN HUFFEL, C., DU, X., BIRDWELL, D., ALEJOS, E., SILVA, M., GALANOS, C., FREUDENBERG, M., RICCIARDI-CASTAGNOLI, P., LAYTON, B. and BEUTLER, B., 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science (New York, N.Y.),* **282**(5396), pp. 2085-2088.

QI, J., QIAO, Y., WANG, P., LI, S., ZHAO, W. and GAO, C., 2012. microRNA-210 negatively regulates LPS-induced production of proinflammatory cytokines by targeting NF-kappaB1 in murine macrophages. *FEBS letters,* **586**(8), pp. 1201-1207.

QIN, J., QIAN, Y., YAO, J., GRACE, C. and LI, X., 2005. SIGIRR inhibits interleukin-1 receptor- and toll-like receptor 4-mediated signaling through different mechanisms. *The Journal of biological chemistry,* **280**(26), pp. 25233-25241.

QUINN, S.R. and O'NEILL, L.A., 2011. A trio of microRNAs that control Toll-like receptor signalling. *International immunology,* **23**(7), pp. 421-425.

QURESHI, S.T., LARIVIERE, L., LEVEQUE, G., CLERMONT, S., MOORE, K.J., GROS, P. and MALO, D., 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *The Journal of experimental medicine,* **189**(4), pp. 615-625.

RAETZ, M., KIBARDIN, A., STURGE, C.R., PIFER, R., LI, H., BURSTEIN, E., OZATO, K., LARIN, S. and YAROVINSKY, F., 2013. Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to Toxoplasma gondii profilin. *Journal of immunology (Baltimore, Md.: 1950),* **191**(9), pp. 4818-4827.

RALPH, P. and NAKOINZ, I., 1977. Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. *Journal of immunology (Baltimore, Md.: 1950),* **119**(3), pp. 950-954.

RANA, T.M., 2007. Illuminating the silence: understanding the structure and function of small RNAs. *Nature reviews.Molecular cell biology,* **8**(1), pp. 23-36.

RASCHKE, W.C., BAIRD, S., RALPH, P. and NAKOINZ, I., 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell,* **15**(1), pp. 261-267.

RATH, D., AMLINGER, L., RATH, D., LUNDGREN, M., 2015. The CRISPR-Cas immune system: biology, mechanisms and applications. *Biochimie*, **117**, pp. 119-128

REHWINKEL, J., TAN, C.P., GOUBAU, D., SCHULZ, O., PICHLMAIR, A., BIER, K., ROBB, N., VREEDE, F., BARCLAY, W., FODOR, E. and REIS E SOUSA, C., 2010. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell,* **140**(3), pp. 397-408.

RHEE, S.H., JONES, B.W., TOSHCHAKOV, V., VOGEL, S.N. and FENTON, M.J., 2003. Toll-like receptors 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages. *The Journal of biological chemistry,* **278**(25), pp. 22506-22512.

RICHEZ, C., BLANCO, P., RIFKIN, I., MOREAU, J.F. and SCHAEVERBEKE, T., 2011. Role for toll-like receptors in autoimmune disease: the example of systemic lupus erythematosus. *Joint, bone, spine : revue du rhumatisme,* **78**(2), pp. 124-130.

ROACH, J.C., GLUSMAN, G., ROWEN, L., KAUR, A., PURCELL, M.K., SMITH, K.D., HOOD, L.E. and ADEREM, A., 2005a. The evolution of vertebrate Toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America,* **102**(27), pp. 9577-9582.

ROACH, J.M., RACIOPPI, L., JONES, C.D. and MASCI, A.M., 2013. Phylogeny of Toll-like receptor signaling: adapting the innate response. *PloS one,* **8**(1), pp. e54156.

ROSAS, M., DAVIES, L.C., GILES, P.J., LIAO, C.T., KHARFAN, B., STONE, T.C., O'DONNELL, V.B., FRASER, D.J., JONES, S.A. and TAYLOR, P.R., 2014. The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal. *Science (New York, N.Y.),* **344**(6184), pp. 645-648.

ROSETTO, M., ENGSTROM, Y., BALDARI, C.T., TELFORD, J.L. and HULTMARK, D., 1995. Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line. *Biochemical and biophysical research communications,* **209**(1), pp. 111-116.

ROSSOL, M., PIERER, M., RAULIEN, N., QUANDT, D., MEUSCH, U., ROTHE, K., SCHUBERT, K., SCHONEBERG, T., SCHAEFER, M., KRUGEL, U., SMAJILOVIC, S., BRAUNER-OSBORNE, H., BAERWALD, C. and WAGNER, U., 2012. Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nature communications,* **3**, pp. 1329.

ROUX, P.P. and BLENIS, J., 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiology and molecular biology reviews : MMBR,* **68**(2), pp. 320-344.

RUIPEREZ, V., ASTUDILLO, A.M., BALBOA, M.A. and BALSINDE, J., 2009. Coordinate regulation of TLR-mediated arachidonic acid mobilization in macrophages by group IVA and group V phospholipase A2s. *Journal of immunology (Baltimore, Md.: 1950),* **182**(6), pp. 3877-3883.

RUIPEREZ, V., CASAS, J., BALBOA, M.A. and BALSINDE, J., 2007. Group V phospholipase A2-derived lysophosphatidylcholine mediates cyclooxygenase-2 induction in lipopolysaccharide-stimulated macrophages. *Journal of immunology (Baltimore, Md.: 1950),* **179**(1), pp. 631-638.

SAHA, S.K., PIETRAS, E.M., HE, J.Q., KANG, J.R., LIU, S.Y., OGANESYAN, G., SHAHANGIAN, A., ZARNEGAR, B., SHIBA, T.L., WANG, Y. and CHENG, G., 2006. Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. *The EMBO journal,* **25**(14), pp. 3257-3263.

SAIJO, K., WINNER, B., CARSON, C.T., COLLIER, J.G., BOYER, L., ROSENFELD, M.G., GAGE, F.H. and GLASS, C.K., 2009. A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. *Cell,* **137**(1), pp. 47-59.

SAITOH, T., YAMAMOTO, M., MIYAGISHI, M., TAIRA, K., NAKANISHI, M., FUJITA, T., AKIRA, S., YAMAMOTO, N. and YAMAOKA, S., 2005. A20 is a negative regulator of IFN regulatory factor 3 signaling. *Journal of immunology (Baltimore, Md.: 1950),* **174**(3), pp. 1507-1512.

SALO, R.J., BLEAM, D.K., GREER, V.L. and ORTEGA, A.P., 1985. Interferon production in murine macrophage-like cell lines. *Journal of leukocyte biology,* **37**(4), pp. 395-406.

SANDIG, H. and BULFONE-PAUS, S., 2012. TLR signaling in mast cells: common and unique features. *Frontiers in immunology,* **3**, pp. 185.

SASAI, M., SHINGAI, M., FUNAMI, K., YONEYAMA, M., FUJITA, T., MATSUMOTO, M. and SEYA, T., 2006. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. *Journal of immunology (Baltimore, Md.: 1950),* **177**(12), pp. 8676-8683.

SATO, A., LINEHAN, M.M. and IWASAKI, A., 2006. Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America,* **103**(46), pp. 17343-17348.

SATO, S., NOMURA, F., KAWAI, T., TAKEUCHI, O., MUHLRADT, P.F., TAKEDA, K. and AKIRA, S., 2000. Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *Journal of immunology (Baltimore, Md.: 1950),* **165**(12), pp. 7096-7101.

SATO, S., SANJO, H., TAKEDA, K., NINOMIYA-TSUJI, J., YAMAMOTO, M., KAWAI, T., MATSUMOTO, K., TAKEUCHI, O. and AKIRA, S., 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nature immunology,* **6**(11), pp. 1087-1095.

SATOH, M., PARENT, A.D. and ZHANG, J.H., 2002. Inhibitory effect with antisense mitogen-activated protein kinase oligodeoxynucleotide against cerebral vasospasm in rats. *Stroke; a journal of cerebral circulation,* **33**(3), pp. 775-781.

SCHEIERMANN, C., KUNISAKI, Y., FRENETTE P.S., 2013. Circadian control of the immune system. *Nat. Rev. Immunol*. **13**(3), pp.190-198

SCHNEIDER, D.S., HUDSON, K.L., LIN, T.Y. and ANDERSON, K.V., 1991. Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the Drosophila embryo. *Genes & development,* **5**(5), pp. 797-807.

SCHOENEMEYER, A., BARNES, B.J., MANCL, M.E., LATZ, E., GOUTAGNY, N., PITHA, P.M., FITZGERALD, K.A. and GOLENBOCK, D.T., 2005. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. *The Journal of biological chemistry,* **280**(17), pp. 17005-17012.

SCHRODER, K., SPILLE, M., PILZ, A., LATTIN, J., BODE, K.A., IRVINE, K.M., BURROWS, A.D., RAVASI, T., WEIGHARDT, H., STACEY, K.J., DECKER, T., HUME, D.A., DALPKE, A.H. and SWEET, M.J., 2007. Differential effects of CpG DNA on IFN-beta induction and STAT1 activation in murine macrophages versus dendritic cells:

alternatively activated STAT1 negatively regulates TLR signaling in macrophages. *Journal of immunology (Baltimore, Md.: 1950),* **179**(6), pp. 3495-3503.

SCHRODER, K., SWEET, M.J. and HUME, D.A., 2006. Signal integration between IFNgamma and TLR signalling pathways in macrophages. *Immunobiology,* **211**(6-8), pp. 511-524.

SCHRODER, K., ZHOU, R. and TSCHOPP, J., 2010. The NLRP3 inflammasome: a sensor for metabolic danger? *Science (New York, N.Y.),* **327**(5963), pp. 296-300.

SEGAWA, K., KURATA, S., YANAGIHASHI, Y., BRUMMELKAMP, T.R., MATSUDA, F. and NAGATA, S., 2014. Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science (New York, N.Y.),* **344**(6188), pp. 1164-1168.

SEONG, S.Y. and MATZINGER, P., 2004. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nature reviews.Immunology,* **4**(6), pp. 469-478.

SHAW, P.J., LAMKANFI, M. and KANNEGANTI, T.D., 2010. NOD-like receptor (NLR) signaling beyond the inflammasome. *European journal of immunology,* **40**(3), pp. 624-627.

SHEEDY, F.J. and O'NEILL, L.A.J., 2007. *The Troll in Toll: Mal and Tram as bridges for TLR2 and TLR4 signaling.*

SHEN, Y.H., GODLEWSKI, J., ZHU, J., SATHYANARAYANA, P., LEANER, V., BIRRER, M.J., RANA, A. and TZIVION, G., 2003. Cross-talk between JNK/SAPK and ERK/MAPK pathways: sustained activation of JNK blocks ERK activation by mitogenic factors. *The Journal of biological chemistry,* **278**(29), pp. 26715-26721.

SHERIDAN, B.S. and LEFRANCOIS, L., 2012. Isolation of mouse lymphocytes from small intestine tissues. *Current protocols in immunology / edited by John E.Coligan ...[et al.],* **Chapter 3**, pp. Unit3.19.

SHI, Z., CAI, Z., WEN, S., CHEN, C., GENDRON, C., SANCHEZ, A., PATTERSON, K., FU, S., YANG, J., WILDMAN, D., FINNELL, R.H. and ZHANG, D., 2009. Transcriptional regulation of the novel Toll-like receptor Tlr13. *The Journal of biological chemistry,* **284**(31), pp. 20540-20547.

SHIBATA, T., TAKEMURA, N., MOTOI, Y., GOTO, Y., KARUPPUCHAMY, T., IZAWA, K., LI, X., AKASHI-TAKAMURA, S., TANIMURA, N., KUNISAWA, J., KIYONO, H., AKIRA, S., KITAMURA, T., KITAURA, J., UEMATSU, S. and MIYAKE, K., 2012. PRAT4A-dependent expression of cell surface TLR5 on neutrophils, classical monocytes and dendritic cells. *International immunology,* **24**(10), pp. 613-623.

SHIMO, T., MATSUMURA, S., IBARAGI, S., ISOWA, S., KISHIMOTO, K., MESE, H., NISHIYAMA, A. and SASAKI, A., 2007. Specific inhibitor of MEK-mediated cross-talk between ERK and p38 MAPK during differentiation of human osteosarcoma cells. *Journal of cell communication and signaling,* **1**(2), pp. 103-111.

SIDHU, K., COX, E., YANEZ, A., HEIPERTZ, E., WALKER, W., 2015. The effects of circadian rhythms on Toll-like receptor responses in murine macrophages. *J. of Immunology*, 194(1), supplement 194.14

SILVER, A.C., ARJONA, A., HUGHES, M.E., NITABACH, M.N. and FIKRIG, E., 2012. Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. *Brain, behavior, and immunity,* **26**(3), pp. 407-413.

SIMS, J.E. and DOWER, S.K., 1994. Interleukin-1 receptors. *European cytokine network,* **5**(6), pp. 539-546.

SLY, L.M., RAUH, M.J., KALESNIKOFF, J., SONG, C.H. and KRYSTAL, G., 2004. LPS-induced upregulation of SHIP is essential for endotoxin tolerance. *Immunity,* **21**(2), pp. 227-239.

SNYDERMAN, R., PIKE, M.C., FISCHER, D.G. and KOREN, H.S., 1977. Biologic and biochemical activities of continuous macrophage cell lines P388D1 and J774.1. *Journal of immunology (Baltimore, Md.: 1950),* **119**(6), pp. 2060-2066.

SONG, X., JIN, P., QIN, S., CHEN, L. and MA, F., 2012a. The evolution and origin of animal Toll-like receptor signaling pathway revealed by network-level molecular evolutionary analyses. *PloS one,* **7**(12), pp. e51657.

SONG, X., JIN, P., QIN, S., CHEN, L. and MA, F., 2012b. The evolution and origin of SPILLER, S., ELSON, G., FERSTL, R., DREHER, S., MUELLER, T., FREUDENBERG, M., DAUBEUF, B., WAGNER, H. and KIRSCHNING, C.J., 2008. TLR4-induced IFN-gamma production increases TLR2 sensitivity and drives Gram-negative sepsis in mice. *The Journal of experimental medicine,* **205**(8), pp. 1747-1754.

STEBBINS, J.L., DE, S.K., MACHLEIDT, T., BECATTINI, B., VAZQUEZ, J., KUNTZEN, C., CHEN, L.H., CELLITTI, J.F., RIEL-MEHAN, M., EMDADI, A., SOLINAS, G., KARIN, M. and PELLECCHIA, M., 2008. Identification of a new JNK inhibitor targeting the JNK-JIP interaction site. *Proceedings of the National Academy of Sciences of the United States of America,* **105**(43), pp. 16809-16813.

STOIBER, D., KOVARIK, P., COHNEY, S., JOHNSTON, J.A., STEINLEIN, P. and DECKER, T., 1999. *Lipopolysaccharide Induces in Macrophages the Synthesis of the Suppressor of Cytokine Signaling 3 and Suppresses Signal Transduction in Response to the Activating Factor IFN-γ.*

SUET TING TAN, R., LIN, B., LIU, Q., TUCKER-KELLOGG, L., HO, B., LEUNG, B.P. and LING DING, J., 2013. The synergy in cytokine production through MyD88-TRIF pathways is co-ordinated with ERK phosphorylation in macrophages. *Immunology and cell biology,* **91**(5), pp. 377-387.

SUN, J., LI, N., OH, K.S., DUTTA, B., VAYTTADEN, S.J., LIN, B., EBERT, T.S., DE NARDO, D., DAVIS, J., BAGIRZADEH, R., LOUNSBURY, N.W., PASARE, C., LATZ, E., HORNUNG, V. and FRASER, I.D., 2016. Comprehensive RNAi-based screening of human and mouse TLR pathways identifies species-specific preferences in signaling protein use. *Science signaling,* **9**(409), pp. ra3.

TADA, H., NEMOTO, E., SHIMAUCHI, H., WATANABE, T., MIKAMI, T., MATSUMOTO, T., OHNO, N., TAMURA, H., SHIBATA, K., AKASHI, S., MIYAKE, K.,

SUGAWARA, S. and TAKADA, H., 2002. Saccharomyces cerevisiae- and Candida albicans-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. *Microbiology and immunology,* **46**(7), pp. 503-512.

TAKEDA, K. and AKIRA, S., 2005. Toll-like receptors in innate immunity. *International immunology,* **17**(1), pp. 1-14.

TAKEUCHI, O. and AKIRA, S., 2010. Pattern recognition receptors and inflammation. *Cell,* **140**(6), pp. 805-820.

TAKEUCHI, O. and AKIRA, S., 2009. Innate immunity to virus infection. *Immunological reviews,* **227**(1), pp. 75-86.

TAKEUCHI, O., HOSHINO, K. and AKIRA, S., 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *Journal of immunology (Baltimore, Md.: 1950),* **165**(10), pp. 5392-5396.

TAMAI, R., SUGAWARA, S., TAKEUCHI, O., AKIRA, S. and TAKADA, H., 2003. Synergistic effects of lipopolysaccharide and interferon-gamma in inducing interleukin-8 production in human monocytic THP-1 cells is accompanied by up-regulation of CD14, Toll-like receptor 4, MD-2 and MyD88 expression. *Journal of endotoxin research,* **9**(3), pp. 145-153.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A. and KUMAR, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution,* **30**(12), pp. 2725-2729.

TANEMURA, S., MOMOSE, H., SHIMIZU, N., KITAGAWA, D., SEO, J., YAMASAKI, T., NAKAGAWA, K., KAJIHO, H., PENNINGER, J.M., KATADA, T. and NISHINA, H., 2009. Blockage by SP600125 of Fcepsilon receptor-induced degranulation and cytokine gene expression in mast cells is mediated through inhibition of phosphatidylinositol 3-kinase signalling pathway. *Journal of Biochemistry,* **145**(3), pp. 345-354.

TANOUE, T., ADACHI, M., MORIGUCHI, T. and NISHIDA, E., 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nature cell biology,* **2**(2), pp. 110-116.

TARANTINO, N., TINEVEZ, J.Y., CROWELL, E.F., BOISSON, B., HENRIQUES, R., MHLANGA, M., AGOU, F., ISRAEL, A. and LAPLANTINE, E., 2014. TNF and IL-1 exhibit distinct ubiquitin requirements for inducing NEMO-IKK supramolecular structures. *The Journal of cell biology,* **204**(2), pp. 231-245.

TEMPERLEY, N.D., BERLIN, S., PATON, I.R., GRIFFIN, D.K. and BURT, D.W., 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. *BMC genomics,* **9**, pp. 62-2164-9-62.

THAISS, C.A., LEVY, M., ITAV, S. and ELINAV, E., 2016. Integration of Innate Immune Signaling. *Trends in immunology,* **37**(2), pp. 84-101.

THORNBERRY, N.A., BULL, H.G., CALAYCAY, J.R., CHAPMAN, K.T., HOWARD, A.D., KOSTURA, M.J., MILLER, D.K., MOLINEAUX, S.M., WEIDNER, J.R. and

AUNINS, J., 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature,* **356**(6372), pp. 768-774.

TOSHCHAKOV, V.Y., FENTON, M.J. and VOGEL, S.N., 2007. Cutting Edge: Differential inhibition of TLR signaling pathways by cell-permeable peptides representing BB loops of TLRs. *Journal of immunology (Baltimore, Md.: 1950),* **178**(5), pp. 2655-2660.

TRINCHIERI, G. and SHER, A., 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nature reviews.Immunology,* **7**(3), pp. 179-190.

TROUTMAN, T.D., BAZAN, J.F. and PASARE, C., 2012. Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K. *Cell cycle (Georgetown, Tex.),* **11**(19), pp. 3559-3567.

TROUTMAN, T.D., HU, W., FULENCHEK, S., YAMAZAKI, T., KUROSAKI, T., BAZAN, J.F. and PASARE, C., 2012. Role for B-cell adapter for PI3K (BCAP) as a signaling adapter linking Toll-like receptors (TLRs) to serine/threonine kinases PI3K/Akt. *Proceedings of the National Academy of Sciences of the United States of America,* **109**(1), pp. 273-278.

TSENG, P.H., MATSUZAWA, A., ZHANG, W., MINO, T., VIGNALI, D.A. and KARIN, M., 2010. Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. *Nature immunology,* **11**(1), pp. 70-75.

UCHIDA, C., GEE, E., ISPANOVIC, E. and HAAS, T.L., 2008. JNK as a positive regulator of angiogenic potential in endothelial cells. *Cell biology international,* **32**(7), pp. 769-776.

UEMATSU, S., FUJIMOTO, K., JANG, M.H., YANG, B.G., JUNG, Y.J., NISHIYAMA, M., SATO, S., TSUJIMURA, T., YAMAMOTO, M., YOKOTA, Y., KIYONO, H., MIYASAKA, M., ISHII, K.J. and AKIRA, S., 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nature immunology,* **9**(7), pp. 769-776.

UEMATSU, S., JANG, M.H., CHEVRIER, N., GUO, Z., KUMAGAI, Y., YAMAMOTO, M., KATO, H., SOUGAWA, N., MATSUI, H., KUWATA, H., HEMMI, H., COBAN, C., KAWAI, T., ISHII, K.J., TAKEUCHI, O., MIYASAKA, M., TAKEDA, K. and AKIRA, S., 2006. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nature immunology,* **7**(8), pp. 868-874.

UNTERGASSER, A., CUTCUTACHE, I., KORESSAAR, T., YE, J., FAIRCLOTH, B.C., REMM, M. and ROZEN, S.G., 2012. Primer3--new capabilities and interfaces. *Nucleic acids research,* **40**(15), pp. e115.

UPHOFF, C.C. and DREXLER, H.G., 2013. Detection of mycoplasma contaminations. *Methods in molecular biology (Clifton, N.J.),* **946**, pp. 1-13.

VABULAS, R.M., AHMAD-NEJAD, P., DA COSTA, C., MIETHKE, T., KIRSCHNING, C.J., HACKER, H. and WAGNER, H., 2001. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *The Journal of biological chemistry,* **276**(33), pp. 31332-31339.

VABULAS, R.M., AHMAD-NEJAD, P., GHOSE, S., KIRSCHNING, C.J., ISSELS, R.D. and WAGNER, H., 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *The Journal of biological chemistry,* **277**(17), pp. 15107-15112.

VAN AUBEL, R.A.M.H., KEESTRA, A.M., KROOSHOOP, D.J.E.B., VAN EDEN, W. and VAN PUTTEN, J.P.M., 2007. Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells. *Molecular immunology,* **44**(15), pp. 3702-3714.

VAN DER VAART, M., SPAINK, H.P. and MEIJER, A.H., 2012. Pathogen recognition and activation of the innate immune response in zebrafish. *Advances in hematology,* **2012**, pp. 159807.

VARINOU, L., RAMSAUER, K., KARAGHIOSOFF, M., KOLBE, T., PFEFFER, K., MULLER, M. and DECKER, T., 2003. Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity. *Immunity,* **19**(6), pp. 793-802.

VINKLER, M., BAINOVA, H. and BRYJA, J., 2014. Protein evolution of Toll-like receptors 4, 5 and 7 within Galloanserae birds. *Genetics, selection, evolution : GSE,* **46**, pp. 72-014-0072-6.

WADSWORTH, T.L. and KOOP, D.R., 1999. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochemical pharmacology,* **57**(8), pp. 941-949.

WAGNER, E.F. and NEBREDA, A.R., 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nature reviews.Cancer,* **9**(8), pp. 537-549.

WALD, D., QIN, J., ZHAO, Z., QIAN, Y., NARAMURA, M., TIAN, L., TOWNE, J., SIMS, J.E., STARK, G.R. and LI, X., 2003. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. *Nature immunology,* **4**(9), pp. 920-927.

WALLOSCHKE, B., FUHRMANN, H. and SCHUMANN, J., 2010. Macrophage cell line RAW264.7 but not P-388D1 is an appropriate in vitro-model for studying oxidative burst as well as cytokine production in context of fatty acid enrichment. *Cellular immunology,* **262**(1), pp. 58-61.

WANG, C., DENG, L., HONG, M., AKKARAJU, G.R., INOUE, J. and CHEN, Z.J., 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature,* **412**(6844), pp. 346-351.

WANG, C., YU, X., CAO, Q., WANG, Y., ZHENG, G., TAN, T.K., ZHAO, H., ZHAO, Y., WANG, Y. and HARRIS, D.C., 2013. Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC immunology,* **14**, pp. 6-2172-14-6.

WANG, L., GORDON, R.A., HUYNH, L., SU, X., PARK MIN, K.H., HAN, J., ARTHUR, J.S., KALLIOLIAS, G.D. and IVASHKIV, L.B., 2010. Indirect inhibition of Toll-like receptor and type I interferon responses by ITAM-coupled receptors and integrins. *Immunity,* **32**(4), pp. 518-530.

WANG, S.C., ROSSIGNOL, D.P., CHRIST, W.J., GELLER, D.A., FREESWICK, P.D., THAI, N.L., SU, G.L. and SIMMONS, R.L., 1994. Suppression of lipopolysaccharide-

induced macrophage nitric oxide and cytokine production in vitro by a novel lipopolysaccharide antagonist. *Surgery,* **116**(2), pp. 339-46; discussion 446-7.

WAUSON, E.M., GUERRA, M.L., BARYLKO, B., ALBANESI, J.P. and COBB, M.H., 2013. Off-target effects of MEK inhibitors. *Biochemistry,* **52**(31), pp. 5164-5166.

WEN, Z., ZHONG, Z. and DARNELL, J.E.,Jr, 1995. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell,* **82**(2), pp. 241-250.

WENINK, M.H., SANTEGOETS, K.C., BROEN, J.C., VAN BON, L., ABDOLLAHI-ROODSAZ, S., POPA, C., HUIJBENS, R., REMIJN, T., LUBBERTS, E., VAN RIEL, P.L., VAN DEN BERG, W.B. and RADSTAKE, T.R., 2009. TLR2 promotes Th2/Th17 responses via TLR4 and TLR7/8 by abrogating the type I IFN amplification loop. *Journal of immunology (Baltimore, Md.: 1950),* **183**(11), pp. 6960-6970.

WERLING, D., JANN, O.C., OFFORD, V., GLASS, E.J. and COFFEY, T.J., 2009. Variation matters: TLR structure and species-specific pathogen recognition. *Trends in immunology,* **30**(3), pp. 124-130.

WESCHE, H., HENZEL, W.J., SHILLINGLAW, W., LI, S. and CAO, Z., 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity,* **7**(6), pp. 837-847.

WHEELER, D.S., CHASE, M.A., SENFT, A.P., POYNTER, S.E., WONG, H.R. and PAGE, K., 2009. Extracellular Hsp72, an endogenous DAMP, is released by virally infected airway epithelial cells and activates neutrophils via Toll-like receptor (TLR)-4. *Respiratory research,* **10**, pp. 31-9921-10-31.

WHITMARSH, A.J. and DAVIS, R.J., 1998. Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. *Trends in biochemical sciences,* **23**(12), pp. 481-485.

WHITMORE, M.M., IPARRAGUIRRE, A., KUBELKA, L., WENINGER, W., HAI, T. and WILLIAMS, B.R., 2007. Negative regulation of TLR-signaling pathways by activating transcription factor-3. *Journal of immunology (Baltimore, Md.: 1950),* **179**(6), pp. 3622-3630.

WIGLEY, P., HULME, S.D., BUMSTEAD, N. and BARROW, P.A., 2002. In vivo and in vitro studies of genetic resistance to systemic salmonellosis in the chicken encoded by the SAL1 locus. *Microbes and infection / Institut Pasteur,* **4**(11), pp. 1111-1120.

WILHELMSEN, K., MESA, K.R., LUCERO, J., XU, F. and HELLMAN, J., 2012. ERK5 protein promotes, whereas MEK1 protein differentially regulates, the Toll-like receptor 2 protein-dependent activation of human endothelial cells and monocytes. *The Journal of biological chemistry,* **287**(32), pp. 26478-26494.

WILHELMSEN, K., XU, F., FARRAR, K., TRAN, A., KHAKPOUR, S., SUNDAR, S., PRAKASH, A., WANG, J., GRAY, N.S. and HELLMAN, J., 2015. Extracellular signal-regulated kinase 5 promotes acute cellular and systemic inflammation. *Science signaling,* **8**(391), pp. ra86.

WILLIAMS, A., FLAVELL, R.A. and EISENBARTH, S.C., 2010. The role of NOD-like Receptors in shaping adaptive immunity. *Current opinion in immunology,* **22**(1), pp. 34-40.

WONG, C.K., CHEUNG, P.F., IP, W.K. and LAM, C.W., 2007. Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils. *American journal of respiratory cell and molecular biology,* **37**(1), pp. 85-96.

WU, G.S., BURNS, T.F., ZHAN, Y., ALNEMRI, E.S. and EL-DEIRY, W.S., 1999. Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. *Cancer research,* **59**(12), pp. 2770-2775.

XU, Y., JAGANNATH, C., LIU, X.D., SHARAFKHANEH, A., KOLODZIEJSKA, K.E. and EISSA, N.T., 2007. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity,* **27**(1), pp. 135-144.

YAMAMOTO, M., SATO, S., HEMMI, H., HOSHINO, K., KAISHO, T., SANJO, H., TAKEUCHI, O., SUGIYAMA, M., OKABE, M., TAKEDA, K. and AKIRA, S., 2003a. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science (New York, N.Y.),* **301**(5633), pp. 640-643.

YAMAMOTO, M., SATO, S., HEMMI, H., SANJO, H., UEMATSU, S., KAISHO, T., HOSHINO, K., TAKEUCHI, O., KOBAYASHI, M., FUJITA, T., TAKEDA, K. and AKIRA, S., 2002a. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature,* **420**(6913), pp. 324-329.

YAMAMOTO, M., SATO, S., HEMMI, H., UEMATSU, S., HOSHINO, K., KAISHO, T., TAKEUCHI, O., TAKEDA, K. and AKIRA, S., 2003c. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nature immunology,* **4**(11), pp. 1144-1150.

YAMAMOTO, M., SATO, S., MORI, K., HOSHINO, K., TAKEUCHI, O., TAKEDA, K. and AKIRA, S., 2002b. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *Journal of immunology (Baltimore, Md.: 1950),* **169**(12), pp. 6668-6672.

YANG, I.V., JIANG, W., RUTLEDGE, H.R., LACKFORD, B., WARG, L.A., DE ARRAS, L., ALPER, S., SCHWARTZ, D.A. and PISETSKY, D.S., 2011. Identification of novel innate immune genes by transcriptional profiling of macrophages stimulated with TLR ligands. *Molecular immunology,* **48**(15-16), pp. 1886-1895.

YANG, X., CHANG, H.Y. and BALTIMORE, D., 1998. Autoproteolytic activation of pro-caspases by oligomerization. *Molecular cell,* **1**(2), pp. 319-325.

YAROVINSKY, F., ZHANG, D., ANDERSEN, J.F., BANNENBERG, G.L., SERHAN, C.N., HAYDEN, M.S., HIENY, S., SUTTERWALA, F.S., FLAVELL, R.A., GHOSH, S. and SHER, A., 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science (New York, N.Y.),* **308**(5728), pp. 1626-1629.

YEE, N.K. and HAMERMAN, J.A., 2013. beta(2) integrins inhibit TLR responses by regulating NF-kappaB pathway and p38 MAPK activation. *European journal of immunology,* **43**(3), pp. 779-792.

YEH, D.W., LIU, Y.L., LO, Y.C., YUH, C.H., YU, G.Y., LO, J.F., LUO, Y., XIANG, R. and CHUANG, T.H., 2013. Toll-like receptor 9 and 21 have different ligand recognition profiles and cooperatively mediate activity of CpG-oligodeoxynucleotides in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America,* **110**(51), pp. 20711-20716.

YI, A.K., YOON, J.G., YEO, S.J., HONG, S.C., ENGLISH, B.K. and KRIEG, A.M., 2002. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. *Journal of immunology (Baltimore, Md.: 1950),* **168**(9), pp. 4711-4720.

YONEYAMA, M. and FUJITA, T., 2008. Structural mechanism of RNA recognition by the RIG-I-like receptors. *Immunity,* **29**(2), pp. 178-181.

YONEYAMA, M., KIKUCHI, M., NATSUKAWA, T., SHINOBU, N., IMAIZUMI, T., MIYAGISHI, M., TAIRA, K., AKIRA, S. and FUJITA, T., 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature immunology,* **5**(7), pp. 730-737.

YOO, J.S., KATO, H. and FUJITA, T., 2014. Sensing viral invasion by RIG-I like receptors. *Current opinion in microbiology,* **20**, pp. 131-138.

YOSHIMURA, A., NAKA, T. and KUBO, M., 2007. SOCS proteins, cytokine signalling and immune regulation. *Nature reviews.Immunology,* **7**(6), pp. 454-465.

YOSHIMURA, A. and YASUKAWA, H., 2012. JAK's SOCS: a mechanism of inhibition. *Immunity,* **36**(2), pp. 157-159.

YUAN, S.C., RUAN, J., PENG, J. and XU, A.L., 2015. Pattern recognition system in amphioxus (Amphioxus Immunity: Tracing the Origins of Human Immunity). *Academic Press,* , pp. 85-120.

YUK, J.M., SHIN, D.M., LEE, H.M., KIM, J.J., KIM, S.W., JIN, H.S., YANG, C.S., PARK, K.A., CHANDA, D., KIM, D.K., HUANG, S.M., LEE, S.K., LEE, C.H., KIM, J.M., SONG, C.H., LEE, S.Y., HUR, G.M., MOORE, D.D., CHOI, H.S. and JO, E.K., 2011. The orphan nuclear receptor SHP acts as a negative regulator in inflammatory signaling triggered by Toll-like receptors. *Nature immunology,* **12**(8), pp. 742-751.

ZAREMBER, K.A. and GODOWSKI, P.J., 2002a. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *Journal of immunology (Baltimore, Md.: 1950),* **168**(2), pp. 554-561.

ZHANG, G. and GHOSH, S., 2002. Negative regulation of toll-like receptor-mediated signaling by Tollip. *The Journal of biological chemistry,* **277**(9), pp. 7059-7065.

ZHANG, W. and LIU, H.T., 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell research,* **12**(1), pp. 9-18.

ZHAO, J., KONG, H.J., LI, H., HUANG, B., YANG, M., ZHU, C., BOGUNOVIC, M., ZHENG, F., MAYER, L., OZATO, K., UNKELESS, J. and XIONG, H., 2006. IRF-8/interferon (IFN) consensus sequence-binding protein is involved in Toll-like receptor

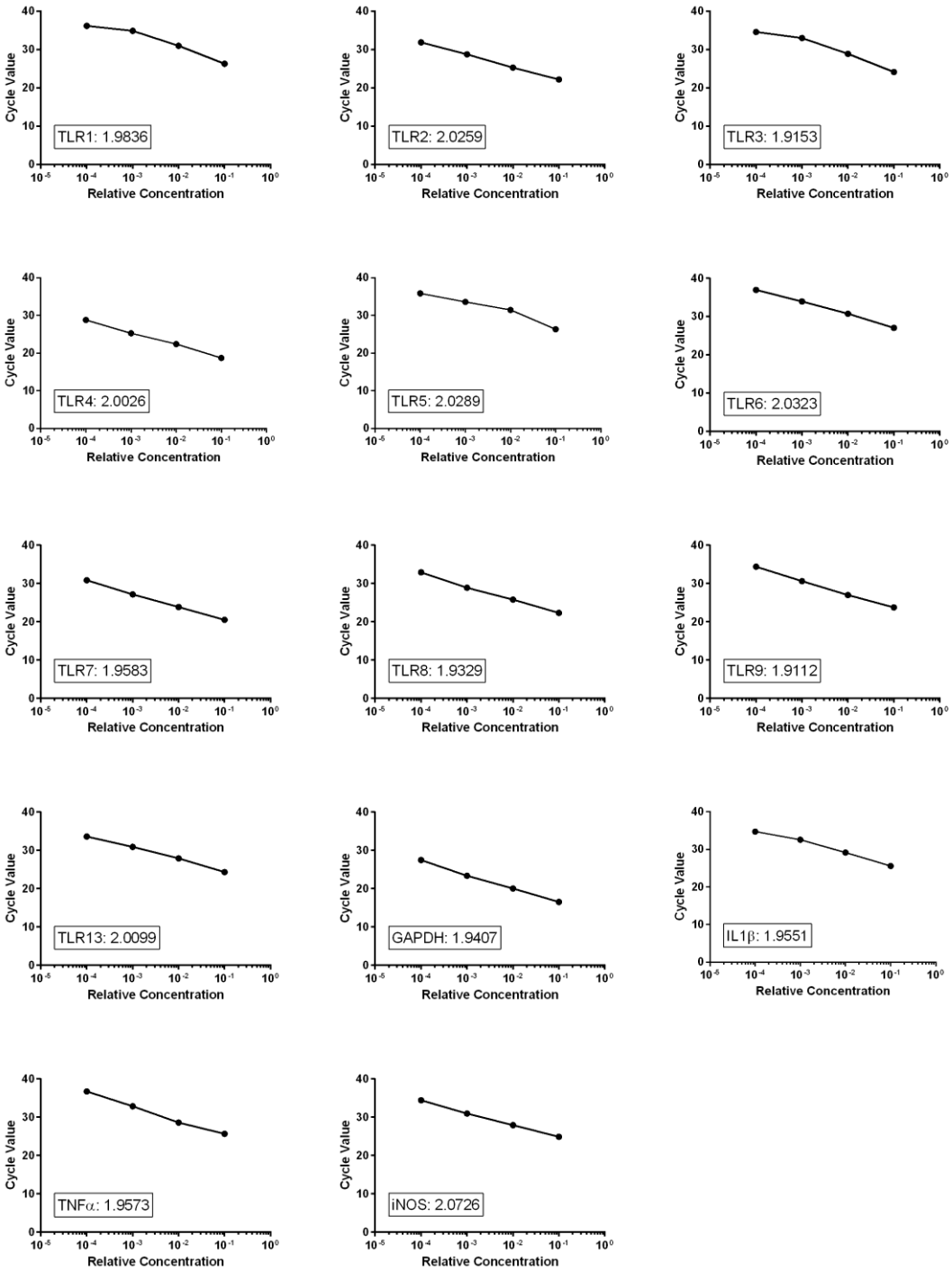
(TLR) signaling and contributes to the cross-talk between TLR and IFN-gamma signaling pathways. *The Journal of biological chemistry,* **281**(15), pp. 10073-10080.

ZHOU, M., WANG, W., KARAPETYAN, S., MWIMBA, M., MARQUES, J., BUCHLER, N.E. and DONG, X., 2015. Redox rhythm reinforces the circadian clock to gate immune response. *Nature,* .

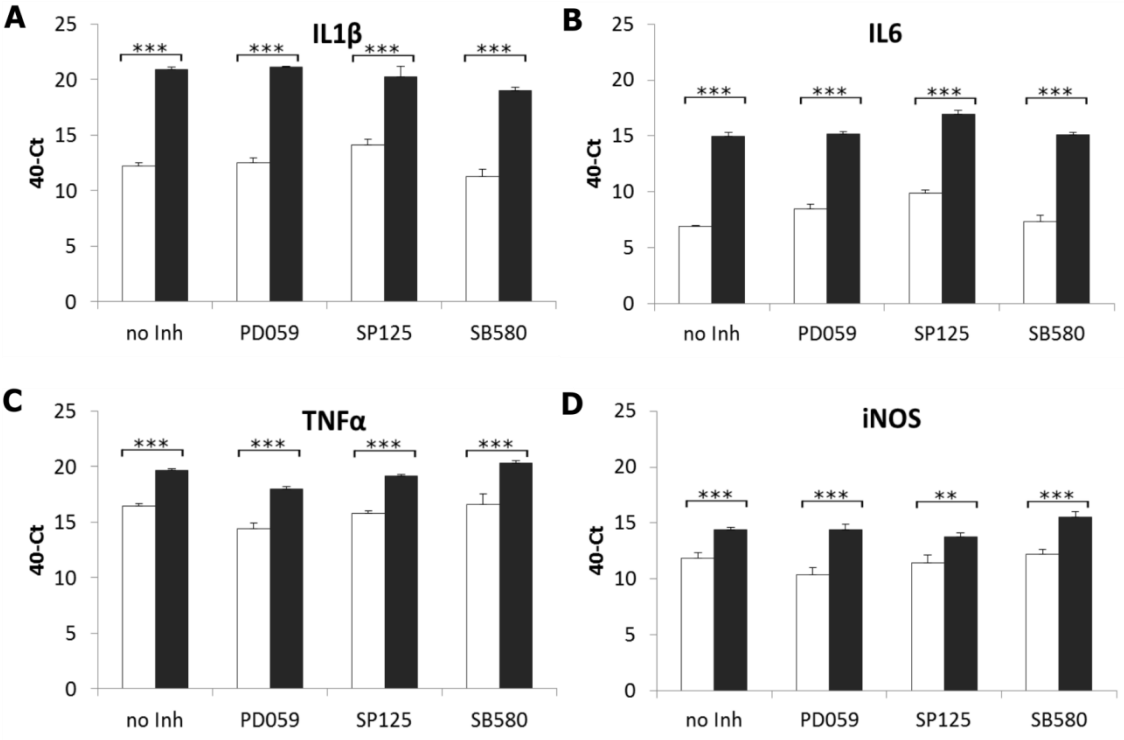
ZHU, W., YANG, W., HE, Z., LIAO, X., WU, J., SUN, J., YANG, Y. and LI, Y., 2011. Overexpressing autoimmune regulator regulates the expression of toll-like receptors by interacting with their promoters in RAW264.7 cells. *Cellular immunology,* **270**(2), pp. 156-163.

Appendix Figures

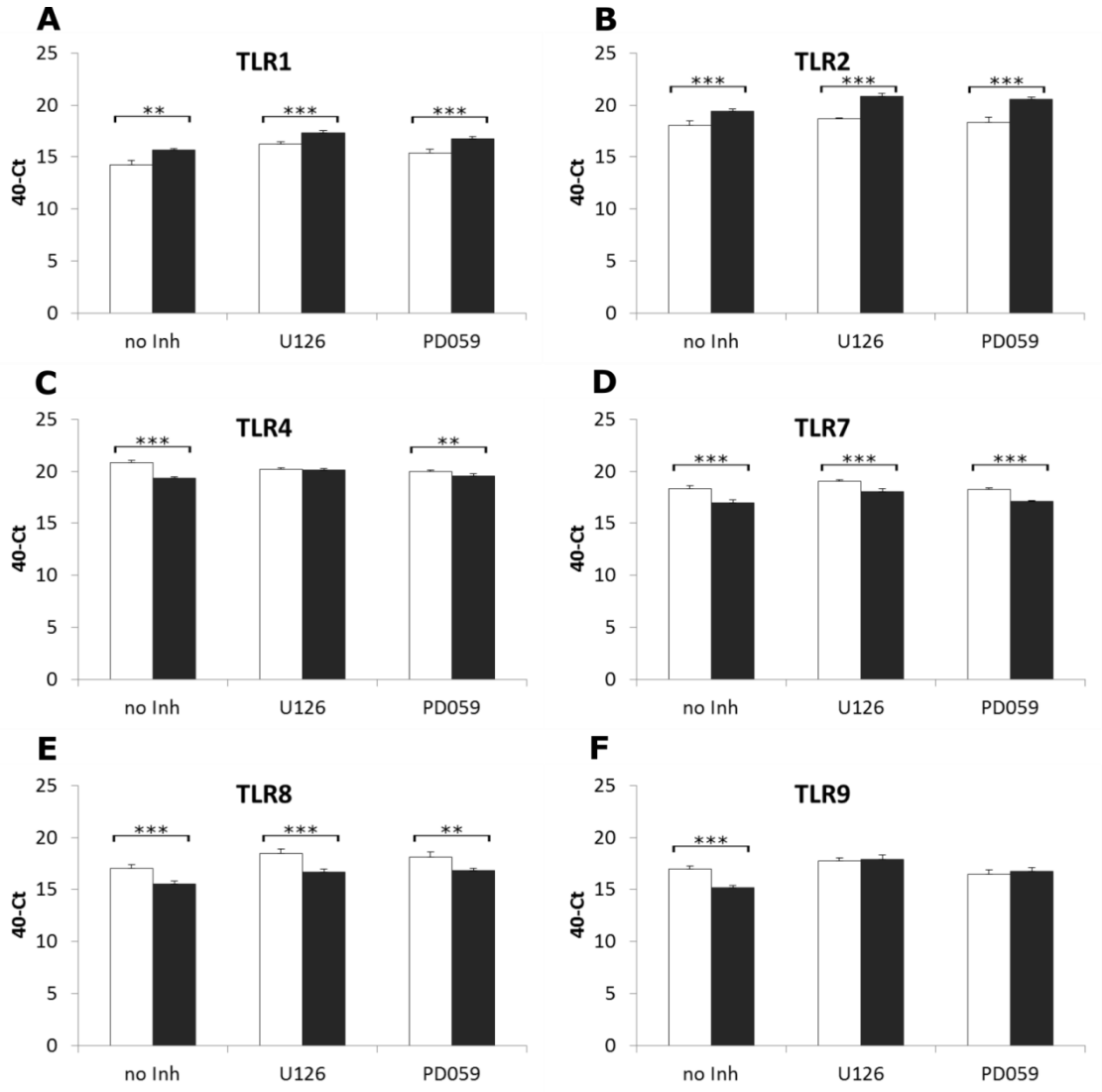
Appendix Figure 1: Standard slopes for optimised TLR qPCR assays. Fold increase per cycle is indicated on each plot, represents the efficiency of replication.



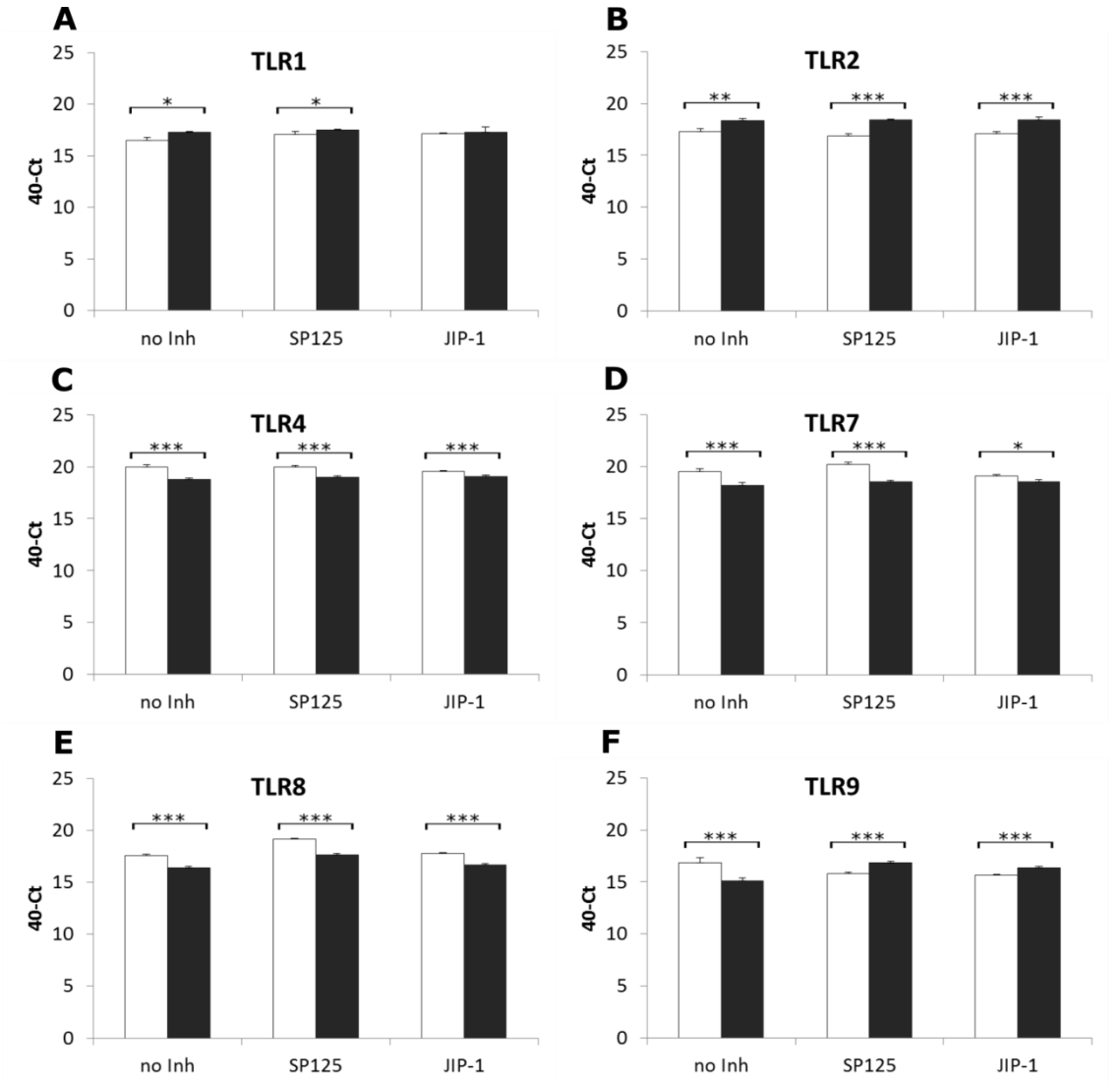
Appendix Figure 2: Effect of MAPK signalling inhibition on PAM-induced pro-inflammatory responses in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent cytokine expression in non-stimulated or inhibitor-treatment only cells; the black bars represent cytokine expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars presented are at 95% confidence intervals and asterisks signify significance levels between adjacent bars: *p< 0.05, **p< 0.01, ***p< 0.001.



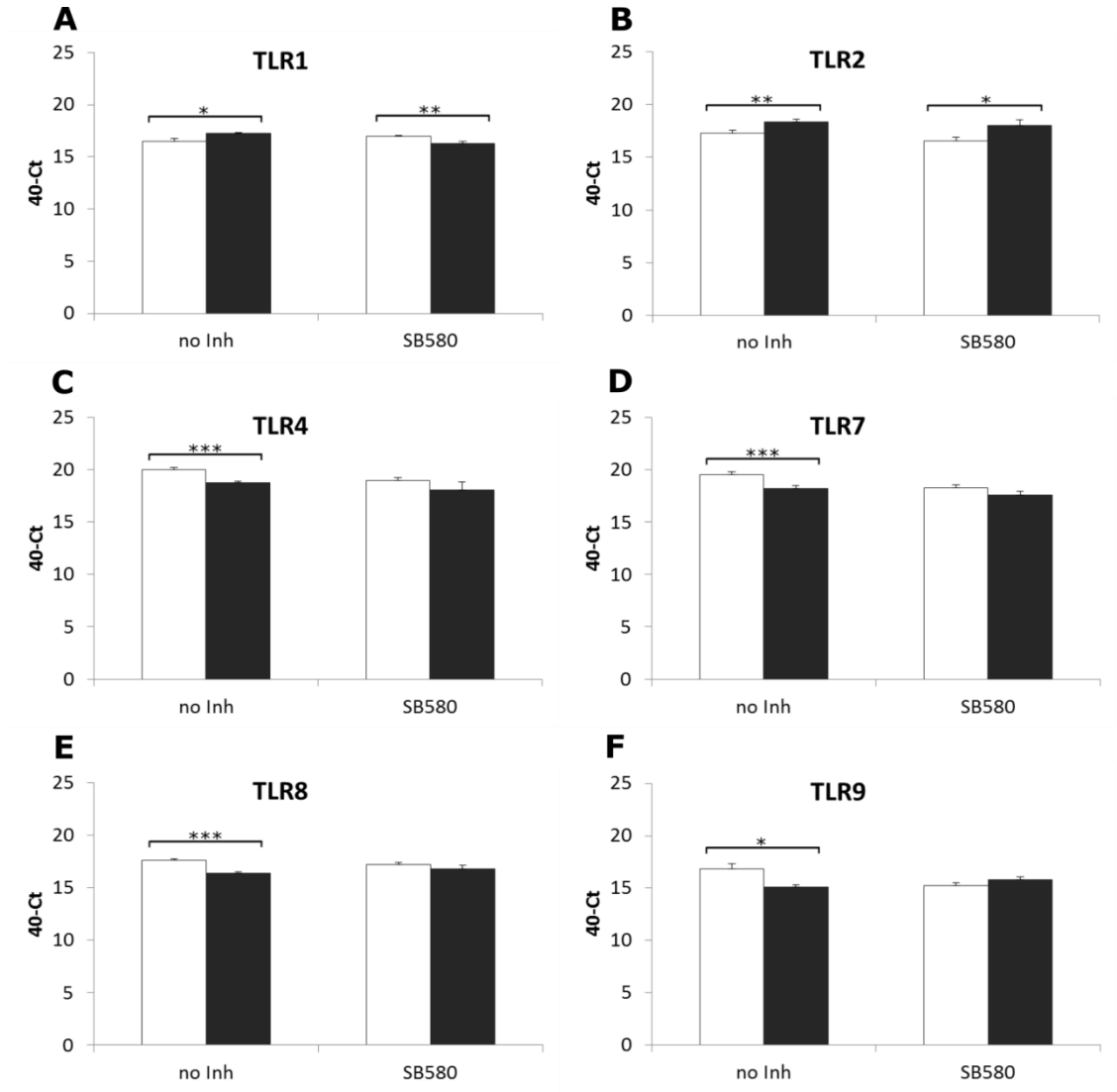
Appendix Figure 3: Effect of ERK/MEK signalling inhibition on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.



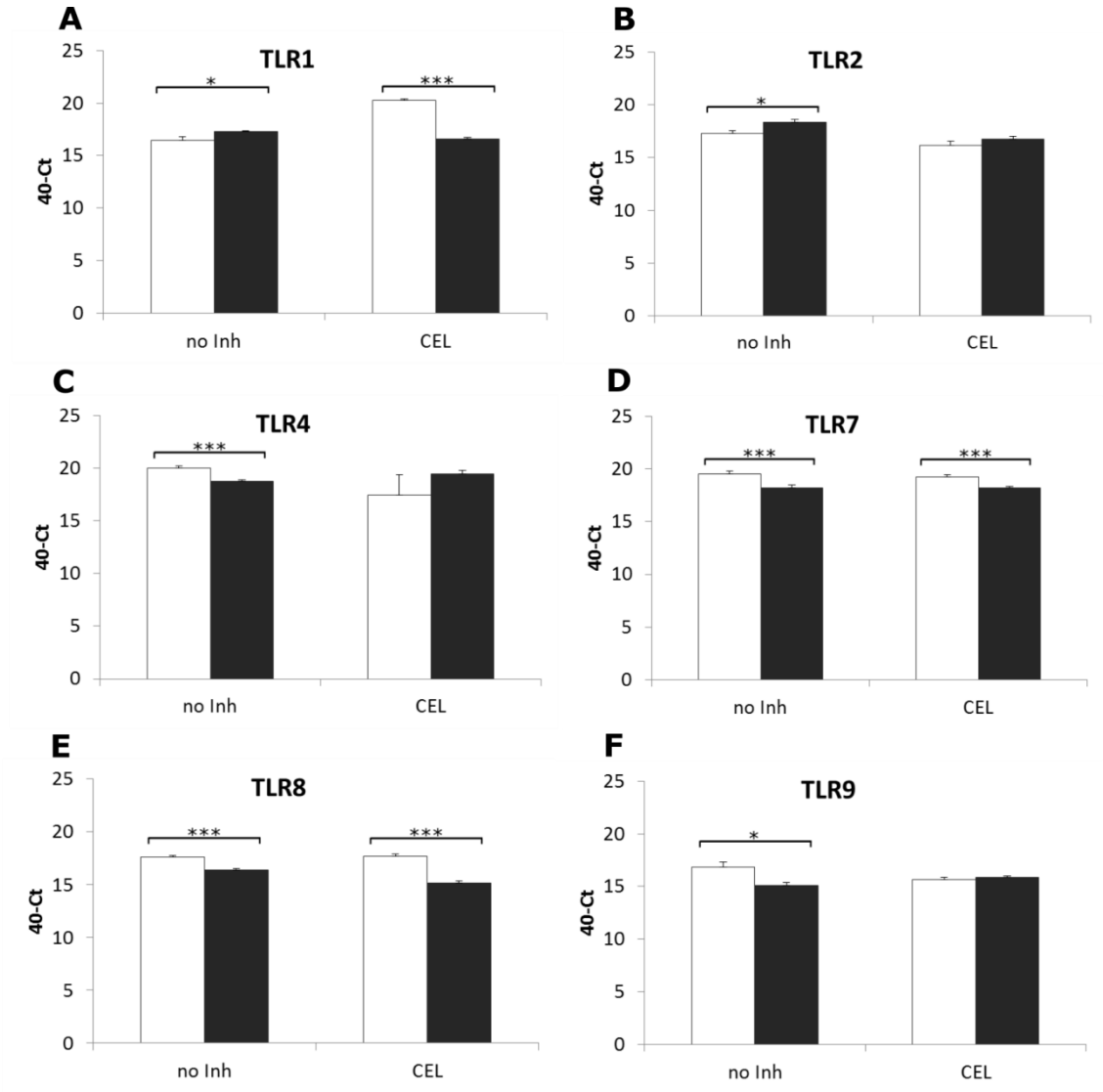
Appendix Figure 4: Effect of JNK signalling inhibition on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.



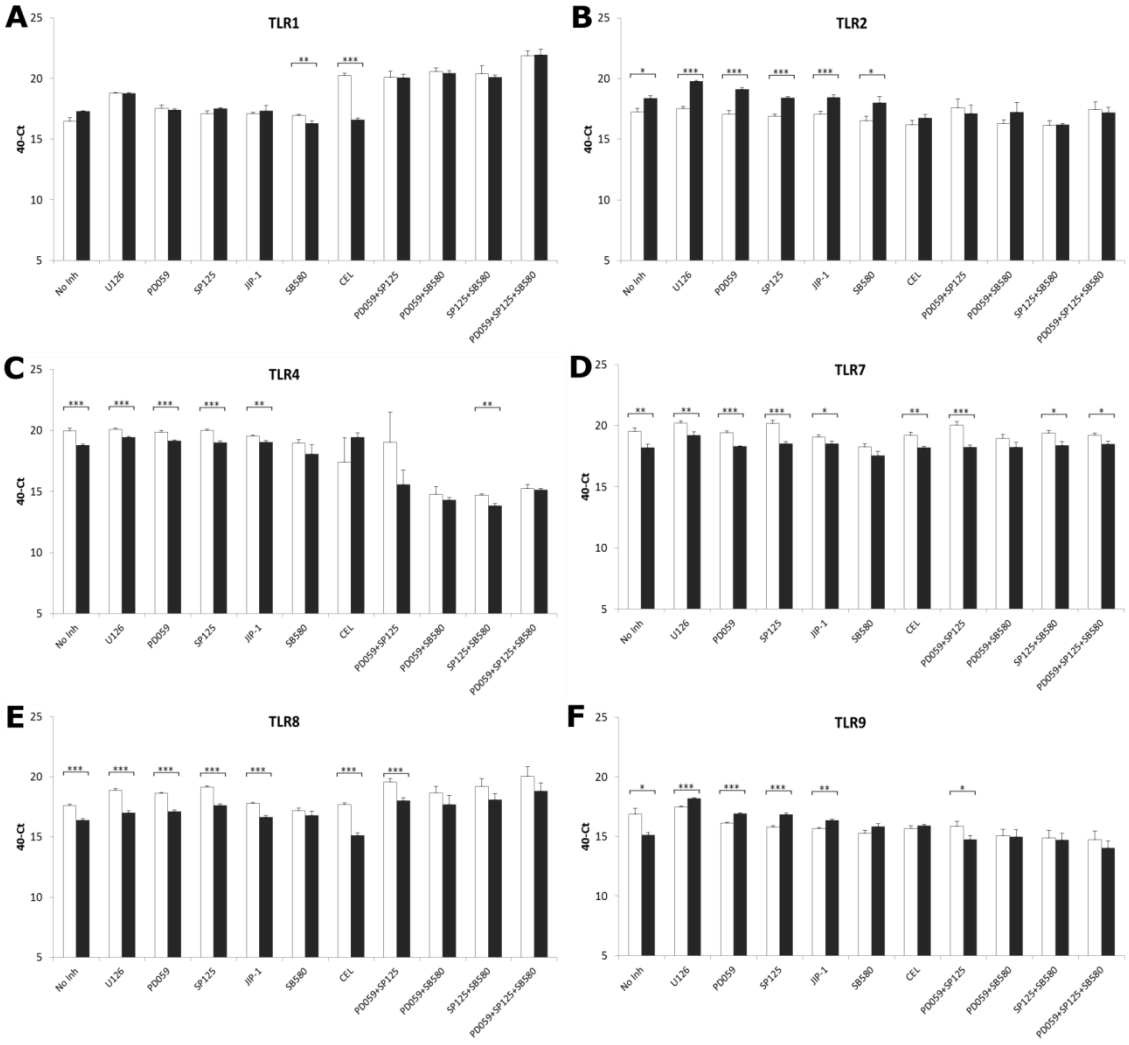
Appendix Figure 5: Effect of P38 signalling inhibition on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.



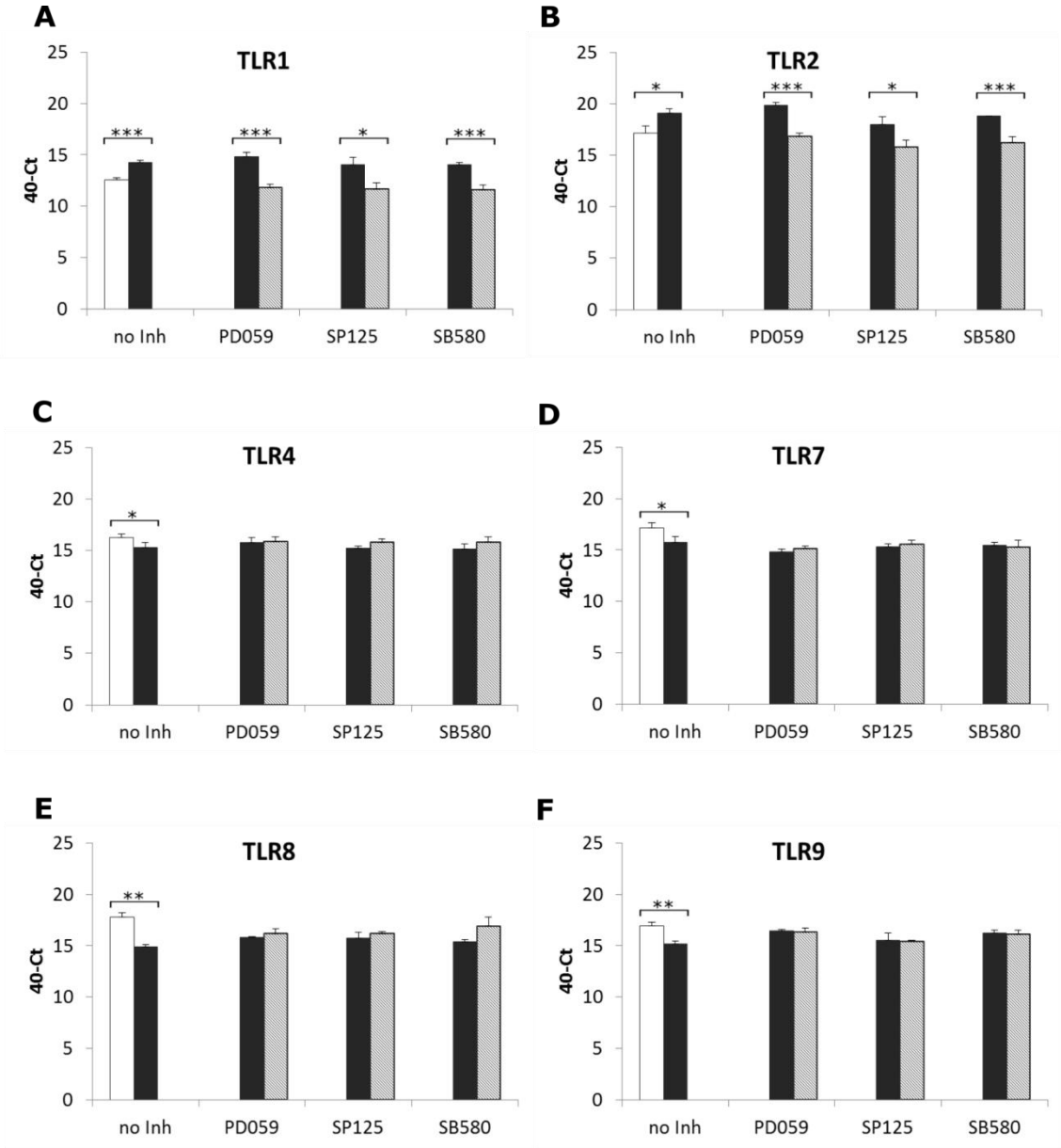
Appendix Figure 6: Effect of NFκB signalling inhibition on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.



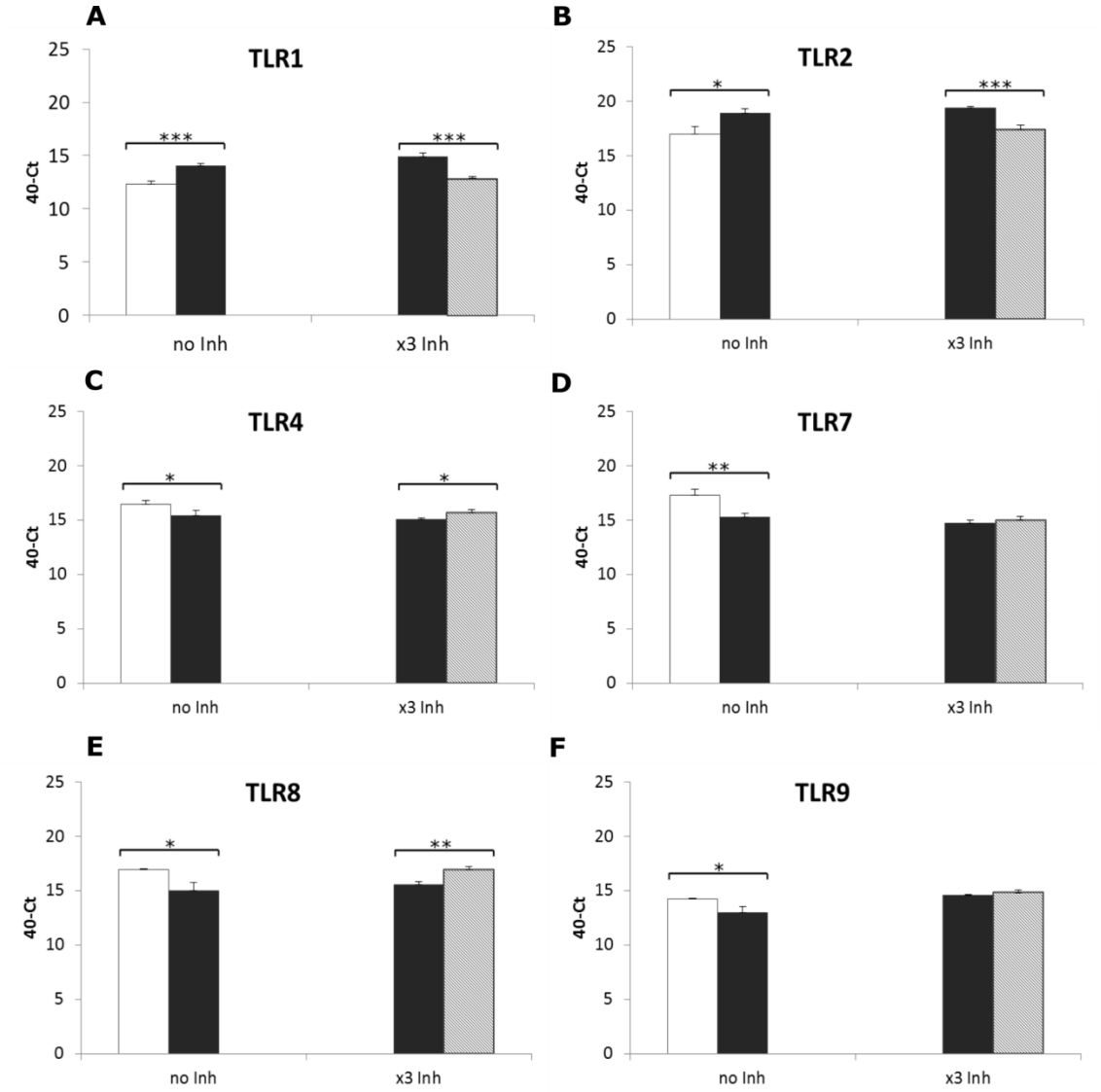
Appendix Figure 7: Effect of inhibiting multiple MAPK signalling cascades on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR expression in non-stimulated or inhibitor-treatment only cells; the black bars represent TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells. Error bars and significance levels denoted by asterisks are the same as previous figures.



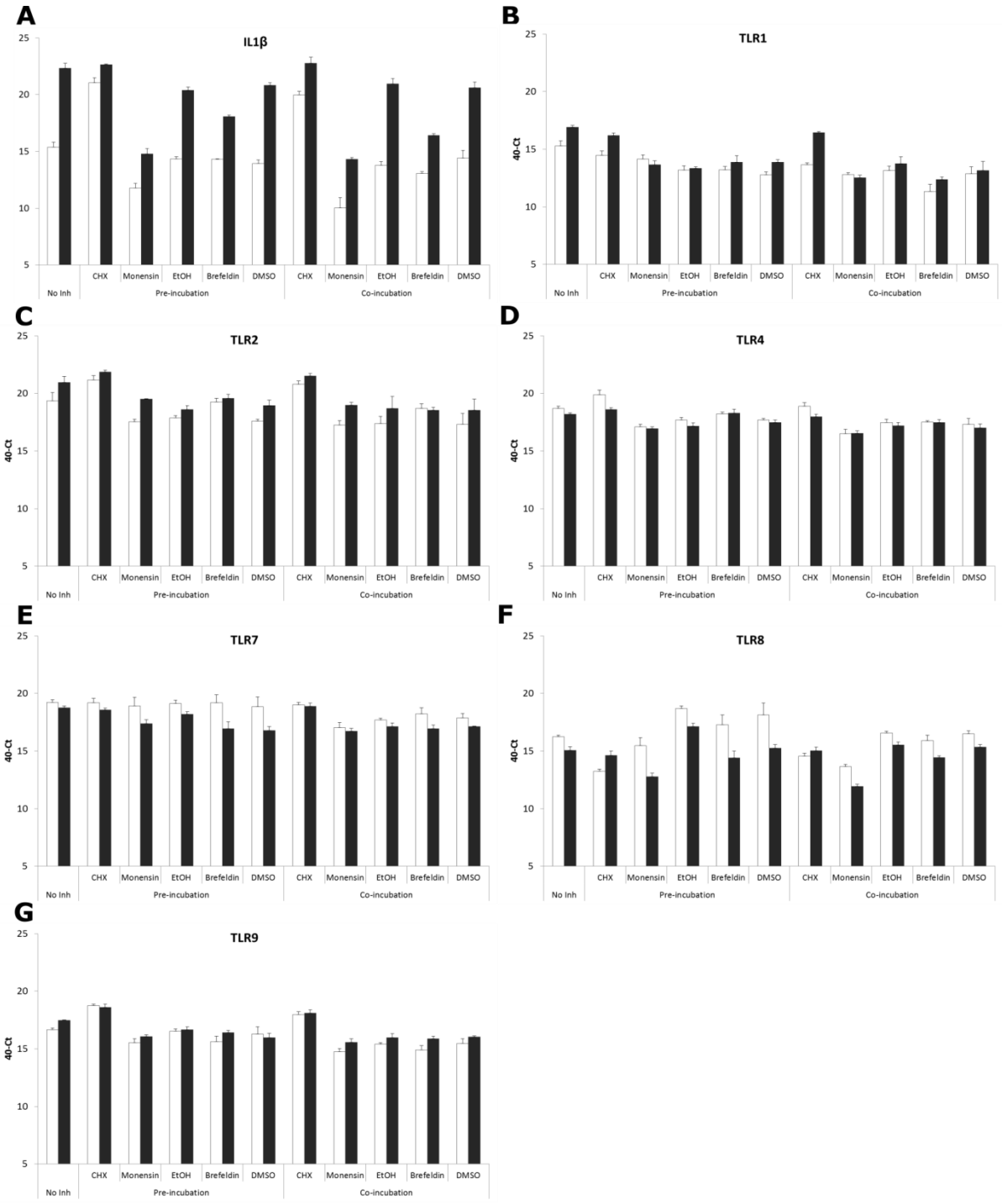
Appendix Figure 8: Effect of inhibiting individual MAPK signalling cascades with/without NFκB pathway on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in PAM-stimulated or PAM and MAPK inhibitor co-incubated cells; the grey bars represent TLR expression in cells treated with both MAPK and NFκB inhibitors in addition to PAM stimulation. Error bars and significance levels denoted by asterisks are the same as previous figures.



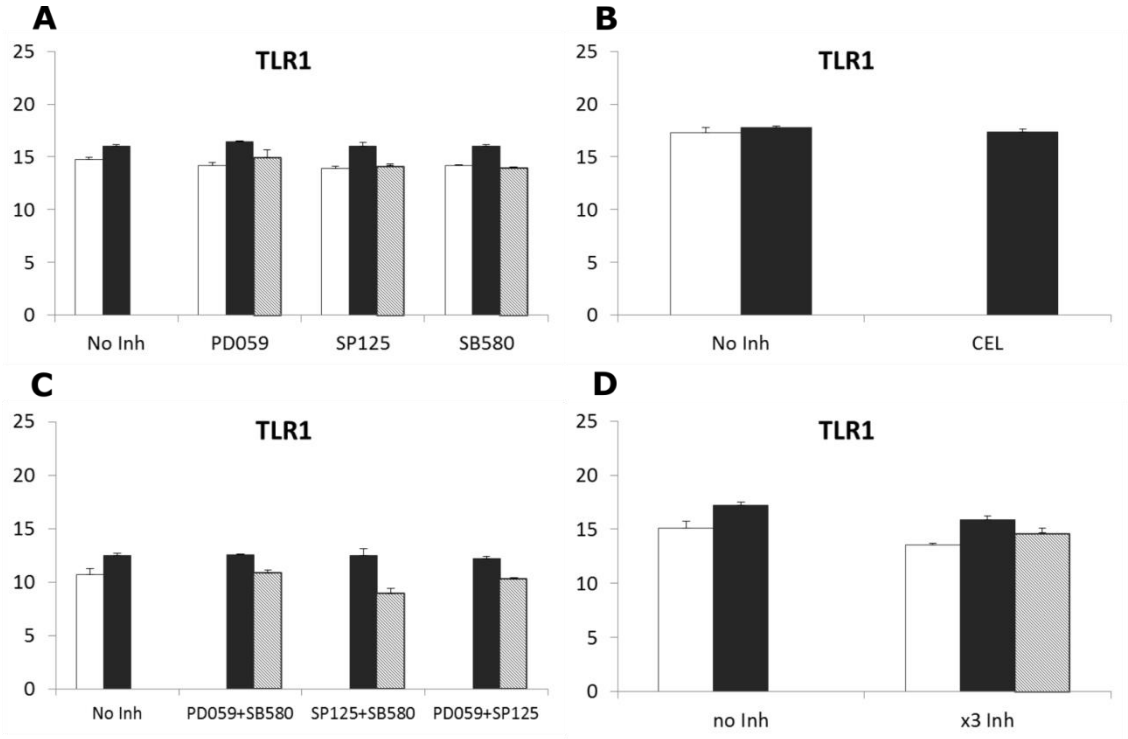
Appendix Figure 9: Effect of inhibiting complete MAPK signalling pathway with/without NFκB pathway on PAM-induced TLR regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR expression in non-stimulated cells; the black bars represent TLR expression in PAM-stimulated or PAM and MAPK inhibitor co-incubated cells; the grey bars represent TLR expression in cells treated with both MAPK and NFκB inhibitors in addition to PAM stimulation. Error bars and significance levels denoted by asterisks are the same as previous figures.



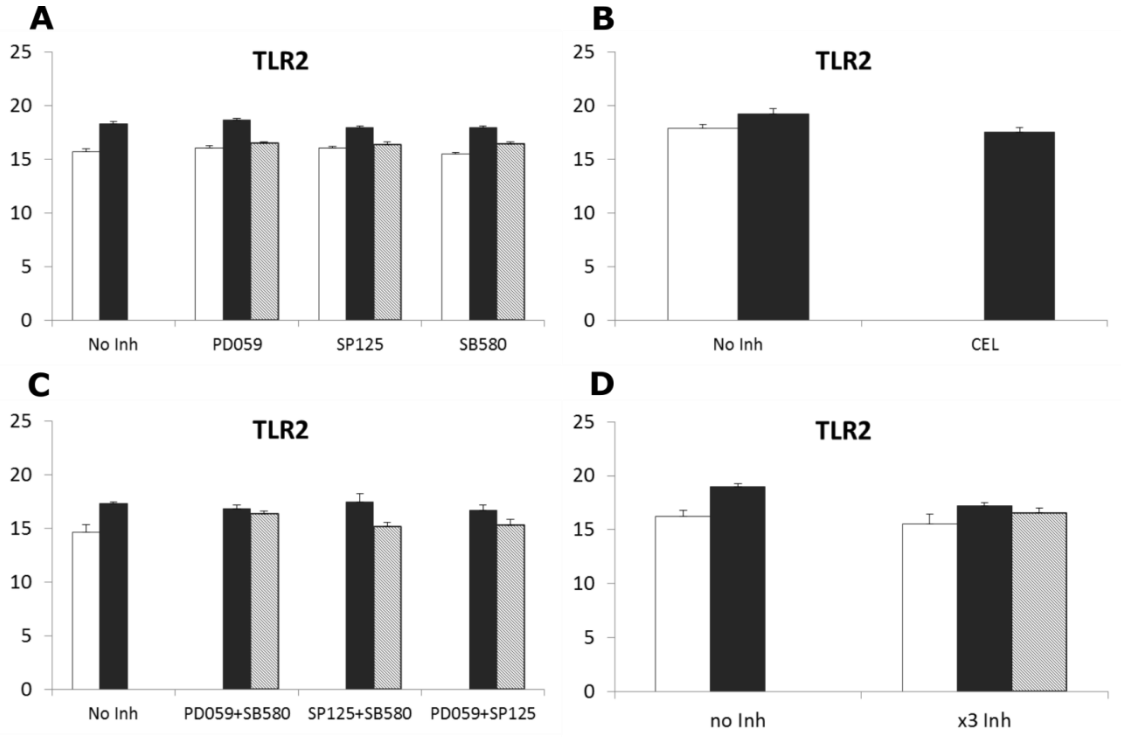
Appendix Figure 10: Effect of inhibiting protein transportation, secretion or translation on PAM-induced IL1 and TLR regulation in RAW cells. The x axis indicates inhibitor or control treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent IL1 or TLR expression in non-stimulated or inhibitor only-stimulated cells; the black bars represent IL1 or TLR expression in PAM-stimulated or PAM and inhibitor co-incubated cells.



Appendix Figure 11: Effect of inhibiting MAPK and NFκB signalling on LPS-induced TLR1 transcriptional regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR1 mRNA expression in non-stimulated cells; the black bars represent TLR1 mRNA expression in LPS-stimulated or LPS and MAPK inhibitor co-incubated cells (B: TLR1 mRNA expression in LPS and NFκB inhibitor co-incubated cells); the grey bars represent TLR1 mRNA expression in cells treated with both MAPK and NFκB inhibitors in addition to LPS stimulation.



Appendix Figure 12: Effect of inhibiting MAPK and NFκB signalling on LPS-induced TLR2 transcriptional regulation in RAW cells. The x axis indicates inhibitor treatments; the y axis indicates mean 40 – Ct values normalised to GAPDH. The clear bars represent TLR2 mRNA expression in non-stimulated cells; the black bars represent TLR2 mRNA expression in LPS-stimulated or LPS and MAPK inhibitor co-incubated cells (B: TLR2 mRNA expression in LPS and NFκB inhibitor co-incubated cells); the grey bars represent TLR2 mRNA expression in cells treated with both MAPK and NFκB inhibitors in addition to LPS stimulation.



Appendix Recipes

Tris-buffered saline (TBS):

50 mM Tris-Cl (pH 7.5)

150 mM NaCl

Tris-NP40 lysis buffer:

25 mM Tris-HCl (pH 7.6)

150 mM NaCl

1% NP40

1% Sodium deoxycholate

0.1% SDS

10% resolving gel:

1.9 ml H_2O

1.7 ml Acrylamide/Bis-acrylamide, 30% solution

1.3 ml 1.5M Tris (pH 8.8)

50 µl 10% Sodium dodecyl sulfate (SDS)

50 µl 10% Ammonium persulfate (APS)

2ul Tetramethylethylenediamine (TEMED)

5% stacking gel:

0.68 ml H_2O

0.17 ml Acrylamide/Bis-acrylamide, 30% solution

0.13 ml 1M Tris (pH 6.8)

10 µl 10% SDS

10 µl 10% APS

1 µl TEMED

Caspase-1 colorimetric assay reaction buffer (pH 7.4):

20 mM HEPES

100 mM NaCl

10 mM DTT

0.1% CHAPS

10% Sucrose

0.5 mM EDTA

Running buffer:

25 mM Tris

250 mM Glycine

0.1% SDS

Make up to 1L with H_2O

Wet transfer buffer:

48 mM Tris

39 mM Glycine

0.037% SDS

20% MeOH

Make up to 1L with H_2O

10x TBE buffer:

1 M Tris base

1 M Boric acid

0.02 M EDTA (disodium salt)

Terrific Broth agar plates:

1.2% Bacto tryptone

2.4% yeast extract

1.6% glycerol

1.5% agar

72 mM K_2HPO_4

17 mM KH_2PO_4