



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

**Polymorphisms in members of the
Toll-like receptor signalling pathway
and their role in infectious disease**

Thesis submitted to the
University of Dublin
for the
Degree of Doctor of Philosophy

By

Caroline Murphy

School of Biochemistry and Immunology

Trinity College Dublin

Ireland

September 2009



thesis 9311

Declaration

This thesis is submitted by the undersigned to the University of Dublin for examination of Doctorate in Philosophy. The work herein is entirely my own and has not been submitted as exercise for a degree to any other university. The library at Trinity College, Dublin, has my full permission to lend or copy this thesis upon request.

11.3.2010

Date

Acknowledgements

First of all, I would like to thank my supervisor Professor Luke O'Neill for all the guidance, help and support over the past four years. I've enjoyed the highs and the lows that goes hand in hand with doing a PhD, I really appreciate your help and advice and have always been stimulated by your passion for immunology and signalling, I hope some of it has rubbed off onto me.

A huge thanks to the greatest bunch of colleagues that could ever fit into a tiny office. Everyone who I have worked with in the LON lab; Anne, Andrea, Cait, Christine, Claire, Dympna, Elaine, Eimear, Emma, Eva, Kathy, Majella, Paulina, Rebecca, Sarah, Seth and Sinead. I have had an absolutely fantastic time working, drinking coffee, gossiping, laughing, singing with you all. To my fellow twilight fanatics; Susan, Fred and Liz, you have been great colleagues and friends to me, I have been so lucky to work alongside you but I probably would have got a lot more done in 2009 if it wasn't for Edward! To Aisling Dunne who has helped me develop my career, without you none of this would have been possible.

To Orla Hanrahan and Barry Moran, I want thank you for all your help with the confocal and FACS.

I want to thank my family at home in Christianstown, to my mother Mary, thank you for being so supportive and putting up with me, thank you to my sisters; Claire, Aisling and Rosemary you are the best in the world and I am so lucky to have you. To Daddy, thank you for all your support over the years. To Glenn (the brother I never had), I'll finally be able to play a proper game of football with you now. To all my uncles; Pauric, Dicky and Tony, you have been a great influence on me over my life and my PhD. Last but not least, George, thank you for your wonderful support, and for all the jokes you told me every morning on the train. You have all been an inspiration to me always encouraging me and keeping me sane though all our ups and downs.

Finally, to Tony I still have not managed to clone you but there is still hope in the future.

TABLE OF CONTENTS

Table of contents	i
List of figures	viii
List of tables	xii
Abbreviations	xiii
Abstract	xix
<i>Chapter 1 INTRODUCTION</i>	1
1.1 The immune system	2
1.2 The innate immune system	2
1.3 Combating infectious diseases-signalling through TLRs	5
1.4 Extracellular TLR signalling (TLR1, 2, 4, 5, and 6)	6
1.5 Intracellular signalling by TLR 3, 7, 8 and 9	10
1.6 Nod-like receptors (NLRs)	11
1.6.1 NOD1 and NOD2	12
1.7 RIG-I like receptors (RLRs)	13
1.8 C-type lectin receptors (CLRs)	14
1.9 Functionally validated single nucleotide polymorphisms (SNPs)	15
1.9.1 SNPs associated with TLR1	16
1.9.2 SNPs associated with TLR2	16
1.9.3 SNPs associated with TLR4	18
1.9.4 SNPs associated with TLR10	20
1.9.5 SNPS associated with TLR5	20
1.9.6 SNPs associated with TLR3	21

1.9.7	SNPs associated with TLR7	21
1.9.8	SNPs associated with TLR8	22
1.9.9	Rare polymorphisms contribute to susceptibility to disease	22
1.10	Intestinal inflammatory diseases associated with NOD1 and NOD2	23
1.11	Structure and characterisation of TIR domain containing proteins	24
1.11.1	Extracellular structural domains of TLRs	24
1.11.2	Intracellular structure of TIR domain containing proteins	25
1.11.3	Structure and characterisation of TLR6	26
1.11.4	TLR2 heterodimerizes with TLR1 and TLR6	27
1.11.5	Characteristics of enzyme cleavage	28
1.11.6	Structure and characterisation of Mal	29
1.12	Infectious diseases	30
1.12.1	Tuberculosis	30
1.12.2	Bacteria in the blood	33
1.12.2.1	Bacteraemia	33
1.12.2.2	Sepsis and septic shock	34
1.12.2.3	Activation of phagocytes causes the initiation of the acute-phase response	35
1.12.3	<i>Streptococcus pneumoniae</i> infections	36
1.12.4	Malaria	36
1.12.4.1	How malaria is transmitted	37
1.12.4.2	Influence of selective pressure on human genome	38
1.12.4.3	The innate immune response to malaria infection	38
1.12.4.4	A fine line between pathology and host protection	39

1.13 Are TLR6 and Mal polymorphisms involved in disease?	40
1.14 Aims and objectives	41
 Chapter 2 MATERIALS AND METHODS	
2.1 Materials	42
2.2 Cell lines	44
2.3 Expression vectors	44
2.4 Reporter vectors	45
2.5 Cell culture	45
2.5.1 Growth of cell lines from frozen stocks	45
2.5.2 Growth and maintenance of cell lines	46
2.5.3 Generation of human peripheral blood mononuclear cells	47
2.5.4 Cryopreservation of cell lines	47
2.6 Plasmid preparation for transient and stable transfections	48
2.6.1 Site directed mutagenesis	48
2.6.2 Plasmid purification for use in transient and stable transfections	49
2.6.3 Mini preparation of plasmid DNA	50
2.6.4 Plasmid purification procedure	51
2.7 Transient transfection	52
2.8 Luciferase gene reporter assay	52
2.8.1 Luciferase gene reporter assay transfection	52
2.8.2 Cellular lysate preparation	53
2.8.3 Measurement of luciferase activity	54

2.9 Reconstitution assay	54
2.10 Western blotting	55
2.10.1 Cell stimulation and extract preparation	55
2.10.2 Cell lysis with sample buffer	55
2.10.3 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis	55
2.10.4 Transfer procedure	56
2.10.5 Blocking the membrane	56
2.10.6 Antibody incubations	57
2.10.7 Stripping and re-probing	57
2.11 Enzyme linked immunosorbent assays (ELISAs)	57
2.11.1 Sample preparation	57
2.11.2 Plate preparation	58
2.11.3 Assay procedure	58
2.12 GST Pulldown	59
2.12.1 Purification of GST-Mal S180 and GST-Mal L180	59
2.12.2 GST protein purification	60
2.12.3 Cell lysate preparation	61
2.12.4 DD loop blocking peptide – <i>in vitro</i>	61
2.12.5 GST pulldown assay FLAG TLR2/GST-Mal interaction	62
2.13 DD loop peptide in cell based assays	63
2.13.1 Peptide blocking – cell based assays	64
2.14 Confocal microscopy	64
2.14.1 Preparation of coverslips	64

2.14.2 Preparation of slides	64
2.15 Membrane fraction assay	65
2.16 SNP genotyping – real time – polymerase chain reaction	66
2.17 Caspase-1 cleavage assay	66
2.18 Generation of stable cell lines	68
2.18.1 Generation of HEK 293T pDUO TLR2/6 and pDUO TLR2/6 Asn30 clonal cell lines	68
2.19 Analysis of stable cell lines using flow cytometry	69
2.19.1 Cell surface analysis of stable cell pools and clones using flow cytometry	69
2.19.2 Intracellular staining of stable cell line clones	70
2.20 PCR of TLR6 from stable cell lines	71
2.20.1 Reverse transcriptase – PCR of cDNA library	72
2.20.2 Generation of human TLR6 using specific primers	72
2.20.3 Insertion of TLR6 and TLR6 Asn30 into pGEM-T easy vector	74
2.21 Co-expression of IRAK4 with Mal L180 and Mal S180	74

Chapter 3 A SINGLE NUCLEOTIDE POLYMORPHISM OF MAL AFFECTS TLR SIGNALLING AND PROVIDES PROTECTION FROM DISEASE

3.1 Introduction	76
3.2 Results	78
3.2.1 Identification of Mal variants reported on dbSNP and their location within functional domains of Mal	74
3.2.2 Genotyping of a healthy Irish population	79

3.2.3 Investigating the structural significance of MalL180 polymorphism	79
3.2.3.1 Secondary structure prediction	79
3.2.3.2 Tertiary structure prediction	80
3.2.4 Mal L180 variant is unable to mediate IκB degradation, P-38 phosphorylation or IL6 production	80
3.2.5 Mal L180 GST does not interact with FLAG TLR2	82
3.2.6 Mal L180 possesses a dominant effect over Mal S180	83
3.2.7 A co-expression assay determined that co-expressing IRAK4 with Mal S180 and Mal L180 causes the degradation of both.	83
3.2.8 Blocking Mal TLR2 interactions using in vitro blocking peptides	84
3.2.9 Blocking IL-6 production using peptides targeted to the region surrounding S180 of Mal	84
3.2.9.1 Addition of S and L form peptides to human peripheral blood mononuclear cells	85
3.2.9.2 Addition of peptides to PolyI:C stimulated U373-CD14 cells	86
3.2.10 Caspase-1 cleavage of Mal is not hindered in the Mal L180 protein	87
3.2.11 Confocal analysis – comparing the localisation of Mal S180 CFP and Mal L180 CFP	88
3.2.11.1 Mal S180-CFP and Mal L180-CFP are localised to the plasma membrane of U373-CD14 cells	88
3.2.11.2 Mal L180-CFP and Mal S180-CFP accumulate in the cytosol upon stimulation with MALP-2 in HEK 293 TLR2 cells	89
3.2.11.3 GFP alone does not accumulate in the cytosol of cells upon stimulation with MALP-2	90
3.3 Discussion	91

Chapter 4 TOLL-LIKE RECEPTOR 6 POLYMORPHISM ASP30ASN, ITS ROLE IN TLR6 SIGNALLING AND ITS IMPACT ON INVASIVE PNEUMOCOCCAL DISEASE

4.1 Introduction	101
4.2 Results	105
4.2.1 Significance of polymorphism Asp30Asn in human TLR6 structure	105
4.2.2 Secondary structure prediction	108
4.2.3 Investigating the cellular location of TLR6 D30 compared to TLR6 N30	108
4.2.3.1 Cellular localisation using membrane fractionation	108
4.2.3.2 Cellular localisation of TLR6 using confocal microscopy	110
4.2.4 Investigating the role of TLR6 N30 in TLR functional assays	111
4.2.5 Development of HEK 293T pDUO TLR2/6 stable cell lines	111
4.2.5.1 Establishing HEK 293T pDUO TLR2/6 D30 and HEK 293T pDUO TLR2/6 N30 stable cell lines	111
4.2.5.2 TLR signalling assays reveal that HEK 293T pDUO TLR2/6 N30 pool of stable cells are activated to a greater extent than TLR2/6 D30 cells in response to TLR2/6 ligand	112
4.2.5.3 Re-sequencing stable cell line pools to ensure sequences are correct	114
4.2.6 Generation of clonal cells	114
4.2.7 Detecting the presence of human TLR6 in the clonal cell population	115
4.3 Discussion	117

Chapter 5 FINAL DISCUSSION AND FUTURE PERSPECTIVES

5.1 Discussion	126
-----------------------	-----

Chapter 6 REFERENCES	133
-----------------------------	-----

Chapter 7 RECORD OF PUBLICATION	145
--	-----

List of figures

Chapter 1

- 1.1 Toll like receptors and their ligands
- 1.2 Downstream signalling of human TLR2 and TLR4
- 1.3 Downstream signalling of intracellular TLRs
- 1.4 NLR mediated signalling cascades
- 1.5 Non-synonymous SNPs in extracellular TLRs
- 1.6 Non-synonymous SNPs associated with intracellular TLRs
- 1.7 Signal peptide cleavage sites of TLRs
- 1.8 Box regions 1, 2 and 3 of the TIR domain
- 1.9 Tuberculous granuloma
- 1.10 The innate immune response to the presence of *P. falciparum*

Chapter 3

- 3.1 SNPs present in functional domains of Mal
- 3.2 Genotyping of a healthy Irish population revealed that the Irish population have the same heterozygosity as the UK population
- 3.3 Secondary structure prediction of Mal
- 3.4 Tertiary structure prediction of Mal
- 3.5 Mal L180 was unable to restore I κ B degradation in Mal deficient MEFs
- 3.6 Mal S180 and Mal L180 were equally expressed
- 3.7 Mal L180 was unable to reconstitute p-38 phosphorylation
- 3.8 Mal L180 could not mediate the production of human IL-6 in Mal-deficient MEFs
- 3.9 Over-expression of HA tagged Mal S180 and HA-Mal L180

- 3.10 Quantification of GST proteins
- 3.11 Mal L180 GST does not interact with FLAF TLR2
- 3.12 Mal L180 possesses a dominant negative effect over Mal S180
- 3.13 Co-expression of IRAK4 with Mal L180 and Mal S180 caused degradation of both proteins
- 3.14 A blocking peptide designed to the Mal S180 region blocks Mal/TLR2 binding
- 3.15 Blocking peptide sequences
- 3.16 DD loop peptide S form does not block IL6 production in MALP-2 stimulated PBMCs
- 3.17 DD loop peptide L form does not block IL-6 production in MALP-2 stimulated PBMDc
- 3.18 DD loop peptide S form does not block IL-6 production in Pam₃CSK₄ stimulated PBMCs
- 3.19 DD loop peptide L form does not block IL-6 production in Pam₃CSK₄ stimulated PBMCs
- 3.20 DD loop peptide S form does not block IL-6 production in Poly:IC stimulated U373s
- 3.21 DD loop peptide L form does block IL-6 production in PolyI:C stimulated U373s
- 3.22 Mal S180 and Mal L180 are cleaved by caspase-1
- 3.23 Mal S180-CFP and Mal L180 CFP in U373-CD14
- 3.24 HEK 293 TLR2 cells transfected with Mal S180-CFP and Mal L180-CFP and stimulated with MALP-2
- 3.25 GFP transfected into HEK 293 TLR2 cells does not accumulated after MALP-2 treatment

Chapter 4

- 4.1 The functional sites of human TLR6.
- 4.2 Signal peptide cleavage site predictions
- 4.3 The N-terminal signal sequence of TLR6
- 4.4 Secondary structure prediction of human TLR6 reveals a change in structure comparing TLR6 D30 and TLR6 N30
- 4.5 Generation of TLR6 N30-FLAG variant
- 4.6 Membrane fractionation studies revealed that TLR6 D30-FLAG and TLR6 N30-FLAG were found in the membrane and not the cytosol. Stimulation with FSL-1 did not alter the location of the proteins.
- 4.7 Signal peptide cleavage prediction of FLAG tagged TLR6 D30 and N30
- 4.8 Membrane fractionation revealed TLR6 N30 was found in both membrane and cytosolic fractions
- 4.9 Confocal microscopy revealed TLR6 D30-YFP and TLR6-YFP are both found in the endomembrane system. TLR6 N30 YFP was found to be more diffuse
- 4.10 TLR6 D30-YFP co-localised with the endoplasmic reticulum.
- 4.11 The TLR6 alone did not activate NF- κ B
- 4.12 TLR6 D30 increased NF- κ B luciferase activity when over-expressed with TLR2-FLAG. TLR6 N30 did not cause an increase in NF- κ B activity
- 4.13 TLR2 was found on the cell surface of stably transfected cells.
- 4.14 NF- κ B luciferase assay revealed HEK 293T pDUO TLR2/6 D30N cells strongly stimulated NF- κ B luciferase.
- 4.15 HEK 293T pDUO TLR2/6 N30 stable cell pool produced more p-P38 than the D30 stable pool.
- 4.16 HEK 293T pDUO TLR2/6 N30 stable cell pool showed more I κ B α degradation compared to the D30 stable pool

- 4.17 pDUO TLR2/6 N30 stables cells produced more hIL8 than pDUO TLR2/6 D30 cells when stimulated with FSL-1
- 4.18 The stable cells lines were re-sequenced to ensure that the sequences were correct.
- 4.19 Sequences of TLR6 D30 and TLR6 N30 from MWG
- 4.20 pDUO TLR2/6 D30 and pDUO TLR2/6 N30 clones were cell surface labelled with anti TLR2 FITC
- 4.21 pDUO TLR2/6 N30 clones showed activation of p38 when stimulated with 5nM MALP-2
- 4.22 HEK 293T pDUO TLR2/6 stables and clones produced high concentrations of hIL8.
- 4.23 HEK 293T TLR2/6 D30 clones do not reveal the presence of TLR6 on the cell surface.
- 4.24 HEK 293T TLR2/6 N30 clones do not reveal the presence of TLR6 on the cell surface.
- 4.25 N terminal antibody of human TLR6 stained cell permeabilized TLR2/6 D30 clones.
- 4.26 N terminal antibody of human TLR6 did not stain cell permeabilized TLR2/6 N30 clones.
- 4.27 The alpha helical region of the signal peptide differs for each form of TLR6
- 4.28 The signal sequence of a protein
- 4.29 Polypeptide synthesis, translocation and membrane insertion.

List of tables

Table 3.1 Summary of action of DD loop blocking peptides on the production of pro-inflammatory cytokine IL-6 in *in vitro*, cell based and primary cell based studies.

Abbreviations

AP-1	Activating protein-1
ATF-2	Activating transcription factor – 2
BIR	Baculovirus-inhibitor of apoptosis repeats
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
DC	Dendritic cell
CARD	Caspase recruitment domain
CD	Crohn's disease
CD	Cluster of differentiation
CFP	Cyan fluorescent protein
CLR	C-type lectin receptors
CpG	Cytosine phosphate guanine
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3- Grabbing Non-integrin
DD	Death domain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
dsRNA	double stranded RNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EV	Empty vector
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GST	Glutathione S transferase
HA	Haemagglutinin
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HRP	Horse radish peroxidase
IAP1	Inhibitor of apoptosis protein-1
IFN	Interferon
Ig	Immunoglobulin
I κ B	Inhibitor of NF- κ B
IKK	I κ B kinase
IL	Interleukin
IPD	Invasive pneumococcal disease
IPTG	Isopropyl- β -D-thiogalactopyranoside
IRAK	Interleukin 1 receptor associated kinase
IRF-3	Interferon response factor-3

ISRE	Interferon stimulated response element
JNK	c-Jun-N-terminal kinase
kDa	kilodalton
LAM	Lipoarabinomannan
LB broth	Luria-Bertani broth
LBP	LPS binding protein
LOAD	Late onset alzheimer's disease
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
MALP-2	Macrophage-activating lipoprotein-2kDa
MDP	Muramyl dipeptide
Mda-5	Melanoma differentiation-associated gene-5
MAPK	Mitogen activated protein kinase
Mal	MyD88 adaptor like
MAVS	Mitochondrial antiviral signalling adaptor
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
MEF	Murine embryonic fibroblast
MKK	MAPK kinase
MyD88	Myeloid differentiation factor 88
NAIP	Neuronal apoptosis inhibitor protein
NCBI	National centre for biotechnology information

NEMO	NF-κB essential modifier
NF-κB	Nuclear factor kappa B
NK	Natural killer
NLR	Nucleotide binding domain leucine rich repeat containing
NO	Nitric oxide
NOD	Nucleotide oligomerisation domain
NOS	Nitric oxide synthase
ODN	Oligodinucleotide
PAGE	Polyacrylamide gel electrophoresis
Pam ₃ CSK ₄	Palmitoyloxy 3 cysteinyl seryl lysine 4
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pDC	plasmacytoid dendritic cell
PGN	Peptidoglycan
PolyI:C	polyinosine-polycytidilic acid
PIP2	phophatydy l inositol 4,5,- bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PMSF	Phenylmethysulphonylfluoride
PRRs	Pattern Recognition Receptors
PS	Polysaccharide

PspA	pneumococcal surface protein A
PVDF	Polyvinylidene difluoride
RHD	Rel homology domain
RIG-I	Retinoic acid-inducible gene-I
RING	Really interesting new gene
RIP	Receptor interacting protein
RLR	RIG-I like receptors
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RSV	Respiratory syncytial virus
SARM	Sterile α - and armadillo-motif containing protein
SDS	Sodium Dodecyl Sulfate
SLE	Systemic Lupus erythematosus
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SRP	Signal peptide recognition protein
ssRNA	single stranded RNA
STAT	Signal transducer and activator of transcription
TA	Transactivation domain
TAB	TAK1 binding protein
TAG	TRAM adaptor with gold domain
TAK	TGF β -activated kinase

TB	Tuberculosis
TBS	Tris buffered saline
TBK-1	Tank binding kinase-1
TEMED	N,N,N',N',-Tetramethylethylenediamine
TH	T helper
TICAM-1	TIR domain containing adaptor-1
TICAM-2	TIR domain containing adaptor-2
TIR	Toll/IL-1 region
TIRAP	TIR domain-containing adaptor protein
TLR	Toll like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
Tollip	Toll/IL-1R interacting protein
TRIF	TIR domain-containing adaptor inducing IFN- β
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adaptor protein
UC	Ulcerative colitis
UTI	Urinary tract infection
WHO	World health organisation
WT	Wild type
YFP	Yellow fluorescence protein

Abstract

Toll-like receptors (TLRs) play an important role in mediating the host's response to invading pathogens. In humans, they comprise of a family of ten pattern recognition receptors (PRRs) located on innate immune cells. They mediate pro-inflammatory signals through their Toll/Interleukin-1/Receptor (TIR) domains by recruiting adaptor molecules through TIR/TIR interactions. Down-stream signals are mediated through a combination of phosphorylation, ubiquitination and degradation events; finally culminating in the transcription of pro-inflammatory genes which initiate the anti-microbial response.

Work presented in this thesis demonstrates that non-synonymous polymorphisms (SNPs) present in TLR6 and the TLR adaptor MyD88 adaptor like (Mal) play a role in the pathogenesis of infectious disease. A serine to leucine SNP at position 180 of Mal provides protection from infectious diseases, malaria, tuberculosis (TB), invasive pneumococcal disease (IPD) and bacteraemia. Individuals who are heterozygous for Mal (S180/L180) have half the risk of suffering from malaria, TB, bacteraemia and IPD with a fourfold decreased risk of severe malaria. A non-synonymous SNP found in TLR6 at position 30 changes aspartic acid to an asparagine, and causes a seven fold increase in susceptibility to IPD and septicaemia. The microbes which mediate these diseases are known to be recognised by extracellular TLRs such as TLR4, TLR2 and TLR6, providing a link between the presence of the invading pathogen and the TLR signalling system.

Utilising this information two questions arose, firstly, "could this genetic information be used to understand more about the biochemistry of Mal and TLR6?" and secondly,

“could a mechanistic explanation be provided for the genetic association of Mal and TLR6 with disease?”

Investigative studies into the functional relevance of these polymorphisms revealed that the Mal L180 displayed decreased signalling when challenged with the TLR2 ligand MALP-2 and showed decreased binding capacity to TLR2 compared to the Mal S180. Peptides based on the S180 region of Mal were used to test the binding capacity of Mal to bind TLR2. The peptides containing S180 disrupted the interaction whereas a scrambled version of the peptide had no effect. Each peptide was tethered to an internalisation sequence and was subsequently tested on its ability to influence TLR-2 induced cytokine production in PBMCs. Inconclusive results were obtained however, due to non-specific effects. Confocal microscopy analysis revealed translocation of both S180 and L180 Mal into the cytosol, possibly due to the ability of both forms to bind MyD88. Defective trafficking does not therefore explain the impairment in function of Mal L180. Investigative studies involving the TLR6 N30 variant found that secondary structure modelling studies revealed a difference in the signal peptide region of the variant compared to TLR6 D30. It was also revealed that a dramatic increase in signalling and cytokine production occurred when the TLR6 N30 variant was stimulated with TLR2/6 ligand compared to TLR6 D30 form in stably transfected cells. This exciting finding suggests that increased signalling by the TLR6 variant may cause increased susceptibility to IPD and septicaemia.

This novel study reveals an important link between variants of TLR6 and Mal with infectious diseases. Utilising information obtained from genetic association studies and linking it with biologically significant inflammatory signalling effects, has provided a possible mechanistic explanation for the development of common

infectious diseases. This thesis contributes to the validation that that TLR signalling pathways are important in the pathogenesis of disease.

Chapter One

Introduction

Chapter 1 Introduction

Human evolution has been greatly impacted upon by infectious diseases, environment, changes in human lifestyles and population densities. Mortality caused by infectious diseases affects reproductive success and so has influenced the human genotype over millions of years. The human genome has been affected by both pre-historic and modern diseases that are now influencing the human population such as malaria, HIV and tuberculosis (Cox, 2002). Consequently variants of genes that are beneficial to the host have been sustained and variants influencing susceptibility deleted out.

The identification of polymorphisms within the innate immune system links our health and our innate immune system with our evolutionary heritage.

Much work has been carried out by immunogeneticists to investigate the role that genetic variants have played in mediating infectious diseases. Assessing the incidence of genetic variants within disease populations only goes so far to address all the questions that remain unanswered in this area. This project utilises a different path to further elucidate the role of genetic variants in infectious disease; by investigating the biochemistry associated with protein variants in the innate immune system.

At the outset of this project, Professor Adrian Hill and colleagues from the Centre for Human Genetics Oxford described two non-synonymous polymorphisms (SNPs) found in genes of the human innate immune system. Both have been found to impact the disease outcome of patients who contract infectious diseases. The first is a polymorphism in the Toll-like receptor (TLR) adaptor MyD88 adaptor like (Mal) and

the second is described in TLR6. The Mal SNP has been found to provide protection from infectious diseases such as malaria, tuberculosis (TB) and invasive pneumococcal disease (IPD). The TLR6 SNP has been found to have the opposite effect in that it causes susceptibility to IPD. This project concerns an effort to identify a functional mechanism within the innate immune system for the protection afforded by the Mal variant and the susceptibility caused by the TLR6 variant to infectious disease.

1.1 The immune system

The mammalian body is susceptible to infection by many forms of pathogens such as, bacteria, viruses and parasites. Throughout evolution the mammalian immune system has developed two arms of defence; the innate and the adaptive immune system. The innate system represents the early host response system which recognises and responds to the presence of invading pathogens. The adaptive immune response involves the response of antigen specific lymphocytes which have been generated by clonal selection to specific antigen. The adaptive immune response also involves the generation of immunological memory allowing enhanced immune response to a previously encountered antigen.

1.2 The innate immune system

The epithelial surface of the vertebrate system keeps pathogens out. It acts as a barrier, providing protection from the colonization of bacteria and viruses by secreting antimicrobial enzymes and peptides. Pathogens that overcome the epithelial surface are faced with the innate immune system (Gibbons, 1992, Krisanaprakornkit et al., 2000). The innate immune system consists of immune effector cells such as macrophages,

neutrophils, dendritic cells (DCs), natural killer (NK) cells and much more. The innate immune cells first detect the presence of microorganisms in the tissue and secondly the pathogen is ingested and destroyed (Aderem and Underhill, 1999). The immune cells detect pathogen-associated molecular patterns (PAMPs), these patterns are evolutionarily conserved molecular patterns present on the surface of the invading microbes which are essential for their survival, and so have not been changed over millions of years (Aderem and Underhill, 1999). The macrophage recognises the pathogen by specific germline-encoded receptors, known as pattern recognition receptors (PRRs). These receptors can discriminate between pathogen and host molecules, enabling the macrophage to engulf the pathogen and destroy it by means of the phagolysosome. The activation of the macrophage leads to the up-regulation of the inflammatory response and the production of pro-inflammatory molecules such as interleukin-1 (IL-1), IL-6 and TNF- α (Svanborg et al., 1999). These molecules cause what is known as the ‘acute phase response’, increasing body temperature through activation of the hypothalamus, causing mobilization of neutrophils to the area of infection and causing increased phagocytosis. TNF- α stimulates the migration of DCs to lymph nodes to allow maturation to occur and to activate the adaptive immune system. The ‘acute phase response’ also activates the ‘complement system’ which activates plasma cells and antibody production (Lowenstein and Matsushita, 2004, Frank and Fries, 1991).

The release of cytokines by activated macrophages also recruits DCs and NK cells to the area of infection. Invasion by intracellular bacteria such as *Listeria monocytogenes* and viruses activates the DCs and NK cells to produce IL-12 and IFN- γ (IFN- γ) respectively. A combination of these cytokines and presentation of bacterial antigens by major histocompatibility class (MHC) II activate naive CD4 T cells which become

committed to becoming activated TH1 cells. Activated TH1 cells mount a T cell mediated immune response. By producing IL-12 they activate cytotoxic CD 8 T cells which block viral replication within cells and target infected cells for destruction. By producing IFN- γ they positively feed back to up-regulate macrophages to produce reactive oxygen species and reactive nitrogen species thus destroying intracellular pathogens. TH1 cells also produce tumor necrosis factor- β (TNF- β) which activates macrophages, inhibits B cells and is cytotoxic to certain cells.

Extracellular antigens such as those from *Staphylococcus aureus* or *Streptococcus pneumonia* tend to stimulate a TH2 response. In the presence of these extracellular pathogens DCs and NK cells produce IL-4 and endothelial cells produce IL-6. These cytokines activate naive CD4 T cells which are then committed to becoming TH2 cells. TH2 cells produce cytokines such as IL-1, IL-5, IL-13 and IL-10. IL-4 and IL-5 activate B cells to produce antibodies which can opsonise and neutralize invading pathogens.

The generation of the innate immune response initiated by PRRs on the surface of immune effector cells is the focus of this project. This project endeavours to generate a greater understanding of how evolution has permitted variations to occur in components of the immune system and to further understand the biochemical implications that these variations have on signalling pathways within the mammalian body.

1.3 Combating infectious diseases – signalling through TLRs

Innate immune effector cells possess PRRs to recognise the presence of invading microorganisms. An important class of PRRs are the Toll-like receptors (TLRs) (Rock et al., 1998). TLRs are class 1 transmembrane receptors which contain an N-terminal leucine-rich repeat (LRR) domain, a transmembrane domain and a C-terminal cytoplasmic domain (West et al., 2006). The cytoplasmic domain shares a high degree of homology with the type-1 IL-1 receptor and hence is termed the Toll/IL-1 receptor (TIR) signalling domain (O'Neill, 2000). At present 10 members of the TLR family have been found in humans. Genetic studies have been employed to reveal the various ligands that the TLRs recognise. Figure 1.1 displays the different ligands that are recognised by the TLRs. Lipopolysaccharide (LPS) from gram negative bacteria is recognised by TLR4 (Poltorak et al., 1998, Hoshino et al., 1999). TLR2 recognises the presence of lipopeptides and lipoprotein from gram positive bacteria and lipopeptide from mycoplasma. When operating in combination with TLR1 or TLR6, TLR2 can expand its repertoire of antigens that it recognises. A heterodimer of TLR1 and TLR2 recognises triacylated lipopeptides and a heterodimer of TLR6 and TLR2 recognises diacylated lipopeptides (Ozinsky et al., 2000). Ligands for other TLRs include; double stranded RNA (dsRNA) which is produced by replicating viruses and is recognised by TLR3 (Alexopoulou et al., 2001); flagellin is a component of flagella from flagellated bacteria and is recognised by extracellular TLR5 (Hayashi et al., 2001). TLR7 recognises single stranded RNA (ssRNA) from viruses and also recognises the synthetic compound imiquimod, TLR8 recognises ssRNA and the agonist 3M002 (Hemmi et al., 2000, Heil et al., 2004, Hemmi et al., 2002, Larange et al., 2009). TLR9 recognises unmethylated CpG DNA motifs and malaria associated haemozoin (Hemmi et al., 2000, Lund et al., 2003, Coban et al., 2005). The ligand for TLR10 is currently unknown.

1.4 Extracellular TLR signalling by TLR 1, 2, 4, 5 and 6

Ligand induced heterodimerization of TLR2 with TLR1 or TLR6 up-regulates downstream signalling, likewise LPS homodimerization of TLR4 up-regulates downstream signalling (Nunez Miguel et al., 2007). The signalling cascades created by TLR2 and TLR4 are shown in figure 1.2. The gram negative cell wall component LPS binds to plasma protein LPS-binding protein (LBP). Subsequently the receptor protein CD14 binds to the LPS bound LBP complex. Both CD14 and LBP have leucine rich repeat domains which form a complex with TLR4 and MD2. Park and colleagues showed that in the absence of LPS, TLR4 and MD2 can associate. However, dimerization of the TLR2/MD2 complex with a second TLR4/MD2 complex only occurs following LPS binding (Park et al., 2009). MD2 binds to the concave surface of the leucine rich repeats of TLR4. LPS consists of six lipid chains, five of the lipid chains become buried within a hydrophobic pocket of MD2 while the remaining lipid chain forms a hydrophobic chain with phenylalanine residues of TLR4. Upon binding of LPS the MD2/TLR4 interaction becomes even more stabilized allowing subsequent dimerization of TLR4/MD2 complexes and downstream signalling (Carpenter and O'Neill, 2009). Dimerization of TLR4 causes a concerted conformational change providing a scaffold that is able to bind downstream signalling adaptor proteins such as myeloid differentiating factor 88 (MyD88), MyD88 adaptor like (Mal) (also known as TIRAP) (Fitzgerald et al., 2001), TIR-related adaptor protein inducing interferon (TRIF) and Trif-related adaptor molecule (TRAM) a fifth adaptor known as sterile α - and armadillo-motif containing protein (SARM) has been found to be a negative regulator of TLR signalling by associating with TRIF and blocking downstream NF- κ B signalling (Carty et al., 2006). Downstream signalling from TLR4 is mediated thorough the MyD88 dependant and independent pathways. The adaptor protein Mal interacts with

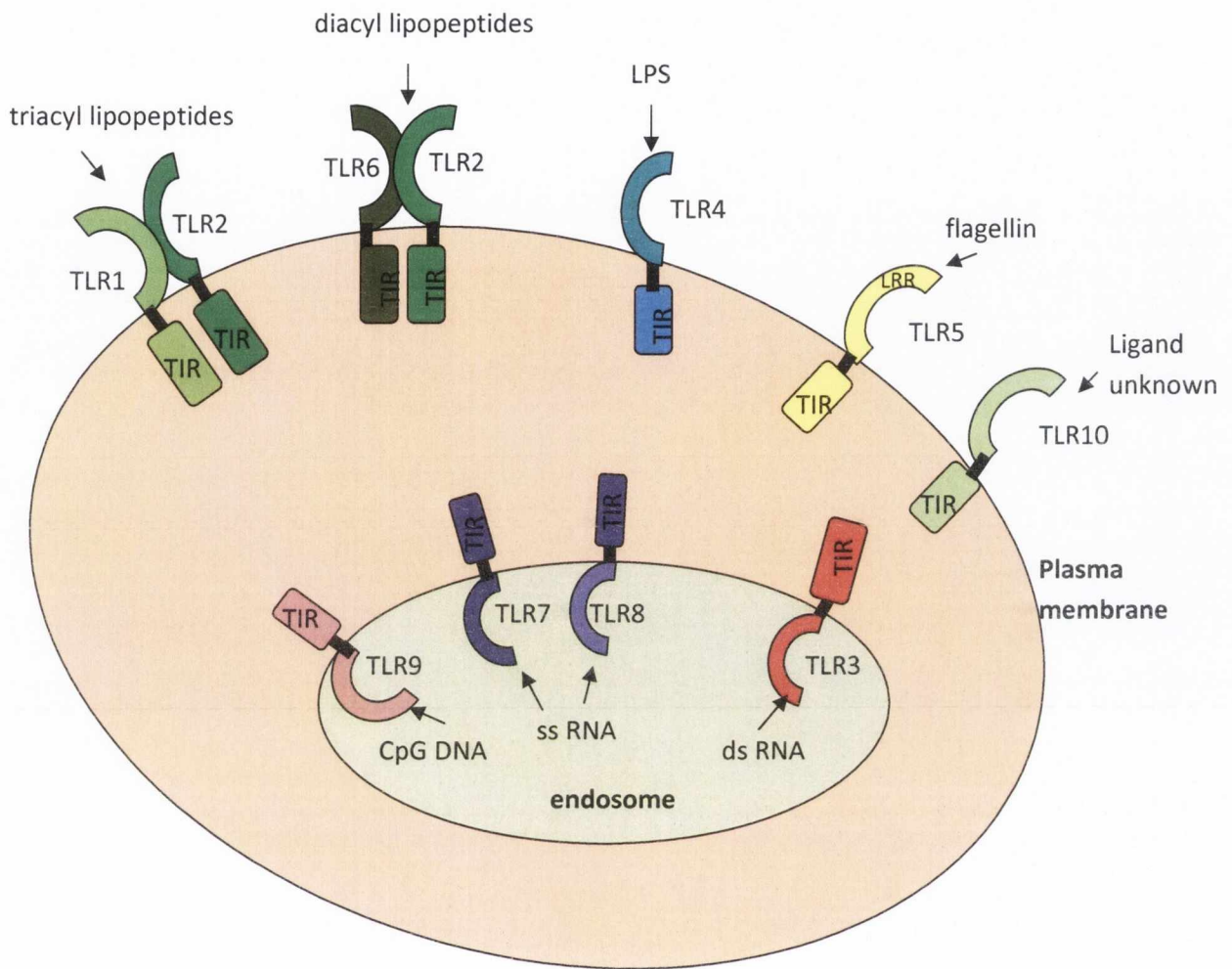


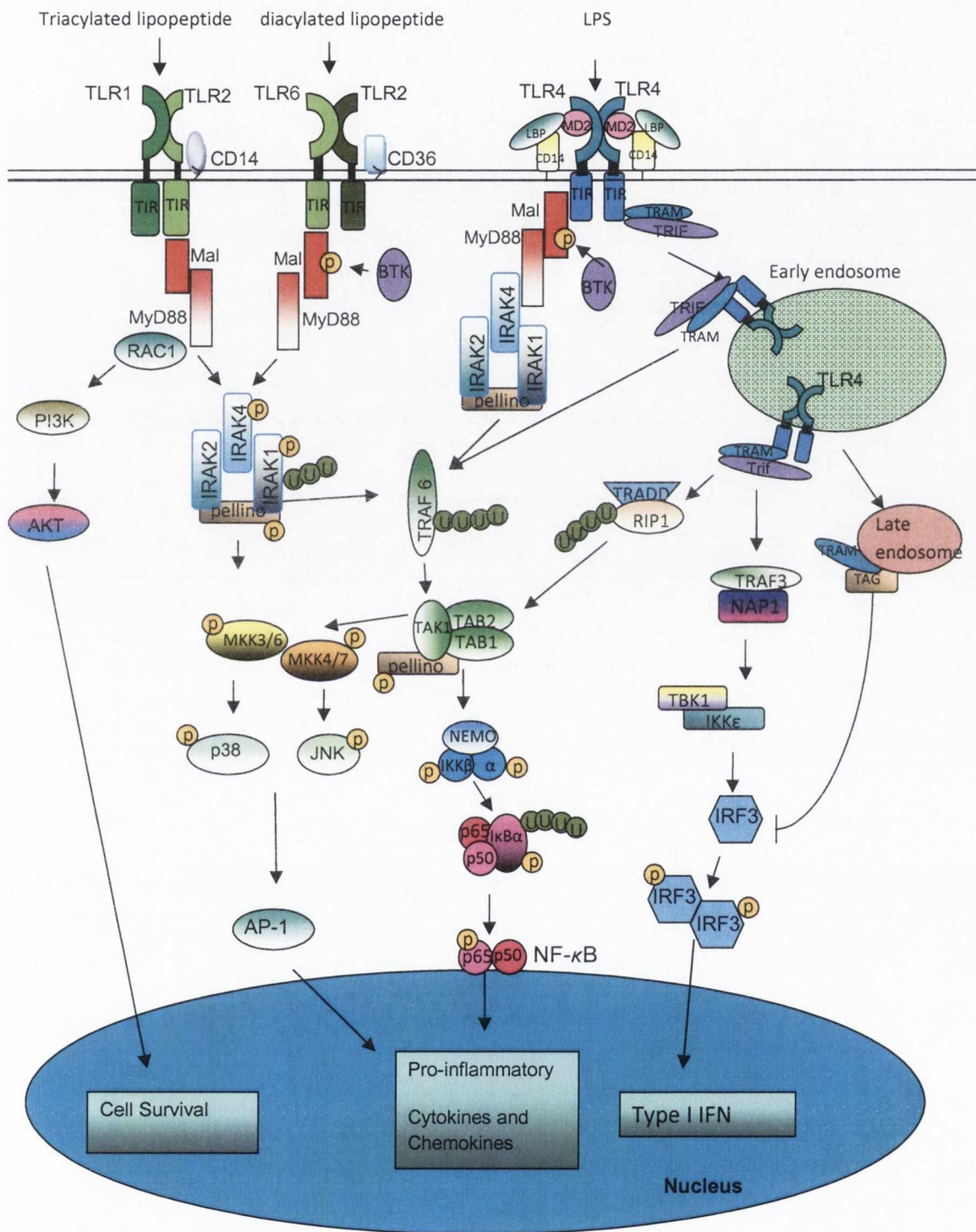
Figure 1.1 Toll like receptors and their ligands

TLRs 1, 2, 4, 5, 6 and 10 are extracellularly located. TLRs 3, 7, 8 and 9 are located on intracellular endosomal membranes. TLR2 recognises lipoproteins and lipopeptides and in conjunction with TLR1 recognises triacyl-lipopeptides and in conjunction with TLR6 recognises diacyl-lipopeptides. TLR4 recognises LPS from gram negative bacteria. TLR5 recognises flagellin from flagellated bacteria. The ligand for TLR10 is as yet unknown. The intracellular TLRs recognise viral and bacterial nucleic acids, TLR3 is required for the recognition of double stranded RNA, TLR7 and 8 respond to single stranded RNA while TLR9 is essential for the recognition of unmethylated CpG DNA.

Figure 1.2 Downstream signalling of human TLR2 and TLR4

Extracellular TLRs interact with adaptor molecules MyD88, Mal, TRIF and TRAM via TIR/TIR interactions to initiate downstream signalling cascades which culminate in the up-regulation of pro-inflammatory transcription factors and anti-viral type I interferon. TLR2 in combination with either TLR1 or TLR6 recognises peptidoglycan, lipopeptide and lipoprotein from gram positive bacteria, lipoarabinomannan from mycobacteria, haemagglutinin protein from measles virus and tGPI-mucin from *Tyrpanasoma*. TLR4 recognises LPS from gram negative bacteria. TLR4 and TLR2 signal downstream via the MyD88 dependent pathway which results in early NF- κ B expression. TLR4 and TLR2 recruit the bridging adaptor Mal which recruits MyD88 via interaction of their TIR domains. MyD88 in turn interacts with IRAKs via their common death domains and up-regulates a series of protein kinases, culminating in the release of NF- κ B into the nucleus up-regulating the transcription of pro-inflammatory cytokines TNF α , IL-6 and IL-12. Following stimulation, TLR2 and TLR4 also signal to the MAPK pathway resulting in the phosphorylation and activation of transcription factor AP-1 which like NF- κ B results in the production of inflammatory cytokines.

TLR4 also signals through the MyD88 independent pathway resulting in late NF- κ B expression. TLR4 sequesters the bridging adaptor TRAM to recruit TRIF via their TIR domains. The TLR4/TRAM/TRIF complex internalises after stimulation with LPS where it recruits TRAF3 which interacts with TBK1 and IKK ϵ mediating phosphorylation of IRF3 and IRF7 leading to the induction of type I IFN (IFN β and IFN α).



TLR4 and recruits MyD88 through its TIR domain while TRAM binds TLR4 recruiting TRIF, forming a TLR4/TRAM/TRIF complex which is then recruited into early endosomes (Kagan et al., 2008).

Signalling through TLR2 occurs in a MyD88 dependent fashion while TLR4 signals in both a MyD88 dependent and independent fashion. Docking studies carried out by Dunne and colleagues suggest that Mal and MyD88 bind to different regions of TLR2 and TLR4 (Dunne et al., 2003). After TLR/adaptor interactions occur, a signal transduction pathway is activated (figure 1.2). Mal is recruited to the TIR domain of TLR2 and TLR4, Mal then recruits MyD88 where they too interact via their TIR domains. MyD88 subsequently mediates signalling to members of the interleukin-1 receptor associated kinase (IRAK) family (Akira et al., 2003). IRAK family members are composed of an N-terminal death domain (DD) and a serine-threonine kinase domain. Currently four IRAKs have been identified IRAK1, IRAK2, IRAK4 and IRAK-M. IRAK 4 interacts with MyD88 via its DD. IRAK4 interacts with IRAK1 and IRAK1 becomes auto-phosphorylated after 30 minutes MALP-2 stimulation (TLR2/6 ligand) (Kawagoe et al., 2008). IRAK4 associates with IRAK2 at a later time point (4-8 hours) (Kawagoe et al., 2008). The newly established members of the TLR signalling pathway known as Pellinos possess E3 ligase activity and have been implicated in K63 linked polyubiquitination of IRAK1. Ubiquitination involves the covalent linkage of ubiquitin molecules to a target protein as monomers (monoubiquitination) or polymers (polyubiquitination). Ubiquitination is carried out by the ubiquitin complex which is composed of three enzymes which operate sequentially: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3). Polyubiquitination occurs when ubiquitin chains are linked by the carboxy-terminal glycine of one ubiquitin to an internal lysine of another ubiquitin. Polyubiquitin chains

can be linked through lysine 48 (K48) or lysine 63 (K63). Polyubiquitination through K48 targets a protein to proteasomal degradation by the 26S proteasome. Polyubiquitination through K63 facilitates various biological processes such as kinase activity, protein trafficking involving endocytosis and lysosomal targeting (Sun and Chen, 2004). It has also been shown that the Pellinos are phosphorylated by IRAK1 and IRAK4 which then undergo K48 linked polyubiquitination targeting them for degradation (Butler et al., 2005, Butler et al., 2007, Moynagh, 2009). The activated IRAKs subsequently dissociate from MyD88 and interact with tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6). TRAF6 contains an N-terminal RING domain that possesses E3 ubiquitin ligase activity. TRAF6 forms a complex with E2, Ubc13 and Uev1A promoting lysine (K) 63-linked polyubiquitination of target proteins (including itself) (Chen, 2005). TRAF6 activates transforming growth factor β -activated protein kinase 1 (TAK1). TAK1 binding protein 2 (TAB2) and TAB3 are capable of recognising K63 linked ubiquitin chains on TRAF6, and they recruit TAK1 to TRAF6. This interaction induces the activation of TAK1 (Chen, 2005). The Pellino proteins have also been found to bind TAK1 and TRAF6, suggesting that Pellinos may play a role in activation of the TAK1/TRAF6/TAB1/TAB2 complex (Jiang et al., 2003). Following phosphorylation by TAK1 the inhibitor of NF- κ B kinase (IKK) is activated. The IKK complex consists of IKK α , IKK β protein kinases and the regulatory molecule IKK γ /NEMO. In un-stimulated cells the transcription factor nuclear factor- κ B is sequestered to the cytoplasm in an inactive form by members of the inhibitor of NF- κ B (I κ B) family. Upon ligand stimulation I κ B α is phosphorylated on serine 32 and serine 36 it then undergoes K48 linked polyubiquitination on K21 and K22 by the IKK complex (Scherer et al., 1995, Chen, 2005). I κ B α is then targeted for proteasomal degradation. The NF- κ B family of proteins can form up to 15 combinations of homo-

or hetero-dimers and regulate expression of genes involved in innate and adaptive immunity, inflammation, anti-apoptosis, proliferation, stress responses and cancer progression (Gilmore, 2006, Karin and Greten, 2005). The prototypical NF- κ B is thought to consist of subunits p65 and p50 (Ghosh and Hayden, 2008). Following I κ B degradation the NF- κ B dimer translocates to the nucleus where it binds to κ B sites in promoters or enhancers of target genes, up-regulating the production of pro-inflammatory mediators such as TNF- α , IL-6, IL-1 β and IL-12.

The activation of TAK1 also mediates the activation of mitogen-activated protein (MAP) kinases such as c-Jun N-terminal kinase (JNK) and p38. The transcription factor activating protein-1 (AP-1) is activated by these MAP kinases (figure 1.2) (Shaulian and Karin, 2002). These MAP kinases are also involved in mRNA stability.

TLR4 also mediates a MyD88 independent pathway (figure 1.2). Database searches have led to the identification of TRAM also known as TICAM2 (Fitzgerald et al., 2003). The TLR adaptor TRAM is essential for the generation of the TLR4 mediated MyD88-independent signalling pathway. TRAM interacts with TLR4 to recruit TRIF. The up-regulation of the MyD88 independent pathway causes the TLR4/TRAM/TRIF complex to form an early endosome from where it signals in a similar manner as an intracellular TLR (Kagan et al., 2008). The sequestering of TRAM and TRIF by TLR4 leads to the induction of type I IFN. Upon stimulation these proteins are phosphorylated by IKKs, Tank-binding kinase 1 (TBK1) and IKK ϵ . This phosphorylation allows interferon response factor 3 (IRF3) and IRF7 to translocate to the nucleus to regulate gene expression of type I IFN; IFN α and IFN β both of which are essential for the initiation of an antiviral state in cells, and for the detection of malignant cells (figure 1.2) (Garcia-Sastre, 2002, Fitzgerald et al., 2003). Recently a splice variant of TRAM known as TAG (TRAM adaptor with gold domain) has been shown to

negatively regulate the MyD88 independent pathway (Palsson-McDermott et al., 2009). TAG is a splice variant which is composed of the TIR domain of TRAM, and a Golgi dynamics domain. Upon LPS stimulation TAG displaces TRAM from the TRIF/TRAM complex. TRAM and TAG localise to the late endosome limiting LPS signalling. Experiments have shown that cells deficient in TAG have enhanced signalling through IRF3 and have increased expression of RANTES but not IL-8, indicating that TAG is a specific inhibitor of the MyD88 independent pathway.

1.5 Intracellular signalling by TLR 3, 7, 8, and 9

Unlike TLR 1, 2, 4, 5 and 6 which are cell surface located; TLR3, 7, 8, and 9 are located in endosomal compartments of immune effector cells such as dendritic cells (DCs) (figure 1.3).

TLR3 responds to dsRNA a molecular signature of RNA viruses. TLR3 is the only TLR that does not signal through the adaptor MyD88. Instead TLR3 utilizes the adaptor TRIF and receptor interacting protein-1 (RIP-1) to up-regulate IFN (figure 1.3) (Bell et al., 2005). TLR7 and TLR8 are similar in sequence and form a subgroup with TLR9 which are evolutionarily related. TLR7 and TLR8 are highly expressed in DCs. They recognise ssRNA from viruses and play a major role in the immune response during viral infection. TLR7 also responds to the synthetic compound imiquimod, while TLR8 responds to the synthetic resiquimod compound. DCs treated with these synthetic compounds have been shown to induce cancer cell death (Sary et al., 2007). TLR7 and 8 activate the adaptor MyD88 through interaction via their TIR domains. MyD88 then signals to the IRAKs which activate both the NF- κ B pathway up-regulating pro-inflammatory mediators, and the IRF pathway which up-regulates type-I

Figure 1.3 Downstream signalling of intracellular TLRs

Endocytosed viruses are detected by endosomally located TLRs 3, 7, 8 and 9. TLR3 signals downstream to activate IRF3. TLR 7 and 8 function to up-regulate IRAK 4 and 2 and signal downstream to degrade I κ B releasing the NF- κ B dimer p50/p65 into the nucleus. NF- κ B up-regulates transcription of pro-inflammatory cytokines. TLR7 and 9 activate the IRAK4/IRAK1 pathway leading to IRF7 activation and IFN- α transcription. Released type I IFN activates interferon receptor (IFNAR1/2). Activated IFNAR recruits the JAKs and STATS which form the ISGF3 complex upon IRF9 recruitment. ISGF3 signals to ISRE9 which transcribes type I IFN. Invading viruses are endocytosed into the cell at the plasma membrane or they inject their nucleic acids into the cytosol. Cytosolic receptors Mda-5 and RIG-I recognise the presence of viral nucleic acids where they signal downstream via their CARD domains to adaptor IPS-1/MAVS. IPS-1 found on the mitochondrial membrane signals to TRAF6 and TRAF3. TRAF6 signals to MEKK1, cfos/Jun and activates transcription factor AP1. TRAF3 signals downstream to up-regulate IRF3 phosphorylation which activates ISRE3, whereby type I IFN is transcribed.

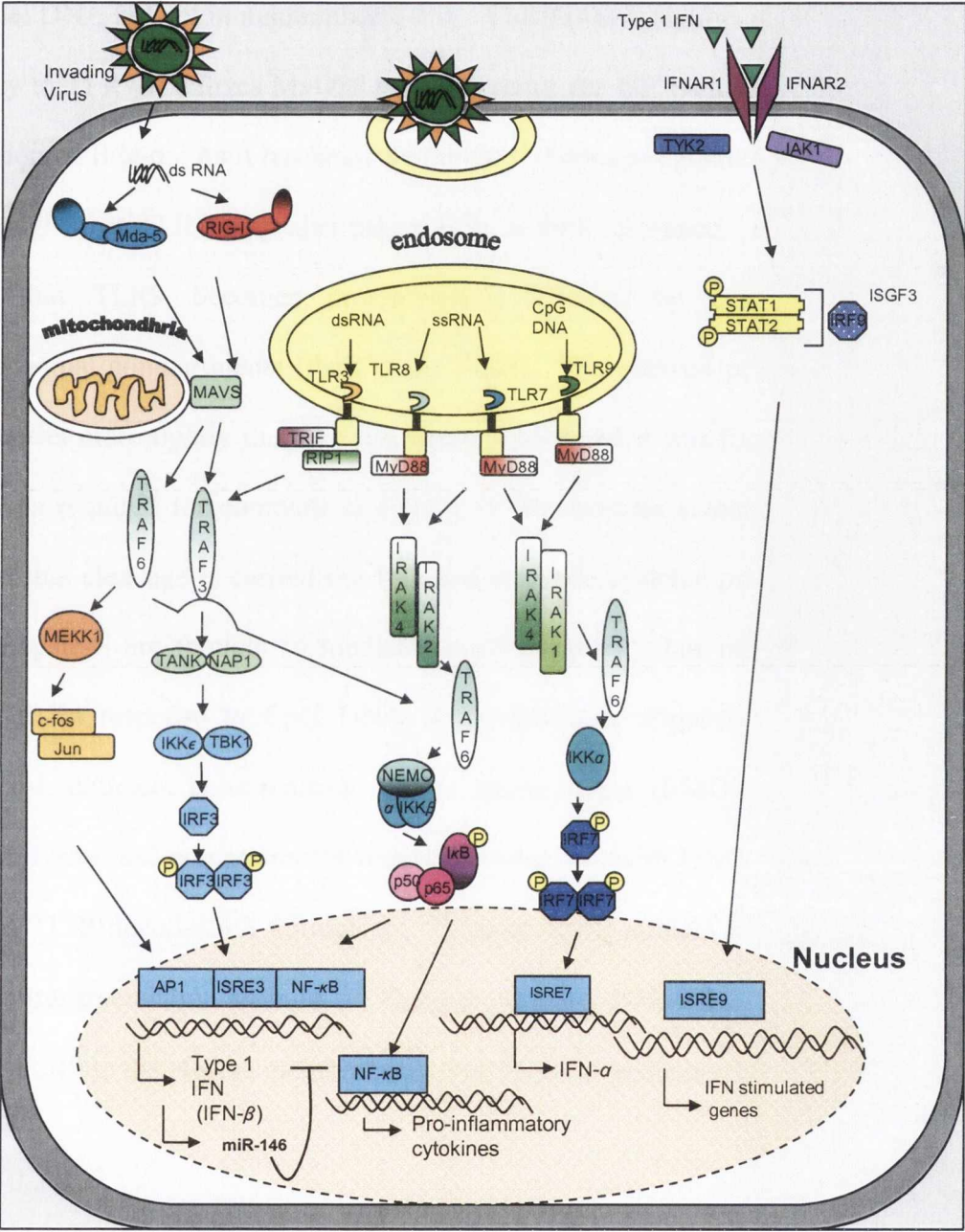


Figure 1.3 Downstream signalling of intracellular TLRs

IFN generating an anti-viral response (figure 1.3). TLR9 recognises the presence of un-methylated CpG DNA motifs. Un-methylated CpG DNA motifs are common in microbial DNA but not in mammalian DNA. TLR9 is also highly expressed in DCs and similarly to TLR7 it utilizes MyD88 to signal down the IRF pathway to up-regulate the production of IFN- α . As it has been shown that TLR9 up-regulates the antiviral IFN- α it is thought that TLR9 may also play a role in viral clearance. It has been recently shown that TLR9 becomes proteolytically cleaved by cysteine proteases in endolysosomal compartments (Park et al., 2008). The cleaved product binds to CpG DNA motifs more tightly than the full length TLR9 and it was found that cleavage of TLR9 was required for complete activation of downstream signalling cascades. It is believed that cleavage is carried out by more than one cysteine protease. Cathepsin-L and cathepsin-S are thought to mediate its cleavage as it has been shown that TNF production in response to CpG DNA is abrogated in response to cathepsin-S and cathepsin-L deficient bone marrow derived macrophages (BMDMs). The delivery of TLR9 to lysosomal compartments requires interaction with UNC93B1. This protein mediates TLR9 localisation through the transmembrane domain of TLR9. Interfering with the transmembrane domain of TLR9 or deleting UNC93B1 was shown to mis-localise TLR9 to the plasma membrane instead of endosomal compartments.

1.6 Nod-like receptors (NLRs)

The NLR (nucleotide-binding domain leucine rich repeat containing) family of proteins are a second arm of pattern recognition receptors (PRRs). They are intracellular receptors which recognise the presence of PAMPs. The NLRs are structurally composed of an N-terminal effector domain which can comprise of caspase recruitment

domains as in the case of the nucleotide oligomerization domain (NODs) proteins, a pyrin domain as in the case of night domain leucine rich repeat and pyrin domain containing protein (NALP) or baculovirus-inhibitor-of-apoptosis repeats (BIR) domain for neuronal apoptosis inhibitor protein (NAIP). NLRs are comprised of a central oligomerization domain and a C-terminal leucine rich repeat (LRR) domain (Tschopp et al., 2003, Inohara et al., 2005). They mediate signalling cascades through homophilic and heterophilic protein-protein interactions.

1.6.1 NOD 1 and NOD 2

Nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 also known as caspase recruitment domain 15 (CARD15) belong to the NLR family. NOD1 and NOD2 are intracellular sensors of bacterial fragments derived from peptidoglycan. NOD2 recognises the presence of muramyl dipeptide (MDP) while NOD1 senses γ -D-*meso*-DAP (iE-DAP) (Chamaillard et al., 2003a, Girardin et al., 2003c, Girardin et al., 2003b, Girardin et al., 2003a). It has been recently demonstrated that NOD1 and NOD2 activating ligands most likely enter by endocytosis through a clathrin-coated pit pathway (Lee et al., 2009). The internalization of the peptides is pH dependant and when formed, the endosomes process the peptides by introducing endosomal enzymes prior to transport out into the cytosol. NOD1 is ubiquitously expressed in adult human tissues while NOD2 is expressed in leukocytes, DCs and epithelial cells (Inohara et al., 1999, Gutierrez et al., 2002). NOD1 and NOD2 interact with inhibitor of apoptosis proteins (cIAP1 and cIAP2) which have been found to possess C-terminal RING-finger domains with E3 ligase activity (figure 1.4) (Reardon and Mak, 2009). Although cIAP proteins have CARD domains the NOD1 and NOD2 interactions occur independently of these; as it was found that when the CARD domains of NOD1, NOD2 and RIP2 were mutated interaction was not abrogated. Indicating that interaction between the NODs,

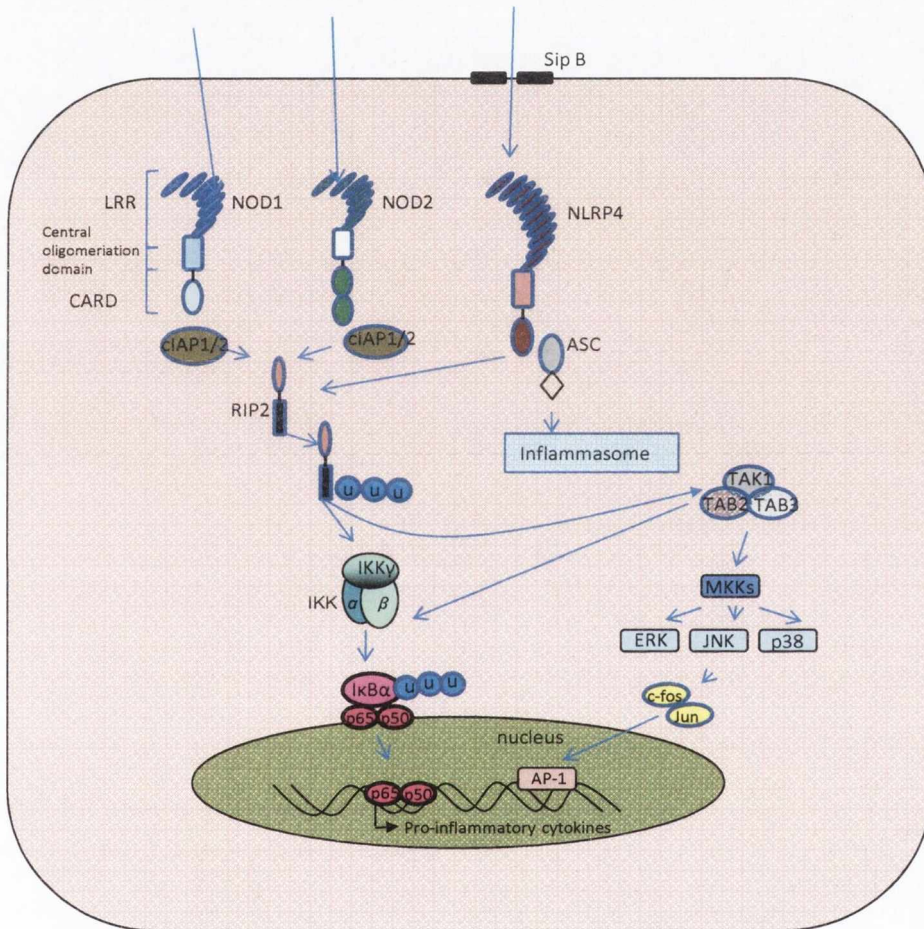
Figure 1.4 NLR mediated signalling cascades

Bacterial components of cytosolic *Listeria monocytogenes* are recognised by intracellular NOD1 and NOD2, *Helicobacter pylori* is identified by NOD1, *Salmonella typhimurium* is recognised by NOD2. NOD1 and NOD2 interact with inhibitor of apoptosis proteins (cIAP1 and cIAP2) which have been found to possess C-terminal RING-finger domains with E3 ligase activity. cIAP1 or cIAP2 bind and ubiquitinate RIP2 adding K63 linked ubiquitin units to the kinase protein. Ubiquitinated RIP2 binds and activates TAK1. The kinase activity of TAK1 activates both IKK γ and MKK which culminates in the translocation of NF- κ B and MAPK into the nucleus to up-regulate the transcription of pro-inflammatory genes and mediate antibacterial effects by the up-regulation of defensins. The bacterial translocation complex SipB mediates the translocation of flagellin across the plasma membrane, which is recognised by NLRP4 (IPAF), and in turn recruits ASC via its N-terminal CARD domain. ASC then recruits and activates caspase-1, forming the NLRP4 inflammasome.

Listeria monocytogenes
Helicobacter pylori

L. monocytogenes
Salmonella

Salmonella typhimurium



cIAPs and RIP2 is independent of their CARD domains. Following recruitment to NOD1 and NOD2, the cIAP proteins function as E3 ligases and mediate lysine (K) 63 linked ubiquitination of RIP2. Ubiquitination of RIP2 accounts for its activation and subsequent binding and activation of TAK1. The kinase activity of TAK1 activates both IKK γ and MAPK kinase (MKK) which culminates in the translocation of NF- κ B to the nucleus and the activation of transcription factors cFos and Jun by MAPK to up-regulate the transcription of pro-inflammatory genes and mediate antibacterial effects by up-regulation of defensins.

1.7 RIG-I like receptors (RLRs)

The detection of viruses can be largely attributed to the presence of intracellular TLRs and retinoic acid inducible gene-I (RIG-I) like receptors (RLRs) (Kawai and Akira, 2008). RLRs such as RIG-I and melanoma differentiation-associated gene-5 (Mda-5) are cytoplasmic helicases and are localised to the cytoplasm (figure 1.3). RIG-I and Mda-5 contain a DExD/H box RNA helicase domain and two CARD-like domains (Kawai and Akira, 2008). They recognise viral RNA and upon recognition of their ligands they signal downstream to NF- κ B, MAP kinases and IRFs which transcribe genes encoding type I IFN and pro-inflammatory cytokines. RIG-I specifically recognises 5'triphosphate containing viral RNA and up-regulates a downstream signalling cascade.

Downstream of RIG-I and Mda5 is an adaptor molecule known as mitochondrial antiviral signalling adapter (MAVS) also known as IPS-1. MAVS has an amino-terminal CARD domain and a carboxy-terminal transmembrane domain that localizes it

to the mitochondrial membrane. MAVS functions to activate IFN- α , IFN- β and NF- κ B promoters in a TBK1 dependent manner (Seth et al., 2005). MAVS signals to downstream TRAF3, TANK and NAP1, where the signal encounters the kinase proteins IKK- ϵ and TBK1. The IKK- ϵ /TBK1 complex activates the inactive IRF-3. Activation of IRF-3 requires the phosphorylation of multiple serine/threonine residues at its carboxy terminus (Lin et al., 1998). The phosphorylation of IRF-3 on serine clusters is carried out by TBK-1 and IKK- ϵ allowing dimerization of IRF3 to occur followed by the accumulation of the IRF3 dimer in the nucleus. The IRF3 dimer forms a complex with transcription factors ATF-2/c-Jun(AP-1) and NF- κ B (p65 and p50 heterodimer) and co-activators CBP/p300 allowing it to bind to DNA in a sequence specific manner (Lin et al., 1998, Lin et al., 1999, Qin et al., 2003, Yoneyama et al., 1998). The up-regulation of IFN protects the host from viral infection.

Recently RIG-I disruption in mice has been implicated by Wang Yi and colleagues in a colitis-like phenotype. RIG-I deficient mice were found to have a reduced body weight which was caused by severe damage and inflammatory infiltration into the mucosa of the colon (Wang et al., 2007).

1.8 C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) are a family of PRRs that are expressed by DCs. They interact with a large number of pathogens by recognising the presence of non-self glycoproteins and stimulate the inflammatory system to produce pro-inflammatory cytokines, promote phagocytosis and elicit the respiratory burst. CLRs C-type lectin domain family (CLEC-1) and DC-associated C-type lectin-1 (Dectin-1) signal through Syk dependent pathways (Fuller et al., 2007). Dectin-1 and DC-specific ICAM3

grabbing non-integrin (DC-SIGN) have been implicated in recognising PAMPs from PRRs, while intracellular CLRs have been found to modulate TLR responses to pathogens and also have been found to exert responses independent to TLRs (van Vliet et al., 2007). Syk activation by Dectin-1 induces caspase recruitment domain 9 (Card9)-dependent activation of NF- κ B. The activation of Dectin-1 by fungal- β glucans leads directly to TLR independent cytokine induction (Gross et al., 2006). Recently DC-SIGN has been shown to modulate TLR4 signalling. Several pathogens including *Mycobacterium tuberculosis* have been found to target DC-SIGN on DCs modulating the immune response promoting their survival. Binding of the mannose-capped lipoarabinomannan (Man LAM) ligand to DC-SIGN inhibits LPS induced TLR4 signalling and increases the production of immunosuppressive cytokine IL-10 (Gringhuis et al., 2007, Geijtenbeek et al., 2004). Man LAM activated DC-SIGN also activates the serine and threonine kinase Raf-1 independently of TLR signalling. Activation of Raf-1 then causes acetylation of NF- κ B subunit p65 but only after it has been activated by TLR signalling. Acetylation of p65 causes prolonged NF- κ B activity and enhanced IL-10 gene transcription (Gringhuis et al., 2007).

1.9 Functionally validated single nucleotide polymorphisms (SNPs)

Mutagenesis studies have identified functionally important amino acids in TLRs and their adaptors. A different approach to identifying functionally important amino acids in the TLRs and the adaptors is to identify the mis-sense SNPs that have been shown to have a possible role in disease progression. An analysis of the literature surrounding the role of SNPs in disease progression reveals much about the important regions within these proteins.

1.9.1 SNPs associated with TLR1

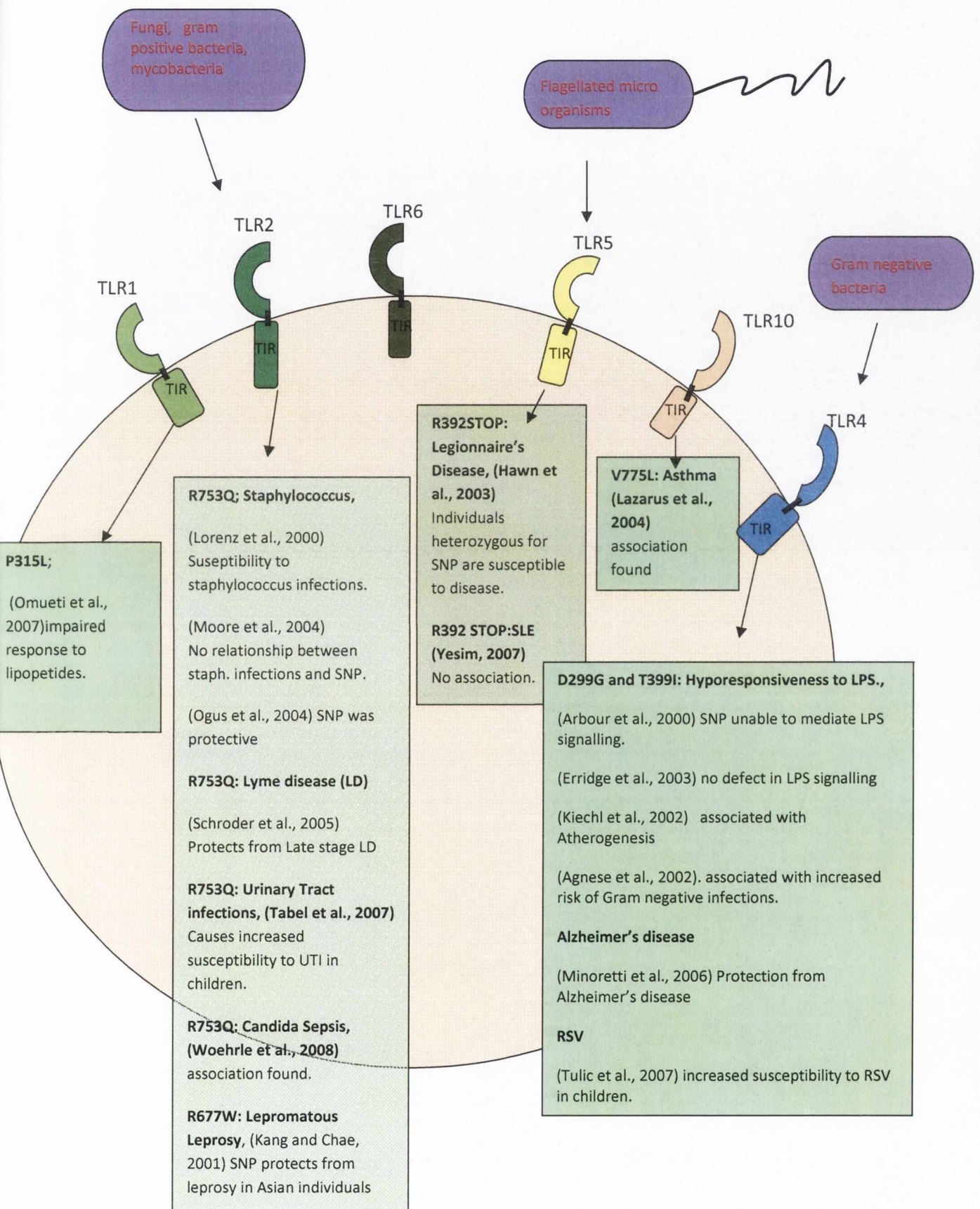
The extracellular receptor TLR1 has two functionally validated non-synonymous SNPs, Pro315Leu and Ile602Ser. SNP Pro315Leu (P315L) is found in the outer loop of LRR 11. The largely hydrophobic leucine residue greatly impairs response to lipopeptides. The outer convex side of the TLR1 extracellular domain provides the interface for interactions with ligand or TLR2 and/or components of the TLR1/TLR2 activation complex (Omueti et al., 2007). Population studies from the National centre for biotechnology information (NCBI) indicate that Pro315Leu is present in approximately 2% of the human population (figure 1.5). The most common TLR1 SNP among Caucasians to affect disease in humans is the Ile602Ser TLR1 SNP present in approximately 50% of the human population. Ile602Ser is associated with decreased incidence of leprosy, a chronic skin and peripheral nerve system disease caused by *Mycobacterium leprae* (figure 1.5) (Johnson et al., 2007). Ile602 is present in the transmembrane domain of TLR1. Individuals who are homozygous for 602SerSer have lower surface expression levels of TLR1 on monocytes compared to 602IleSer heterozygous individuals or 602IleIle homozygous individuals. A diminished TLR1 surface expression and function is protective against clinical leprosy. In this case a dampened immune response provides protection from disease.

1.9.2 SNPs associated with TLR2

The mis-sense mutations of TLR2 are highly studied and many laboratory groups are in disagreement as to the role these SNPs play in diseases (figure 1.5). In 2000 Lorenz and colleagues found a polymorphism in the *TLR2* gene Arg753Gln (present in 3% of the human population) (Lorenz et al., 2000). They found that compared to the wild type *TLR2* gene the variant form was significantly less responsive to bacterial peptides from

Figure 1.5 Non-synonymous SNPs in extracellular TLRs

TLRs 1, 2, 5, 10 and 4 have all been found to possess non-synonymous SNPs which have been implicated in either susceptibility to or protection from disease. TLR4 polymorphisms D299G and T399I are found in the extracellular domain have been implicated in hypo-responsiveness to LPS and are associated with increased gram negative infections. TLR2 polymorphism R753Q is found in the TIR domain has been found to be associated with *Staphylococcus* infections. A polymorphism in the extracellular region of TLR1 P315L showed impaired response to lipopeptides to which it responds to in conjunction with TLR2. The I602S polymorphism of TLR1 is found in the transmembrane domain and was shown to protect from leprosy. TLR5 possesses a polymorphism that replaces an arginine at position 392 with a stop codon causing TLR5 to become truncated; it was found to cause individuals to be susceptible to Legionnaire's disease. A TLR10 polymorphism located in the TIR domain was found to have an association with asthma. Associated references are found in the figure.



Borrelia burgdorferi and *Treponema pallidum*. They found that the Arg753Gln may predispose individuals to staphylococcal infection. In 2004 Moore and colleagues found contradictory evidence, they did not find a relationship between Arg753Gln and staphylococcal infection (Moore et al., 2004). The same year Ogus and colleagues showed that the SNP was found at a greater frequency in the control group than the staphylococcal diseased group, indicating that the SNP was protective (Ogus et al., 2004).

An association of Arg753Gln SNP and urinary tract infections was found by Tabel and colleagues (Tabel et al., 2007). Urinary tract infections (UTI) can be caused by *Escherichia coli*, *Klebsiella*, *Proteus*, *Staphylococcus*, *Enterococcus* and *Pseudomonas*. The frequency of UTI was higher in the Arg753Gln allele carriers than the wild type carriers. Asymptomatic urinary tract infection was also higher in the variant group. They also found that the risk of contracting a second UTI from gram positive bacteria was also higher. They concluded that the Arg753Gln SNP caused an increased susceptibility to UTI in children. Patients with candida sepsis and who are heterozygous for the Arg753Gln (R753Q) SNP have altered cytokine production levels. Woehrle and colleagues found that patients with the Arg753Gln SNP have an increased level of TNF- α and reduced production of IFN- γ and IL-8 in candida sepsis but not gram positive sepsis (figure 1.5) (Woehrle et al., 2008). Patients with the Arg753Gln SNP had an increase in apoptosis of monocytes. A subsequent study found that individuals heterozygous for Arg753Gln had impaired immune activation by *Borrelia burgdorferi* and protects the individuals from late stage Lyme disease (Schroder et al., 2005). Blood from heterozygous individuals had lower TNF- α and IFN- γ compared to the wild type. The Arg753Gln occurs at a significantly lower frequency in the Lyme disease patients than the healthy controls. The SNP may protect from development of

late stage Lyme disease due to the reduced signalling via TLR2/1 signalling pathway. Clinical symptoms of late stage Lyme disease are mediated by the presence of inflammatory mediators such as TNF- α and IFN- γ and heterozygosity for the Arg753Gln SNP impairs the release of these cytokines. The arthritic effects of the cytokines are reduced while the bacteria are still being eliminated. The presence of these SNPs in individuals could explain the sustained prevalence of these SNPs in populations.

The TLR2 SNP Arg677Trp has been detected in patients suffering from lepromatous leprosy (figure 1.5) (Kang and Chae, 2001). It was shown that the TLR variant was functionally impaired in the recognition of lipopeptides. Arg677Trp has only been found in Asian populations.

1.9.3 SNPs associated with TLR4

Two extracellular TLR4 SNPs Asp299Gly and Thr399Ile are co-segregating SNPs located in loops 10 and 15 respectively and were found to attenuate TLR4 receptor signalling (present in 8.6% of human population) (figure 1.5). They were found to be associated with LPS hypo-responsiveness in response to the inhalation of endotoxin (Arbour et al., 2000). The Asp299Gly TLR4 mutant was unable to mediate LPS signalling in primary airway epithelial cells and acted in a dominant fashion with respect to wild type TLR4. A study by Agnese and colleagues looked at gram negative infections and found that Asp299Gly and Thr399Ile (found in 4.2% of population) were found to increase the incidence of infection (Agnese et al., 2002).

Following from this, various studies have found evidence to the contrary. In 2003, a report by Erridge and colleagues was published which disagreed with Arbour's claims (Erridge et al., 2003). The group found no defect in LPS signalling. The difference lay in the fact that Erridge carried out his studies in monocytes whereas Arbour's study concerned epithelial cells. A further study by Kiechl examined the pathogenesis of atherogenesis he found that Asp299Gly is associated with a decreased risk of atherosclerosis, a degenerative disease of the arteries (Kiechl et al., 2002). The following year a study was carried out by Zee which disagreed with Kiechl and found that Asp299Gly SNP did not have any associated risk to atherogenesis (Zee et al., 2005).

In 2006 Minoretti and colleagues found that Asp299Gly may be protective from the development of late onset Alzheimer's disease (LOAD) (Minoretti et al., 2006). The Asp299Gly has been reported to have attenuated response to stimuli and may increase the risk of acute severe infections, but it may exert beneficial effects when considering the development of LOAD. The weakened TLR4 variant may not be as efficient as the wild type TLR4 in producing oxidised low-density lipoproteins (which are produced in response to endotoxin) which have been suggested to cause neurodegeneration.

A study carried out by Tulic and colleagues found that human bronchial epithelial cells expressing Asp299Gly or Thr399Ile displayed normal levels of intracellular TLR4 but failed to efficiently translocate the TLR4 receptor to the cell surface when stimulated (Tulic et al., 2007). The inability to translocate the mutated receptor to the surface was associated with reduced NF- κ B signalling, reduced production of IFN, IL-8, IL-10, IL-12p35, IL-18, and an absence of acute phase TNF- α . When Tulic examined PBMCs from children with both co-segregating polymorphisms, he found that their responses to

respiratory syncytial virus (RSV) were dampened. Tulic concluded that reduced surface expression caused reduced ligand binding conferring increased susceptibility to severe infection with RSV.

1.9.4 SNPs associated with TLR10

TLR10 is a highly polymorphic gene with as many as 15 non-synonymous SNPs reported on NCBI, this could possibly be due to the redundancy of the receptor.

The SNP Val775Leu (found in 17.3% of the human population) is found in the TIR signalling domain of TLR10 (figure 1.5). This SNP has been found to be associated with asthma (Lazarus et al., 2004). An excess of minor homozygous alleles 775LeuLeu were found among individuals with asthma. A change in the TIR domain of TLR10 could provide a plausible explanation for the attenuated signalling found. However the whole region is characterised by strong linkage disequilibrium and so it is possible to mediate this association to asthma to a nearby variant not yet characterised.

1.9.5 SNPs associated with TLR5

TLR5 is an extracellular TLR which recognises a PAMP found on the flagella of flagellated micro-organisms known as flagellin (figure 1.5). The SNP Arg392STOP is associated with susceptibility to legionnaires' disease and Crohn's disease. The cytosine to thymidine base change causes an arginine residue to be replaced by a STOP codon in position 392. The introduced STOP codon causes the loss of the transmembrane domain and the entire intracellular signalling domain. Arg392 is found in the extracellular domain of TLR5 and regulates the host's response to flagellated bacteria. Hawn and colleagues found that the Arg392STOP SNP abolished flagellin signalling through TLR5 (Hawn et al., 2003). The SNP acts in a dominant fashion as individuals

who are heterozygous for the SNP are more susceptible to legionnaires' disease caused by *Legionella pneumophila* than homozygous individuals who do not have the SNP. A study by Gewirtz (Gewirtz, 2007) found that the TLR5-STOP polymorphism protected individuals from developing Crohn's disease (CD). The deficiency in TLR5 caused a decrease in flagellin specific IgG and IgA antibodies. A Jewish population was studied and it was found that carriage of the TLR5-STOP polymorphism was significantly lower in individuals with CD.

In 2007 Demirci and colleagues investigated whether the variants TLR5 SNP Arg392STOP and TLR9 SNP (-1237T-C) were associated with systemic lupus erythematosus (SLE). No major influence of these variants was found on susceptibility or resistance to SLE (Demirci et al., 2007).

1.9.6 SNPs associated with TLR3

Intracellular TLR3 recognises dsRNA and synthetic Poly:IC. Hidaka and colleagues found a non-synonymous mutation in intracellular TLR3 (Hidaka et al., 2006) (figure 1.6). Phe302Ser was found in a patient with influenza-associated encephalopathy. The Phe303Ser mutant is found in the 11th LRR region of TLR3 and was confirmed as a loss of function mutant when examining NF- κ B and IFN- α reporter assays.

1.9.7 SNPs associated with TLR7

TLR7 is an intracellular TLR which responds to ssRNA from viruses such as hepatitis C virus (HCV). Two studies by Schott and colleagues published in 2007 and 2008 suggested that a SNP found in the intron of TLR7 c.1-120G offers protection from the development of inflammation and fibrosis in male patients with chronic HCV infection

(Schott et al., 2007, Schott et al., 2008) (figure 1.6). Fibrosis occurs as a result of defective repair of damaged liver caused by inflammation. It was also found that the Gln11Leu TLR7 is predictive of an unfavourable outcome to IFN- α therapy for HCV patients. The TLR7 Gln11Leu variant impairs the host's immune response to HCV.

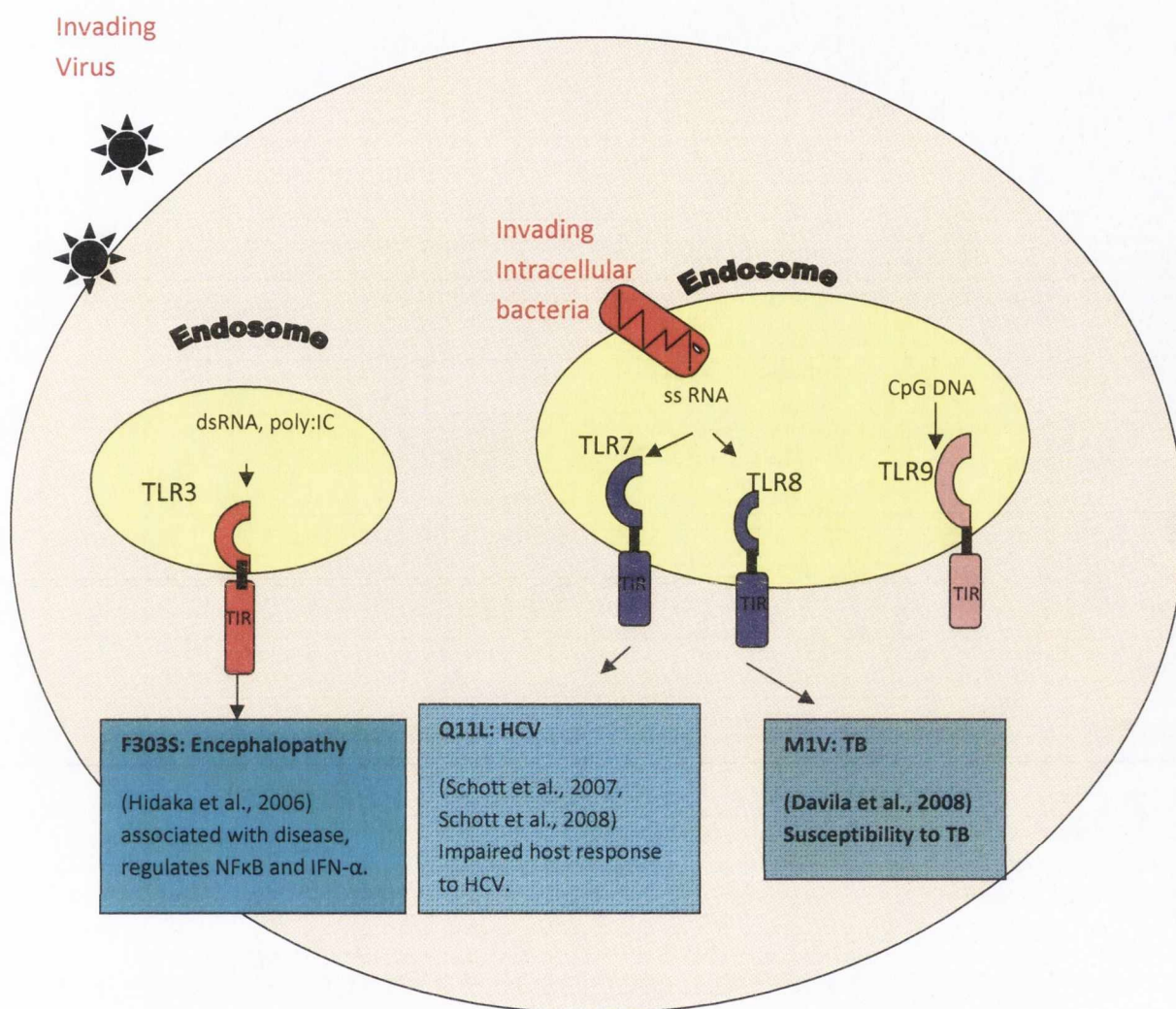
1.9.8 SNPs associated with TLR8

In 2008 Davilla and colleagues discovered that the TLR8 associated single nucleotide polymorphism Met1Val provided protection from tuberculosis (Davila et al., 2008) (figure 1.6).

To date no non-synonymous polymorphisms of the TLR 6, or 9 genes have been characterised. Tao and colleagues examined a SNP located in the first intron TLR9. The SNP in TLR9 was examined in relation to SLE in a Japanese population and found that this SNP predisposes humans to an increased risk of SLE (Tao et al., 2007).

1.9.9 Rare polymorphisms contribute to susceptibility to disease

A novel study was carried out on susceptibility to TB in individuals who carried rare polymorphisms in TLR1, 6 and 10 (Ma et al., 2007). They grouped all the rare SNPs in these TLRs and found that they were significantly over-represented in African/American TB cases. They carried out functional analysis using NF- κ B luciferase assays, using 300ng TLR6 and 6ng TLR2 transfected into HEK 293 cells. They examined polymorphisms in TLR6 L251end, Thr255Ile and Asn690Thr, which were all found to have reduced signalling towards NF- κ B. These rare polymorphisms are thought to be maintained at a low level by the individuals reduced ability to fight infection and so have been maintained at a low level by natural selection. The rare



1.6 Non-synonymous SNPs associated with intracellular TLRs

Non-synonymous SNPs of intracellular TLRs have been found to be associated with disease. A polymorphism in TLR3 has been found to be associated with the LRR region of the TLR as the F303S mutant was incapable of generating a signalling cascade to NF- κ B or type I IFN. TLR7 which recognises ssRNA from invading pathogens was found to possess a polymorphism at position 11 in its signal sequence. It showed an impaired response to hepatitis C virus (HCV). Recently a polymorphism in the first amino acid of TLR8 M1V found that individuals were susceptible to TB. Associated references are found in the figure.

variants are thought to exist in the population by general random mutation (Ma et al., 2007).

A study by a Swedish group in 2005 found that variants in the TLR6/TLR1/TLR10 gene cluster were associated with a risk of prostate cancer (Sun et al., 2005). A second group, Chen and colleagues failed to find the same association (Chen et al., 2007).

1.10 Intestinal inflammatory diseases associated with NOD1 and NOD2

Helicobacter pylori is a major cause of gastric ulcers. Microbial peptide components of *H. pylori* are recognised by NOD1 (figure 1.4) (Viala et al., 2004), while NOD2 deficient mice are more susceptible to oral infection by *Listeria monocytogenes* (Kobayashi et al., 2005). NOD2 is found abundantly in ileal Paneth cells, which are an important producer of anti-microbial peptides. A subgroup of anti-microbial peptides known as cryptidins are significantly diminished in NOD2 deficient cells (Kobayashi et al., 2005).

CD has a complex pathology as it is caused by a combination of genetic factors, dysregulated interaction with enteric microflora and immune mediated tissue injury. It is important to maintain the balance between harmful pathogenic bacteria and safe symbiotic intestinal bacteria within the intestine. NOD2/CARD15 is a susceptibility gene associated with CD. CD is characterised by chronic granulomatous inflammation of various interstitial areas, ulcers of the mucosa and it can also lead to arthritis, uveitis and skin lesions. Three polymorphisms of NOD2 have been found to be associated with CD including Arg702Trp, Gly908Arg and Leu1007fsinsC. These variants are all located within the LRR domain of NOD2 which is involved in sensing of bacterial product MDP. These “loss of function” mutants are associated with decreased NF- κ B activity in response to MDP, and are associated with lack of bacterial clearance

(Chamaillard et al., 2003b, Chamaillard et al., 2003a, Girardin et al., 2003a, Inohara et al., 2003). Individuals with the Leu1007fsinsC frame shift mutation have been shown to have an inability to signal in response to MDP, and an impaired response to intracellular *Salmonella*, indicating that NOD2 plays an integral role in the protection against intracellular bacteria (Hisamatsu et al., 2003). A NOD2 gain of function mutation is also associated with a chronic systemic inflammatory disorder known as “Blau syndrome”, in which NOD2 (2932iC) is constitutively active and can cause ligand independent activation of NF- κ B. A murine version of the NOD2 (2932iC) gain of function mutant was found to exhibit enhanced NF- κ B activation, increased apoptosis and increased secretion of IL-1 β (Maeda et al., 2005).

1.11 Structure and characterization of TIR domain containing proteins

This project involves the investigation of the effect of polymorphisms within TLR6 and the adaptor Mal and their influence on biochemical pathways within the innate immune system. The TLR6 polymorphism resides at the N-terminal domain of TLR6 while the Mal polymorphism is found in the TIR domain. A comprehensive look at the structure of these regions will be undertaken to gain insight into the role polymorphisms may exert on the structure of these proteins.

1.11.1 Extracellular structural domains of TLRs

The nascent polypeptides of TLRs all contain an N-terminal signal peptide cleavage site which is cleaved off upon translocation through the endoplasmic reticulum. The signal sequence directs the protein to its functional location, directing the extracellular TLRs to the plasma membrane while directing the intracellular TLRs to endosomal

compartments. Figure 1.7 shows the signal sequences of the TLRs which were identified using signalP an online web-based computer programme. The extracellular region of the TLR family contain 16-28 LRRs (Matsushima et al., 2007). The individual LRR is 20-30 amino acids long and is comprised of a conserved “LxxLxLxxN” motif (Kobe and Kajava, 2001, Kajava, 1998). The LRR region of all proteins has a common horse-shoe like structural shape (Gay and Gangloff, 2007). The “LxxLxLxxN” motifs are located in the inner concave surfaces of the horse-shoe like shape. Upon ligand binding the TLRs undergo dimerization. The TLR1/2 heterodimer and the TLR3 homodimer is induced by binding their agonistic ligands and forming a common ‘m’ shaped architecture. The C termini of each extracellular domain come together bringing the intracellular TIR domains in close proximity (Jin and Lee, 2008).

1.11.2 Intracellular structure of TIR domain containing proteins

The Toll-Interleukin-1 receptor (TIR) domain is a structurally conserved region which is involved in protein-protein interactions. The TIR domains of the TLRs are approximately 200 amino acids long and contains three highly conserved regions known as box1, box2 and box3 (figure 1.8). The box regions were shown to be involved in signalling by Kuno and colleagues in 1993 using mutagenesis studies (Kuno et al., 1993). The TIR domain consists of a central five stranded parallel β -sheet (β A- β E). A loop connects each β -sheet to an α -helix. The BB loop connects strand β B and helix α B (Xu et al., 2000). The loops are the most variable regions of the TIR domains and have been shown to be involved in TIR-TIR interactions. The insertion of different amino acids into these loop regions can effect signalling (Xu et al., 2000, Dunne et al., 2003). Recently the crystal structure of human TLR10 cytoplasmic domain has been resolved (Nyman et al., 2008). Nyman and colleagues found that TLR10 exists as a dimer, and

that its interaction surface contains residues from the BB-loop, DD-loop and α C-helix. They found that the interaction surface was extensive and that it contains a central hydrophobic patch surrounded by polar residues. They found that in monomer A the tip of the DD loop residue R741 makes polar contact with the α C residue E709 of monomer B and that residues Y742, L745 and K746 act to stabilize the DD loop. They concluded that amino acids surrounding the DD loop stabilise the interaction between the dimer and that mutations in these stabilizing residues would therefore inhibit the possibility for R741 to participate in the dimer interaction. A study carried out by Dunne and colleagues predicted that Mal would interact with TLR2 via its DD and DE loops. Modelling studies carried out by Núñez and colleagues found that Mal and TRAM bind to the same region in the TIR domain the TLR4 dimer interface (Nunez Miguel et al., 2007). They also found that Mal binds to the TLR4 homodimer with closer proximity and with a greater number of direct contacts than TRAM. This finding may explain why Mal/MyD88 pathway triggers early NF- κ B expression and TRAM/TRIF signalling results in late NF- κ B expression.

1.11.3 Structure and characterisation of TLR6

Human TLR6 was first discovered in 1999 by Akira and colleagues (Takeuchi et al., 1999). They found that it was most similar to human TLR1 with a 69% similar homological identity. They found that it constitutively activated NF- κ B and c-Jun N-terminal kinase (JNK) similar to already previously identified TLRs. Human TLR6 belongs to the TLR2 family along with TLR1 and 10, which are all found on chromosome 4. TLR6 protein is 796 amino acids in length with a signal peptide sequence from 1-31, an extracellular LRR region from 32-585, a transmembrane that

Figure 1.7 Signal peptide cleavage sites of TLRs

The signal peptide cleavage sites of the human TLRs were found by entering the protein sequence of each TLR into signalP an online signal peptide cleavage prediction site. Each of the signal cleavage sites are situated approximately around the 20th amino acid. While the cleavage site of TLR6 was situated furthest away from the N-terminus. No signal cleavage site was identified for TLR4.

Signal Peptide Cleavage sites of TLRs

Human TLR1	MTSIFHFAIFMLILQIRIQ [↓] SESEFLVDRSKNGLIHVP
	22-23
Human TLR2	MPHTLWMVWVLGVII [↓] SLSKEESSNQASLSCDRNGICKGSS
	18-19
Human TLR6	MTKDKEPMKSFHFVCLMIIIVGTRI [↓] QSDGNEFAVDKSK
	31-32
Human TLR10	MRLIRNIYIFCSIVMTA [↓] EGDAPELPEERELMTNCSNMSLR
	19-20

Human TLR4	None found
Human TLR5	MGDHLDLLLGVVLMAGPVE [↓] GPSCSFDGRIAFYRFCNLTQ
	20-21

Human TLR3	MRQTLPCNYFWGQLLPFGMLCASSI [↓] TKCTVSHVADCSHL
	26-27
Human TLR7	MVFPMWTLKRQLLFNII [↓] LSKLGARWFPKTLPCDVTL
	26-27
Human TLR8	MENMFLOSSMLT [↓] CFLLSGSCELCAEENFSRSYPCDEKK
	26-27
Human TLR9	MGFCRSALHPLSLVQA [↓] IMLAMTLA [↓] GTLPAFLPC [↓] ELOPH
	25-26

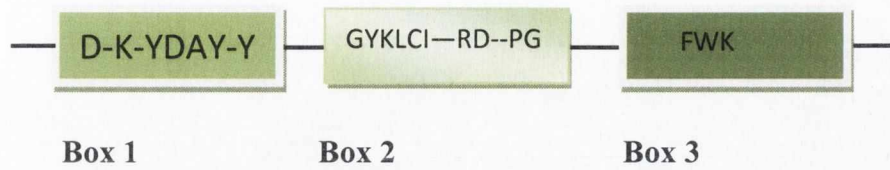


Figure 1.8 Box regions 1, 2 and 3 of the TIR domain

The box regions of the TIR domain are highly conserved regions and have been critically implicated in mediating TLR signalling.

spans the plasma membrane from position 568-606 and an intracellular TIR region from 607-796.

1.11.4 TLR2 heterodimerizes with TLR1 and TLR6

Of all the TLRs, TLR2 has possibly the largest repertoire of ligands which it can recognise because of its ability to heterodimerize with TLR1 and TLR6 (figure 1.2). TLR2 ligands include; components of gram positive bacteria such as lipoteichoic acid (Schwandner et al., 1999), lipoarabinomannan (Means et al., 1999) and lipoproteins (Kim et al., 2002, Takeuchi et al., 2002). The TLR2 and TLR1 heterodimer recognises triacylated lipoproteins and the TLR2 and TLR6 heterodimer recognises diacylated lipoproteins. TLR2 has also been shown to associate with CD36 in response to diacylated lipoproteins (figure 1.2). In 2006 Triantafilou and colleagues found that TLR2 forms heterodimers with TLR1 and TLR6 and that these heterodimers are pre-formed and are not induced by ligand. It is only upon stimulation with specific ligand these heterodimers are recruited within lipid rafts. It was found that the TLR2/TLR6/CD36 complex is not pre-formed and must be ligated by ligand (Triantafilou et al., 2006). They also found that upon stimulation with FSL-1 (TLR2/6 ligand) TLR2/6 and CD36 are all targeted to the Golgi apparatus. This suggests that the preformed clusters are all internalized upon ligand activation. However internalization was not required for downstream NF- κ B activation. The internalization was not found to be CD36 dependent. A second accessory molecule CD14 has been found to be required for the TLR2/6 signalling response to diacylated lipopeptides and lipoteichoic acid (Jiang et al., 2005). Two TLR2 variants have been identified that cause a mis-localised expression of TLR2. TLR2 Cys30 Ser and Cys36Ser variants were found to

be expressed intracellularly when transfected into HEK 293 cells. The variants were unable to induce NF- κ B activation upon ligation with macrophage-activating lipopeptide-2 (MALP-2) (Triantafilou et al., 2006).

1.11.5 Characteristics of enzyme cleavage

Part of this project pertains to putative enzyme cleavage and proteolytic processing of TLR6. Proteolytic cleavage is an important mechanism used to produce biologically active proteins and peptides. Catalytic cleavage of proteins is carried out by proteases and the most common proteolytic enzymes are known as serine proteases as they commonly catalyse sites that contain the serine residue. Most enzyme cleavage in the body occurs during the digestion of food and three enzymes are most commonly used, chymotrypsin which is specific for bulky hydrophobic residues, trypsin which is specific for positively charged residues and elastase which is specific for small neutral residues.

Other than food digestion proteolytic processing is also important in inflammation, blood clotting and many other processes, most peptide processing required for these events occurs within the cell, and more specifically within the Golgi apparatus. In the Golgi apparatus paired basic residues such as arginine, lysine and histidine provide the site of choice for proteolytic cleavage (Steiner et al., 1984). The protein progastrin is cleaved at a lysine-lysine site to form gastrin, the mature form of the protein (Steiner et al., 1980). Tryptic proteases have high selectivity but on rare occasions can cleave at 'trypsin like' sites; including leucine-alanine, leucine-serine, tyrosine-alanine (Steiner et al., 1980). The secondary and tertiary folding structure of proteins can also influence

cleavage (Steiner et al., 1984). Proteins which are processed in the Golgi are packaged for release as secretory granules.

The cleavage site of interest in this project is situated near the N-terminus of TLR6 and so may be involved in the signal peptide cleavage site. Signal peptides direct the protein of interest to the endoplasmic reticulum for processing and for packaging to their eventual intra or extracellular destination. A signal peptide is approximately 20 amino acids long and it generally consists of a positively charged N region, a hydrophobic H region at the core and a neutral polar C region. The signal sequence is cleaved after the C region leaving the remainder of the polypeptide to fold into its 3D structure. The cleavage at signal peptide sites will be discussed further in chapter 4 (von Heijne, 1983, Claros et al., 1997).

1.11.6 Structure and characterisation of Mal

The second protein that is the focus of this project is Mal. Mal also known as TIRAP is a TLR2 and TLR4 associated adaptor protein (figure 1.2). It is 256 amino acids long and it is the most polymorphic of all the adaptor proteins. Mal is highly characterised and has been shown to have many functional domains, as it links TLR4 and 2 to MyD88 by its TIR domain. Mal has also been shown to have a putative TRAF6 motif within its TIR domain and it is possible that it binds it around amino acid 190, as mutagenesis studies have found that Mal Glu190Ala was unable to bind TRAF6 compared to wild type (Mansell et al., 2004). Mal contains a C-terminal phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain which localizes it to the plasma membrane. A mutated PIP2 binding domain abrogates the ability of Mal to recruit MyD88 to the membrane and LPS signalling through TLR4 is inhibited (Kagan and Medzhitov, 2006).

Mal is also subjected to cleavage by caspase-1 before it is activated. When Mal is cleaved by caspase-1 a 4kDa portion of Mal is removed allowing signalling through TLR2 and TLR4 to occur (Miggin et al., 2007). Bruton's tyrosine kinase (BTK) phosphorylates Mal at positions Tyr86 and Tyr187. Mutant forms of these residues act as dominant negative inhibitors of LPS induced signalling to NF- κ B. A second study found that residues Tyr86, Tyr106 and Tyr159 are important in Mal phosphorylation. Mutation studies have found that these residues are important for Mal's interaction with BTK and that they affect downstream signalling (Piao et al., 2008). The phosphorylation of Mal is thought to cause a conformational change in the TIR region of Mal which plays a role in its interaction with other proteins (Gray et al., 2006). Mal is thought to be negatively regulated by suppressor of cytokine signalling-1 (SOCS-1). SOCS-1 mediates polyubiquitination of Mal on two N-terminal lysine residues which targets it for degradation by the 26S proteasome (Mansell et al., 2006).

1.12 Infectious diseases

The two polymorphisms under investigation in this project cause either the susceptibility or protection from infectious disease. Among the disease groups investigated were individuals with tuberculosis, malaria and invasive pneumococcal disease (IPD) caused by *Streptococcus pneumonia* infections.

1.12.1 Tuberculosis

Tuberculosis (TB) is a common and deadly infectious disease caused by *Mycobacterium tuberculosis*. *M. tuberculosis* is an intracellular pathogen capable of surviving within host mononuclear cells. Over one third (approximately 2 billion) of the world's

population people now carry the TB bacterium and new infections are occurring at a rate of one per second. TB infection primarily results from airborne particles of *M. tuberculosis*, made airborne as a result of coughing. On average a patient with TB will infect seven close contact individuals, but not all individuals will develop TB and this is thought to be due to genetic variation in individuals. TB can affect many areas of the body, the most commonly affected are the lungs, which manifests as pulmonary TB. TB can also affect the central nervous system, lymphatic system, circulatory system, genitourinary system, bones and joints. (Tuberculosis Fact Sheet, World Health Organization (WHO) 6 October 2006).

For infection to occur the airborne *M. tuberculosis* particles must escape the upper respiratory defences and become deposited deep within the lung alveoli. The tubercle bacilli must then be ingested by alveolar macrophages. At this stage most tubercle bacilli are killed by the macrophage, however any bacilli that are not killed by CD8 T cells replicate within the phagosome of the macrophage and ultimately kill the host macrophage. The TB bacterium has developed survival strategies over during its time infecting mammals. By preventing the phagosome fusing with the lysosome it blocks phagosome acidification, protecting itself from the bactericidal components of the lysosome, the TB bacillus has also developed a thick bacterial cell wall which helps to avoid destruction (Ferrari et al., 1999). Infected macrophages migrate to the regional lymph nodes where they can access the blood stream. From here the bacilli can hematogenously disseminate to any part of the body, leading to infection of different areas of the body including, bones, kidneys, meninges and different regions of the lungs. Tuberculous granulomas can appear in these regions, developing necrotic centres wherein the bacilli can survive for many years. The tuberculosis granuloma is seen as a trademark of *M. Tuberculosis* infection. Tuberculous granulomas are complex bodies

composed of different layers of immune cells and have highly orchestrated and defined biochemical pathways associated with them (Apt and Kondrat'eva, 2008) (figure 1.9). Upon microbial detection the production of cytokines initially induces the migration of neutrophils, where they become the centre of the granuloma, followed by mononuclear phagocytes. These phagocytes differentiate to become tissue macrophages, where they capture mycobacteria that has been released by the initially infected alveolar macrophages. This process continues, forming the highly characteristic TB granuloma (figure 1.9).

The granuloma effectively 'wall-off' pathogens whereby they can resist destruction, the infection can remain here and may or may not ultimately be resolved by the immune system. The infectious granulomas of the lungs leave behind fibronodular scars in the upper region of the lungs, and may leave calcified cars from the primary infection. (The Merck Manual – Online medical library, <http://www.merck.com>)

Control and resolution of the infection is the responsibility of the innate and the adaptive immune systems of the body. The macrophage has multiple functions in combating tuberculosis, including antigen processing and presentation, transport to deeper tissues and dissemination of infection and effector cell functions. The macrophage sequesters the pathogen and produces mycobacterial products such as lipoarabinomannan (LAM), soluble tuberculosis factor and 19-kDa lipoproteins. The released mycobacterial products signal to cell surface TLR2, TLR4 and DC-SIGN and to intracellular TLR9 found in macrophages, dendritic cells and epithelial cells. This interaction induces the production of IFN γ , IL-12, IL-23, TNF, lymphotoxins, CD40 and nitric oxide which are essential for eradication of the mycobacteria (Brightbill et al., 1999). Infected macrophages also produce accessory molecules which act to recruit T cells to the site of infection. Recently Fremond and colleagues discovered that MyD88

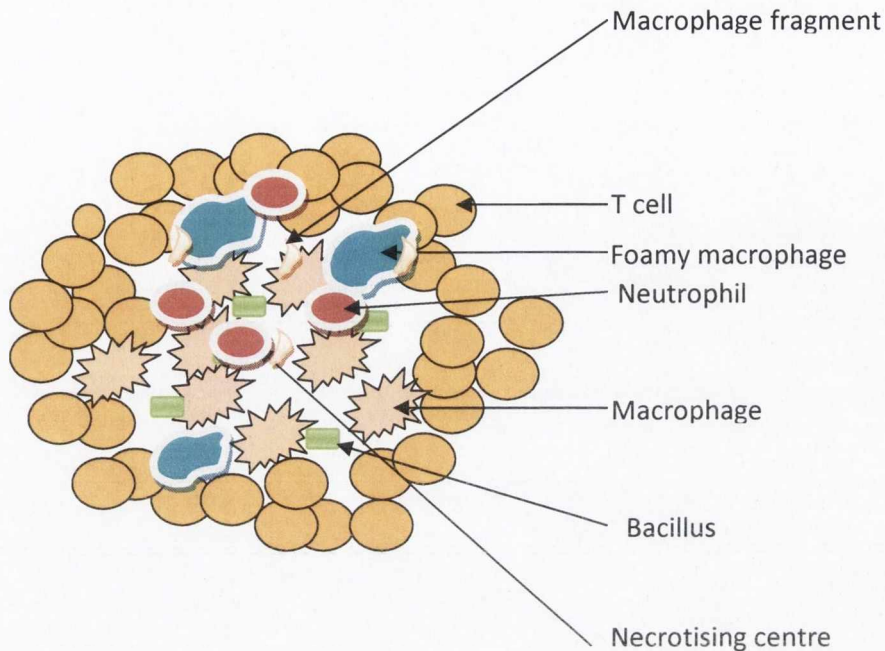


Figure 1.9 Tuberculous granuloma

Upon macrophage phagocytosis of *M. tuberculosis* bacilli, the macrophage produce $\text{TNF-}\alpha$, IL-1, IL-18 and chemokines to recruit neutrophils to the site of infection, whereby they release bactericidal enzymes to destroy the bacteria. More macrophages and common mononuclear phagocytes are recruited to the area, and a granuloma is formed. Entities known as foamy macrophages are also recruited to the granuloma, these macrophages are unable to phagocytose free mycobacteria but they are still capable of acting as antigen presenting cells. The recruitment of T cells occur post infection. The bacterial peptides produced by the macrophages are presented through MHC Class II to CD4^+ T cells, which in turn produce $\text{IFN-}\gamma$, which induces direct anti-mycobacterial activity in macrophages. During this the interior of the granuloma becomes necrotized while the exterior layer remains immunologically active, giving the different layers associated with granuloma maturation.

is required in the control of acute infection with *M. tuberculosis* while TLR2, TLR4 and TLR9 are required for the protection against long-term *M. tuberculosis* infection (Fremond et al., 2007).

Testing for the presence of the disease can be carried out by using chest x-rays, sputum examination, skin testing and DNA based testing. (The Merck Manual – Online medical library, <http://www.merck.com>)

1.12.2 Bacteria in the blood

1.12.2.1 Bacteraemia

Bacteraemia is caused by the presence of bacteria circulating within the blood stream. Bacteraemia can occur frequently throughout our lifetime, it can result from dental procedures and vigorous toothbrushing, from catheterization of an infected urinary tract and can also occur during surgical treatments of abscesses or infected wounds (The Merck Manual – Online medical library, <http://www.merck.com>). Bacteraemia can be caused by both gram positive and gram negative bacteria. Bacteraemia is mainly caused by gram negative and gram positive bacteria, anaerobes and fungi; staphylococcal bacteraemia is commonly found in injection drug users. The activation of endothelial cells by the presence of bacteria causes the expression of proteins involved in blood clotting, which causes blood flow to be cut off in small blood vessels, this acts to prevent the spread of bacterial infection throughout the body (Krishnaswamy et al., 1999). There are usually few symptoms in cases of bacteraemia, however, when the bacterial infection spreads the immune system cannot control the rate of the spread of the bacteria. If symptoms of trachypnea, shaking chills, fever, abdominal pain, nausea,

vomiting and diarrhea occur, this usually suggests the onset of more serious sepsis or septic shock. (The Merck Manual – Online medical library, <http://www.merck.com>).

1.12.2.2 Sepsis and septic shock

Sepsis and septic shock result when there is a systemic inflammatory response to a bacterial infection. Sepsis manifests as an acute inflammatory reaction which is associated with release of numerous inflammatory mediators such as pro-inflammatory cytokines, adhesion molecules and chemokines into the bloodstream. On occasion sepsis can develop to 'severe sepsis' which indicates a progression of symptoms including the failure of at least one organ. The final stage of sepsis is the onset of septic shock. Septic shock can develop when the systemic release of $\text{TNF-}\alpha$ causes vasodilation and loss of plasma volume due to increased vascular permeability. Septic shock is also accompanied by blood coagulation, leading to blood clotting in small blood vessels and causing a dysregulated clotting to occur throughout the body. This can lead to failure of the vital organs, such as the heart, kidneys, liver and lungs (Rigato et al., 1996)

Most cases of septic shock are caused by hospital acquired gram negative bacilli or gram positive cocci. Septic shock mostly occurs in immuno-compromised patients. The pathophysiology of sepsis and septic shock occurs when an inflammatory stimulus such as the bacilli triggers the production of pro-inflammatory mediators such as $\text{IL-1}\beta$ and $\text{TNF-}\alpha$. These cytokines act on endothelial tissue and neutrophils to mediate neutrophil adhesion and activate clotting mechanisms and generate microthrombi. They further act to release immune mediators including leukotrienes, lipoxigenase, histamine, bradykinin, serotonin and IL-2. Vasoactive mediators cause blood flow to

bypass capillary vessels. Poor blood flow through capillary vessels and the presence of microthrombi causes the decrease of oxygen supply, the impairment of carbon dioxide and waste product removal. Decreased oxygen supply and build up of waste products can cause organ dysfunction and in severe cases organ failure. (Merck manual online: Sepsis and septic shock, critical care medicine, 2007).

1.12.2.3 Activation of phagocytes causes the initiation of the acute-phase response

Cytokines released from activated macrophages release cytokines which can have far reaching effects throughout the body to orchestrate the body's response to invading pathogens. The most potent of the released cytokines are IL-1, IL-6 and TNF- α . These three cytokines can act as endogenous pyrogens causing an increase in body temperature by acting on the hypothalamus, inducing fever. During an infection fever is beneficial to the body, as bacteria prefer to grow at lower temperature, most adaptive immune responses work more efficiently and host cells are protected from the dangerous effects of TNF- α at higher temperatures (Ramadori and Christ, 1999). The action of IL-1, IL-6 and TNF- α on hepatocytes in the liver causes a dramatic increase in proteins released from the liver. Proteins which are induced by TNF- α , IL-6 and IL-1 are called 'acute-phase proteins'. These proteins have a broad specificity for binding PAMPs, for example, C-reactive protein binds to the phosphorylcholine region of pathogen associated cell-wall lipopolysaccharide. The C-reactive protein opsonises the bacteria and activates the classical complement cascade by binding to C1q (Ramadori and Christ, 1999). IL-1, IL-6 and TNF- α also act other body organs, such as bone marrow epithelium whereby neutrophils are mobilized to ultimately phagocytose microbes present in the body. Fat tissue and muscle are also acted upon by these

cytokines, releasing energy and proteins to aid in the maintenance of increased body temperature. Finally, TNF- α acts on DCs to stimulate their migration to the lymphatic system (Ramadori and Christ, 1999, Bopst et al., 1998).

1.12.3 *Streptococcus pneumoniae* infections

Streptococcus pneumoniae is a gram-positive cocci, it is the most common cause of community acquired pneumonia, bacterial meningitis, bacteraemia, and otitis media. *S. pneumoniae* infection is also an important cause of sinusitis, septic arthritis, osteomyelitis, peritonitis, and endocarditis. *S. pneumoniae* is an extracellular bacterial pathogen, whose microbial components are recognised by the PRR TLR2. *S. pneumoniae* is estimated to cause more than one-third of the 2 million deaths due to acute respiratory infections. Virtually every child in the world is colonized with one or more strains of the bacteria. Capsular polysaccharides (PS) on the surface of *S. pneumoniae* are the primary virulence factors. Other virulence factors include pneumolysin, which leads to pore formation and osmotic lysis of epithelial cells, autolysin and pneumococcal surface protein A (PspA), which interfere with phagocytosis and immune function of the host.

1.12.4 Malaria

Malaria is a vector borne infectious disease that is widespread in tropical and subtropical regions. It infects between 300 and 500 million people every year and causes between one and three million deaths annually. Malaria is endemic in a broad band surrounding the equator, in South America, South and Southeast Asia, parts of the Middle East, Oceania and much of Africa. The parasite *Plasmodium falciparum*, kills

over 1 million children in Africa alone annually (Cox, 2002). It is thought that malaria has been a human pathogen for the entire history of the human species and it is thought that these highly virulent pathogens cause a high mortality rate among pre-productive individuals and that genetic selection has developed to confer resistance to malaria infection and disease.

Malaria is a disease caused by the infection of the protozoan of the genus *Plasmodium*. The most serious diseases symptoms are caused by *Plasmodium falciparum* and *Plasmodium vivax*. Other forms of the disease are caused by *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*.

1.12.4.1 How malaria is transmitted.

Malarial parasites are transmitted by female *Anopheles* mosquitoes. The mosquito injects malarial sporozoites into the host blood stream. From the blood stream the sporozoites move to the liver and rapidly infect liver hepatocytes. When in the hepatocytes, the parasites rapidly divide and multiply and are now termed merozoites. Both the initial blood and liver stages do not have any pathological implications. The merozoites are then released into the blood stream and it is from this point that the infection is noticeable. The merozoites infect erythrocytes of the blood stream. The parasites multiply within the erythrocytes, causing symptoms that include symptoms of anemia (light headedness, shortness of breath), fever, shivering, flu-like illness, arthralgia (joint pain), and convulsions and in severe cases malaria can cause coma and death. Given the severe impact malaria has had on the human population causing high levels of mortality and morbidity it has become the greatest selective pressure on the human genome in recent history (Kwiatkowski, 2005).

1.12.4.2 Influence of selective pressure on the human genome

The selective pressure caused by the high mortality and morbidity rates has caused many genetic variants to become prevalent within human populations. The effect can be seen clearly in individuals who are heterozygous for hemoglobin S (HbS). Heterozygous individuals are protected against infection and do not succumb to sickle cell anaemia while those who are homozygous for HbS suffer from sickle cell anaemia (Allison, 1954). Secondly individuals with polymorphisms present in the glucose 6 phosphate dehydrogenase (G6PD) gene are protected from malaria as the plasmodium cannot efficiently grow inside the red blood cell due to a lack of host G6PD (Ganczakowski et al., 1995). A build up of oxidative products caused by the absence of G6PD is toxic to the parasite, hence the parasite is unable to divide and multiply. The reduced parasite replication is believed to be the mechanism of protection (Luzzatto et al., 1969).

1.12.4.3 The innate immune response to malaria infection

Macrophages and dendritic cells play a large role in the identification of a protozoan infection and the initiation of an immune response. In addition to the role of antigen presenting cell the macrophage will also employ scavenger receptor CD36 to recognise parasite infected erythrocytes and to subsequently engulf them, in the absence of malarial antibodies (Serghides et al., 2003) (figure 1.10). TLRs found on the surface of macrophages and DCs have been shown to be involved in the initial recognition of the protozoan parasites as well as early resistance to infection, development of acquired immunity as well as pathology observed during acute infection. During early infection TLRs present on the surface of macrophages, dendritic cells and neutrophils recognise the protozoan parasite and produce pro-inflammatory cytokines and express co-

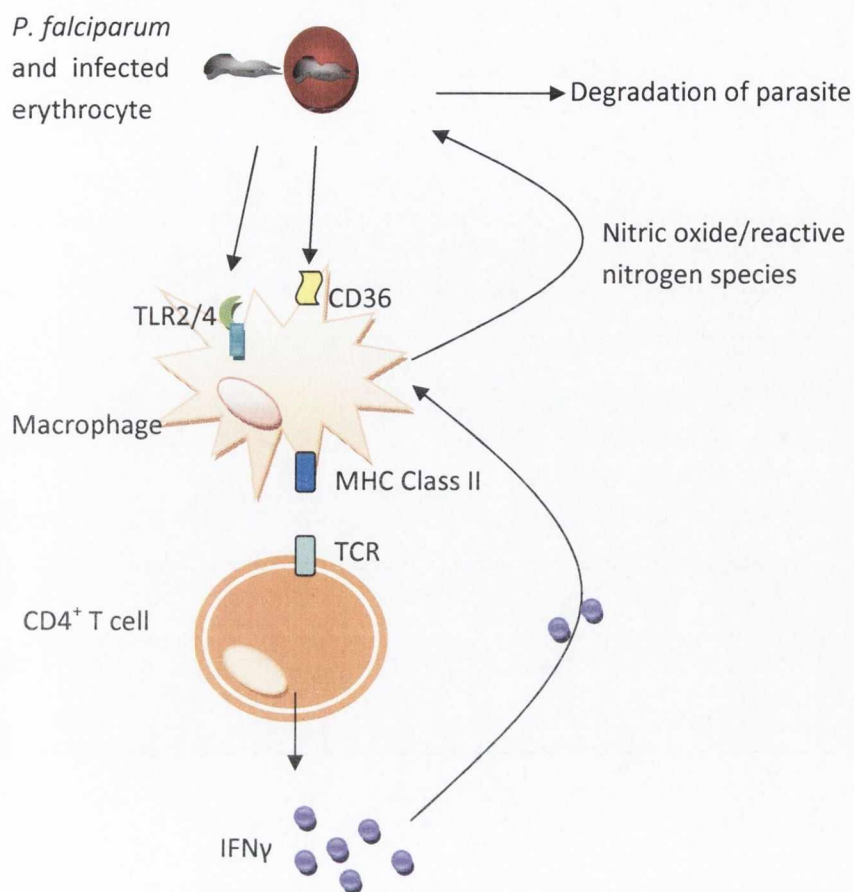


Figure 1.10 The innate immune response to the presence of *P. falciparum*

Glycosylphosphatidylinositol (GPI) molecules from *P. falciparum* activate TLR2 and TLR4 on macrophages. Macrophages also employ CD36 to bind and initiate phagocytosis of plasmodium infected erythrocytes. Activated macrophages signal to CD4⁺ T cells, where the potent anti-microbial mediator IFN- γ is generated. IFN- γ feeds back to the macrophage to up-regulate the production of reactive oxygen and nitrogen species which kill the invading parasite.

stimulatory molecules. TLR2 and TLR4 have been implicated in recognising the malaria associated glycosylphosphatidylinositol (GPI) molecule. The activation of TLR2 and TLR4 causes the up-regulation of pro-inflammatory cytokines TNF, IL-1, IL-6, IL-12, and iNOS (Schofield and Hackett, 1993, Tachado et al., 1997). TLR9 expressed by plasmacytoid DCs and B cells in humans has been implicated in the recognition of the haemoglobin derivative haemazoin. TLR9 recognises the presence of haemazoin in a strictly MyD88 dependent manner, whereby it up-regulates the production of IFN- γ (Coban et al., 2005). Upon phagocytosis of the malaria parasite the macrophage and DC present microbial peptides to CD4⁺ T cells through MHC class II molecules, activating CD4⁺ T cells to produce IFN γ . IFN γ then feeds back to the macrophage to induce the production of nitric oxide and reactive nitrogen species to destroy the malaria parasite (figure 1.10) (Good and Doolan, 1999).

CD8 cytotoxic T cells are also important for the clearance of immune cells that have been infected with protozoa. It has been hypothesised that the lack of MHC I molecules on red blood cells allow the plasmodium to survive undetected.

1.12.4.4 A fine line between pathology and host protection

A rapid and vigorous response to the invading malaria pathogen is important for the elimination of the pathogen. This response is most beneficial to the host at the initial primary infection, however persistent infection and the over-production of high levels of cytokines in malaria is thought to cause the pathology associated with the disease. The immune system is protecting the host by up-regulating the immune response but is also causing the severe pathology that is associated with malaria such as high fever during the progression of the disease. Evidence to support this hypothesis has been shown in cases of *Plasmodium berghei* infection where an overproduction of IFN- γ and TNF/LT-

α is associated with disease pathology (Engwerda et al., 2002). It is thought that in the case of malaria, that the immune response is both beneficial and harmful. It is important to maintain an even balance between eradicating the parasite and triggering harmful disease pathology.

1.13 Are TLR6 and Mal polymorphisms involved in disease?

Professor Adrian Hill and colleagues from the University of Oxford found that two polymorphisms in proteins of the innate immune system were associated with infectious diseases. They found that the Mal Ser180Leu variant was found to protect individuals from invasive pneumococcal disease, bacteraemia, malaria and tuberculosis. When looking at populations from the UK, Vietnam and several African countries that individuals that were heterozygous for this polymorphism were protected. They also carried out studies on individuals that were susceptible to invasive pneumococcal disease and found that a rare polymorphism in TLR6 is associated with increased risk of *S. pneumoniae* infection.

1.14 Aims and objectives

This study is an examination of the role of Mal polymorphism Ser180Leu in TLR2 signalling and to investigate the role of the TLR6 polymorphism Asp30Asn in TLR6 signalling.

The specific goals were as follows:

- Identify the presence of the Mal polymorphism in a healthy Irish population.
- Investigate effects of Mal polymorphism in TLR2 signalling.
- Identify the sub-cellular localisation of Mal Ser180 to Mal Leu180
- Identify innate immune signalling effects of the TLR6 polymorphism.
- Address the sub-cellular localisation that may occur due to the TLR6 Asn30 polymorphism.

The aim of this investigation is to determine whether Mal Ser180Leu polymorphism plays a role in abrogating signalling through TLR2 thus providing protection from infectious disease, and to test whether Asp30Asn polymorphism affects TLR6 signalling which may ultimately play a role in causing susceptibility to disease.

This investigation will assess any role that these polymorphisms may have in innate immune signalling by carrying out functional assays. Identifying a mechanistic role for these polymorphisms in innate immunity will lend further evidence to the role these polymorphisms play in the pathogenesis of infectious disease. Additionally it will be interesting to investigate whether this genetic information can be used to understand more about the biochemistry of Mal and TLR6.

Chapter Two

Materials and methods

Chapter 2 Materials and methods

2.1 Materials

Lipopolysaccharide (LPS) was purchased from Alexis (Braunschweig, Germany). PolyI:C was purchased from Amersham (GE Healthcare UK LTD, Calfont St. Giles, Bucks). MALP-2 was purchased from Alexis (Braunschweig, Germany). FSL-1 and Pam₃CSK₄ were purchased from Invivogen. The human IL-6 and human IL-8 DuoSet ELISA kits were purchased from RnD Systems (Minneapolis, MN). TaqMan Allelic discrimination system was purchased from Applied Biosystems (Foster City, CA, USA).

Maxi prep endotoxin free plasmid purification kits were purchased from Qiagen Ltd. (Fleming Way, Crawley, West Sussex, U.K.). Mini prep kits were purchased from Promega (Madison, Wisconsin, USA). The site directed mutagenesis kit and XL1-blue supercompetent cells were purchased from Stratagene Technopath. RNA extraction kit was purchased from Qiagen Ltd. (Fleming Way, Crawley, West Sussex, U.K.). The fix and perm kit was purchased from ADG (An Der Grub) Bio research GmbH (Germany).

I κ B and p-P38 antibodies were purchased from Cell Signalling (NEB, Ipswich, UK). The beta-actin antibody was purchased from Sigma (Poole, Dorset, UK). Anti-HA antibody was purchased from Abcam (Cambridge, UK). MAb hTLR2-FITC was purchased from Invivogen. Goat anti-hTLR6 was purchased from Santa Cruz Biotechnology Inc (Heidelberg, Germany). Secondary antibody anti-goat-FITC was purchased from Santa Cruz. Anti-FLAG antibody and anti-GFP antibody were purchased from Sigma (Poole, Dorset, UK). Deep red CellMaskTM plasma membrane stain and propidium iodide nucleic acid stain were purchased from Molecular Probes (Invitrogen, Biosciences LTD. Dun Laoghaire, Ireland). RNaseA was purchased from

Sigma (Poole, Dorset, UK). Cell culture materials, DMEM containing L-glutamine and sterile PBS were purchased from Gibco (Biosciences, Dun Laoghaire, Dublin). Ficoll-paque was purchased from Amersham (GE Healthcare Life Sciences, Buckinghamshire, UK). Foetal calf serum was purchased from Biosera (East Sussex, UK). Penicillin streptomycin and trypsin were purchased from Sigma (Poole, Dorset, UK). General laboratory chemicals were purchased from Sigma (Poole, Dorset, UK) with the exception of blasticidin and hygromycin which were obtained from Invivogen (Toulouse, France). GeneJuice^(R) was purchased from Novagen (Merck, Darmstadt, Germany). Glutathione-sepharose 4B beads were purchased from Amersham (GE Healthcare Life Sciences, Buckinghamshire, UK), colentrastazine was purchased from Argus Fine Chemicals (Vernio, Italy), passive lysis buffer was purchased from Promega (MSC, Mulhuddart, Dublin 15), prestained molecular weight marker from New England Biolabs Ltd (Ipswich, UK) and polyacrylamide was purchased from National Diagnostics Ltd. (Biosciences, Dun Laoghaire). DD loop in vitro peptides were obtained from Genscript USA Inc. Metafectene was purchased from Biontex laboratories GmbH (Germany). Recombinant human caspase-1 and caspase-1 inhibitor were purchased from Calbiochem^(R) (Merck Biosciences, San Diego). Enhanced chemiluminescence (20X LumiGLO Reagent and 20X peroxide) was purchased from Cell Signalling Technology^(R) (New England Biolabs, Ipswich, U.K.). All DNA sequencing was carried out by Eurofins MWG. DD loop S and L form peptides were manufactured by Pepscan Presto (Lelystad, The Netherlands).

2.2 Cell lines

Wild type MyD88 adaptor like (Mal) murine embryonic fibroblasts (MEFs) and Mal deficient MEFs were a gift from Dr. Kate Fitzgerald (University Massachusetts, Worcester, MA 01605, USA). Cell lines used in the project were human embryonic kidney cell line (HEK) 293 cell line which was obtained from Tularik Inc, San Francisco, CA 94080. The HEK 293T and U373 human astrocytoma cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC) (Salisbury, U.K.). Cell lines stably transfected with plasmids such as, HEK-293 TLR2, HEK293 TLR2-YFP, HEK293 TLR4 MD2 CD14 and U373-CD14 cells were a gifts from Dr. Kate Fitzgerald (University Massachusetts, Worcester, MA 01605). Whole blood was obtained from the Irish Blood Transfusion Service and PBMCs were isolated in the laboratory.

2.3 Expression vectors

Empty vector pcDNA3.1 was obtained from Invitrogen (Carlsbad, CA). HA-Mal plasmid was cloned in house by Dr. Aisling Dunne (Trinity College Dublin) and site directed mutagenesis was carried out on the HA-Mal plasmid to make HA Mal 180Leu plasmid. pGEX-Mal Ser180 (GST-Mal S180) and pGEX-Mal Leu180 were both made by Dr. Aisling Dunne in house.

Mal Ser180-CFP was received as a gift from Dr. Constantinos Brikos (Trinity College Dublin) and site directed mutagenesis was carried out on the plasmid to generate Mal Leu180-CFP.

TLR6-FLAG and TLR6 YFP was received from Dr. Kate Fitzgerald (University of Massachusetts, USA) and site directed mutagenesis was carried out to generate the TLR6-Asn30 FLAG and the YFP-TLR6 Asn30 construct. pDuo TLR2/6 vector was purchased from Invivogen (Toulouse, France) and site directed mutagenesis was employed to generate the pDuo TLR2/6-Asn30 vector. The plasmid encoding ER-CFP was a kind gift from Harald Husebye (Institute of Cancer Research and Molecular Medicine, NTNU, N-7489 Trondheim, Norway). TLR2-FLAG construct was a gift from Dr. Kate Fitzgerald. pGEM-T Easy vector system was purchased from Promega ((MSC, Mulhuddart, Dublin 15).

2.4 Reporter vectors

The dGL3-5κB plasmid was a gift from Dr. R Hofmeister (Universitat Regensburg, Germany). The phRL-TK vector was purchased from Promega.

2.5 Cell culture

2.5.1 Growth of cell lines from frozen stocks

Cell lines are stored in boxes in liquid nitrogen. All cells are stored at a concentration of 1×10^7 cells/ml in 90% FCS and 10% DMSO in plastic cryo-vials. In order to thaw the cells the cells were removed from the liquid nitrogen and immediately placed at 37°C. U373, HEK293, HEK 293 TLR4 MD2 CD14, HEK293 TLR2, HEK 293 TLR2-YFP, pDUO TLR2/6 Asp30 and Asn30 cell lines, Mal deficient MEFS and Mal WT MEFs were placed in 10ml of DMEM (10% FCS). The cells were centrifuged at 1000g for 3 minutes. The supernatant was removed and the pellets were re-suspended in 10ml of

complete medium. The cells were then placed in a T25 flask. The flasks were placed in a 37°C incubator at 5% CO₂ and grown until confluent.

2.5.2 Growth and maintenance of cell lines

U373, HEK293, HEK 293 TLR4 MD2 CD14, HEK293 TLR2, HEK 293 TLR2-YFP, pDUO TLR2/6 Asp30 and Asn30 cell lines, Mal deficient MEFS and Mal WT MEFs were placed in 10ml of DMEM (10% FCS, penicillin streptomycin 0.1mg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5%CO₂. To use cell lines in specific assays cells were grown up in T75 or T175 flasks. When the cells were confluent they were typically seeded at 1×10^5 cells/ml in 96 well plates, 24 well, 6 well plates or 10cm dishes 24 hours prior to use in any assay. For continuing growth of cell cultures, cells were typically seeded at 1×10^5 cells/ml DMEM and were sub-cultured approximately twice a week. Cell viability was determined each time using trypan blue dye. Trypan blue is not taken up by healthy cells. Cells were counted using a haemocytometer and a light microscope. U373, HEK293, HEK 293 TLR4 MD2 CD14, HEK293 TLR2, HEK 293 TLR2-YFP, pDUO TLR2/6 Asp30 and Asn30 cell lines, Mal deficient MEFS and Mal WT MEFs were removed from the flask by removing DMEM media and washing with 5-10ml sterile PBS, and by incubating the cells at 37°C for 5 minutes with 5ml trypsin-EDTA (0.005mg/ml). Following trypsinisation 10ml of DMEM-10%FCS was added to the flask and the contents of the flask were placed into a 30ml sterilin tube. The tube was centrifuged at 1000 x g for 3 minutes. The supernatant was removed from the pellet and 1ml of DMEM-10%FCS was added to re-suspend each pellet. For continuing growth the cells were divided 1 in 4 or 1 in 3 depending on the requirements of the next experiment.

2.5.3 Generation of human peripheral blood mononuclear cells

Blood was obtained from a healthy donor (20mls) or from the Irish blood transfusion service (IBTS) blood bank. The blood was transferred into a 50ml falcon tube and diluted 1:2 with phosphate buffered saline (PBS). Ficoll paque was used to separate the blood into its constituent parts; red blood cells, PBMCs, and serum. The blood was slowly added to 20mls of ficoll paque. The tubes were centrifuged at 1600 x g for 30 minutes. The white blood cell ring was transferred into a new 50ml tube using a pasteur pipette. The volume was adjusted to 50mls and the samples were centrifuged again this time at 1600 x g for 10 minutes. The supernatant was removed. This step was repeated a second time and the pellet was re-suspended in 10mls of PBS. The cells were counted and seeded in 96 well plates at a concentration of 2×10^6 cells/ml. The cells were incubated for 24 hours at 37°C with 5% CO₂. The following day they were pre-incubated with varying concentrations of peptide for 1 hour. The cells were then stimulated with various ligands. The cells were incubated for a further 24 hours. The media was removed from the cells and used in IL-6 ELISA assays.

2.5.4 Cryopreservation of cell lines

Cells were grown to a state of sub-confluency and were harvested and counted as described in section 2.5.2. The cells were centrifuged at 1000 x g for 3 minutes and were re-suspended in FCS and dimethyl sulfoxide (DMSO) (9:1). Aliquots of the cells at 2×10^6 cells/1ml DMSO-FCS were placed in 1.5ml cryovials. The tubes were placed in a room temperature cryopreservation container containing 40ml isopropanol. The container was placed a -80°C freezer to allow the temperature of the cells to freeze at an

2.6.1 Site directed mutagenesis

The underlined tcg which encodes a serine was mutated to a ttg encoding leucine.

Original sequence 5' acc atc ccc ctg ctg **tcg** ggc ctc agc aga gct 3'

Mutated sequence (Forward Primer) 5' acc atc ccc ctg ctg ttg ggc ctc agc aga gct 3'

Reverse Compliment (Reverse Primer)5' agc tct gct gag gcc caa cag cag ggg gat ggt 3'

Site directed mutagenesis was also used to mutate Asp (D) at position 30 of TLR6 to Asn (N) of the TLR6-FLAG, TLR6-YFP and pDUO TLR2/6 plasmids using the following primers.

Mutated sequence (Forward primer) 5'-TCA AAG GTA TCT TTT AAA CCC-3'

Reverse complement (Reverse primer) 5'-CTA AAT GCC TGA AAC TCA CAA-3'

Sample reactions were prepared as indicated below:

5μl 10X reaction mix

2μl (5ng/μl) plasmid dsDNA

125ng forward primer

125ng reverse primer

1µl of dNTP mix

Made to final volume of 50µl ddH₂O, then added 1µl Pfu DNA polymerase (2.5U/µl).

(A range of DNA concentrations were tried from 5-50ng DNA to optimize the yield.)

The cycling parameters for the site directed mutagenesis were as follows:

Cycles	Temperature and time
1	95°C for 30 seconds
16 cycles of -	95°C for 30 seconds
	55°C for 1 minute
	68°C for 8 minutes

Following the cycling reactions the reactions were placed on ice for 2 minutes to reduce temperature. The reactions were then incubated at 37°C and 1µl of *Dpn I* restriction enzyme (10U/µl) was added directly to each mix. The reaction mixture was incubated at 37°C for 1 hour to allow digestion of the parent supercoiled dsDNA. Plasmids were stored at -20°C until transformed.

2.6.2 Plasmid purification for use in transient and stable transfections

Samples prepared using site directed mutagenesis were transformed into XL1-Blue supercompetent cells from Stratagene Technopath. The supercompetent cells were thawed gently on ice. 50µl of cells were placed in a 1.5ml eppendorf tube and placed on ice. 1µl of reaction mix from the site directed mutagenesis was placed into the 50µl of cells. The transformation reaction was swirled gently and incubated on ice for 30 minutes. The transformation was heat pulsed by placing in a 42°C incubator for 45

seconds and then placed back on ice for 2 minutes. 1ml of LB broth (no antibiotic) was added to the reaction and the tube was placed at 37°C for 1 hour. Following this incubation period, the cells were centrifuged at 1000 x g for 2 minutes and the cells were re-suspended in 50µl LB broth (no antibiotic). The cells were then plated onto an agar plate containing the antibiotic ampicillin. The transformation plates were incubated at 37°C for 24 hours.

2.6.3 Mini preparation of plasmid DNA

DNA plasmids from bacterial colonies were isolated using wizard plus SV miniprep DNA purification system (Promega, Madison WI, USA). Bacterial colonies were isolated and placed in 15ml tubes along with 3ml LB broth. The samples were incubated for 24 hours at 37°C. The following day 1ml of the bacterial cultures were placed in a 1.5ml eppendorf tube and the bacterial cells were pelleted by centrifugation. The bacteria were resuspended in 250µl cell resuspension solution. The bacterial cells were incubated at room temperature for 5 minutes upon addition of 250µl cell lysis solution. Following this incubation, 350µl of neutralization solution was added to the eppendorf tube. The mixture was centrifuged at room temperature for 10 minutes at 13,000 x g. The cleared lysate was added to a spin column inserted into a 2ml collection tube. The spin column was centrifuged at 13,000 x g for 1 minute to allow the lysate move through the spin column and into the collection tube. The contents of the collection tube were discarded and 750µl and then 250µl of wash solution were added to the spin column. The tube was centrifuged again for 2 minutes and the contents of the collection tube were discarded. A 1.5ml eppendorf tube was placed under the spin column and 80µl nuclease free H₂O was placed in the spin column. The

tube was centrifuged and the DNA was eluted into the 1.5ml eppendorf tube. The insert was then sequenced to verify that the selected clone possessed the required mutation.

2.6.4 Plasmid purification procedure

Plasmids were purified using endofree plasmid maxi kit from Qiagen. 100ml of bacteria containing the plasmid of interest was cultured in a 250ml conical flask overnight at 37°C shaking at 200 rpm. Before cells were harvested glycerol stocks could be made aseptically by mixing 900µl bacterial culture with 100µl glycerol and then freezing the bacterial cells at -80°C for long term storage. The remainder of the cell culture was centrifuged at 6000 x g for 15 minutes at 4°C. The pellet was then re-suspended in 10ml buffer P1. 10ml of buffer P2 was added to the suspension, buffer P2 contained SDS to lyse the cells. The suspension was vigorously inverted 4-6 times and incubated at room temperature for 5 minutes to allow lysis to occur. Meanwhile, a cap was screwed into the outlet nozzle of the QIAfilter maxi cartridge and placed onto a rack. After the 5 minute incubation, 10ml of buffer P3 (neutralization buffer) was added to the suspension and the tube was inverted 4-6 times to ensure thorough mixing. The bacterial lysate was poured into the maxi cartridge and incubated at room temperature for 10 minutes. The cap was then removed and the lysate was pushed through the cartridge using a plunger into a 50ml falcon tube. 2.5ml of buffer ER (endotoxin removal) was added to the tube and mixed by inverting, and the tube was incubated on ice for 30 minutes. During the 30 minutes the QIAGEN-tip was equilibrated by adding 5ml equilibration buffer and allowing it to flow through by gravity. After the 30 minute incubation the lysate was added to the QIAGEN-tip and was allowed to flow through by gravity flow. The tip was washed using 2 x 15ml buffer QC. A 50ml falcon tube was placed beneath the tip and 15ml of buffer QN was

added to the tip for elution of the DNA. The DNA was precipitated by adding 10.5ml room temperature isopropanol to the eluted DNA. The tube was centrifuged at 15,000 x g for 30 minutes at 4°C. Following this, the supernatant was decanted off carefully. The pellet was washed in 5ml endotoxin free room temperature 70% ethanol and centrifuged for another 10 minutes at 15,000 x g. The supernatant was carefully removed and the pellet was left to air dry. The dried pellet was resuspended in 500µl buffer TE or nuclease free water.

2.7 Transient transfection

GeneJuice^(R), the liposomal based transfection reagent from Novagen was used in the transfection of HEK 293 TLR2, HEK 293 TLR4 MD2 CD14, HEK 293T pDUO TLR2/6 WT and HEK 293T pDUO TLR2/6 D30N cells. Metafectene was used to transfect wild type and Mal deficient MEFs. Cells to be transfected were seeded at 2×10^5 cells/ml in either 96 well, 6 well, 24 well or 10 cm dishes. As a rule, a ratio of 3:1 of GeneJuice^(R) to plasmid was used (3µl of GeneJuice per 1µg of plasmid). This ratio could be altered to optimize the transfection efficiency.

2.8 Luciferase gene reporter assay

2.8.1 Luciferase gene reporter assay transfection

Cells to be transfected were seeded at 1×10^5 cells/ml in 96 or 24 well plates. The cells were incubated for 24 hours at 37°C with 5% CO₂. The Mal WT and Mal deficient MEFs were transiently transfected with different concentrations of plasmid (a) 50ng

Mal S180 + 50ng EV, (b) 50ng Mal L180 + 50ng EV and (c) 50ng Mal S180 + 50ng Mal L180.

In all cases (MEFs, HEK 293s and HEK 293T cells) the amount of DNA used per transfection was normalised using the appropriate concentration of relevant empty vector control. Plasmids used were the κ B reporter plasmid and the TK renilla normalization control plasmid. 0.8 μ l of GeneJuice was mixed with 9.2 μ l serum free DMEM per transfection and incubated at room temperature for 5 minutes. 30 μ l of this mixture was added to triplicate amounts of plasmid. The mixture was incubated for 15 minutes at room temperature. 10 μ l of DNA/GeneJuice(or Metafectene)/DMEM mix was added per well to the cells. The cells were left to recover for at least 24 hours at 37°C before harvested and a luciferase assay carried out.

2.8.2 Cellular lysate preparation

Cells were transfected in 96 well plates as described in section 2.7.1. The following day the medium was removed from the cells. The dish was patted onto tissue paper to ensure the removal of all the media. The cells were lysed for 15 minutes at room temperature with 50 μ l (96 well plates) 1X Passive Lysis Buffer (Promega) and 80 μ l passive lysis buffer for 24 well plates. The cells were observed under the microscope to ensure that full lysis had occurred. The lysates were placed onto a white 96 well plate conducive for luminescent readings. When doing an assay on a 96 well plate 20 μ l of the lysed cell mix was taken and tested for firefly luciferase activity and 20 μ l of the lysed cell mix was tested for renilla luciferase activity. When using a 24 well plate for the luciferase assay 35 μ l of lysed cell mix was tested for luciferase and TK renilla activity.

2.8.3 Measurement of luciferase activity

Firefly luciferase activity for a 96 well plate was assayed by the addition of 40 μ l of luciferase assay mix (20mM tricine, 1.07mM (MgCO₃)₄Mg(OH)₂.5H₂O, 2.67mM MgSO₄, 0.1M EDTA, 33.3mM DTT, 270mM coenzyme A, 470mM luciferin, 530mM ATP) to the sample and renilla luciferase was read by the addition of 40 μ l of a 1:1000 dilution of colentrastazine in PBS. Luminescence was read using Mediators PHL luminometer. Firefly luminescence readings were corrected for renilla activity and expressed as luciferase activity over un-stimulated empty vector control.

2.9 Reconstitution assay

Wild type murine embryonic fibroblasts (MEFs) and Mal deficient MEFs were set up into 6 well plates at 1.0x10⁵cells/ml DMEM. The cells were incubated overnight at 37°C to allow for recovery and were transiently transfected after 24 hours with 1 μ g empty vector (EV)/well, 1 μ g Mal S180/well and 1 μ g Mal L180/well 6 well plate. The cells were incubated overnight at 37°C with 5% CO₂. The cells were also seeded into 24 well plates at 1.0x10⁵cells/ml DMEM. After 24 hours the cells were transfected with 500ng/well plasmid DNA. The cells were incubated overnight at 37°C with 5% CO₂. The following day the cells were stimulated with TLR ligands, LPS, MALP-2, Pam₃CSK₄ or PolyI:C for a number of different time points. Subsequently the media on the cells was harvested to carry out enzyme linked immunosorbent sandwich assays (ELISAs) and the cells were lysed using sample buffer to carry out western blotting analysis.

2.10 Western blotting

2.10.1 Cell stimulation and extract preparation

MEFs were seeded at 1×10^5 cells/ml DMEM in 6 well plates and stimulated with various TLR ligands at different time points. The removal of growth media and the addition of cold PBS to the wells terminated the reactions. The cells were washed a total of 3 times in cold PBS. The cells were lysed directly using sample buffer.

2.10.2 Cell lysis with sample buffer

Cells in a 6 well plate which had been washed in cold PBS, were lysed directly using 80 μ l 5X sample buffer (62.5mM Tris-HCl pH6.8, 2%w/v SDS, 10% glycerol, 50mM DTT, 0.1% bromophenol blue in 20ml ddH₂O). The cells were scraped from the well and transferred into 1.5ml eppendorfs. The samples were then sonicated, boiled for 5 minutes and centrifuged for 5 minutes at 13,000 x g. The cell lysates were subsequently run on SDS-PAGE gels or stored at -20°C until required for use.

2.10.3 Sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (PAGE)

Samples were resolved on sodium dodecylsulfate (SDS) polyacrylamide gel using constant current of 25mA per gel. Samples were first run through stacking gel (1ml 30% bisacrylamide mix, 0.75ml 1M Tris pH 6.8, 60 μ l 10% (w/v) ammonium persulfate and 5 μ l TEMED made up to 6ml with H₂O) to condense the protein. The protein was then resolved according to size using 8- 12% polyacrylamide gels (30% bisacrylamide mix, 3.75ml 1.5M Tris pH 8.8, 150 μ l 10% (w/v) ammonium persulfate, 6 μ l TEMED

made up to 15ml with H₂O). Samples were resolved with pre-stained protein markers (New England Biolabs) as molecular weight standards.

2.10.4 Transfer procedure

The resolved proteins were transferred to nitrocellulose membrane or polyvinylidene difluoride (PVDF) (Sigma) using a wet transfer system with all components soaked first in transfer buffer (25mM Tris-HCL pH8.0, 0.2M glycine, 20% methanol). The gel was immersed in 1X transfer buffer and placed onto a layer of filter paper and sponge. The gel was covered in nitrocellulose paper or PVDF. A second piece of filter paper was placed on top followed by the second sponge. The entire assembly was placed in a cassette and an electric current of 150mA was applied for two hours.

2.10.5 Blocking the membrane

Once the proteins from the gel had transferred onto the membrane it was removed and placed in 50ml of blocking solution. Blocking solution comprised of 5% w/v Marvel (non-fat dried milk, 5g of marvel was placed into 100ml of Tris Buffered Saline TBS/Tween) or varying percentages (1, 3, 5%) of bovine serum albumin (BSA) in TBS/Tween depending on the requirements of the antibody being used. The nitrocellulose membrane in blocking solution is placed on a gentle rocker for 1 hour at room temperature. After the hour the blot is washed once in TBS/Tween before adding antibody.

2.10.6 Antibody incubations

The blot was placed into a 50ml tube containing 4ml of primary antibody of interest at 1:500 to 1:5000 dilutions, depending on the antibody. The tube was left to roll for 1 hour at room temperature. The blot was then removed and washed 3 times in TBS/Tween for 5 minutes each.

The blot was then placed into a 50ml tube containing 4ml of an appropriate secondary antibody. The tube was rolled for 1 hour at room temperature; the blot was removed and washed 3 times in TBS/Tween for 5 minutes each. The blots were developed by enhanced chemiluminescence (ECL) (Cell signalling technologies) according to manufacturer's instructions.

2.10.7 Stripping and re-probing

A blot can be probed for more than one protein if the membrane is stripped of the initial antibody and re-probed with the new antibody of interest. The blot was washed once in 1x TBS/Tween, to remove the chemiluminescent agent. The membrane was striped by placing it in 4ml of 1x re-blot plus solution (Chemicon International), it was left to rock for 15 minutes at room temperature. The membrane was blocked again in 50ml Marvel or BSA/TBS-Tween for 1 hour and probed with primary and secondary antibodies and then developed as before using enhanced chemiluminescence.

2.11 Enzyme linked immunosorbent assays (ELISAs)

2.11.1 Sample preparation

U373-CD14 human astrocytoma cell lines and HEK-293 pDUO TLR2/6 Asp30 and Asn30 cells were seeded at 1×10^5 cells/ml into 96 well plates (150 μ l/well) and were incubated overnight at 37°C with 5% CO₂. After 24 hours cells the medium on the

U373-CD14 cells was removed and replaced with fresh medium. The media on the HEK 293T pDUO cells was untouched. After 24 hours the cells were either treated or left untreated with appropriate ligand or peptide depending on the assay. A further 24 hours later the media was removed to a new sterile 96 well plate and either assayed the same day or stored at -80°C. A DuoSet ELISA kit from RnD systems was used to carry out human interleukin-6 (IL-6) human interleukin-8 (IL-8) sandwich ELISAs. The ELISA kits were used to measure natural human IL-6 and IL-8 in cell culture supernatants.

2.11.2 Plate Preparation

Each capture antibody was diluted in PBS to a working concentration.

Human IL-6 capture antibody working concentration; 2µg/ml

Human IL-8 capture antibody working concentration; 4µg/ml

A high binding flat bottomed 96 well plate was coated with 100µl per well of the diluted capture antibody. The 96 well plate was sealed and incubated overnight at room temperature. Each plate was rinsed three times by placing in a bath of wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4), and then blotted on clean tissue to remove excess wash buffer. Complete removal of the wash buffer was essential for consistent results. The plate was blocked by adding 300µl of reagent diluent (1%BSA in PBS, pH 7.2-7.4) to each well. The plate was then incubated at room temperature for a minimum of 1 hour. The wash step was repeated.

2.11.3 Assay procedure

The IL-6 standard (600-0pg/ml), IL-8 standard (2000-0pg/ml) and samples were prepared. Each sample or standard was diluted in growth medium (10%FCS in DMEM)

and 100µl was added per well. The plate was covered with an adhesive strip and incubated for two hours at room temperature. The wash step was repeated as in section 2.11.2. Detection antibody was diluted in reagent diluent and 100µl was added to each well.

Human IL-6 detection antibody working concentration: 200ng/ml

Human IL-8 detection antibody working concentration: 20ng/ml

A new adhesive strip was applied to the plate and the plate was incubated at room temperature for two hours. The wash step was repeated. Streptavidin-HRP was diluted 1:200 to a working concentration in reagent diluent and 100µl was added to each well. The plate was covered in an adhesive strip, placed away from direct light and the plate was incubated for 20 minutes. The wash step was repeated. Substrate solution was added at 100µl per well and incubated for 20 minutes at room temperature. The plate was placed away from direct light. In order to stop the reaction 50µl of stop solution (2N H₂SO₄ diluted in H₂O) was added to each well. The plate was gently tapped to ensure thorough mixing. The optical density of each well was determined immediately using a microplate reader set to the appropriate wavelength (450nm). A standard curve is obtained by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis a curve is drawn through the points on the graph the concentration of the samples are extrapolated from the standard curve.

2.12 GST Pulldown

2.12.1 Purification of GST-Mal S180 and GST-Mal L180

Bacterial cultures of pGEX, pGEX Mal-S180 and pGEX Mal-L180 were cultured overnight at 37°C in a shaker incubator. 5ml of each culture was transferred into a 2 litre conical flask and 250ml of LB broth was added to the flask. The cultures were

grown at 30°C in a shaker. The optical density 600 (OD600) of the cultures were checked after 2 hours. When the OD600 reached 0.5 50µl of 1mM isopropylthio-β-D-galactoside (IPTG) was added to each flask to induce the recombinant protein expression. The flask was incubated at 30°C for 1 hour with vigorous shaking. The cells were harvested by centrifugation at 10,000 x g. The pellets were either stored at -20°C or they were lysed to extract proteins immediately.

2.12.2 GST protein purification

The bacterial culture pellets were re-suspended in 30ml NETN buffer (20mM Tris, 100mM NaCl, 1mM EDTA, 0.5% NP40, pH 8.0) with protease inhibitors (leupeptin 10mg/ml, aprotinin, PMSF 10mg/ml). The cells were disrupted using a sonicator. The suspension was put into a 50ml tube and the tube was placed on ice. The sonicator probe was placed into the cell suspension and the cells were sonicated for 5 minutes. The sonicated solution was placed into a 50ml centrifuge tube and the suspension was centrifuged for 30 minutes at 39000 x g. 20ml NETN buffer was added to 1ml of GST beads to wash the beads. The beads were centrifuged at 2200 rpm for 5 minutes. The NETN buffer was removed using a pipette. A small volume of NETN buffer was left in the tube. The beads were mixed with the NETN buffer and 300µl of beads were added to each cell suspension. The tube was incubated with a rolling action at 4°C for 2 hours. The solution was centrifuged at 680 x g for 5 minutes. The NETN was aspirated off and 30mls of NETN buffer was added to the pellet. The pellet was re-suspended and the solution was centrifuged at 680 x g for 5 minutes. The wash step was repeated four times. 10µl of beads was added to 15µl 5X sample buffer (5ml glycerol, 10% SDS 10ml, bromophenol blue 10mg, Tris HCl 1M pH 6.8 6.25ml, H₂O 28.75ml, β-mercaptoethanol 250µl per ml) the samples were boiled and were resolved

on a 12% SDS-PAGE gel. The gel was stained with coomassie blue dye overnight and de-stained for 1 hour to approximately assess the concentration of protein present on the beads.

2.12.3 Cell lysate preparation

HEK 293 cells were seeded into seven ten centimetre dishes at 1.5×10^5 cells per 1 ml of media. The cells were incubated overnight at 37°C. The following day the cells were transfected with 6µg FLAG-tagged TLR2 plasmid per well. Each 10cm dish was lysed using 800µl high stringency buffer (Hepes pH 7.5 50mM, NaCl 150mM, EDTA 2mM, 1% NP40 and 0.5% Sodium deoxycholate) containing protease inhibitors (leupeptin 1µl/ml [stock 100mg/ml], aprotinin 5µl/ml, PMSF 10µl/ml [stock 100mM]). The cells were lysed at 4°C for 1 hour using a rolling motion. The GST and GST-Mal S180 were diluted in NETN buffer so they were at the same concentration as the GST-Mal L180. The cells lysates were pooled together into a 30ml tube. The lysates were incubated with the glutathione coupled GST, GST-Mal S180 and GST-Mal L180 for 2 hours at 4°C. The samples were resolved on 12% SDS-PAGE gels and immuno-blotting was carried out.

2.12.4 DD loop blocking peptide – *in vitro*

Blocking peptides were synthesized by Genscript Corp. against amino acids in DD-loop region of Mal. Control peptides which are scrambled version of the blocking peptides were also synthesised.

Peptide 1: TIPLLSGLSR

Peptide 2: LSRGSPTLIL

HEK 293 cells were seeded at 1×10^5 cells per ml DMEM into 6 well plates. The cells were transiently transfected with 2 μ g FLAG-TLR2 per well. After 24 hours, a GST pull-down assay was performed, cells were washed with 10ml cold PBS and the cells were lysed in high stringency buffer containing protease inhibitors (leupeptin 1 μ l/ml [stock 100mg/ml], aprotinin 5 μ l/ml, PMSF 10 μ l/ml [stock 100mM]). The FLAG TLR2 lysates were incubated over night at 4°C with purified glutathione-coupled GST alone, Mal S180 GST with DD loop peptide and Mal S180 GST with scrambled peptide. The GST samples were centrifuged at 320 x g for 5 minutes and the beads were washed with 1ml high stringency buffer three times and in 1ml PBS once. All buffer was removed from the beads and 40 μ l sample buffer (1ml sample buffer 50 μ l DTT) was added to each sample. The samples were resolved on a 12% SDS-PAGE gel using constant current of 25mA per gel. Each protein gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature with 5% marvel and the probed for FLAG tagged TLR2.

2.12.5 GST pulldown assay FLAG TLR2/GST-Mal interaction

HEK 293 cells were seeded at 1×10^5 cells per ml DMEM into 10cm dishes. After 24 hours the cells were transfected with 6 μ g FLAG TLR2 plasmid. After 24 hours the cells were washed with 10ml cold PBS and the cells were lysed in high stringency buffer containing protease inhibitors. The FLAG tagged TLR2 lysates were incubated over night at 4°C with purified glutathione-coupled GST, GST-Mal S180 and GST-Mal L180. The samples were washed three times in high stringency buffer and 40 μ l of sample buffer containing 5% DTT was added to the beads. The samples were resolved on a 12% SDS- PAGE using constant current of 25mA per gel. Each protein gel was

transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature in 5% marvel and probed for FLAG tagged TLR2

2.13 DD loop peptide in cell based assays

Peptides were designed to the 9 amino acid sequence surrounding S180 of Mal. Two sets of peptides were generated, one that contained the serine and a second that contained the leucine. The peptides were tethered to a nine arginine repeat sequence which aids in the cellular internalisation of the peptide. Scrambled forms of the peptides were also generated and were used as controls. The S form peptide and the scrambled S form peptide had a high purity value at 93.28% and 96.55% respectively; the amino acid change in the L form peptide and the scrambled L form peptide lowered their purity values to 73.44 and 70.15%. The soluble peptides were manufactured by Pepscan Presto (Lelystad, The Netherlands).

Sequences:

PEP-052: acetyl-TIPLLSGLSRRRRRRRRRR-amide 93.28% purity- S form

PEP-053: acetyl-LPSGLTILSRRRRRRRRRR-amide 96.55% purity- Scrambled S form

PEP-054: acetyl-TIPLLLGLSRRRRRRRRRR-amide 73.44% purity- L form

PEP-055: acetyl-LPIGLSLTLRRRRRRRRRR-amide 70.15% purity- Scrambled L form

The reduced purity in the L form peptides was due to the presence of a shorter version of the peptide that was missing an arginine residue.

Experiments were carried out in U373-CD14 human astrocytoma cell line and human peripheral blood mononuclear cells (PBMCs).

2.13.1 Peptide blocking – cell based assays

U373 human astrocytoma cells stably transfected with CD14 (U373-CD14), cells were seeded at 1×10^5 cells/ml DMEM-10%FCS in 96 well plates. The cells were incubated over night at 37°C. Blocking peptides of the DD loop of Mal Ser180 region were synthesized.

Blocking peptides were added to the cells at concentration; 100µM 500µM, 25 µM, 12.5µM, 6.25µM, 3.125M and 0µM. The cells were incubated at 37°C for 1 hour. After 1 hour the cells were stimulated with TLR ligands; Pam₃CSK₄ (10µg/ml TLR1/2 ligand), MALP-2 (5nM, TLR2/6 ligand) and PolyI:C (10µg/ml; TLR3 ligand). The cells were incubated for 24 hours. The media was collected and a human IL-6 ELISA and was performed on the supernatants.

2.14 Confocal microscopy

2.14.1 Preparation of coverslips

Sterile coverslips were coated with 1mg/ml poly-L-lysine. Excess poly-L-lysine was aspirated off after 1 minute. The cover-slips were rinsed once with 2 ml sterile PBS and left to dry for 1 hour in a laminar flow hood.

2.14.2 Preparation of slides

Pre-prepared coverslips from section 2.14.1 were placed in a 6 well plate and HEK 293T and WT MEFs were seeded onto the plates at concentration of 1×10^5 cells/ml DMEM. After 24 hours wells were transfected with 2µg of either Mal Ser180-CFP, Mal Leu180-CFP plasmid, EV-GFP, TLR6 Asp30-YFP or TLR6 Asn30-YFP. The cells were incubated overnight at 37°C and 5% CO₂. After 24 hours cells were rinsed

gently twice in PBS. The cells were fixed in 2% formaldehyde for 15 minutes at room temperature. The cells were washed three times with 3ml PBS. In order to permeabilise the cells 0.2% (v/v) Triton X 100 in PBS was added and the cells were left on ice for 10 minutes. If using an antibody in the experiment the cells were blocked in 50%BSA-PBS solution for 30 minutes. A fluorescently labelled antibody or labelled secondary antibody was diluted in 5%BSA-PBS and was added to each well for 1 hour at room temperature. The 6 well plate was placed in the dark during the incubation. If staining the nuclei with propidium iodide the cells were incubated for 20 minutes at 37°C with RNaseA, washed and incubated with propidium iodide for 5 minutes before washing. The coverslips were washed three times in 5%BSA-PBS and subsequently dipped into distilled water. In the dark each coverslip was mounted onto a slide with 4µl of mounting solution (1ml 90% glycerol 10% PBS) containing 50mg/ml propyl gallate 'anti-fade' reagent. The coverslip was sealed with clear nail varnish to prevent drying and movement under the microscope. The cells were observed with a confocal microscope.

2.15 Membrane fractionation assay

HEK293T cells were seeded at 1×10^5 cells/ml in 10 cm dishes and incubated over night at 37°C with 5% CO₂. The cells were transfected with 6µg Mal Ser180-CFP, Mal Leu180-CFP, TLR6-Asp30 YFP or TLR6 Asn30-YFP plasmid per 10cm dish. The cells were harvested and washed three times with PBS. Pellets were re-suspended in 500µl membrane re-suspension buffer and subjected to 35 strokes of a tight fitting Dounce homogenizer. Samples were centrifuged in an ultracentrifuge at 97,000 x g for 1 hour at 4°C. The supernatant (cytosolic fraction) was removed and 100µl sample buffer with 5% DTT was added. 50µl of sample buffer was added to the pellets

(membrane fraction). The samples were examined by Western blotting (section 2.10) using primary anti-GFP and anti murine secondary antibody.

2.16 SNP genotyping – real time – polymerase chain reaction (RT- PCR)

Genotyping of the Irish population was performed using TaqMan Pre-Designed Genotyping Allelic Discrimination System (Applied Biosystems, Foster City, CA). Four hundred and ninety four healthy individuals aged ≤ 18 years were studied. Applied Biosystems have designed genotyping assays for over 2.5 million SNPs that were found by the Hap Map project and so a probe for the Mal Leu180 SNP was ordered. The system used 25 μ l reactions in a 96 well plate.

For a well of a 96 well plate use 12.5 μ l TaqMan universal PCR master mix, 1.25 μ l TaqMan SNP genotyping assay mix, 11.25 μ l genomic DNA diluted in water (diluted to between 1-20ng). The PCR was carried out as follows; initial step 10 minutes at 95°C, denaturing step 15 seconds at 92°C and annealing/extending step is 1 minute at 60°C. The denaturing and annealing/extending step was repeated for 40 cycles. Genotypes were determined using ABI Prism (Applied Biosystems, Foster City CA).

2.17 Caspase-1 cleavage assay

HEK 293T cells were seeded into 10 cm dishes at 1×10^5 cells/ml DMEM and were incubated at 37°C at 5% CO₂ for 24 hours. After 24 hours the cells were transiently transfected with 6 μ g/ml HA-Mal Ser180, HA-Mal Leu180 and HA-EV. The cells were incubated for a further 24 hours before harvesting. The cells were washed in 2ml cold PBS and were lysed in 300 μ l low stringency buffer (Hepes pH 7.5 50mM, NaCl 100mM, ETDA 1mM, glycerol 10%, NP40 0.5%) with protease inhibitors (leupeptin

1µl/ml [stock 100mg/ml], aprotinin 5µl/ml, PMSF 10µl/ml [stock 100mM]). The cellular lysates were scraped into 1.5ml eppendorf tubes and were rolled at 4°C for 15 minutes to ensure complete lysis had occurred. The samples were prepared as follows; different volumes of lysates were used to optimise the assay. A hepes buffer was used in this assay (hepes 100mM, sucrose 10%, Chaps 0.1%, pH to 7.5, add DTT 10mM before use). Human recombinant caspase-1 (Calbiochem) was used at 200 units (2µl) in this assay. Caspase-1 inhibitor in this assay was used at 100µM.

Sample 1: HA Mal Ser180 10µl lysate, 10µl buffer

Sample 2 : HA Mal Ser180 10µl lysate, 10µl buffer, + recombinant caspase-1

Sample 3 : HA Mal Ser180 10µl lysate, 10µl buffer, + recombinant caspase-1, + caspase-1 specific inhibitor

Sample 4 : HA Mal Leu180 10µl lysate, 10µl buffer

Sample 5 : HA Mal Leu180 10µl lysate, 10µl buffer, + recombinant caspase-1

Sample 6 : HA Mal Leu180 10µl lysate, 10µl buffer, + recombinant caspase-1, + caspase-1 specific inhibitor

The samples were incubated for 90 minutes at 30°C. After incubation the samples were placed on ice. The reaction was stopped by adding 20µl 5X sample buffer to each sample. The samples were boiled for 5 minutes and were resolved on a 12% SDS-PAGE gel at 25mA. The gel was transferred to a nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature in 5% marvel and probed with anti-HA antibody (1:1000 dilution) for 2 hours at room temperature, the blot was washed three times with TBS-Tween with 5 minute washes. Anti-mouse HRP secondary antibody was added to the blot for 1 hour at room temperature. The

blot was washed 3 times using 5 minute washes in TBS-Tween and the blot was developed using enhanced chemiluminescence.

2.18 Generation of stable cell lines

HEK 293T cells were stably transfected with pDUO TLR2/6 vector and pDUO TLR2/6 Asn30 vector which has been generated using site directed mutagenesis (section 2.6.1). The cells were seeded into 10cm dished at 1×10^5 cells/ml DMEM and were incubated for 24 hours at 37°C with 5% CO₂. The plasmids were transfected into the cells using GeneJuice (section 2.7). The cells were incubated for three days at 37°C with 5% CO₂ to allow transfection to occur. After 3 days incubation, the cells were trypsinized from the plates and placed into 24 well plates to establish an antibiotic killing curve. The pDUO vector possesses a blasticidin resistance gene and so any cell which took up the plasmid was resistant to blasticidin. Blasticidin was added to the cells at concentrations 0µg/ml, 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml in DMEM. The cells with 10µg/ml Blasticidin-DMEM were allowed to divide and cells which were resistant to blasticidin survived. The cells were grown for 2 weeks in antibiotic media. After two weeks the cells were analysed by flow cytometry (section 2.19) using anti TLR2-FITC antibody from Invivogen. The amount of TLR2 present was identified in control HEK 293T cells compared to HEK 293T pDUO TLR2/6 Asp30 and HEK 293T pDUO TLR2/6 Asn 30 cells.

2.18.1 Generation of HEK 293T pDUO TLR2/6 Asp30 and pDUO TLR2/6 Asn 30 clonal cells

Pools of cells identified as having TLR2 present (section 2.19, section 2.18) were diluted so as to have 100 cells in 20ml of DMEM. 200µl was placed into each well of a

96 well plate, in the hope that 1 cell was present in each well. The cells were allowed to grow for a week at 37°C 5%CO₂. During the week the cells were inspected for growth of a single cell colony. Any well that had growth from a single cell colony was marked and grown until confluent. Each clone was moved to a 24 well plate with 10µg/ml blasticidin 500 µl DMEM; then to a 6 well plate with 10µg/ml blasticidin in 3ml DMEM. The cells were subsequently analysed for the presence of TLR2 and TLR6 using fluorescently labelled antibodies.

2.19 Analysis of stable cell lines using flow cytometry

2.19.1 Cell surface analysis of stable cell pools and clones using flow cytometry

HEK 293T, HEK 293T pDUO TLR2/6 Asp30 stable pool HEK 293T pDUO TLR2/6 Asn30 stable cell pool, HEK 293T pDUO TLR2/6 Asp30 clone 1, 6 and 11, HEK 293T pDUO TLR2/6 Asn 30 clones 5,6 and 7 were cell surfaced analysed for the presence of TLR2 and TLR6. Cells were trypsinized from T75 flasks and were counted (section 2.5.2). 1×10^6 cells were used per sample. The cells were blocked in 50% FCS PBS for 30 minutes at room temperature. Following this, the cells were placed in FACS tubes (1×10^6 cells per tube) and were placed on ice. Monoclonal anti hTLR2-FITC (stock at 0.1mg/ml, working concentration at 2µg/ml) (Invivogen) was added to one sample of each cell type, the cell and antibody mix was given a gentle swirl and incubated on ice for 20 minutes. The second sample of each cell type was not incubated with antibody. Following the incubation, the cells were washed with 3ml FACS wash buffer (2%BSA-PBS) and centrifuged at 1000 x g for 3 minutes. The supernatant was discarded and the cells were re-suspended in 500µl sheath fluid (PBS). The cells were now ready for analysis using the Cyan ADP flow cytometer.

The cells were also analysed for the presence of cell surface TLR6. The cells were blocked with FACS blocking buffer (50% BSA-PBS) for 30 minutes at room temperature. Each set of cells were divided in two, so as to have 1×10^6 cells per FACS tube. Anti-TLR6 antibody (Santa Cruz) was added to the second tube nothing was added to the first tube. After the antibody was added to the cells the tube was swirled gently to ensure complete mixing. Both tubes were incubated on ice for 30 minutes. Following the incubation the cells were washed with 3ml FACS buffer (2%BSA-PBS) and were centrifuged at $1000 \times g$ for 3 minutes. The supernatant was discarded and the cells were re-suspended in FACS buffer. The secondary antibody was added to the cells goat anti-mouse FITC for 20 minutes, the cells were incubated in the dark on ice. After the incubation, the cells were washed with 3ml FACS buffer and centrifuged at $1000 \times g$ for 3 minutes. The cells were re-suspended in 500 μ l sheath fluid (PBS) and analysed on a Cyan ADP flow cytometer.

2.19.2 Intracellular staining of stable cell line clones

HEK 293T, HEK 293T pDUO TLR2/6 clones 1, 6 and 11 and HEK 293T pDUO TLR2/6 Asn30 clones 5, 6 and 7 were stained intracellularly for the presence of TLR6. The cells were counted and 1×10^6 cells per 100 μ l FACS buffer (2% BSA-PBS) were used per sample. The cells were blocked using 50% BSA-PBS for 30 minutes at room temperature. The samples were fixed and permeabilized using a fix and perm kit from ADG (An Der Grub, Germany). The blocking solution was washed from the cells by adding 3ml FACS buffer and centrifuging the cells at $1000 \times g$ for 3 minutes. The supernatant was removed and the cells were re-suspended in FACS buffer. Solution A from the fix and perm kit (containing methanol) was added to the cells so as to have 1:1 ratio of FACS buffer and fixing solution. The cells were incubated at room temperature

for 15 minutes at room temperature. Following fixing the cells were washed with 3ml FACS buffer and centrifuged as before. The supernatant was poured off carefully and the cells were re-suspended in FACS buffer. Solution B (permeabilization solution) was added to the cells in a 1:1 ratio (FACS buffer to solution B) and the cells were incubated for a further 15 minutes at room temperature. The cells were subsequently washed with 3ml FACS buffer. The supernatant was removed and the cells were once again re-suspended in FACS buffer. The cells were now ready for the addition of antibodies. The cells of each cell type were divided up so that there was 1×10^6 cells/100 μ l FACS buffer. Sample 1 was untreated, sample 2 received primary and secondary-FITC antibody while sample 3 received secondary-FITC antibody only. The first sample was untreated while anti-human TLR6 N-terminal antibody (Santa Cruz) was added to the second sample at 1:50 dilution (2 μ l in 100 μ l FACS buffer). The cells were incubated on ice for 20 minutes. The cells were washed twice with 2ml cold FACS buffer and the secondary antibody (anti-mouse FITC) was applied to the second and third samples. The cells were incubated on ice for 20 minutes and then washed twice with 2ml cold FACS buffer. The cells were centrifuged at 1000 x g for 3 minutes and the cells were re-suspended in sheath fluid (PBS) awaiting analysis using the Cyan ADP flow cytometer.

2.20 PCR of TLR6 from stable cell lines

HEK 293T, HEK 293T pDUO TLR2/6 and HEK 293T pDUO TLR2/6 Asn30 stable pools seeded into 10 cm dishes with 10 μ g/ml blasticidin DMEM. When confluent the cells were lysed and RNA was extracted from the cell lines using RNA extraction kit from Qiagen.

2.20.1 Reverse transcriptase – PCR of cDNA library

A cDNA library was constructed from the extracted RNA from section 2.20 using random primers. mRNA from the cell lines was made using Improm II kit (Promega). The concentration of mRNA per sample was established and it was found that the concentration of each sample was approximately 500ng/μl.

Reverse Transcriptase- PCR (Step 1):	mRNA	3μl
	Random primers	1μl

The RT-PCR step 1 was then carried out on a thermocycler:

70°C	5 minutes
4°C	5 minutes

Reverse Transcriptase-PCR (Step 2), the following volumes were used per sample:

H ₂ O	5.5μl
Reaction buffer	4.0μl
MgCl ₂	3.0μl
dNTP	1.0μl
RNAsin	0.5μl
Reverse Transcriptase	1.0μl

RT-PCR Step 2 was then carried out on a thermocycler:

25°C	5 minutes
42°C	60 minutes
70°C	15 minutes

2.20.2 Generation of human TLR6 using specific primers

Following on from the generation of the cDNA library, TLR6 was generated from the cDNA library using TLR6 specific primers. Human TLR6 forward and reverse primers which made to generate a 350bp product.

Forward 5'- atg acc aaa gac aaa gaa cc -3'

Reverse 5'- caa tag gat ggc agg ata tc -3'

A PCR was carried out and the following volumes were used per sample:

10X buffer	5µl
dNTPs	5µl
Target (cDNA)	2µl
Taq polymerase	0.4µl
Sterile deionised water	35.6µl
Forward TLR6 primer	1µl
Reverse TLR6 primer	1µl

The samples were run on a thermocycler using the following cycles:

Step 1	94°C	3 minutes
Step 2	94°C	45 seconds
Step 3	55°C	45 seconds
Step 4	72°C	1.5 minutes
Step 5	Return to step 2 x 35 times	
Step 6	72°C	5 minutes
Step 7	Hold at 4°C	

Following the PCR the products were resolved on a 1% agarose (TE Buffer) gel (100ml TE, 10µl ethidium bromide) using 100V constant current. The PCR products were visualised under UV light. The PCR products were cut from the gel and were purified using a mini prep DNA isolation system from Promega.

2.20.3 Insertion of TLR6 and TLR6 Asn30 into pGEM-T easy vector

The TLR6 and TLR6 Asn30 products were ligated into the pGEM-T easy vector so as to allow for product sequencing.

The pGEM-T easy vector ligation for each sample was carried out as follows:

2X rapid ligation buffer	5µl
pGEM-T easy vector	1µl
PCR product	10µl
T4 DNA ligase	1µl

The reaction mixtures were incubated for 24 hours at room temperature.

The following day the reaction mixtures were transformed into competent DH5α *E.Coli* cells. The following day the bacterial cells were plated onto X-Gal (20mg/ml) plates and were incubated for 24 hours at 37°C. After 24 hours the plates were observed for growth of colonies. White colonies indicated the presence of the insert. The colonies were picked from the agar plates and grew up in 3ml LB broth over night at 37°C with shaking (200rpm). The DNA was isolated from the bacterial clones the following day (section 2.6.3) and the DNA was sent to Eurofins MWG for sequencing.

2.21 Co-expression of IRAK4 with Mal L180 and Mal S180

HEK 293T cells were seeded at 1×10^5 cells/ml DMEM into 6 well plates. The cells were incubated at 37°C with 5% CO₂ for 24 hours. After 24 hours the cells were transiently transfected with 125ng/well Mal L180 or Mal S180 and increasing concentrations of IRAK4 (125ng, 250ng, 500ng, 1µg/well). Each set of cells were normalised with empty vector control so as to have a total of 1125ng of DNA per well. The cells were incubated for 24 hours, the cells were washed with 2ml cold PBS and placed in 1.5ml eppendorf tubes. The cells were lysed in 80µl sample buffer with DTT

(1ml sample buffer 50µl 1M DTT). The samples were sonicated for 3 seconds and boiled for 5 minutes. The samples were resolved on a 12% SDS-PAGE gel using constant current of 25mA per gel. Each protein gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature in 5% marvel-TBST and probed for HA. The blot was developed by enhanced chemiluminescence.

Chapter Three

Single nucleotide polymorphism of Mal affects TLR signalling and provides protection from disease

Chapter 3

3.1 Introduction

At the outset of this project our collaborator Professor Adrian Hill and colleagues carried out genotyping analysis on the Mal gene. Thirty three single nucleotide polymorphisms (SNPs) were genotyped in a case controlled study of 6106 individuals from Gambia, Kenya, United Kingdom and Vietnam. Importantly a SNP was found which was predicted to convert a serine at position 180 to a leucine. The serine to leucine variant was assessed and heterozygous carriage of this gene was found to be independently associated with invasive pneumococcal disease (IPD), bacteraemia, malaria and tuberculosis (TB).

Mal is a 256 amino acid long protein. There are seven non-synonymous or mis-sense SNPs Ala9Pro, Arg13Trp, Ser55Asn, Asp96Asn, Ser180Leu, Val197 Ile and STOP222Trp associated with Mal. Professor Hill found Ser55Asn and Ser180Leu significantly associated with IPD in Caucasians but in the Gambian study only markers Arg13Trp and Ser180Leu were associated with malaria. The Arg13Trp, Ser180Leu and Val197Ile were genotyped in the Kenyan bacteraemia studies. Arg13Trp was not detected in 1000 individuals and Val197Ile was not found to be associated with bacteraemia. The variant Ser180Leu was found to be associated with general bacteraemia. The Ser180Leu variant was shown to be independently associated with each disease population studied.

Microbial ligands associated with malaria causing *Plasmodium falciparum* such as glycoso-phosphatidyl inositol (GPI) lipids have been shown to activate TLR2, TLR6 and TLR4. Similarly microbial lipopeptides from *Mycobacterium tuberculosis* and *Streptococcus pneumonia* have been found to activate TLR2 and TLR6. Each of these

TLRs utilizes Mal as its intracellular adaptor protein, allowing the TLRs to mediate downstream pro-inflammatory signalling.

This chapter investigates a single variant of Mal which has been shown to half the risk of malaria, TB bacteremia and IPD, with a four fold decreased risk of severe malaria.

It is scientifically important to attempt to identify variants at a genetic level which may influence the immune system at a biochemical level.

In this chapter TLR2 signalling was hindered in the presence of the Mal Ser180Leu variant compared to wild type Mal.

3.2 Results

3.2.1 Identification of Mal variants reported on dbSNP and their location within functional domains of Mal.

All single nucleotide polymorphisms (SNPs) associated with Mal were identified using the on line SNP database dbSNP from NCBI. The Mal SNPs were arranged in the Mal sequence to identify the functional regions that they are situated in. All Mal SNPs Ala9Pro, Arg13Trp, Ser55Asn, Asp96Asn, Ser180Leu and Val197Ile are displayed in figure 3.1 and are highlighted in red. The Arg13Trp mutant is situated in close proximity to the SOCS-1 ubiquitination site which consists of two lysines. The SOCS-1 ubiquitination site becomes important for Mal in TLR signalling after TLR2 and TLR4 stimulation when it becomes phosphorylated by BTK at Tyr86, Tyr106, Tyr159 and Tyr187. Consequently Mal interacts with SOCS-1, this interaction causes the polyubiquitination of Mal and its subsequent degradation (Mansell et al., 2006). The Ser55Asn SNP is situated within the PEST domain (amino acids 40-56). Mansell and colleagues have found that the 'PEST' domain present in Mal is rich in proline, glutamate, serine and threonine residues. It was found that Mal is a short lived protein which gets degraded 30 minutes after stimulation with various TLR ligands and that the 'PEST' domain is required in this process. A mutation in this region could hinder the degradation process and result in an accumulation of Mal occurring within stimulated cells. The Asp96Asn SNP is situated in box 1 of the TIR domain (figure 1.8). The Ser180Leu SNP also lies within the TIR domain. The Val197Ile SNP is situated within the caspase-1 cleavage site (D-198) of YXXD. Mal is cleaved at position 198 by the cysteine protease caspase-1, releasing a 4kDa fragment from the C-terminus of Mal (Miggin et al., 2007). A polymorphism in this region could hinder cleavage rendering

Mal inactive. As stated above, of these SNPs only Ser180Leu has been shown to be significantly associated with infectious disease.

3.2.2 Genotyping of a healthy Irish population

As Professor Adrian Hill had carried out genotyping analysis on an English population, the identification of the presence of the Mal Ser180Leu variant in the Irish population would be of interest. Genotyping analysis was conducted on 459 healthy individuals (≥ 18 years). The genotyping analysis revealed that the Ser180Leu SNP was present at 24.26% which is very similar to the UK Caucasian population which Adrian Hill found to be present in 26% of the population (figure 3.2).

3.2.3 Investigating the structural significance of Mal L180 polymorphism

3.2.3.1 Secondary structure prediction

As it had been determined that the Mal polymorphism afforded some protection against disease, a model of the secondary structure of the Mal Leu180 (Mal L180) variant was compared to Mal Ser180 (Mal S180) wild type protein. This was carried out to assess if there was a change in the structure of Mal that lead to an alteration in TLR signalling.

The amino acid change from serine (S) to leucine (L) causes the polarity of the amino acid in position 180 to alter. The serine residue (molecular weight 105.09) has a structural formula of $\text{HO-CH}_2\text{-CH(NH}_2\text{)-COOH}$ which is smaller than the leucine $(\text{CH}_3)_2\text{-CH-CH}_2\text{-CH(NH}_2\text{)-COOH}$ (molecular weight 131.17). The serine to leucine change caused a polar hydrophilic neutral residue to be changed to a non polar hydrophobic residue.

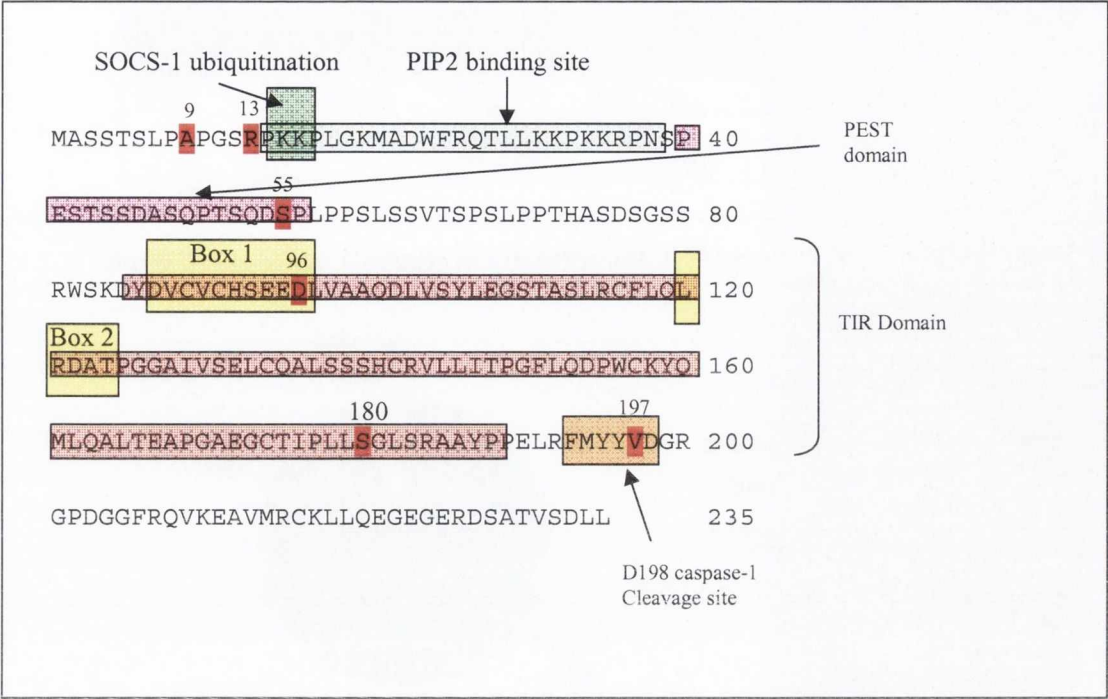


Figure 3.1 SNPs present in functional domains of Mal

The human Mal protein consists of 235 amino acids. It is an intracellular TLR adaptor protein consisting of many functional domains as indicated. SNP Ala9Pro and Arg13Trp are present at the N terminus of Mal. A phosphatidylinositol 4,5-biphosphate (PIP2) binding domain which mediates Mal recruitment to the plasma membrane is located from amino acids 15-35. Ser55Asn is present in the PEST domain (purple) between amino acid 40-56. The TIR domain of Mal shown in pink from amino acid 86to188 and contains the SNPs Asp96Asn and Ser180Leu. The SNP Val197Ile is present in the D198 caspase-1 cleavage site.

Population	Status	Genotype distribution. Number of individuals (%)		
		Ser/Ser	Ser/Leu	Leu/Leu
Irish	Control	324 (70.89%)	113 (24.62%)	22 (4.79%)

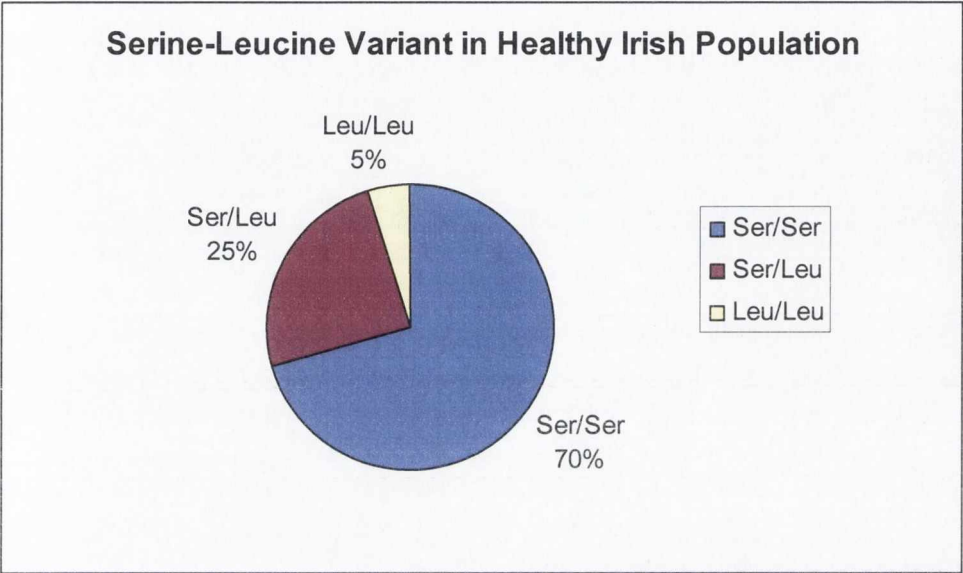


Figure 3.2 Genotyping of a healthy Irish population revealed that the Irish population have the same heterozygosity value as the UK population.

Genotyping of the Mal Ser180Leu polymorphism was performed using TaqMan pre-designed SNP genotyping assay. Genotyping analysis was carried out in 495 healthy Irish individuals aged ≥ 18 years. Genotyping reactions were verified using positive sequenced controls. The Real Time - Polymerase Chain Reaction (RT-PCR) was set up according to Applied Biosystem’s instructions.

70% (324 individuals) of the population were found to be homozygous for Ser/Ser. 25% (113 individuals) of the population were heterozygous for the Ser/Leu variant and 5% (22 individuals) of the population were homozygous for Leu/Leu.

The amino acid sequences of both forms of Mal S180 and Mal L180 were submitted into an online secondary structure prediction programme called PSIPRED from EMBL/Swiss Prot. Figure 3.3 displays the graphical output that was obtained. Amino acid 180 was found to be present in the TIR domain of Mal, between amino acids 86 and 188. The position of the amino acid change is marked by a black square in figure 3.3. No change in secondary structure was observed comparing both forms of Mal.

3.2.3.2 Tertiary structure prediction

Using the computational utility - Molecular Operating Environment, tertiary models of the Mal S180 and Mal L180 were generated (figure 3.4). The Mal S180 structure shows the protruding DD loop structure is composed of a random coil structure. When the serine is changed to a leucine (shown in red) in the L180 Mal model, the protruding DD loop structure changes slightly. The variant leucine form gives it an alpha helical structure and causes the DD loop structure to become more solvently exposed and protrude further out of the protein. This change in tertiary structure could limit Mal's ability to bind potential binding partners such as TLR2, TLR4, IRAK4 or MyD88.

3.2.4 Mal L180 variant is unable to mediate I κ B degradation, p-38 phosphorylation or IL-6 production.

In order to elucidate whether the Mal L180 mutant affected TLR signalling, reconstitution experiments were carried out. The experiments required reconstituting Mal deficient murine embryonic fibroblasts (MEFs) with empty vector or plasmids encoding Mal S180 and the variant L180.

As shown in figure 3.5 wild type MEFs were transfected with empty vector and were found to show I κ B degradation after 30 minutes stimulation with MALP-2. Mal deficient MEFs were transfected with empty vector and showed impaired I κ B α degradation. The absence of degradation after 30 minutes indicates that without the presence of Mal S180 the TLR2 signal transduction pathway is impaired. After reconstitution with Mal S180 I κ B degradation is restored. The Mal deficient MEFs were transfected with Mal L180, stimulated with MALP-2 and were analysed for I κ B degradation. I κ B degradation was not restored after reconstitution with the Mal L180 indicating that the TLR2 signalling pathway requires Mal Ser180.

The inability of the Mal L180 to restore signalling relative to Mal S180 was not due to the difference in expression of each protein as shown in figure 3.6.

Analysis of p38 activation was then carried out. As shown in figure 3.7 wild type MEFs were transfected with empty vector, p38 phosphorylation was demonstrated after 5 minutes stimulation with MALP-2. Mal-deficient MEFs were transfected with empty vector and it was found that MALP-2 was unable to induce phosphorylation of p38. Mal-deficient MEFs were reconstituted with Mal S180 and were shown to restore TLR2 signalling when ligated with MALP-2. When Mal-deficient MEFs were reconstituted with Mal L180, stimulation with MALP-2 did not restore p-38 phosphorylation.

Induction of cytokine IL-6 production was tested using the reconstitution assay. Mal-deficient MEFs were reconstituted with empty vector and plasmids encoding Mal S180 and Mal L180. WT Mal MEFs were also transfected with empty vector as a control. After 24 hours the transfected Mal-deficient MEFs were stimulated with TLR4 ligand LPS (100ng/ml) and TLR2/6 ligand MALP-2 (5nM). As shown in figure 3.8, after stimulation the WT MEFs produced 120pg/ml IL6 with the LPS signal and 140pg/ml

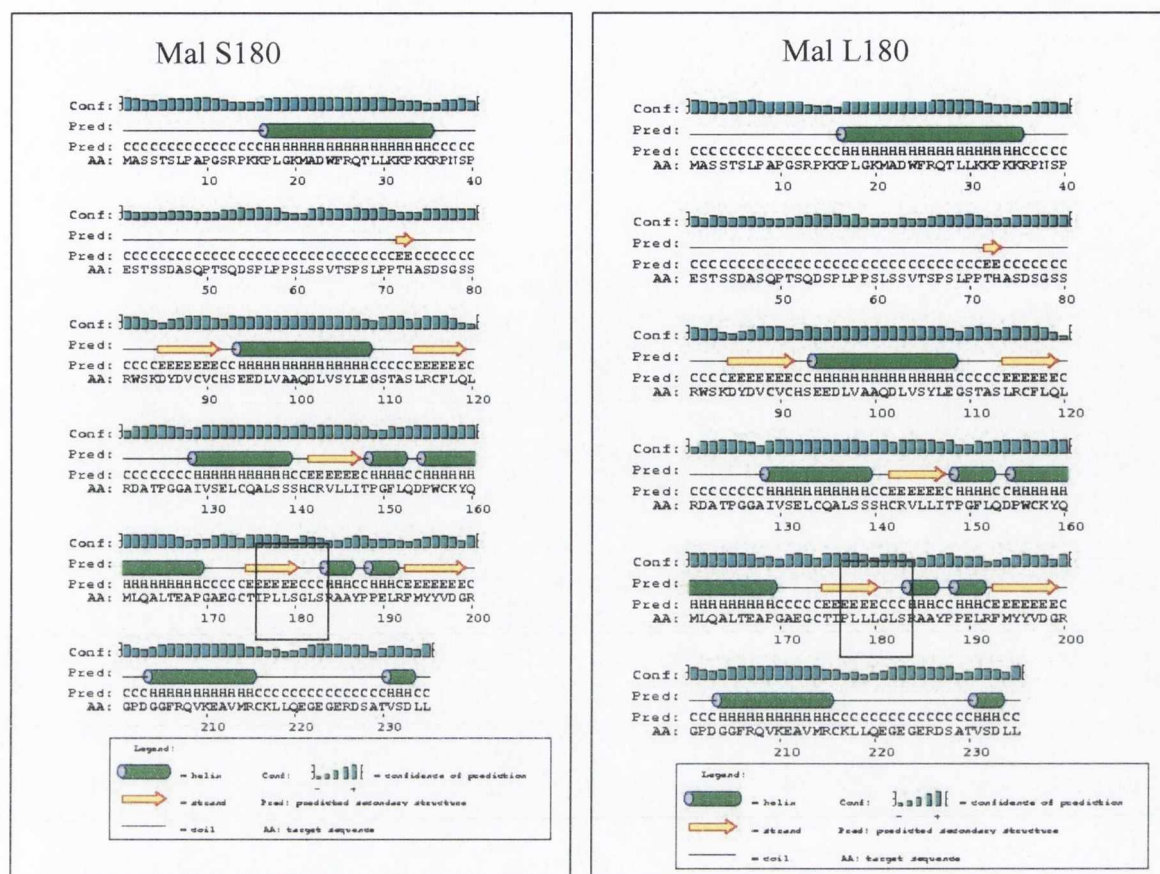
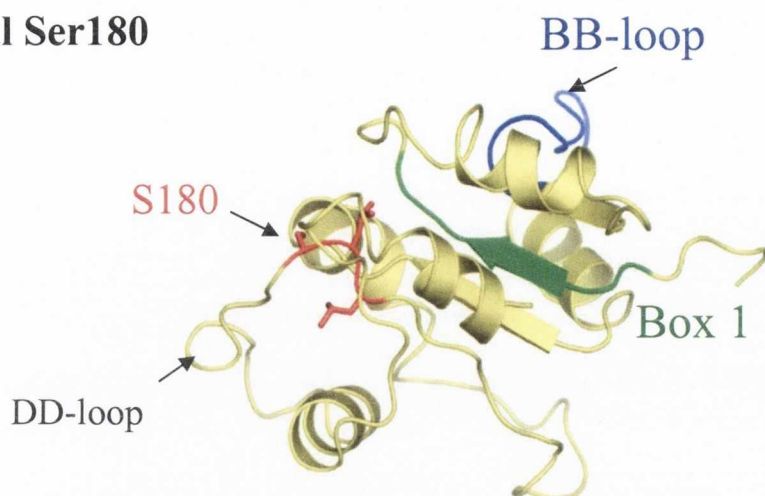


Figure 3.3 **Secondary structure prediction of Mal**

The Predict Protein programme is a computer-based programme that predicts the alpha helical (green cylinder), beta strand (yellow arrow) and random coil (straight line) structure of protein sequences that have been input into the software (bioinf.cs.ucl.ac.uk/psipred/). The TIR domain of Mal is situated between amino acids 86 to 188 and is predicted to have 5 alpha helices and 4 beta sheets present in its TIR domain. There are four alpha helices and two beta sheets present outside the TIR domain. The Mal Leu180 (Mal L180) polymorphism is indicated within a black square. The prediction programme did not reveal any conformational change to the predicted secondary structure when compared to the wild type Mal Ser180 (Mal S180) protein.

Mal Ser180



Mal Leu180

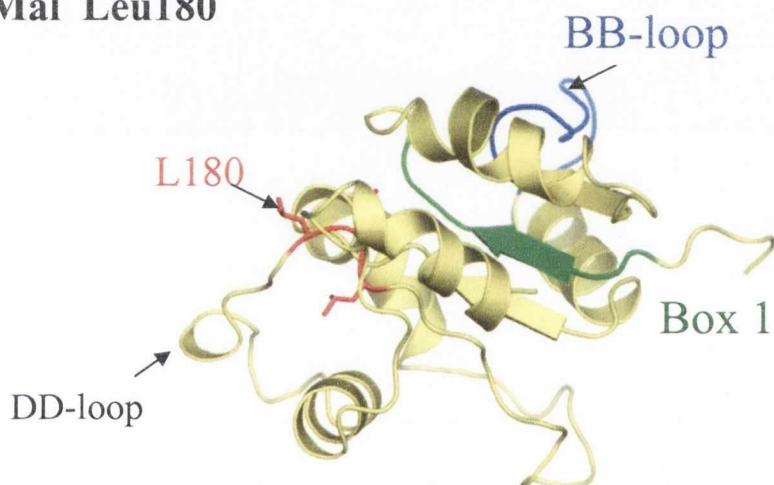


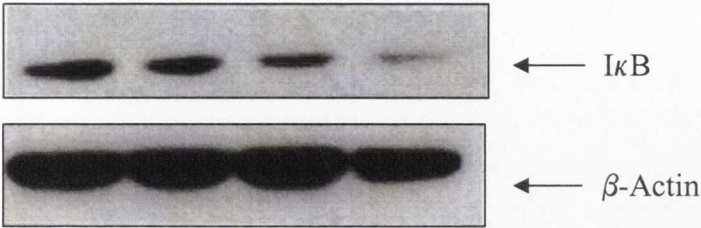
Figure 3.4 Tertiary structure prediction of Mal

The computational utility Molecular Operating Environment Tertiary was used to generate 3D models of the Mal Ser180 and Mal Leu180. The Mal S180 structure revealed that the protruding DD loop structure is composed of a random coil structure. A serine to leucine change at position 180 (shown in red in the Mal L180 model), revealed a change in the structure, causing the protruding DD loop to change slightly. Altering the serine to a leucine caused the DD loop to change its structure and become more alpha helical, and thus caused it to become more solvently exposed.

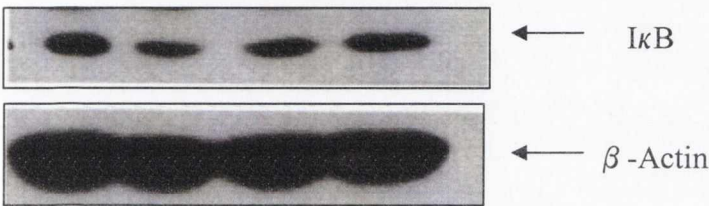
Figure 3.5 Mal L180 was unable to restore I κ B degradation in Mal deficient MEFs

Wild type (WT) and Mal deficient MEFs were seeded in 6 well plates at 1×10^5 cell/ml DMEM and were cultured overnight at 37°C with 5% CO₂. Each well was transfected with 2 μ g plasmid of either empty vector, Mal S180 or Mal L180 and incubated for a further 24 hours. The cells were stimulated with 5nM MALP-2 at 50, 30, 10 and 0 minute time points. The media was removed from the cells. The cells were washed with 2ml cold PBS and 100 μ l sample buffer containing DTT (1ml Sample Buffer, 50 μ l 1M DTT) was added to each well. The cells were scraped from each well and put into 1.5ml eppendorf tubes. The cells were sonicated for 3 seconds and boiled for 5 minutes. The samples were resolved on 12% Sodium Dodecylsulphate (SDS) polyacrylamide gels using constant current of 25mA per gel. Each protein gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature with 5% Marvel and then probed for I κ B. The membranes were also probed for β -Actin control to test for equal loading of samples. The blots were developed by enhanced chemiluminescence. The results are representative of three experiments.

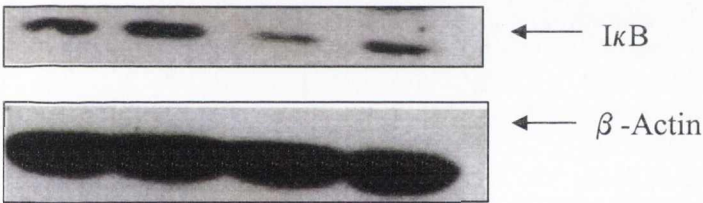
WT MEFs transfected with empty vector and stimulated with 5nM MALP-2



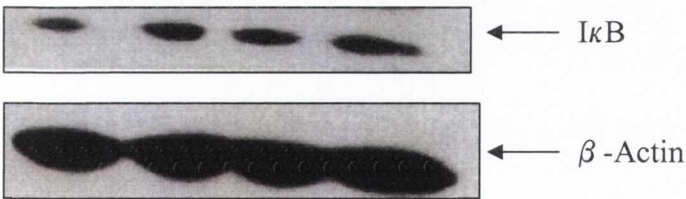
Mal deficient MEFs transfected with empty vector and stimulated with 5nM MALP-2



Mal deficient MEFs transfected with Mal S180 and stimulated with 5nM MALP-2



Mal deficient MEFs transfected with Mal L180 and stimulated with 5nM MALP-2



0 10 30 50 minutes

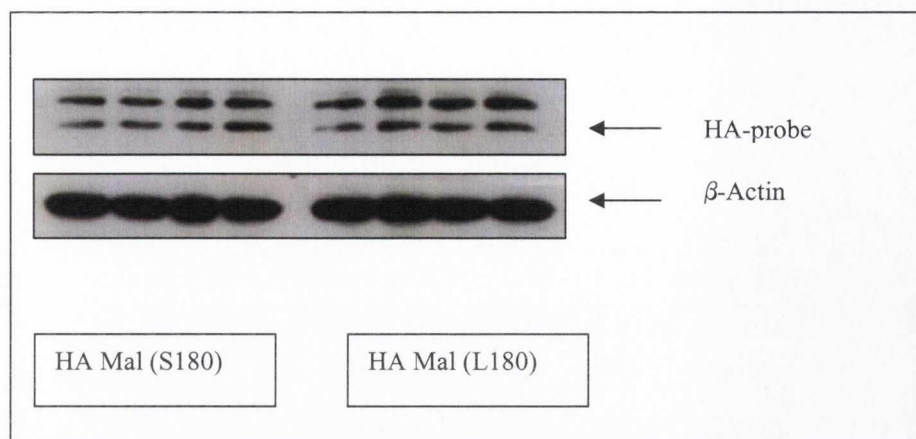


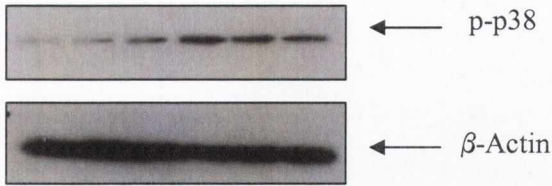
Figure 3.6 Mal S180 and Mal L180 were equally expressed.

Lysates from the $I\kappa B$ experiment (figure 3.5) were resolved on 12% SDS-PAGE gel using constant current of 25mA per gel. Each protein gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature in 5% Marvel and then probed for HA tagged Mal S180 and L180. A β -Actin loading control shows equal loading. The blots were developed by enhanced chemiluminescence.

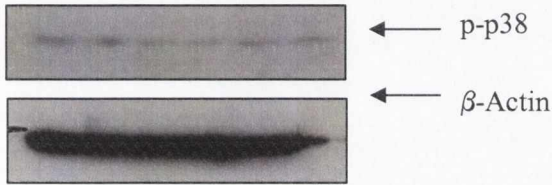
Figure 3.7 Mal L180 was unable to reconstitute p-38 phosphorylation

Wild type (WT) and Mal-deficient MEFs were seeded in 6 well plates at 1×10^5 cell/ml and were cultured overnight at 37°C with 5% CO₂. The cells were transfected with 2µg plasmid of either empty vector, Mal S180 or Mal L180 and incubated at 37°C with 5% CO₂ for 24 hours. The cells were stimulated with 5nM MALP-2 at 60, 45, 30, 15, 10 and 0 minute time points. The medium was removed from the cells and the cells were washed with 2ml cold PBS. Each sample was lysed in 100µl sample buffer (1ml sample buffer 50µl 1M DTT). The cells were scraped from each well and placed in 1.5ml eppendorf tubes. The cells were sonicated for 3 seconds and boiled for 5 minutes. The samples were resolved on 12% SDS-PAGE gels using constant current of 25mA per gel. Each protein gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature with 5% Marvel and probed over night with anti p-p38 at 4°C in 2% BSA-PBS. The membranes were also probed for β -Actin control to test for equal loading of samples. The blots were developed by enhanced chemiluminescence. The results are representative of 3 experiments.

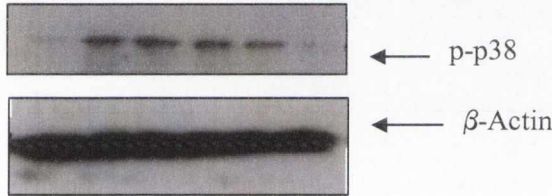
WT MEFs transfected with empty vector and stimulated with 5nM MALP-2



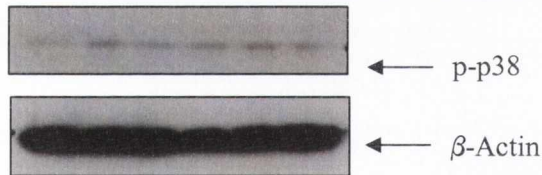
Mal deficient MEFs transfected with empty vector and stimulated with 5nM MALP-2



Mal deficient MEFs transfected with Mal S180 and stimulated with 5nM MALP-2



Mal deficient MEFs transfected with Mal L180 and stimulated with 5nM MALP-2



0 10 15 30 45 60 minutes

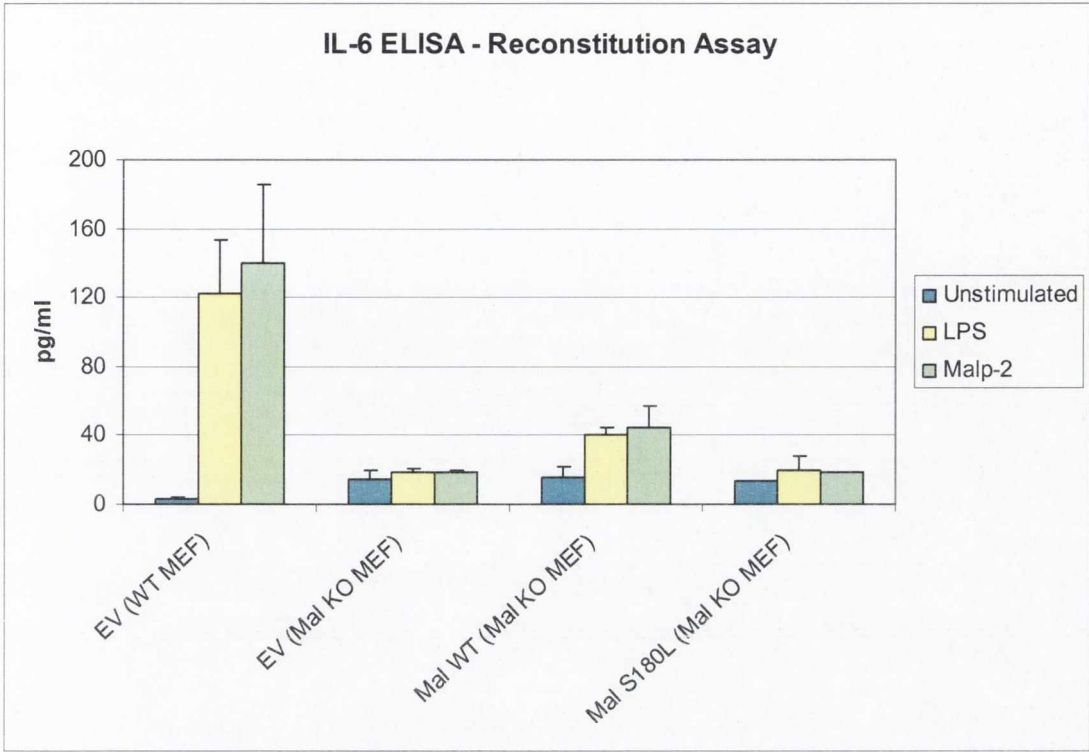


Figure 3.8 Mal L180 could not mediate the production of human IL-6 in Mal-deficient MEFs

Wild type (WT) and Mal-deficient MEFs were seeded in 24 well plates at 1×10^5 cell/ml DMEM and were cultured overnight at 37°C with 5% CO₂. The cells were transfected with 500ng plasmid per well as indicated and incubated at 37°C overnight. After 24 hours, the cells were stimulated with LPS (100ng/ml) and MALP-2 (5nM). The media was removed from the cells and 100µl plated onto a detection antibody coated ELISA plate to measure hIL6 production. The results are representative of four experiments. Results shown are the standard deviation of triplicate determinants.

with the MALP-2 stimulus. Mal deficient-MEFs reconstituted with empty vector did not produce any signal indicating that TLR4 and the TLR2 pathways are impaired without Mal present. Reconstitution with WT Mal restored a signal and IL-6 was produced. Reconstitution of the Mal-deficient MEFs with Mal L180 did not restore a signal and no IL-6 was produced with either an LPS or MALP-2 stimulus. A Western blot shown in figure 3.9 shows the presence of HA Tagged Mal S180 and HA tagged Mal L180 transfected into the deficient MEFs. Indicating that the same concentration of HA Mal S180 and Mal L180 were present in each sample.

3.2.5 Mal L180 GST does not interact with FLAG TLR2

In order to elucidate whether TLR2 could bind to Mal at the S180 region and to identify whether a change to this binding area could hinder binding and hence prevent the downstream signal, a GST pull-down assay was performed. Initially, a known concentration of GST protein (1 μ g) was resolved on an SDS-PAGE gel along with 1:10 diluted proteins GST, Mal S180-GST and Mal L180-GST (figure 3.10). The concentration of the proteins had now been approximated and these proteins were subsequently used in GST pulldown experiments. Lysates prepared from HEK 293 cells transfected with FLAG-TLR2 plasmid were incubated with Mal S180 or Mal L180 bound to GST Beads. Figure 3.11 displays the results of the GST pulldown assay. GST alone did not bind to FLAG-TLR2 (lane 1), Mal S180 did bind to TLR2 (lane2) but Mal L180 did not bind to FLAG TLR2 (lane 3). This result indicates that the L180 variant may be impacting TLR2 signalling by not binding to the receptor and hindering signalling. This would explain the failure of the Mal L180 to restore signalling in the Mal deficient MEFs.

3.2.6 Mal L180 possesses a dominant effect over Mal S180

Having demonstrated that Mal L180 could not reconstitute signalling in Mal deficient cells, the question arose as to whether Mal L180 could interfere with WT Mal S180 signalling in a dominant negative fashion (figure 3.12). Mal deficient MEFs were transfected with various combinations of Mal S180 and L180. Over-expression of Mal S180 showed a two fold increase in activity over empty vector. The Mal L180 form did not show any increase in NF- κ B luciferase activity over empty vector. When the Mal S180 and Mal L180 were transfected simultaneously the effect of the S180 was inhibited, indicating a negative effect of the Mal L180.

3.2.7 A co-expression assay determined that co-expressing IRAK4 with Mal S180 and Mal L180 causes the degradation of both.

TLR2 and TLR4 signalling recruits Mal to its TIR domain which in turn recruits MyD88, mediating the MyD88 dependent pathway. These proteins form a complex which recruits serine/threonine kinase, interleukin-1 receptor associated kinase-4 (IRAK4). The adaptor Mal is constitutively phosphorylated, possibly by BTK, and in the presence of IRAK4 becomes degraded. Degradation of both forms of Mal was examined. HEK 293T cells were seeded into 6 well plates the cells were transiently transfected with 125ng/well Mal L180 or Mal S180 and increasing concentrations of IRAK4 (125ng, 250ng, 500ng, 1 μ g/well). Figure 3.13 shows that both Mal S180 and Mal L180 are degraded similarly; indicating that IRAK4 degradation does not impact on the differential signalling between Mal L180 and Mal S180.

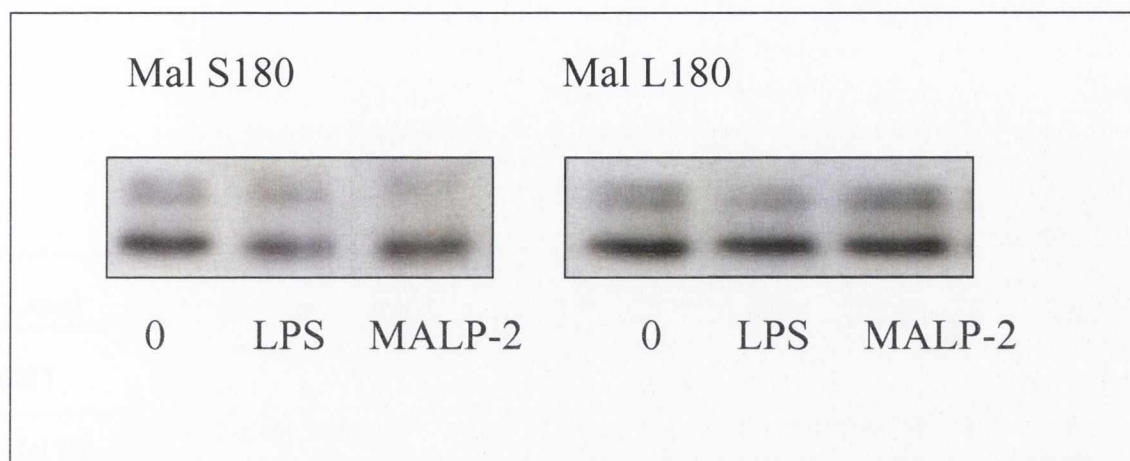


Figure 3.9 Over-expression of HA tagged Mal S180 and Mal L180-HA

Lysates from the hIL6 ELISA (figure 3.8) were resolved on a 12% SDS-PAGE gel using constant current of 25mA per gel. Each protein gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature in 5% Marvel-TBST and then probed for HA tagged Mal S180 and L180 overnight at 4°C in 5% Marvel-TBST.

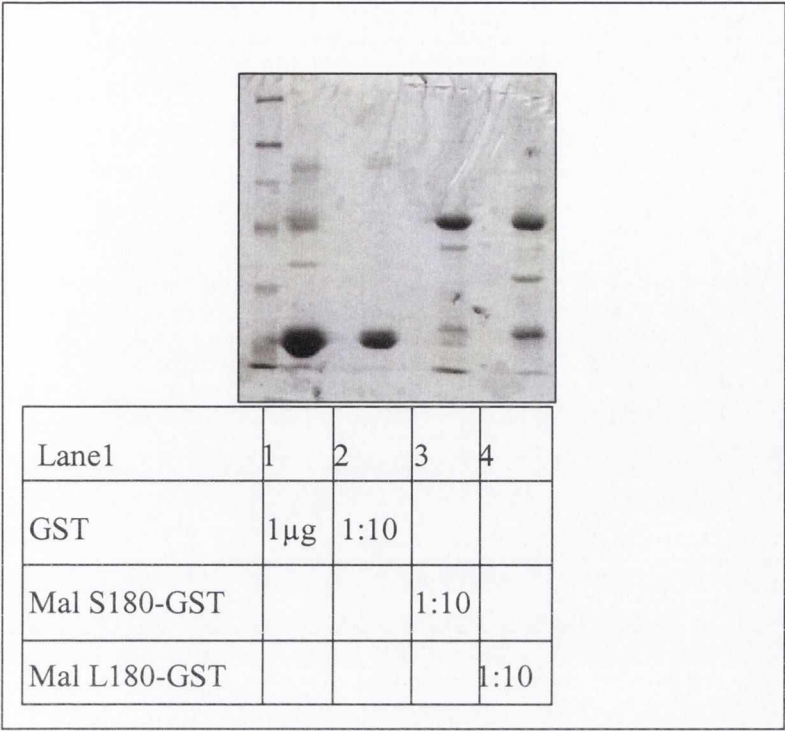


Figure 3.10 Quantification of GST proteins

To identify the approximate quantity of protein to be added during GST pulldown experiments, a known quantity of GST protein was resolved along with Mal S180-GST and Mal L180-GST. 1µg of GST protein alone was run in lane 1, this was then diluted 1:10 and run in lane 2. Mal S180-GST and Mal L180-GST were also diluted 1:10 and were resolved on the gel and are displayed in lanes 3 and 5, giving an indication of the concentration of protein present (approximately 0.1µg).

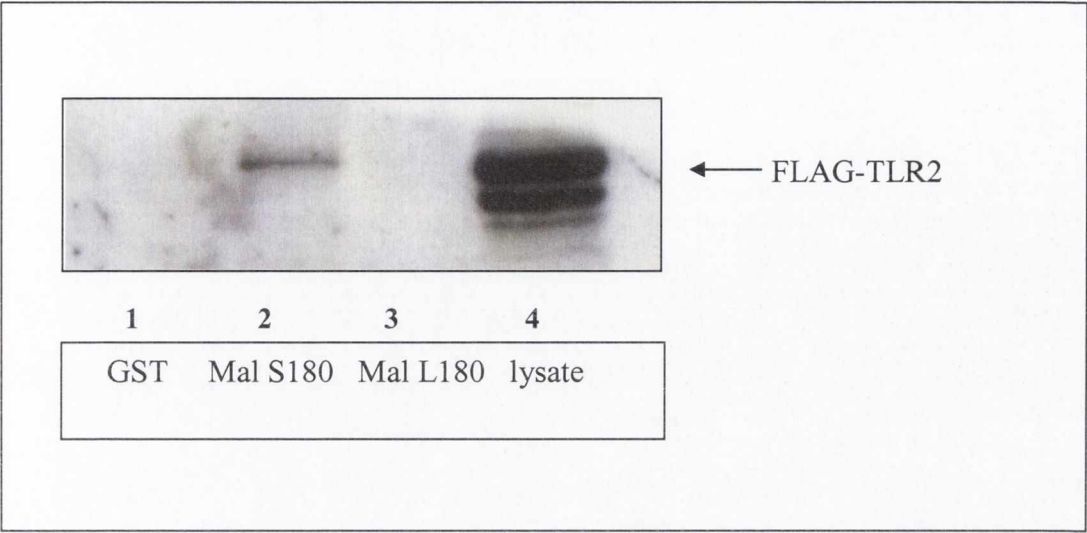
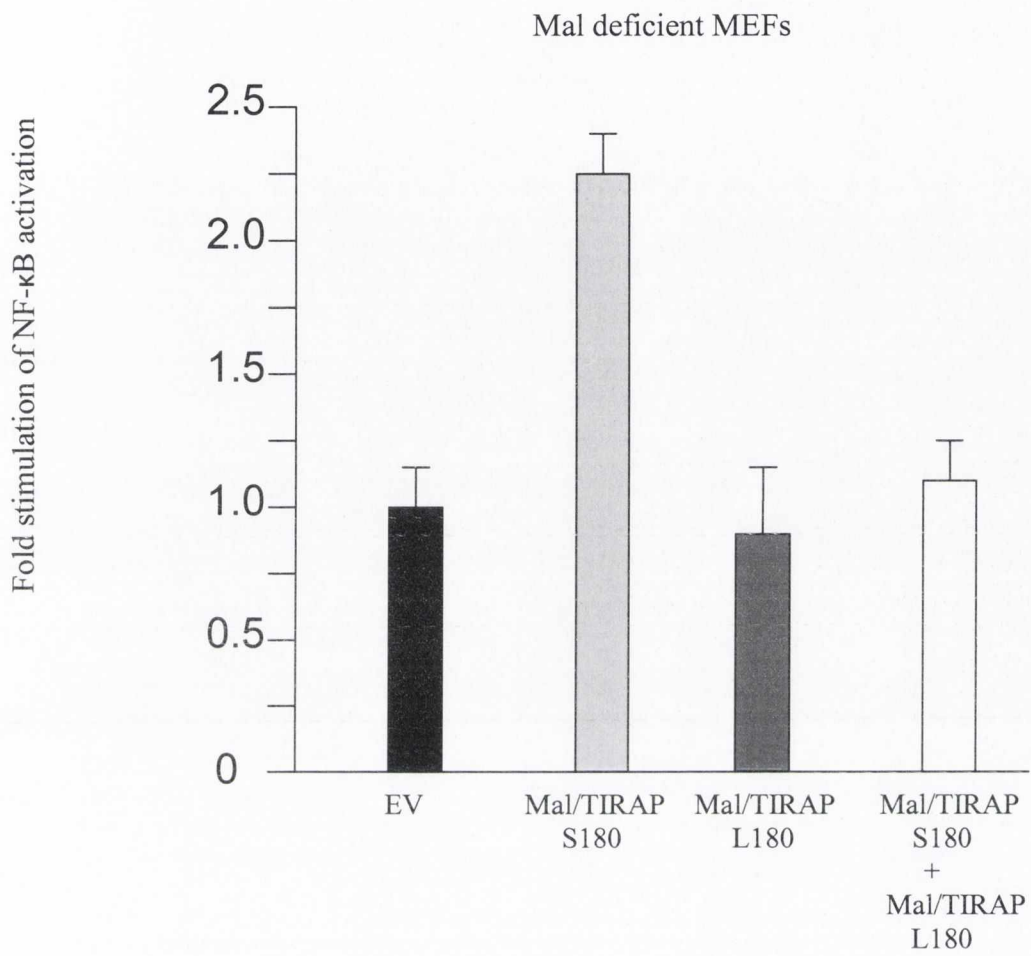


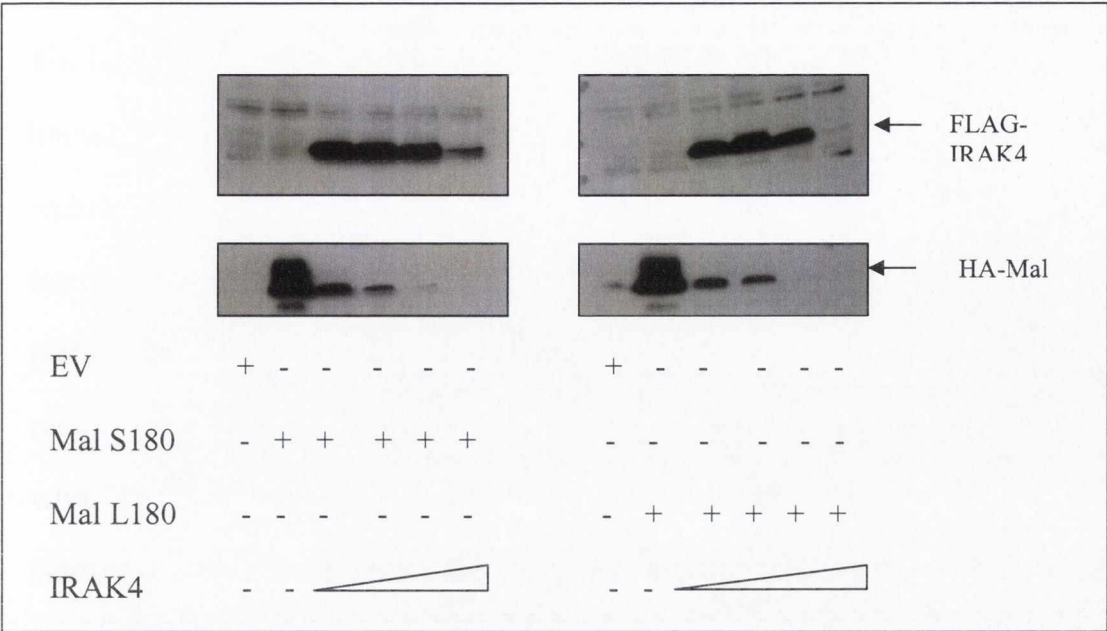
Figure 3.11 Mal L180 GST did not interact with FLAG TLR2

HEK 293 cells were seeded at 1×10^5 cells per ml DMEM into 10cm dishes. The cells were incubated at 37°C with 5% CO₂. After 24 hours, the cells were transfected with 6µg FLAG TLR2 plasmid. After 24 hours, the cells were washed with 10ml cold PBS and the cells were lysed in high stringency buffer containing protease inhibitors. The FLAG tagged TLR2 lysates were incubated over night at 4°C with GST, GST-Mal S180 and GST- Mal L180. The samples were resolved on a 12% SDS-PAGE gel using constant current of 25mA per gel. The protein from each gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature with 5% Marvel and probed for FLAG tagged TLR2. The blot was developed by enhanced chemiluminescence. GST alone did not bind to FLAG-TLR2 (lane 1), Mal S180 did bind to TLR2 (lane2) but Mal L180 did not bind to FLAG TLR2 (lane3). This blot is representative of three experiments.

Figure 3.12 Mal L180 possessed a dominant negative effect over Mal S180

Mal deficient MEFs were cultured overnight at 1×10^5 cells per ml in 96 well plates. The cells were transfected with (a) 100ng EV alone, (b) 50ng Mal S180 and 50ng EV, (c) 50ng Mal L180 and 50ng EV and (d) 50ng Mal S180 and 50ng Mal L180 mutant. The cells were transfected with TK renilla, a control plasmid. The levels of TK renilla should be constant in each well, and therefore all results were normalized to the TK renilla values. The cells were co-transfected with NF- κ B reporter plasmid. After 24 hours, incubation the cells were lysed for 20 minutes at room temperature with 50 μ l passive lysis buffer. The cells were observed microscopically to ensure lysis had occurred. 20 μ l of the cell lysate was transferred into 96 well plates and tested for firefly luciferase activity. 20 μ l of the cell lysate was tested for renilla luciferase activity. Firefly luminescence readings were corrected for renilla activity and were expressed as fold stimulation over un-stimulated empty (EV) control. Error bars represent the standard deviation of the mean of triplicate determinants. This result is representative of four experiments.





3.13 Co-expression of IRAK4 with Mal L180 and Mal S180 causes degradation of both proteins

HEK 293T cells were seeded at 1×10^5 cells/ml DMEM into 6 well plates. The cells were incubated at 37°C with 5% CO₂ for 24 hours. After 24 hours, the cells were transiently transfected with 125ng/well Mal L180 or Mal S180 and increasing concentrations of IRAK4 (125ng, 250ng, 500ng, 1µg/well). Each set of cells were normalised with empty vector control so as to have a total of 1.125µg of DNA per well. The cells were incubated for 24 hours, the cells were washed with 2ml cold PBS and placed in 1.5ml eppendorf tubes. The cells were lysed in 80µl sample buffer with DTT (1ml sample buffer 50µl 1M DTT). The samples were sonicated for 3 seconds and boiled for 5 minutes. The samples were resolved on a 12% SDS-PAGE gel using constant current of 25mA per gel. Each protein gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature with 5% Marvel-TBST and probed for HA. The blot was developed by enhanced chemiluminescence. This blot is representative of three experiments.

3.2.8 Blocking Mal TLR2 interactions using in vitro blocking peptides

The inability of Mal L180 to bind TLR2 suggested that the region surrounding L180 is important for their interaction. Consequently a peptide was designed based on this region, and experiments were carried out to determine its effect on both (a) TLR2's interaction with Mal and (b) TLR2 signalling. GST pulldown assays were first performed to determine if a peptide (termed the DD loop peptide) designed to this region of Mal could block interaction. Figure 3.14a shows GST- Mal S180 interacting with FLAG-TLR2 (lane 2). After the addition of DD loop peptide the interaction of GST-Mal S180 and TLR2 was hindered (lane 3). The control peptide (a scrambled version) did not hinder the interaction (lane 4). This finding further emphasises the importance of this region for the Mal/TLR2 interaction.

3.2.9 Blocking IL-6 production using peptides targeted to the region surrounding S180 of Mal

To understand further the importance of the region surrounding the S180 amino acid blocking peptides were used to disrupt the TLR2/Mal interaction were tested on induction of IL-6 by TLR2/TLR6 ligands. Two blocking peptides were designed, one that included the serine and one that included a leucine. They are called the S form DD loop peptide and the L form DD loop peptide. A nine arginine repeat sequence was tethered to the DD loop peptide to aid with internalisation of the peptide. Figure 3.15 displays the sequence of the peptides used in the peptide experiments.

3.2.9.1 Addition of S and L form peptides to human peripheral blood mononuclear cells

To investigate whether these peptides could block IL-6 production in cell based assays, the peptides were added to human peripheral blood mononuclear cells (PBMCs) before stimulation with TLR2 ligands. The cells were set up in 96 well plates and various concentrations of S and L form peptides were added to the cells (0-100 μ M). The cells were subsequently analysed with and without TLR1/2 ligand Pam₃CSK₄ and TLR2/6 ligand MALP-2. When stimulated with 5nM MALP-2 the S form peptides did not exert a blocking effect on the production of IL-6 (figure 3.16). The L form peptide was then added to the PBMCs and stimulated with 5nM MALP-2 the sandwich ELISA revealed a reduction in IL-6 production at 25 μ M, 50 μ M and 100 μ M peptide addition (figure 3.17). However, in the same experiment the IL-6 production was also lowered when the scrambled version of the peptide was added to the cell and so the reduction of the IL-6 cannot be attributed to the L form peptide. This indicates that the action of the L form peptide is non-specific. Human PBMCs also were pre-incubated with S and L form peptides and stimulated with 10 μ g/ml Pam₃CSK₄ (figures 3.18 and 3.19). Upon addition of increasing concentrations of S form peptide, there was no impact on production of IL-6 compared to the scrambled S form. Upon addition of increasing concentration of the L form of the peptide to the PBMC cells a decrease in IL-6 production is seen at the 25 μ M point. However, the error bar is high and so the result could be open to misinterpretation. 100 μ M of L peptide was added to the cells and both the scrambled and the L form were found to reduce signalling (figure 3.19).

Figure 3.14 A blocking peptide designed to the Mal S180 region blocked Mal/TLR2 binding

HEK 293 cells were seeded at 1×10^5 cells per ml DMEM into 6 well plates. The cells were transiently transfected with $2 \mu\text{g}$ TLR2-FLAG per well. After 24hrs, a GST pull-down assay was performed, cells were washed with 10ml cold PBS and the cells lysed in high stringency buffer containing protease inhibitors. The FLAG tagged TLR2 lysates were incubated over night at 4°C with GST alone, Mal S180 GST with DD loop peptide and Mal S180 GST with the scrambled control peptide. The GST samples were centrifuged at 2000 rpm for 5 minutes and the beads were washed with 1ml high stringency buffer three times and in 1ml PBS once. All buffer was removed from the beads and $40 \mu\text{l}$ sample buffer (1ml sample buffer $50 \mu\text{l}$ DTT) was added to each sample. The samples were resolved on a 12% SDS-PAGE gel using constant current of 25mA per gel. The protein from each gel was transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature with 5% Marvel-TBST and the probed for FLAG tagged TLR2. This blot is representative of two experiments

Part (a) represents the protein interactions;

lane 1 - GST alone

lane 2 - GST Mal S180 interacted with FLAG TLR2

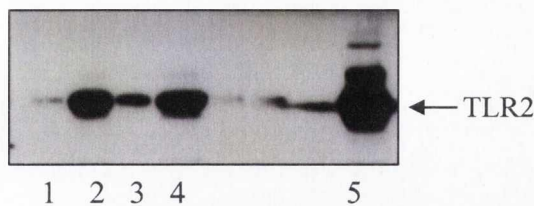
lane 3 - GST Mal S180 interaction with FLAG TLR2 was hindered in presence of DD loop peptide

Lane 4 - GST Mal S180 interaction with FLAG TLR2 was not hindered by the presence of scrambled control peptide

Lane 5 - TLR2 lysate

(b) DD-Loop peptide and control peptide amino acid sequence.

(a)



Lane 1 = GST alone
Lane 2 = GST Mal and FLAG TLR2
Lane 3 = GST Mal and FLAG TLR2
with DD loop peptide
Lane 4 = GST Mal and FLAG TLR2
with DD loop control peptide
Lane 5 = TLR2 Lysate

(b)

DD Loop Peptide	TIPLLSELSR
DD Loop Control Peptide	LSRGSP TLIL

PEP-052: acetyl-TIPLL <u>S</u> GLSRRRRRRRRR-amide	93.28% purity- S form
PEP-053: acetyl-LPSGLTILSRRRRRRRRR-amide	96.55% purity-Scrambled S form
PEP-054: acetyl-TIPLL <u>L</u> GLSRRRRRRRRR-amide	73.44% purity- L form
PEP-055: acetyl-LPIGLSLTLRRRRRRRRR-amide	70.15% purity- Scrambled L form

Figure 3.15 Blocking peptide sequences

Blocking peptides were designed to the 9 amino acid sequence surrounding S180 of Mal. Two sets of peptides were generated, one that contained the serine and a second that contained the leucine. Scrambled forms of the peptides were also generated and were used as controls. A nine arginine repeat sequence was tethered to the DD loop peptide to aid with internalisation of the peptide. The S form peptide and the L form peptide had a high purity value at 93.28% and 96.55% respectively; the amino acid change in the second set of peptides lowered their purity values to 73.44 and 70.15%. The reduced level of purity was due to the presence of a product that has one less arginine residue than the original L form and scrambled L form peptides.

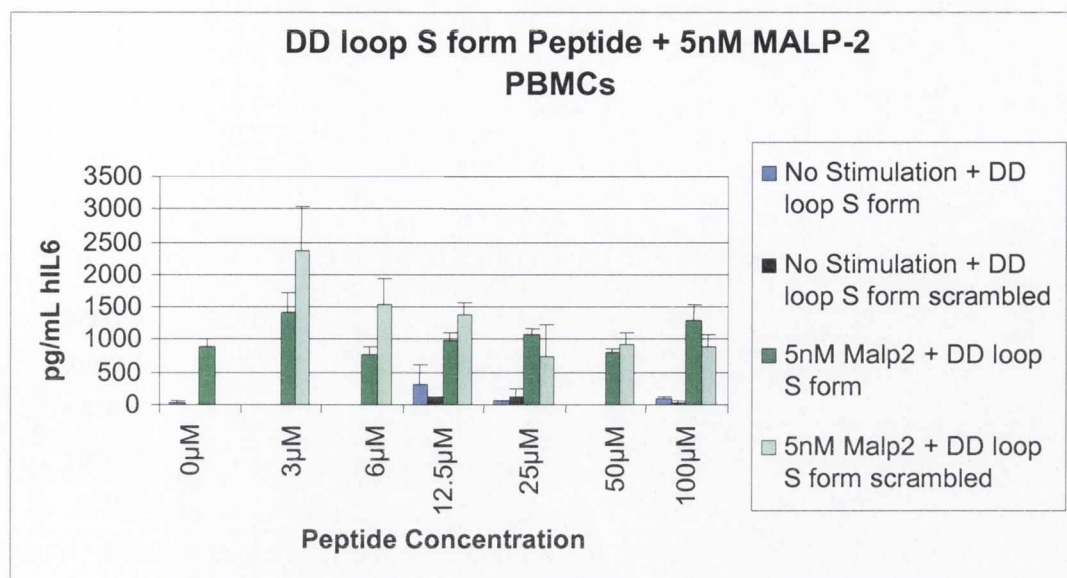


Figure 3.16 DD loop peptide S form did not block IL-6 production in MALP-2 stimulated PBMCs

Human PBMCs were isolated from whole blood and were cultured in 96 well plates at 1×10^6 cell/ml DMEM. The cells were incubated overnight at 37°C with 5% CO_2 . S form peptide and scrambled S form peptide were added to the cells at increasing concentrations $0\mu\text{M}$, $3.125\mu\text{M}$, $6.25\mu\text{M}$, $12.5\mu\text{M}$, $25\mu\text{M}$, $50\mu\text{M}$ and $100\mu\text{M}$. The cells were incubated for one hour before stimulating with 5nM MALP-2. After 24 hours, the supernatants were removed from the cells and were analysed for IL-6 production by sandwich ELISA. Experimental points were carried out in biological triplicate and the standard deviation is represented as error bars.

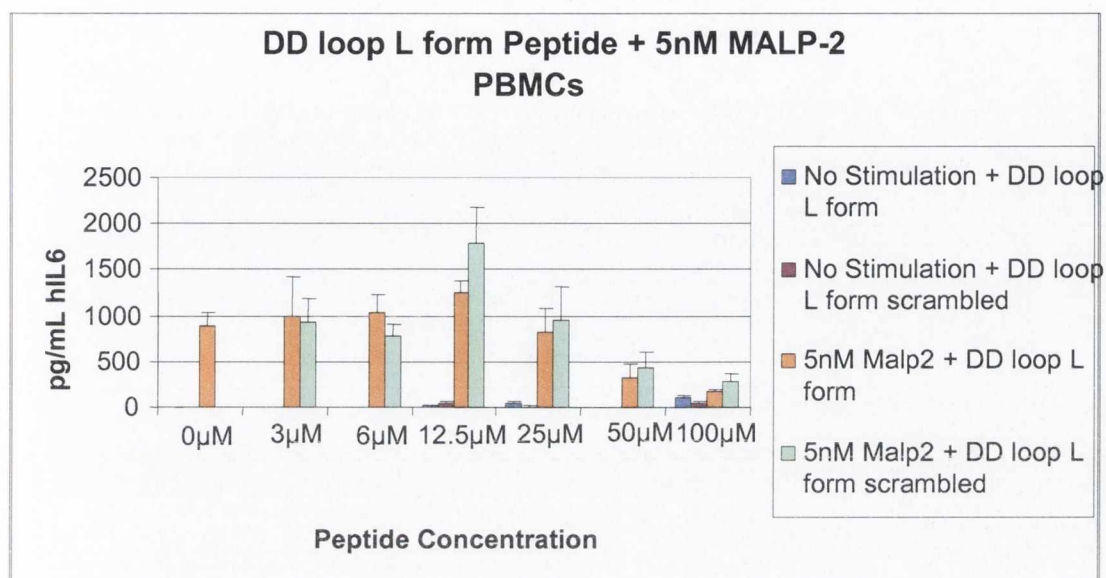


Figure 3.17 DD loop peptide L form does not block IL-6 production in MALP-2 stimulated PBMCs

Human PBMCs were isolated from whole blood and were cultured in 96 well plates at 1×10^6 cell/ml DMEM. The cells were incubated overnight at 37°C with $5\% \text{ CO}_2$. L form peptide and scrambled L form peptide were added to the cells at increasing concentrations $0\mu\text{M}$, $3.125\mu\text{M}$, $6.25\mu\text{M}$, $12.5\mu\text{M}$, $25\mu\text{M}$, $50\mu\text{M}$ and $100\mu\text{M}$. The cells were incubated for one hour before stimulating with 5nM MALP-2. After 24 hours, the supernatants were removed from the cells and was analysed for IL-6 production by sandwich ELISA. Experimental points were carried out in biological triplicate and the standard deviation is represented as error bars.

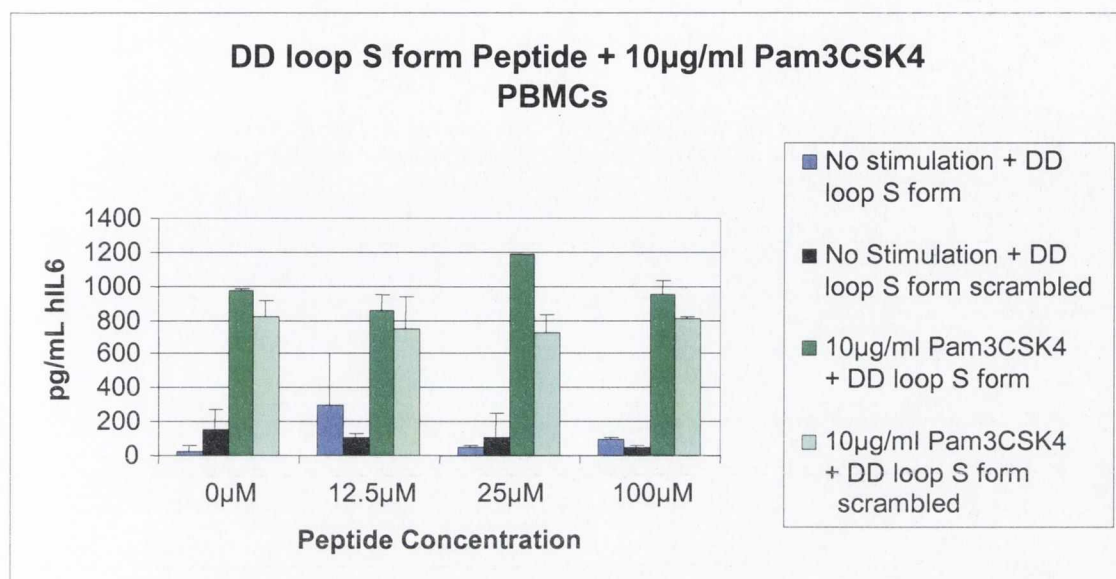


Figure 3.18 DD loop peptide S form does not block IL-6 production in Pam₃CSK₄ stimulated PBMCs

Human PBMCs were isolated from whole blood and were cultured in 96 well plates at 1×10^6 cell/ml DMEM. The cells were incubated overnight at 37°C with 5% CO₂. S form peptide and scrambled S form peptide were added to the cells at increasing concentrations 0µM, 3.125µM, 6.25µM, 12.5µM, 25µM, 50µM and 100µM. The cells were incubated for one hour before stimulating with 10µg/ml Pam₃CSK₄. After 24 hours, the supernatants were removed from the cells and was analysed for hIL-6 production by sandwich ELISA. Experimental points were carried out in biological triplicate and the standard deviation is represented as error bars.

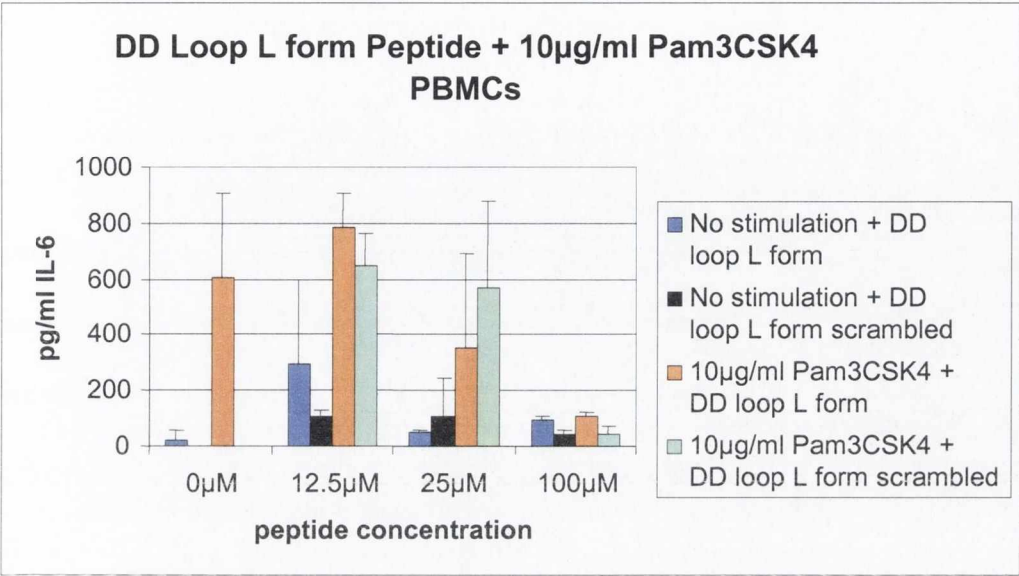


Figure 3.19 DD loop peptide L form does not block IL-6 production in Pam₃CSK₄ stimulated PBMCs

Human PBMCs were isolated from whole blood and were cultured in 96 well plates at 1×10^6 cell/ml. The cells were incubated overnight at 37°C with 5% CO₂. L form peptide and scrambled L form peptide were added to the cells at increasing concentrations 0µM, 3.125µM, 6.25µM, 12.5µM, 25µM, 50µM and 100µM. The cells were incubated for one hour before stimulating with 10µg/ml Pam₃CSK₄. After 24 hours, the supernatants were removed from the cells and was analysed for IL-6 production by sandwich ELISA. Experimental points were carried out in triplicate and the standard deviation is represented in the error bars.

3.2.9.2 Addition of peptides to PolyI:C stimulated U373-CD14 cells

To assess whether the peptides could block IL-6 production using ligands to other TLRs, the TLR3 ligand PolyI:C was used. U373-CD14 cells were treated with S form and L form peptides and the cells were stimulated with 10µg/ml PolyI:C. A high concentration of IL-6 (approximately 10ng/ml) is produced when U373-CD14 cells are stimulated with PolyI:C. As shown in figures 3.20 the S form of the peptide or the scrambled version of the peptide did not block IL-6 production at any concentration of peptide. The L form of the peptide was also tested on U373-CD14 cells and it is shown in figure 3.21 that the scrambled form of the L peptide was able to inhibit TLR3 signalling. The L form was able to inhibit signalling while the S form was not, indicating that the S form is not involved in TLR3 and the L form is operating non-specifically.

The U373-CD14 cells were also stimulated with 5nM MALP-2 (TLR2/6 ligand) to investigate if the peptide could possibly block TLR2 signalling, unfortunately U373-CD14 cells do not respond to MALP-2. Different cells lines would have to be used to further investigate if the peptides could block TLR2 signalling.

This set of experiments did not reveal whether the S form could block TLR2/Mal signalling. The issue may lie in the ability of the peptides to gain entry to the cells and to block signalling. The quantity that gains access to the cell may be important also as too much peptide may have a damaging effect on the viability of the cells and hinder any production of cytokine that may occur as a result of ligation of ligand. The L form of the peptide did not hinder TLR2/6 or TLR2/1 signalling but did play a non-specific role in TLR3 signalling however this could also be attributed to a high concentration of peptide being added.

To identify whether the peptides were causing cell death, MTT cell viability assays were carried out on HEK 293 cells. The tests indicated that upon addition of between 0-5 μ M DD loop peptide S form a 20% decrease in cell viability was observed (data not shown). This indicates that the reason for the decrease in cytokine production could be due to a decrease in cell viability upon addition of both the S and L form and the S and L scrambled peptides. As a result of this is difficult to interpret whether the S or L form DD loop peptides are having an effect in these cell based assays.

In the GST pull-down experiment the S form peptide was able to disrupt TLR2/Mal interactions suggesting that this region may be important in the TLR2/Mal interface but this could not be expanded upon using cell based assays. A summary of the actions of the peptides is displayed in table 3.1.

3.2.10 Caspase-1 cleavage of Mal is not hindered in the Mal L180 protein

During this project, collaborators from Cambridge University expressed an interest in this study. Prof. Nick Gay and Dr. Joyce Wong carried out 3D crystallization studies of the Mal S180 and Mal L180 protein. They found that the structure of Mal S180 differs from Mal L180 in the caspase-1 cleavage area of Mal at YYVD 195-198.

It was revealed through a personal communication that the caspase-1 cleavage site of Mal is buried within the protein structure of Mal L180 and therefore may not be cleaved.

A caspase-1 cleavage assay was carried out to identify if Mal S180 and Mal L180 differed in their ability to be cleaved by caspase-1. Figure 3.22 displays the ability of caspase-1 to cleave Mal S180 and Mal L180. The blots were probed with HA antibody. The HA tag of Mal is on the C terminal end and so it is removed when caspase-1 cleaves Mal. Lane 1 shows Mal S180-HA without the presence of caspase-1 while lane

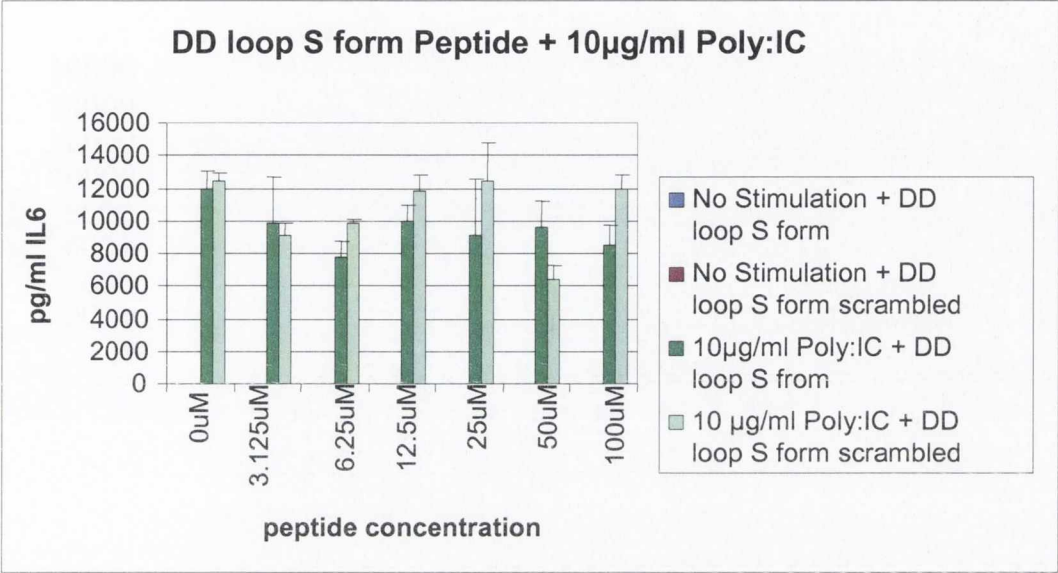


Figure 3.20 DD loop peptide S form does not block IL-6 production in Poly:IC stimulated U373s.

U373 human astrocytoma cell line cells were cultured in 96 well plates at 1×10^5 cell/ml and were incubated overnight at 37°C with 5% CO₂. S form peptide and scrambled S form peptide were added to the cells at different concentrations 0µ, 3.125µM, 6.25µM, 12.5µM, 25µM, 50µM and 100µM. The cells were incubated for one hour before stimulating with 10µg/ml Poly:IC. After 24 hours, the supernatants were removed from the cells and was analysed for IL-6 production by sandwich ELISA. Experimental points were carried out in biological triplicate and the standard deviation is represented as error bars.

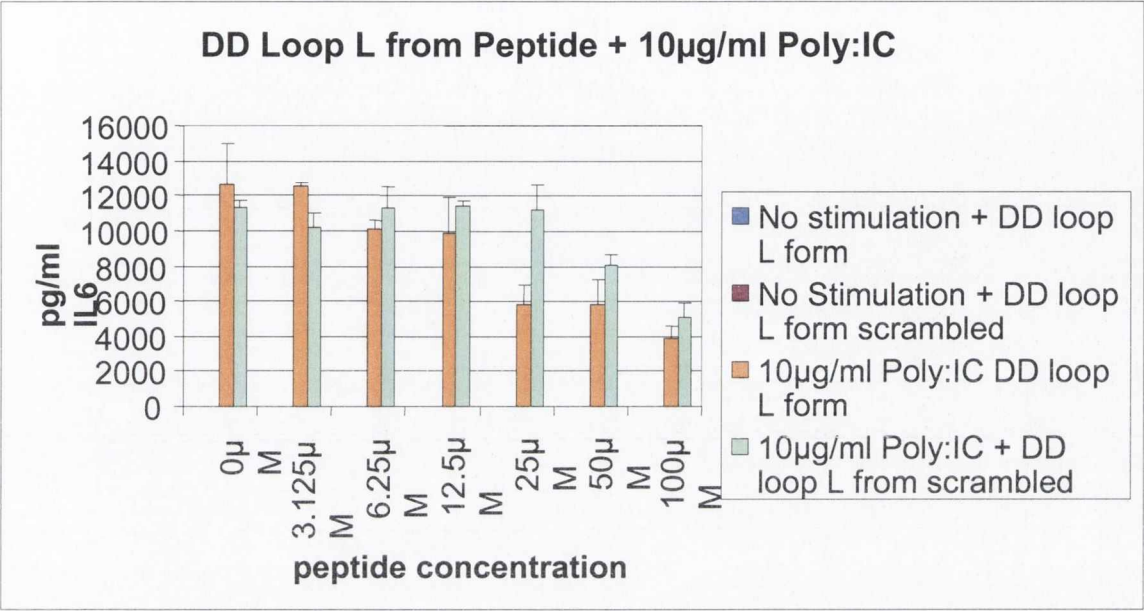


Figure 3.21 DD loop peptide L form does not block IL-6 production in Poly:IC stimulated U373s.

U373 human astrocytoma cell line cells were cultured in 96 well plates at 1×10^5 cell/ml DMEM and were incubated overnight at 37°C with 5% CO₂. L form peptide and scrambled L form peptide were added to the cells at different concentrations 0µ, 3.125µM, 6.25µM, 12.5µM, 25µM, 50µM and 100µM. The cells were incubated for one hour before stimulating with 10µg/ml Poly:IC. After 24 hours, the supernatants were removed from the cells and was analysed for IL-6 production by sandwich ELISA. Experimental points were carried out in biological triplicate and the standard deviation is represented as error bars.

	DD S form Peptide	DD S scrambled form	DD L form Peptide	Scrambled
In Vitro Studies				
TLR2/Mal	Yes, caused inhibition	No inhibition	Not tested	Not rested
Primary Cell Based Studies				
MALP-2	No inhibition	No inhibition	Yes, caused inhibiton	Yes, caused inhibiton
Pam ₃ CSK ₄	no inhibition	No inhibition	no inhibition	no inhibition
Cell Based Studies				
Poly:IC	No inhibition	No inhibition	Yes, caused inhibition	Yes, caused inhibition

Table 3.1 **Summary of action of DD loop blocking peptides on the production of pro-inflammatory cytokine IL-6 in *in vitro*, cell based and primary cell based studies.**

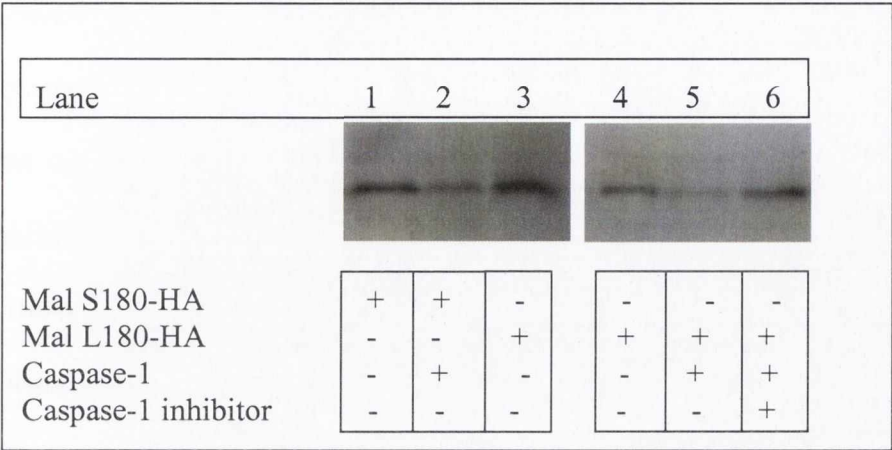


Figure 3.22 Mal S180 and Mal L180 are cleaved by caspase-1

HEK 293T cells were seeded into 10cm dishes at 1×10^5 cells/ml DMEM. The cells were incubated at 37°C at 5% CO₂ for 24 hours. The following day the cells were transiently transfected with 6µg/ml Mal S180-HA and Mal L180-HA. The cells were incubated for a further 24 hours before harvesting. The cells were washed in 2ml cold PBS and were lysed in 300µl low stringency buffer with protease inhibitors. The samples were rolled at 4°C for 15 minutes. The samples were prepared; 2µl (200Units) human recombinant caspase-1 was added to 10µl of lysed sample. 100µM of caspase-1 inhibitor was added to Mal L180-HA and Mal S180-HA. The samples were incubated for 90 minutes at 30°C and were resolved on a 12% SDS-PAGE gel using constant current of 25mA per gel. The proteins on the gel were transferred to nitrocellulose membrane for 1.5 hours at 200mA. The membrane was blocked for 1 hour at room temperature with 5% Marvel and probed for HA for 2 hours at room temperature. The blot was developed by enhanced chemiluminesence. The results are representative of three separate experiments.

2 shows that Mal S180-HA incubated with caspase-1 and cleavage has occurred. Lane 3 shows the presence of Mal L180-HA and when caspase-1 is present, Mal L180 is cleaved at the YYVD caspase-1 cleavage site, removing the HA tag. The antibody detects less protein. A caspase-1 specific inhibitor is used to ensure that the cleavage that occurs is due to the presence of caspase-1 alone. Lanes 3 and 5 display the inhibitory action of the caspase-1 inhibitor on the caspase-1 cleavage action on Mal S180-HA and Mal L180-HA, thus revealing that the cleavage is specific for caspase-1. Caspase-1 is capable of cleaving both Mal S180-HA and Mal L180-HA, as shown by Miggin and colleagues (Miggin et al., 2007) the cleavage of Mal is required for downstream signalling however in this project the inability of Mal-L180 to signal downstream is not due to caspase-1 cleavage.

3.2.11 Confocal analysis – comparing the localisation of Mal S180-CFP and Mal L180-CFP.

The cellular localisation of Mal S180 compared to Mal L180 was investigated using confocal microscopy.

3.2.11.1 Mal S180-CFP and Mal L180-CFP are localised to the plasma membrane in U373-CD14 cells

It was examined whether the differential signalling results that were observed between Mal S180 and Mal L180 were studied to see if these differences were due to the mislocalisation of Mal L180. Mal S180 and Mal L180 were both tagged with fluorescent cyan fluorescent protein (CFP). These constructs were transfected into U373-CD14 cells. Figure 3.23 displays Mal S180 CFP and Mal L180 CFP present in membrane similar to the findings by Kagan and Medzhitov (Kagan and Medzhitov, 2006) who

found Mal present in membrane ruffles of macrophages. They found that Mal binds to PIP2 containing plasma membranes by its N terminal PIP2 binding domain to recruit MyD88 to initiate TLR2 and TLR4 signalling. The ability of the Mal to localise to the membrane is not dependent on its TIR domain and so the Mal L180 variant should localise to membranes similar to Mal S180.

3.2.11.2 Mal L180-CFP and Mal S180-CFP accumulate in the cytosol upon stimulation with MALP-2 in HEK 293 TLR2 cells

To test if the observation found by Kagan et al., with TLR4 occurs within a TLR2 activated pathway, HEK 293 TLR2 cells were seeded into 6 well plates and transfected with 2 μ g DNA per well, the cells were stimulated with 5nM MALP-2 48 hours later. The cells were stimulated at time points 0-45 minutes (figure 3.24). The cells were fixed and permeabilized, and the nuclei were stained red with PI. It was observed that Mal S180 CFP and Mal L180 CFP were present in the membrane at the zero time point and that after 5 minutes stimulation with 5nM MALP-2, Mal S180 CFP and Mal L180 CFP accumulate in the cytosol. At the later time points 30 and 45 minutes the Mal S180 CFP and Mal L180 CFP has dissipated and no longer formed an accumulation. Although the results did not indicate a difference between Mal S180 and Mal L180 distribution in the cell, the translocation of both forms was of interest as this was a novel finding. Although previously in this project it was found that Mal L180 does not interact with TLR2, in this confocal experiment the Mal L180 is able to translocate after stimulation. A recent study by Kenny and colleagues (Kenny et al., 2009) have found using yeast-two-hybrid that TLR2 interacts with MyD88 directly and that Mal acts to increase efficacy of TLR2 signalling at lower concentration of TLR2 ligand. It has also

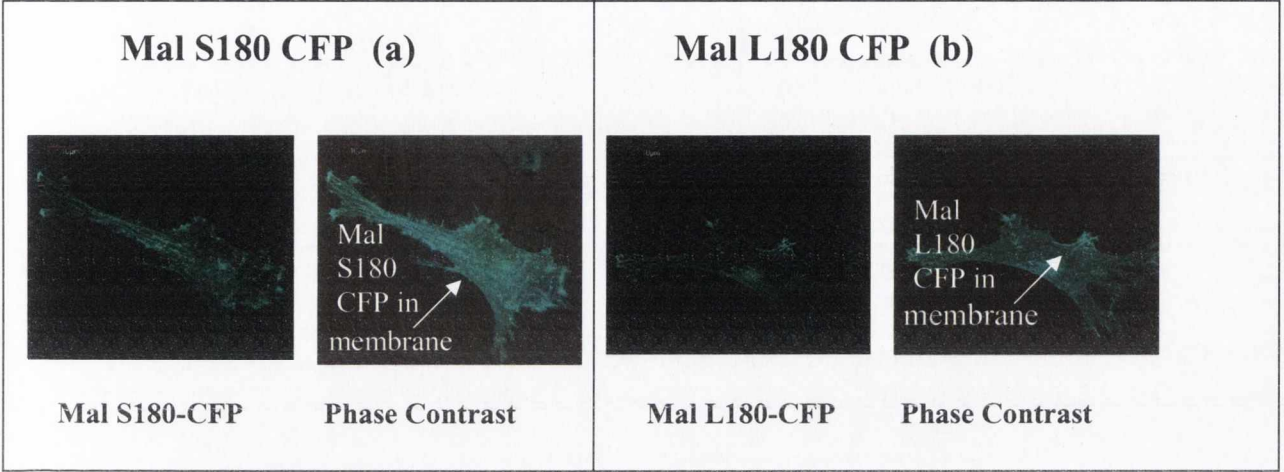


Figure 3.23 Mal S180 CFP and Mal L180 CFP in U373-CD14

U373-CD14 human astrocytoma cells were seeded onto poly-L lysine coated coverslips and placed into 6 well plates at 1×10^5 cells/ml DMEM. The cells were incubated overnight at 37°C with 5% CO₂. After 24 hours, the cells were transfected with 2µg of Mal S180 CFP and Mal L180 CFP per well of 6 a well plate. The cells were washed with 2ml PBS, fixed and permeabilized and mounted on microscope slides. Panel (a) shows Mal S180 CFP in cyan and panel (b) shows Mal L180 in cyan. The phase contrast image overlaid with the CFP image gives an indication as to the health of the cell at the time of fixing.

All these images were taken with the same laser intensity.

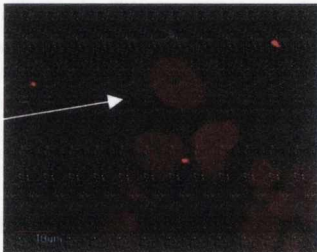
Figure 3.24

HEK 293 TLR2 cells were transfected with Mal S180 CFP and Mal L180 CFP and stimulated with MALP-2.

HEK 293 TLR2 cells were seeded onto poly-L lysine coated cover-slips and placed into 6 well plates at 1×10^5 cells/ml. The cells were incubated overnight at 37°C with 5% CO₂. After 24 hours, the cells were transfected with 2 µg of Mal S180 CFP and Mal L180 CFP per well of 6 well plate. After a further 24 hours the cells were stimulated with 5nM MALP-2 for 0, 5, 30 and 45 minutes. Following stimulation the cells were washed with 2ml PBS, fixed and permeabilized. The cells were incubated with RNase A for 20 minutes at 37°C and subsequently nuclear stained with propidium iodide (PI) for 3 minutes. The cells were washed three times with PBS and mounted on microscope slides and analysed by confocal microscopy. Panel (a) displays Mal S180 CFP in cyan and panel (b) shows Mal L180 in cyan. Nuclei are stained red. All these images were taken with the same laser intensity.

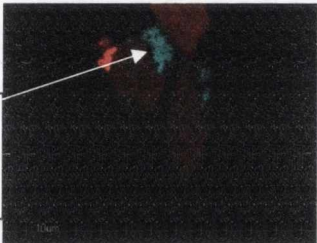
Mal S180 CFP (a)

Mal S180
Membrane
localised



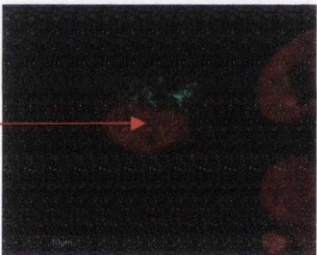
0 min

Mal S180
Accumulation
in cytosol



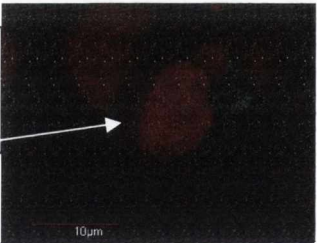
5 min

Nucleus



30 min

Mal S180
dissipation

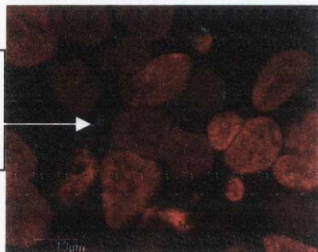


45 min

- HEK293 TLR2 Cells,
- 5nM MALP-2
- Mal CFP - cyan
- Nuclei - red

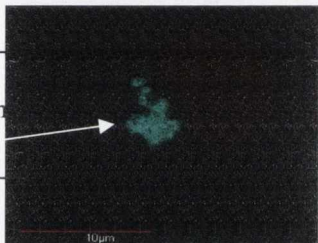
Mal L180 CFP (b)

Mal L180
Membrane
localised



0 min

Mal S180
Accumulation
in cytosol

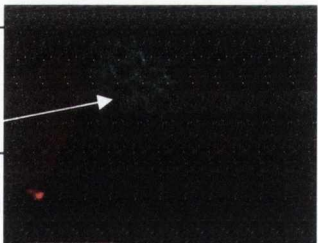


5 min



30 min

Mal L180
dissipation



45 min

- HEK293 TLR2Cells,
- 5nM MALP-2
- Mal CFP – cyan
- Nuclei - red

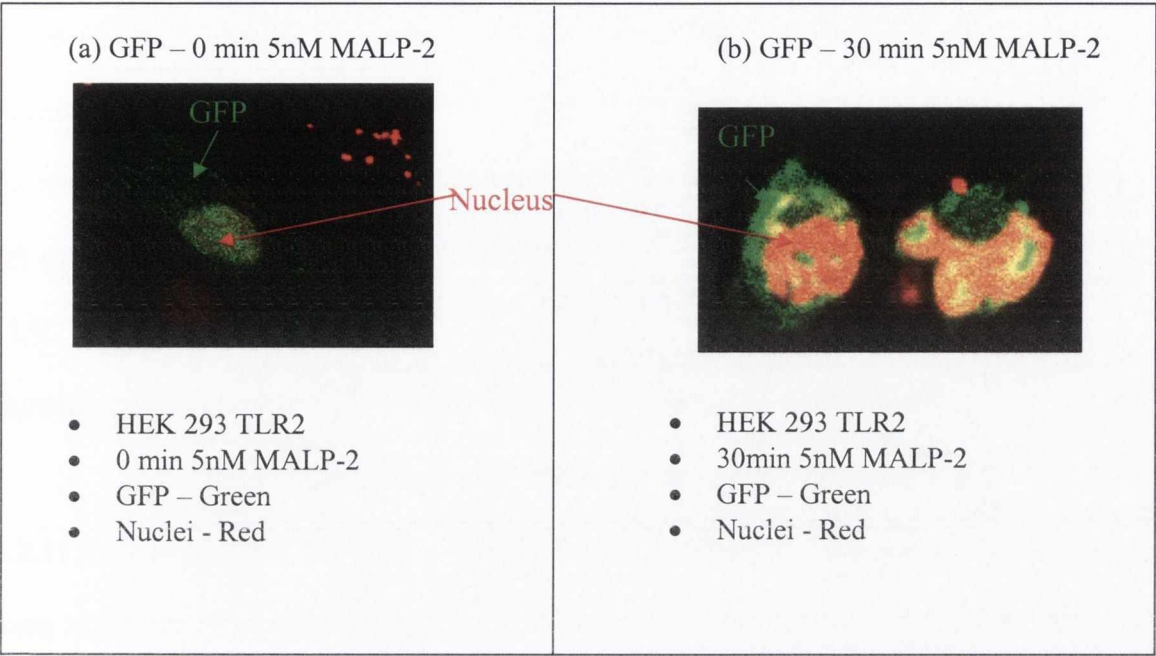


Figure 3.25 GFP transfected into HEK 293 TLR2 cells does not accumulate in the cytosol after treatment with MALP-2.

1.5mm thick cover glass slides were coated in 500µl poly-L-lysine and rinsed with 1ml PBS. The slides were placed in 6 well tissue culture plates. HEK 293 TLR2 cells were cultured at 0.5x10⁵cells/ml. The cells were incubated for 24 hours at 37°C with 5% CO₂. The cells were transfected with 1.5µg/ml GFP per well and incubated for 48 hours. After 48 hours, the cells were stimulated with 5nM MALP-2 for 0 and 30 minutes. Following stimulation the cells were washed in 2ml PBS, fixed and permeabilized and the nuclei were stained with PI.

been found by a colleague that Mal L180 interaction with MyD88 is not hindered and so it may be because of this that that Mal L180 accumulates in the cytosol. Studies by Triantafilou and colleagues found that TLR2 and TLR6 are recruited to lipid rafts when stimulated by ligand and that after stimulation they are internalized and are directed to the Golgi apparatus. This action was found to occur in the absence of signalling to NF- κ B (Triantafilou et al., 2006). This suggests that Mal may be recruited along with TLR2 into the Golgi apparatus; however this action does not automatically cause signalling to NF- κ B.

3.2.11.3 GFP alone does not accumulate in the cytosol of cells upon stimulation with MALP-2

To ensure that it is the Mal protein that is causing the accumulation and it is not the CFP forming aggregates when the cell is stimulated, HEK 293 TLR2 cells were transfected with green fluorescent protein (GFP). 24 hours after transfection the cells were stimulated with 5nM MALP-2. Figure 3.25 displays GFP transfected into HEK 293 TLR2 cells. Panel (a) shows GFP in green is found dispersed throughout the cell and when stimulated with MALP-2 the GFP appears to move to the nucleus, it does not form aggregates in the cytoplasm as observed by Mal L180.

3.3 Discussion

This project was initiated by a study carried out by Professor Adrian Hill and colleagues of the Centre for Human Genetics Oxford. They chose to look at some SNPs associated with members of the TLR signalling pathway focusing on the TLR 2 and 4 adaptor Mal.

A case controlled genetic association study of 4510 individuals from Gambia, Kenya, United Kingdom and Vietnam was carried out. Four major infectious diseases, invasive pneumococcal disease (IPD), bacteraemia, malaria and tuberculosis were studied. The presence of Mal SNPs, Ser55Asn, Ser180Leu, Arg13Trp and Val197Ile of the TLR adaptor Mal was investigated in the four populations. It was found that the Mal SNP Ser180Leu was independently associated with all four infectious diseases.

The presence of SNPs in the Mal gene was investigated using dbSNP from NCBI. It was found that Mal was associated with seven non-synonymous SNPs. The location of the SNPs within the Mal gene were noted and their presence in any functionally significant regions of Mal were recognised. Ser180Leu was found to be present in the functionally significant signalling region called the TIR domain. Ala9Pro and Arg13Trp were located at the N terminus of Mal. The Arg13Trp mutant was situated in close proximity to the SOCS-1 ubiquitination site as described by Mansell and colleagues (Mansell et al., 2006). Following Mal phosphorylation by Bruton's tyrosine kinase (BTK), Mal is subsequently polyubiquitinated by SOCS-1 and degraded. The change from a hydrophilic arginine to a hydrophobic tryptophan residue may have an impact on the structure of the N terminal region of Mal hindering SOCS-1 associated polyubiquitination. The Ser55Asn SNP is situated within the PEST domain (amino acids 40-56). Mansell and colleagues found a 'PEST' domain present in Mal. They found that Mal is a short lived protein which gets degraded 30 minutes after stimulation with various TLR ligands and that the 'PEST' domain is required in this process

(Kobayashi et al., 2006), a finding that was confirmed in this study. A mutation in the 'PEST' region could hinder the degradation process and result in an accumulation of Mal occurring within stimulated cells. The Asp96Asn SNP was found to be situated within the structurally conserved region of box 1 of the TIR domain. A recent study by Nagpal and colleagues found that the Asp96Asn polymorphism hindered MyD88 binding to Mal and found that this polymorphism showed reduced signalling to MyD88 (Nagpal et al., 2009). The Val197Ile SNP was found to be situated within the caspase-1 cleavage site (D-198) of YXXD. Mal is thought to be cleaved at position 198 by the cysteine protease caspase-1, releasing a 4kDa fragment from the C-terminus of Mal. The 4kDa cleaved fragment removes the EE loop and the last E α -helix of the TIR. Miggin and colleagues found that cleavage of Mal was required at D198 before signalling to downstream signalling partners could occur (Miggin et al., 2007). A seventh SNP STOP222Trp was found on dbSNP however this SNP must be present on a different isoform of the Mal gene as there is no stop codon at position 222 in the wild type Mal gene.

Due to the disease associations of SNP Ser180Leu a functional reason for the protection afforded by the Mal variant to individuals with this polymorphism was endeavoured to be found.

It was hypothesised that the serine to leucine variant provided protection from the four infections as the Mal variant would hinder signalling through TLR2 and TLR4. As a result of this, the production of inflammatory cytokines would be muted in these individuals resulting in a reduced disease pathology. When mortality rate is lower, reproduction increases allowing the variant gene to become more prevalent in a population.

The Hill study did not examine an Irish population and so genotyping analysis of 415 healthy Irish individuals was assessed. It was found that the Irish population had the same heterozygosity value as the UK Caucasian population. Irish population was found to have Ser/Ser at 70%, Ser/Leu at 25% and Leu/Leu individuals at 5%. The presence of the Ser180Leu variant in the Irish population indicates that the Irish population would have the same degree of protection from the infectious diseases as the U.K. population. A recent study by Ferwerda and colleagues carried out distribution analysis of the Mal L180 in different populations (Ferwerda et al., 2009). Ferwerda and colleagues found that by studying the mitochondrial DNA evidence from European populations that Irish people are genetically descended from western European populations, and more directly that they are descended from predecessors of the Basque region in northern Spain. Ferwerda looked at a Netherlands population and a UK population which he found to have a S180L frequency of 25% and 26.3% respectively. Similarly in this project it was found that the Irish population showed 25% heterozygous carriage of the S180L variant. They also looked at a small Basque population to which Irish people are thought to be most closely descended to as they belong to the same R1b haplotype group (Campbell, 2007). The percentage of S180L in this population was 35%, which was quite high compared to what the Irish population was found to be in this study, this however could be attributed to the small number of individuals genotyped in the Ferwerda study (110 individuals).

The goal of this project was to determine differences between Mal Ser180 (S180) and Mal Leu180 (L180) in order to gain insight into why heterozygous individuals would be protected against the infectious diseases.

Initially protein homology modelling was utilized to identify any possible change to the protein structure upon changing a serine to leucine at position 180. The secondary structure prediction programme (PSIPRED) from EMBL did not reveal any differences. However the tertiary structure prediction programme revealed differences in a region known as the DD loop of Mal. Altering the serine to a leucine, a small hydrophilic residue to a larger hydrophobic residue caused the DD loop of Mal to become more exposed. A study by Nyman and colleagues which resolved the structure of the cytoplasmic domain of TLR10 dimer found that residues surrounding the DD loop tip of the TIR domain act to stabilize the TIR/TIR interactions (Nyman et al., 2008). Serine 180 lies upstream of the DD loop and could be acting as a stabilizing residue for the tip of the DD loop.

Data presented in this chapter found that the Mal L180 variant influences signalling through TLR2. Reconstitution assays were employed to ascertain the signalling potential of both Mal S180 and Mal L180. When the Mal deficient MEFs were reconstituted with Mal S180 TLR2, signalling was restored, and this was shown by the initiation of I κ B degradation, p38 activation and IL-6 production. When Mal deficient MEFs were reconstituted with Mal L180 activation was not restored. Importantly in these reconstitution experiments the transfected protein was shown to be equally over-expressed, indicating that the difference in signalling was not due to differential expression. The study by Ferwerda and colleagues also looked at the production of cytokines in individuals with the serine to leucine polymorphism (Ferwerda et al., 2009). They found that like the Khor and colleagues study the individuals were protected from disease (Khor et al., 2007), Ferwerda and colleagues looked at experimental endotoxemia and found that individuals with the S180L polymorphism were protected. When doing cytokine analysis of the individuals they found that the

individuals that were challenged with LPS and MALP-2 produced more TNF and IL-6 cytokines than S180S individual. This study also found that PBMCs for L180L individuals produced more IFN than S180S individuals or heterozygotes. This may be as a result of a small number of patients being assessed. Furthermore, the induction of IFN α by TLR2 has not been shown to require Mal. However since there was a clear association with severe sepsis in subjects carrying the L180 variant, overall this study reveals a role for Mal in susceptibility to infectious diseases. Furthermore, inefficient Mal signalling could cause redundant pathways to compensate for Mal.

Using a GST pull-down assay to ascertain the binding capacity of Mal S180 to TLR2 was compared to Mal L180. It was found that Mal S180 bound readily to FLAG tagged-TLR2 but Mal L180 was unable to bind. Further pull-down experiments were carried out to investigate the binding potential of Mal S180 and Mal L180 to MyD88 by colleague Dr. Aisling Dunne. It was found that Mal S180 and Mal L180 were found to bind MyD88 equally. Mal L180 was also able to bind Mal S180. Therefore the defect in signalling appears to be in the potential of the Mal L180 form to inefficiently bind TLR2.

As it was observed that Mal S180 had the potential to bind Mal L180, it led to the question of whether Mal L180 could hinder Mal S180 function in a dominant negative fashion. This indeed appeared to be the case. Mal L180 when transfected into Mal deficient cells did not activate NF- κ B, unlike Mal S180, however when co-transfected Mal L180 blocked the effect of Mal S180. These results suggest that Mal L180 might have two effects: firstly, an inability to interact with TLR2 and secondly an ability to block Mal S180 action. A complex of TLR2, Mal S180 and Mal L180 may occur in the heterozygotes which therefore inhibit TLR2 signalling.

Blocking peptides were next designed to the region surrounding serine 180. A GST pull-down assay found that Mal bound to TLR2 and that this interaction was blocked by the peptide TIRILLSGLS. A scrambled version of the peptide did not hinder the Mal/TLR2 interaction. A possible explanation for this is that the serine peptide form (the same as the WT Mal) would bind to TLR2 in the same position that the Mal would usually bind and hinder the interaction of TLR2 and wild type Mal.

Further cell based studies were carried out to test the ability of the peptide to block TLR2 signalling. This time an S form TIPLLSGLSRRRRRRRRR and L form TIPLLLGLSRRRRRRRRR of the peptide was used to block TLR2 signalling. The peptides for the cell based assays had a nine arginine repeat sequence to aid their internalisation into cells. The peptides were synthesized to the 9 base pair region surrounding the Ser180. Scrambled versions of these peptides were also made. PBMCs were obtained to investigate if a decrease in IL-6 production could be observed in primary cells. L and S form peptides were added to the cells, and the cells were stimulated with TLR2/1 ligand Pam₃CSK₄ and TLR2/6 ligand MALP-2. Addition of the L and S form peptides did not block IL-6 production. In one example the L form of the peptide was added to the PBMCs and stimulated with Pam₃CSK₄ the production of LPS was lowered, however this was mirrored by the scrambled version which caused a reduction in IL-6 production.

The putative blocking peptides did not have any effect on the primary PBMC cells and the blocking of IL-6 production in cell lines is difficult to reproduce. It is unclear whether the peptides are accessing the cells and it is not known if the peptides are being degraded by resident peptidases within the PBMCs. The quantity of peptide that gains

access to the cell may be important also as too much peptide may have a damaging effect on the viability of the cells and hinder any production of cytokine that may occur as a result of ligation of ligand.

PolyI:C stimulation of PBMCs found that the L form was able to block TLR3 signalling, however it was found that both the L form and the scrambled version blocked signalling. The S form of the peptide did not block TLR3 signalling indicating that any blocking that did occur was non-specific.

Three dimensional structural modelling of the Mal structure was carried out by colleagues from Cambridge University. The 3D crystalisation structure of Mal L180 was compared to Mal S180. The location of the caspase-1 cleavage site within the Mal L180 form was more embedded within the protein compared to the Mal S180 form. Previous studies by Miggin and colleagues found that Mal is specifically cleaved by caspase-1 before it is fully functional (Miggin et al., 2007). The caspase-1 cleavage assay in this chapter was carried out to determine if there was an inability of caspase-1 to cleave Mal L180 and consequently inhibit signalling. Caspase-1 was added to Mal S180 and Mal L180 and it was found that caspase-1 was able to cleave both forms of Mal. A specific caspase-1 inhibitor was added to the fifth sample and it was found that it reduced the cleavage of the Mal L180 and Mal S180, indicating that cleavage was specific for caspase-1. In this case the inability of Mal-L180 to signal downstream is not due to caspase-1 cleavage.

To assess the sub-cellular localisation of Mal S180 CFP compared to Mal L180 CFP confocal microscopic analysis was carried. It was found that the Mal S180 and Mal

L180 were both localised to the plasma membrane, as described by Kagan and Medzhitov (Kagan and Medzhitov, 2006).

HEK 293 TLR2 cells were transiently transfected with Mal S180-CFP and Mal L180-CFP and stimulated 5nM MALP-2 to investigate further the localisation of the proteins post stimulation. An accumulation of Mal S180 CFP and Mal L180 CFP was observed after five minutes stimulation. As Kagan had discovered that TLR4 travels to early endosomal membranes, Mal was similarly found to travel intracellularly upon stimulation of TLR2 with MALP-2.

It was necessary to clarify that it was not the CFP tag that was causing the accumulation of the Mal protein. As no CFP plasmid was available in the laboratory transfected HEK 293 TLR2 cells were transiently transfected with GFP. (GFP and CFP only differ in a few amino acids which only causes them to fluoresce a different colour) GFP transiently transfected cells were stimulated for 30 minutes with 5nM MALP-2. At time zero the GFP was present in the cytosol and in the nucleus. Upon stimulation the GFP appeared to become more concentrated in the nucleus. This finding clarifies that GFP does not clump together post stimulation. A study published by Nishiya and colleagues in 2007 found that MyD88-YFP was present in intracellular vesicles; they were concerned that the MyD88 was clumping due to the presence of the tag. They carried out gel filtration analysis and separated lysates based on their molecular size. They found MyD88 was present at 67kDa and 132kDa corresponding to monomeric and homodimeric forms of MyD88 (Nishiya et al., 2007).

It therefore appears that Mal translocates from the membrane upon stimulation by MALP-2. Whether there is a serine or a leucine at position 180 does not affect this. A study by Kenny and colleagues shows that MyD88 binds directly to TLR2 and that Mal only functions to increase efficacy of signal when there is a low level of ligand.

Therefore the movement of Mal into the membrane can be explained by its binding to MyD88 as it has been shown by Dr. Aisling Dunne that both Mal-S180 and Mal-L180 can bind to MyD88. Mal L180 does not signal in Mal deficient MEFs whereas Mal S180 does. Mal L180 also has a dominant negative effect over the ability of Mal S180 to signal. Peptide blocking studies help to understand the region of the Mal S180 that is being hindered by the presence of Mal L180.

The current interpretation would therefore be that the Mal L180 will translocate but fails in its ability to mediate signalling further down-stream as it is unable to form an active signalling complex.

A large body of research has been carried out since the discovery that Mal Ser180Leu provides protection from infectious disease. Many research groups have investigated whether Mal 180 Leu provides protection in their sets of samples. A group from Germany argued that the MyD88 and Mal did not play a role in the protection of individuals from TB in a Russian population. They argued that Mal and MyD88 are dispensable in sensing *Mycobacteria tuberculosis* in mice (Reiling et al., 2008). However it has been counter-argued that the discrepant findings could be as a result of heterogeneity of association in different populations which has been previously described for many immunogenetic polymorphisms. Importantly it was noted that the age of the individuals differed from the Khor study and that with a younger mean age it is unclear how many of these control individuals have been exposed to *M. tuberculosis* or will develop clinical TB in the future. Importantly another group found that Mal Ser180Leu played a protective role against the development of systemic lupus erythematosus (SLE) and TB (Castiblanco et al., 2008). A recent study by Nagpal and colleagues analysed the ability of all the Mal polymorphisms to signal. They found that Mal Leu180 was capable of mediating TLR2 and TLR4 signalling in HEK 293s and did

not act as a dominant negative; however endogenous Mal in HEK 293 cells may be mediating the signal instead. They also found that in Mal knock out cells that the Mal Leu180 variant was able to signal. The reason for the difference is not clear, although as Nagpal and colleagues state, it is possible that the Mal Ser180Leu mutation is hypomorphic, indicating that there may be a blunting of activity which can be overcome if Mal Ser180Leu is over-expressed. Furthermore they saw that I κ B degradation was restored in Mal deficient MEFs when reconstituted with Mal Leu180; however, they used double the concentration of ligand than used in this project. Investigative studies by Kenny and colleagues have shown that Mal may not be required in signalling at higher doses of ligand (Kenny et al., 2009). Since separate studies have found disease associations associated with Mal Ser180Leu, it seems likely that Mal is important in the pathogenesis of these diseases. Further studies confirming a role for Mal Ser180Leu in infectious diseases can be anticipated.

Chapter Four

Toll like receptor 6 polymorphism Asp30Asn, its role in TLR6 signalling and its impact on invasive pneumococcal disease

Chapter 4

4.1 Introduction

S. pneumonia causes an estimated death toll of one million children under five annually (O'Brien et al., 2009). It commonly colonises the mucosal surface of the upper respiratory tract and does not harm most individuals. However, invasive infection can become established by the disruption of the respiratory epithelial membrane. Signalling through TLR2 induces an inflammatory response which is thought to contribute to decreased in trans-epithelial resistance to bacteria, allowing bacterial migration across the epithelial barrier (Beisswenger et al., 2007). *S. pneumonia* is the most common cause of community acquired pneumonia. The morbidity and mortality rate of with *S. pneumonia* infection is associated with the occurrence of septic complications and septic shock (Meister, 2000, Georges et al., 1999). It has been said, that most cases of IPD remain genetically and immunologically unexplained (Ku et al., 2007). A study in 2003 investigated TNF- α and IL-10 and the role they may play in susceptibility to IPD (Schaaf et al., 2003) The group was unable to show an association between acute phase protein TNF- α and sepsis severity; however they found that a polymorphism in the immuno-suppressive cytokine IL-10 which caused increase IL-10 release, predisposing individuals to severe pneumococcal disease. Overproduction of IL-10 also dampened the pro-inflammatory response, allowing the *S. pneumonia* microbe to divide and multiply causing severe pneumococcal infection and increasing the risk of development of septic shock.

Mannose binding lectin (MBL) is a key mediator of innate host immunity. It is produced in the liver during the acute phase response. MBL is a serum secreted PRR belonging to the calcium-dependent lectin family. It recognises high mannose and *N*-acetyl-glucosamine oligosaccharides which are present on a range of microorganisms

including *S. pneumoniae*. It activates the complement pathway and directly opsonises some infectious pathogens (Turner, 1998). *MBL* is a heterogenous gene that is associated with susceptibility to several infectious diseases. *MBL* is derived from the gene *MBL2* on chromosome 10, exon 1 contains three single-base substitutions which independently cause low expression levels of serum *MBL*. There are also several nucleotide substitutions in the promoter region which affect the *MBL* serum levels (Madsen et al., 1998). Individuals who were homozygous for the *MBL* variant had less than 2% of the *MBL* than controls and individuals who were heterozygous had approximately 20% lower *MBL* levels than controls. Studies looking at heterogenous *MBL* found that it had no effect on susceptibility to *S. Pneumoniae* associated disease while the homozygotes had increased susceptibility to IPD (Kronborg et al., 2002, Roy et al., 2002)

A recent study investigated a possible link between nitric oxide synthase 2A (NOS 2A) polymorphisms and invasive pneumococcal disease (Payton et al., 2009). NOS2A is a gene which is responsible for the production of nitric oxide in the defence against bacterial infection. The group investigated nine polymorphisms within the NOS2A gene and found that there was no association between polymorphic variation within NOS2A and IPD susceptibility or protection.

In 2007 a study by Ku and colleagues looked at two patients with primary immunodeficiencies (PIDs). They were found to show poor antibody responses to pneumococcal carbohydrates. They also showed decreased inflammatory responses to IL-1R and TLR stimulants, suggesting genetic defects in TLR/NF- κ B mediated immunity. The investigation found that the first patient had two heterozygotic mutations in an intron in *IRAK4* located between exons 10 and 11. These two mutations were not found in 60 healthy individuals tested. The second patient was found to have a

mutation in *NEMO* cDNA R173G was found in exon 4 and this mutation was not found in 120 controls analysed (Ku et al., 2007).

A study by Chapman and colleagues found an association between genes in members of the I κ B family members, *NFKB1A* and *NFKB1E*. Mutations in these genes are quite rare and are associated with susceptibility to IPD (Chapman et al., 2007). Research into variants of TLR2 and TLR4 by Moens and colleagues looked a possible link to streptococcal disease. Ninety nine Caucasians with IPD and 178 healthy controls were genotyped. They found that SNPS in TLR2 (R579H, P631 and R753Q) and a SNP in TLR4 (D299G) were not differentially distributed between the two groups, indicating that in this study there was no association between TLR2 or TLR4 and IPD (Moens et al., 2007).

Investigative studies into genetic associations in IPD seem to suggest that any association that has been found is related to genes within the TLR and NF- κ B pathways. Several studies have revealed that both TLR2 and TLR4 play a role in the detection of *S. Pneumonia*.

Dysregulation of TLRs plays a role in the susceptibility of the host to acute and chronic inflammatory processes. Acute over-reactions to pathogens can manifest as sepsis which is defined as a systemic inflammatory response syndrome (SIRS) and occurs when the body as a whole over-reacts to infection whether it be bacterial, viral, fungal or parasitic. Animals lacking TLR4 do not develop septic shock when challenged with bacterial LPS (Hoshino et al., 1999, Brandtzaeg and van Deuren, 2002).

A rare polymorphism in human TLR6 has been identified by Professor Adrian Hill and his group. The presence of this TLR6 variant in individuals has been shown to be associated with a 7 fold increased risk of contracting invasive pneumococcal disease (IPD). Human TLR6 is a type 1 transmembrane pathogen receptor. It is found on the surface of immune cells such as macrophages and dendritic cells and can recognise the presence of microbial products by their extracellular leucine rich repeat (LRR) motifs. More specifically TLR6 in combination with TLR2 recognise the presence of diacylated lipopeptides and peptidoglycan both of which are present on the surface of *S.pneumonia*. Activation of these TLRs by pneumococcal derived particles serves to up-regulate downstream signals to activate pro-inflammatory NF- κ B and mitogen activated protein kinases (MAPKs) (figure 1.2).

The polymorphism in question causes an amino acid change at position 30, causing the replacement of aspartic acid (D) with an asparagine (N) residue (figure 4.1). Individuals who possess the mutation are far more likely to succumb to IPD than the individuals with the wild type gene. The amino acid change is located in the N terminal region of TLR6 and more specifically may reside in the signal sequence of human TLR6.

The aim of this project was to investigate any role that human TLR6 Asp30Asn may play in TLR2/TLR6 signalling and its effect on TLR6 cellular localisation. A biochemical link between this variant and IPD will also be explored.



Figure 4.1 The functional sites of human TLR6.

Human TLR6 is a cell surface TLR containing 19 leucine rich repeats utilised in the detection of pathogen associated molecular patterns. TLR6 functions in tandem with TLR2 to recognise diacylated lipoproteins. TLR6 contains a transmembrane domain and an intracellular TIR domain. Ligand induced dimerization of the TLRs is thought to allow protein protein interactions of the TIR (Toll-interleukin-1 receptor) domains creating a scaffold to allow the recruitment of TLR adaptor molecules. Recruitment of the adaptor molecules activates the NF- κ B signalling cascade.

4.2 Results

4.2.1 Significance of polymorphism Asp30Asn in human TLR6 structure

The starting point in the TLR6 investigation was to address whether the Asp 30Asn polymorphism in TLR6 lay in a position within the protein that was significant to its structure and whether it was near any functionally relevant areas of TLR6.

In 1999 Takeuchi identified TLR6 as a novel member of the TLR family (Takeuchi, 1999). Takeuchi predicted that human TLR6 contained both an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic Toll/IL-1 receptor (IL-1R) like region (TIR) domain. Takeuchi predicted that the N-terminal signal peptide of TLR6 was from amino acid 1 to 29 that it contained 19 leucine rich repeats, and that it possessed a transmembrane region from amino acids 583-606 (figure 4.1).

The TLR6 polymorphism under investigation in this thesis lies at position 30, in the vicinity of the signal peptide region and so may affect the membrane localisation of TLR6.

The N terminal signal peptide region of the proteins controls the proteins entry into the secretory system of the cell. The signal peptide controls selective targeting, translocation and/or insertion into the plasma membrane or the endomembrane system.

Using signalP, an online computer signal peptide prediction programme, both TLR6 D30 and TLR6 N30 protein sequences were input into the database. SignalP was developed in 1997 by Neilsen and colleagues. They compared 3379 different N-terminal peptide regions from human, eukaryote, *E. coli*, gram negative and gram positive bacteria. They only considered proteins sequences which had experimentally pre-determined signal peptide cleavage sites. By aligning all these

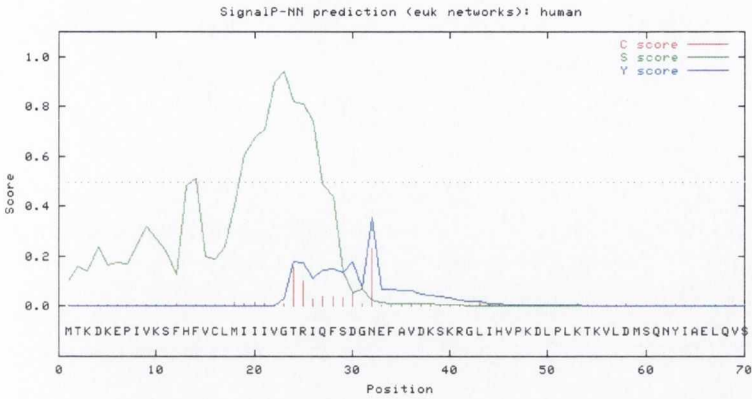
peptide sequences, they developed an algorithm to help predict the signal peptide cleavage of all proteins (Nielsen et al., 1997). The signalP programme predicted that the signal peptide cleavage site of TLR6 D30 was between position 31 and 32, the predicted cleavage site of TLR6 N30 was at the same position between amino acid 31 and 32 (figure 4.2). C-scores (shown in red) were obtained for both proteins and Y and S scores which are derived from the C-score were also identified. The Y value determines the mean of the C scores and which results in a better cleavage prediction value. The S value determines the length of the signal sequence and whether this is a secretory or non-secretory protein. Both TLR6-N30 and TLR6-D30 were predicted to be non-secretory proteins.

It has been previously shown that two mutants found at the N-terminus of TLR 2 affected its localisation (Nakao et al., 2005). TLR2 Cys30Ser and TLR2 Cys36Ser were both found to be expressed intracellularly in HEK 293 cells and that they failed to activate proinflammatory transcription factor NF- κ B upon activation with TLR2/6 ligand MALP-2. The sequence of TLR2 was input into the signalP signal peptide cleavage prediction programme, the signal peptide cleavage site was predicted to be at position 19 (figure 4.2). This suggests that the inability of the TLR2 Cys30Ser and Cys36Ser to reach the plasma membrane is unrelated to its signal peptide.

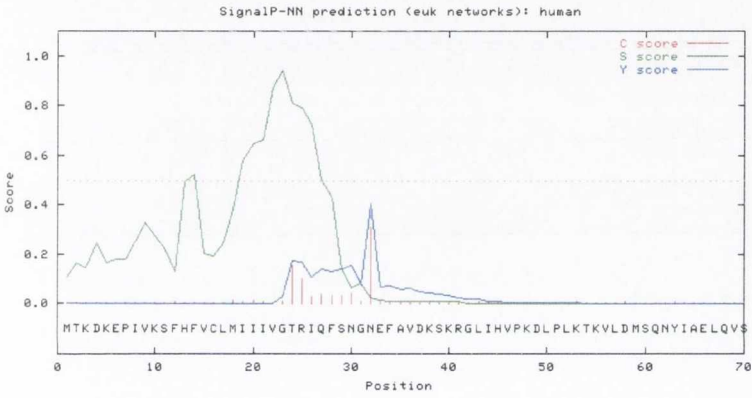
The nascent polypeptide is targeted to the endoplasmic reticulum (ER) by the ribosome, a signal recognition particle recognises the N terminal signal peptide and directs it to the membrane bound translocon complex. The translocon complex is normally closed and only when a protein possessing a fully functional signal peptide is allowed to bind, will it open allowing the entry of the nascent polypeptide (Hegde and Kang, 2008). The signal peptide is composed of three regions which are displayed in figure 4.3 (Nielsen et al., 1997). They are commonly described as a

Figure 4.2 Signal peptide cleavage site predictions.

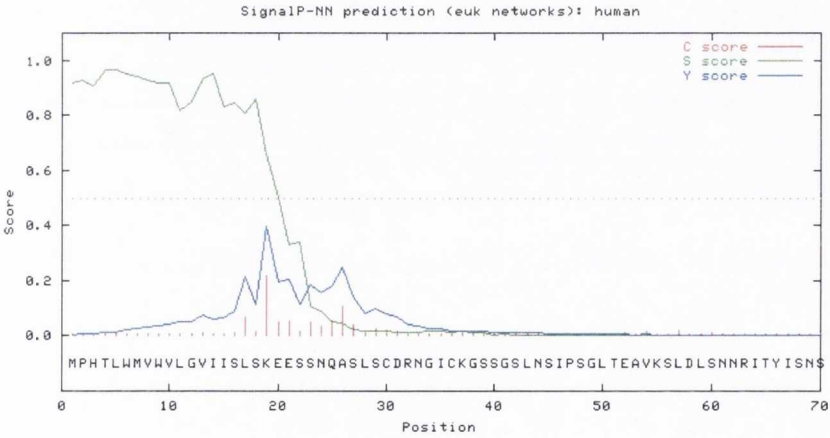
The signal peptide cleavage site of human TLR6 D30, human TLR6 N30 and human TLR2 are displayed. The predicted cleavage site of WT TLR6 was found between positions 31-32; the predicted cleavage site of TLR6 N30 is also between positions 31-32. The predicted cleavage site of human was found at position 19 of TLR2. The C score shown in red indicates the cleavage site score, the residue with the highest score is the most favourable cleavage site.



Human TLR6 D30



Human TLR6 N30



Human TLR2

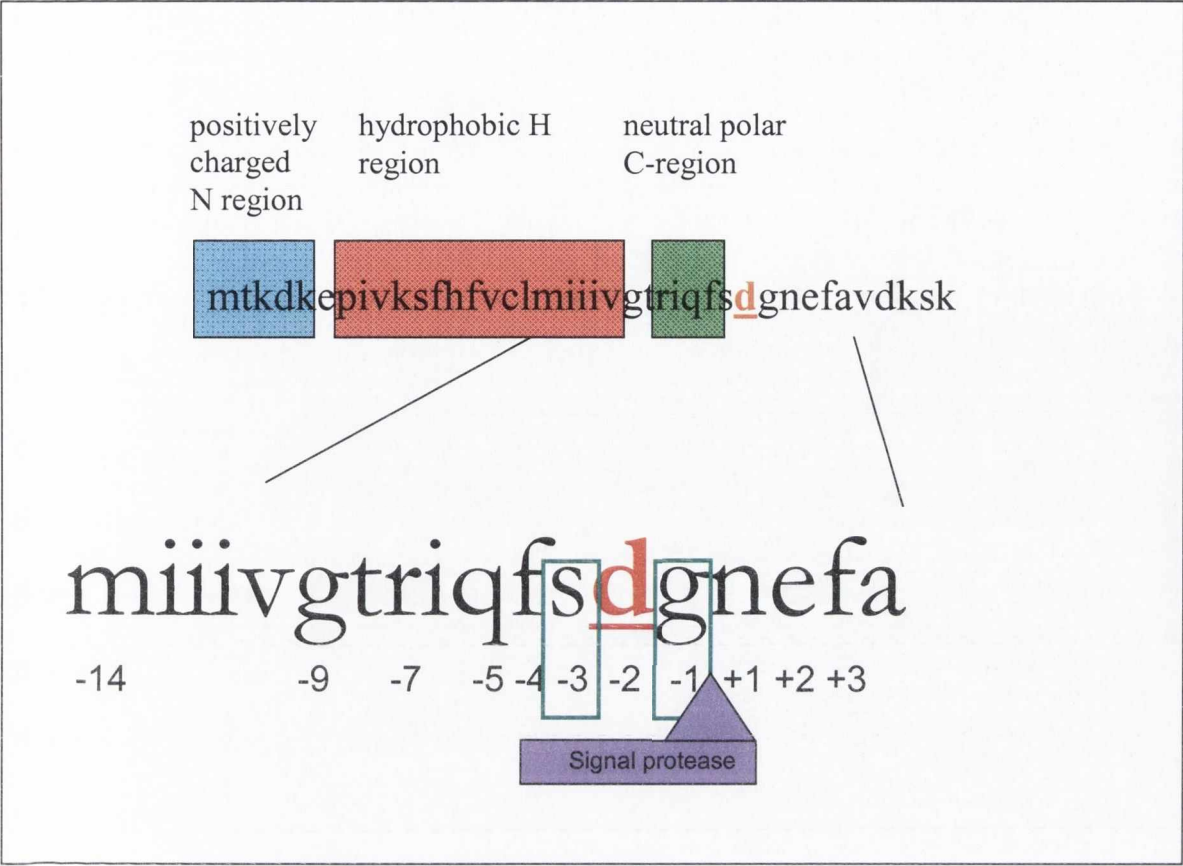


Figure 4.3 The N-terminal signal sequence of TLR6.

The N-terminal signal sequence of TLR6 is composed of three regions, a positively charged N region shown in blue which contains the starting methionine, a hydrophobic H region displayed in red, and a neutral polar C region shown in green. The aspartic acid which becomes mutated is located at position -2 in relation to the signal peptide cleavage site.

positively charged N-region shown in blue a hydrophobic H-region (red) and a neutral but polar C-region (green). The hydrophobic signal peptide binds to the translocon and the remainder of the polypeptide is moved through the pore. A signal protease cleaves the polypeptide at the signal peptide cleavage site, in the case of TLR6 it is between amino acid 31 (glycine) and 32 (asparagine). A common nomenclature has been devised which describes the amino acids either side of the cleavage site as -1 and +1 (figure 4.3). The system describes the amino acids from the cleavage site to the starting methionine as negative numbers, in the case of TLR6 from -1 to -31 and the amino acids which form the mature protein as positive numbers. The aspartic acid at position 30 is described as -2 using this system (figure 4.3). It has been found that it is important that amino acids at position -1 and -3 are small and neutral for cleavage to occur correctly. The aspartic acid at amino acid 30 resides in the -2 position and amino acids situated at position -2 are highly varied. This may lead to the conclusion that an amino acid change at this position would not lead to any great variability in the translocation of the protein. However, as the amino acid change is in such near proximity to the cleavage site, further investigation was carried out.

The cleavage of signal peptides depends heavily on the charge of the amino acids. Alterations in the charge of amino acids in the translocon influences the orientation and stability of the polypeptide chains that interact with it (Hegde, 2008): and so it may be postulated that a change in the charges of the polypeptide chain may affect its binding to the translocon. In the case of the TLR6 polymorphism, a negatively charged hydrophilic aspartic acid is replaced by a hydrophobic neutral asparagine, causing the loss of a charged amino acid to occur.

4.2.2 Secondary structure prediction

All signal peptides are predicted to be composed of alpha helical hydrophobic regions. To gain further insight into the secondary structure of wild type TLR6 D30 compared to the TLR6 N30, the sequence of human TLR6 was input into an online secondary structure prediction programme (bioinf.cs.ucl.ac.uk/psipred/). The graphical output is shown in figure 4.4. As can be seen in figure 4.4, the alpha-helical hydrophobic core of the signal peptide region is shown in green. The structure of the TLR6 D30 and TLR6 N30 are compared the secondary structure is altered in the sequence with the mutation. The hydrophobic alpha helix remains the same but then after two amino acids a second shorter alpha helix forms. This structural change is located at the C region of the signal peptide and may influence the cleavage of the peptide.

4.2.3 Investigating the cellular location of TLR6 D30 compared to TLR6 N30

4.2.3.1 Cellular localisation using membrane fractionation

As it was predicted that the TLR6 D30 and TLR6 N30 may have different localisation properties due to their structural differences in the region close to the peptide cleavage site TLR6 N30-FLAG and TLR6 N30-YFP variant were synthesized using site directed mutagenesis. The FLAG tag of TLR6 is located within the N terminal signal peptide of TLR6 (figure 4.5). The plasmid was used in membrane fraction assays to identify the location of the TLR6 FLAG (figure 4.6). It was found that the TLR6-D30 FLAG and TLR6-N30 FLAG were membrane bound

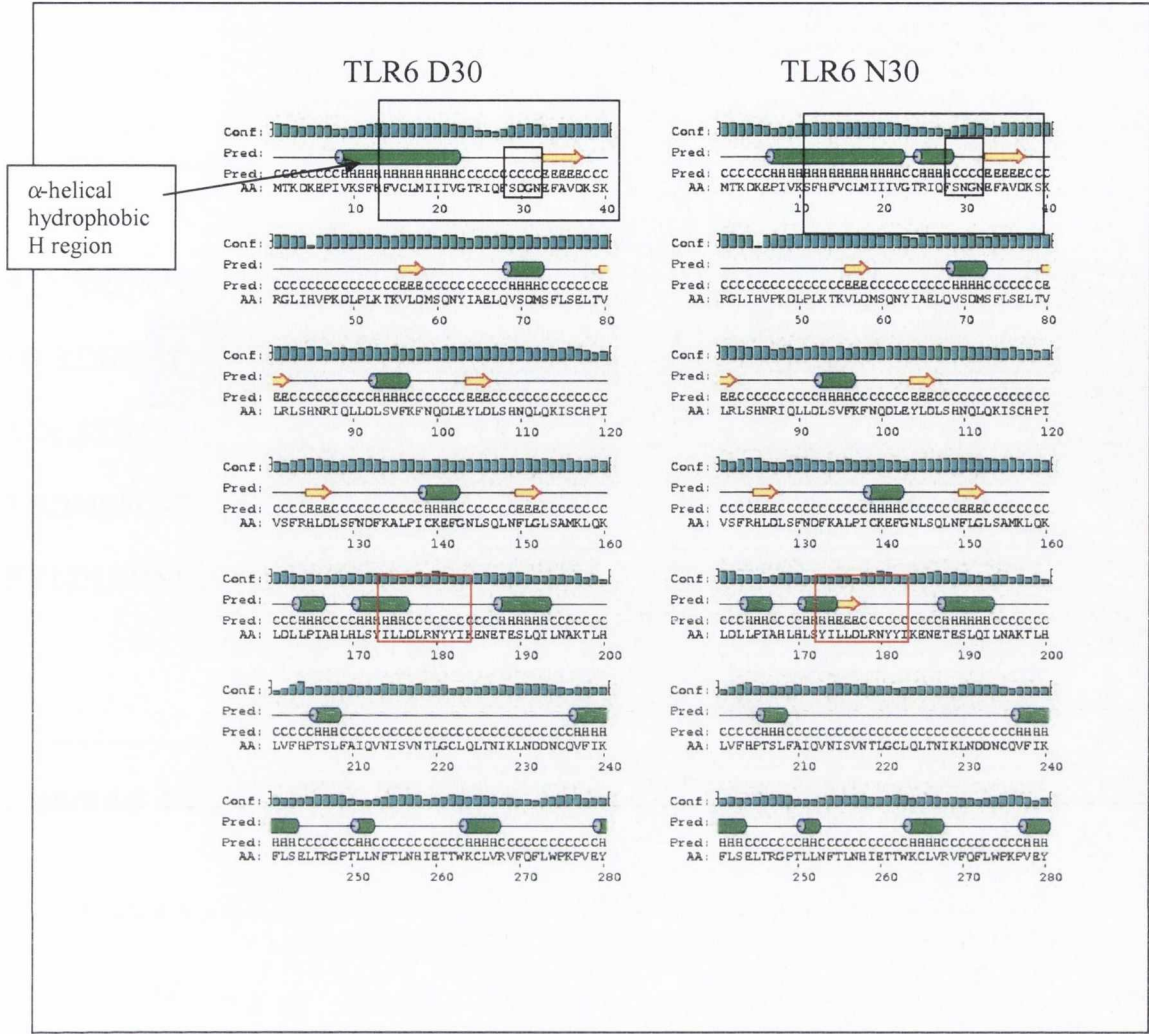


Figure 4.4 Secondary structure prediction of human TLR6 revealed a change in structure comparing TLR6 D30 to TLR6 N30.

The Predict Protein programme is a computer-based programme that predicts the alpha helical (green cylinder), beta strand (yellow arrow) and random coil (straight line) structure of protein sequences that have been input into the software (bioinf.cs.ucl.ac.uk/psipred/). The aspartic acid was changed to an asparagine at position 30, the structure of TLR6 N terminus became altered. The asparagine at position 30 caused a longer alpha helix to form at the N terminus. A second change to the secondary structure is indicated by a red box, it is situated away from the area of interest.

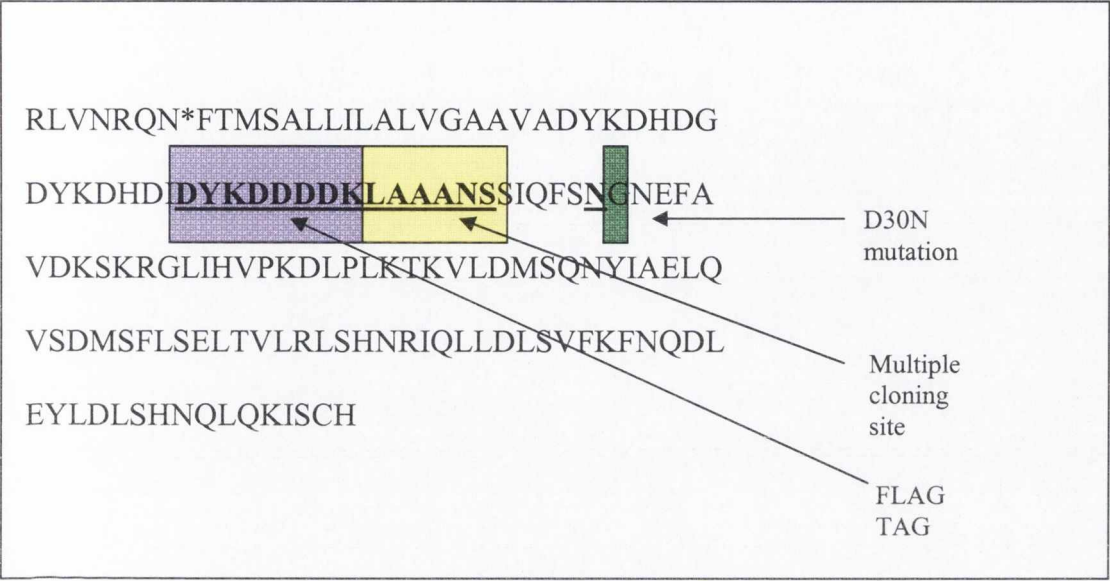
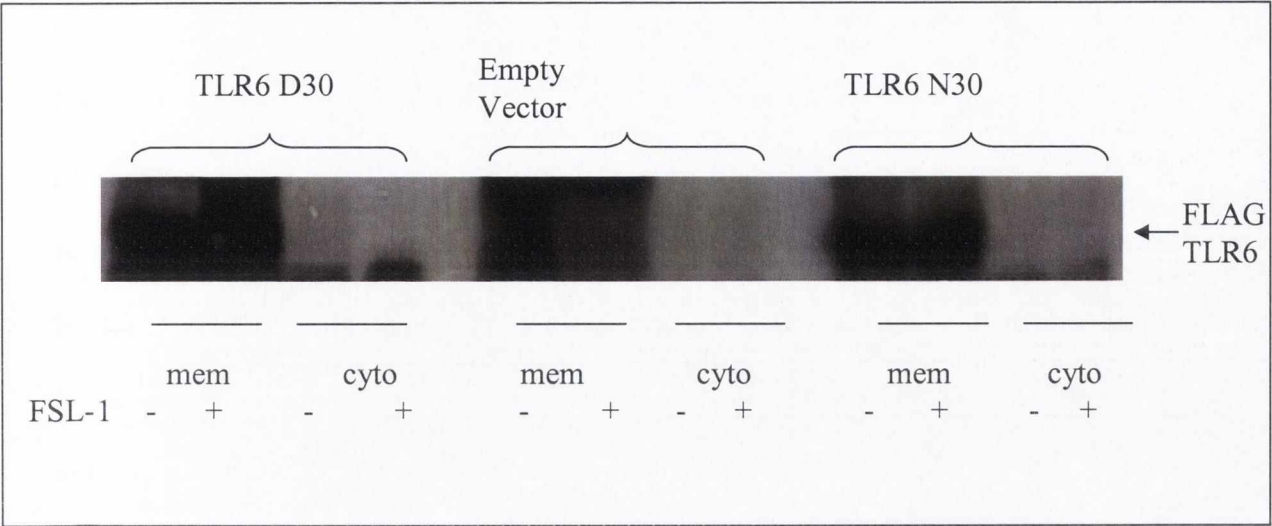


Figure 4.5 Generation of TLR6 N30-FLAG variant.

Site directed mutagenesis was carried out, which mutated the aspartate at position 30 to an asparagine. The FLAG tag is situated at the N terminus of TLR6.

Figure 4.6 Membrane fractionation studies revealed that TLR6 D30-FLAG and TLR6 N30-FLAG were found in the membrane and not the cytosol. Stimulation with FSL-1 did not alter the location of the proteins.

HEK 293T cells were seeded at 1×10^5 cells/ml into 10cm dishes and incubated at 37°C with 5% CO₂ overnight in DMEM. After 24 hours, the 10cm dishes were transiently transfected with 6µg EV-FLAG, TLR6 D30-FLAG or TLR6 N30-FLAG and incubated for a further 24 hours. The cells were washed in 2ml cold PBS twice and were lysed in 400µl membrane resuspension buffer. The cells were homogenised with a dounce homogeniser using 35 strokes. The lysed cells were centrifuged in an ultracentrifuge at 100,000g for 1 hour. The supernatant was removed from the pellet and 80µl sample buffer with DTT was added to the supernatant/cytosol fraction. The pellet was removed and 100µl sample buffer with DTT was added to the pellet/membrane fraction. The samples were sonicated for 3 seconds and boiled for 5 minutes. The samples were resolved on a 12% sodium dodecylsulfate (SDS) polyacrylamide gel using a constant current of 25mA per gel. Each protein gel was transferred to PVDF membrane for 2 hours at 150mA. The membrane was blocked in 5% Marvel in TBST for 1 hour at room temperature. The membrane was probed with anti GFP antibody 1:500 dilution in 5% Marvel in TBST and rolled overnight at 4°C. The membrane was re-probed for β -Actin loading control. The blot was developed by enhanced chemiluminescence. The results are representative of two experiments.



and none of the protein was mis-localised to the cytoplasm (figure 4.6). No bands appeared in the empty vector lanes. These cells were treated with the TLR2/6 ligand FSL-1 at 100ng/ml, it was found that stimulation did not alter the location of the proteins (figure 4.6). The N-terminal sequence of the FLAG-TLR6 D30 and N30 were entered into the signalP signal peptide cleavage prediction program and it was found that no cleavage site was identifiable in this sequence (figure 4.7). The C-score shown in red shows the cleavage site prediction score, the score for this was very low at 0.131, no Y or S score found either indicating that no cleavage would occur. This was something that was found in the membrane fraction assay as the N-terminal tag was still present on both forms of TLR6 (figure 4.6)

Site directed mutagenesis was also carried out on the TLR6-YFP plasmid changing the aspartate at position 30 to asparagine. HEK 293T cells were transfected with empty vector, TLR6 D30-YFP, TLR6 N30-YFP plasmid and cytosolic and membrane fractions were extracted. Figure 4.8 compares membrane and cytosolic fractions, TLR6 D30 is found in the membrane fraction and TLR6 N30 is found both the membrane and cytosolic fractions.

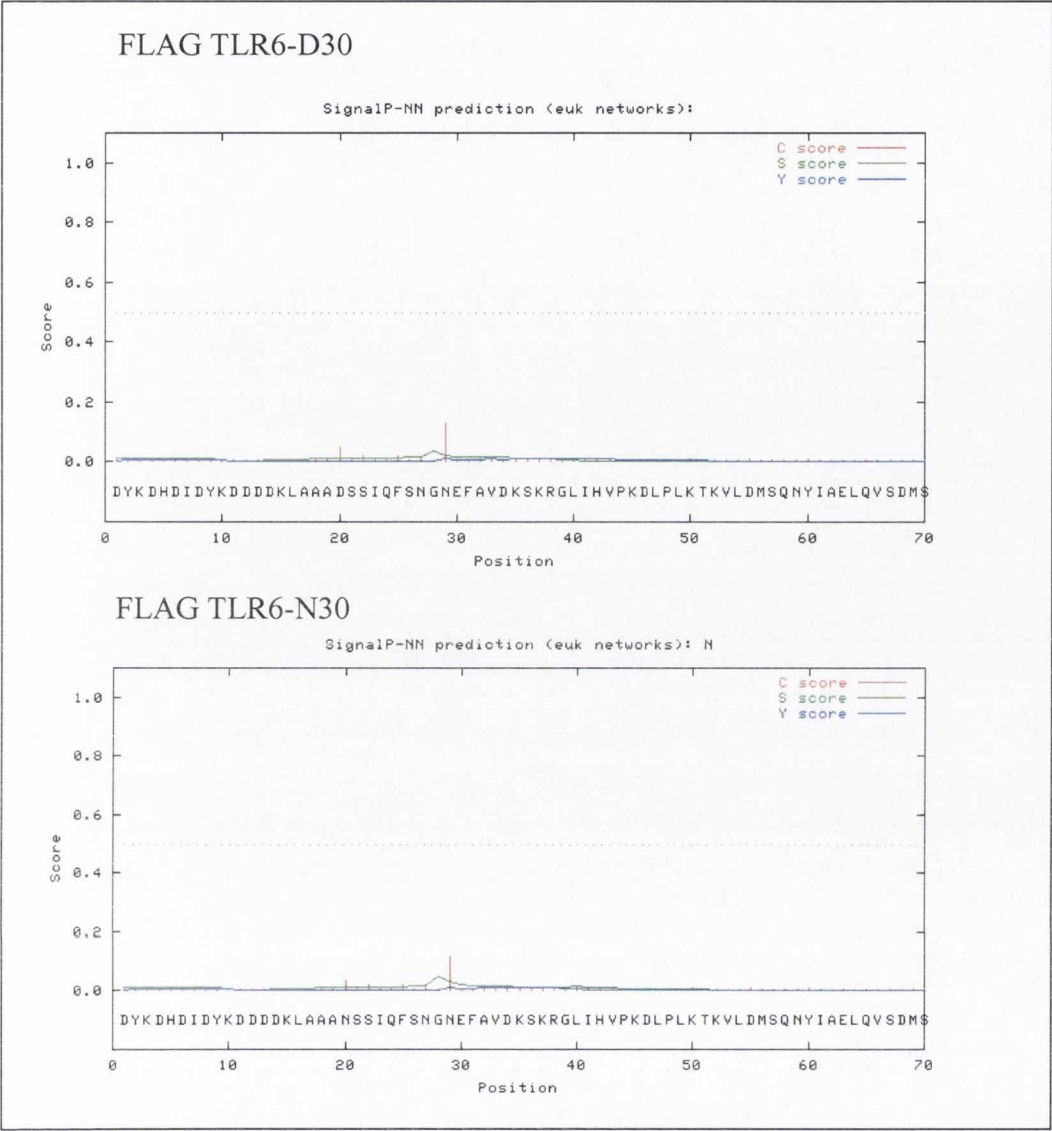
To further confirm the presence of the variant in the cytosol, a control lane which probed for a purely membrane bound protein in the cytosol should have been used this would indicate that no contamination of the cytosolic fraction with the membrane fraction occurred. A different method used in this project to further understand the localisation of the protein, was to analyse the presence of the protein by confocal microscopy.

4.2.3.2 Cellular localisation of TLR6 using confocal microscopy

As it was found that the TLR6 N30 variant was found in both the membrane and the cytosolic fraction, the location of the variant was analysed by confocal microscopy. HEK 293T cells were transfected with both TLR6-D30 YFP and TLR6-N30 YFP. The confocal imagery revealed that the TLR6 D30-YFP and TLR6 N30-YFP were localised around the nucleus (figure 4.9). The membrane stained in red revealed that most of the TLR6 was localised within the cell and not as prominent on the plasma membrane. These images also suggest that although the TLR6 N30-YFP is found on the endomembrane system, TLR6-N30 YFP is also found diffused throughout the cytosol (figure 4.9), this follows from section 4.2.3.1 which displayed that TLR6 N30-YFP was found in the cytosolic fraction of the membrane fractionation assay.

Experiments were carried out to determine whether by stimulating the cells with TLR2/6 ligand TLR6 D30-YFP or TLR6 N30-YFP would become more plasma membrane localised. The images reveal on figure 4.9 that indeed upon stimulation the TLR6 D30-YFP becomes plasma membrane localised as shown by the yellow colour that appears when the green of the TLR6-YFP is localised with the red plasma membrane stain.

To further understand the cellular location of TLR6 D30-YFP and TLR6 N30-YFP co-localisation assays were performed (figure 4.10). Both TLR6 plasmids were transfected together with ER-CFP marker into HEK-293T cells. The images reveal that TLR6 D30-YFP and TLR6 N30-YFP were present on the endoplasmic reticulum of HEK 293T cells.

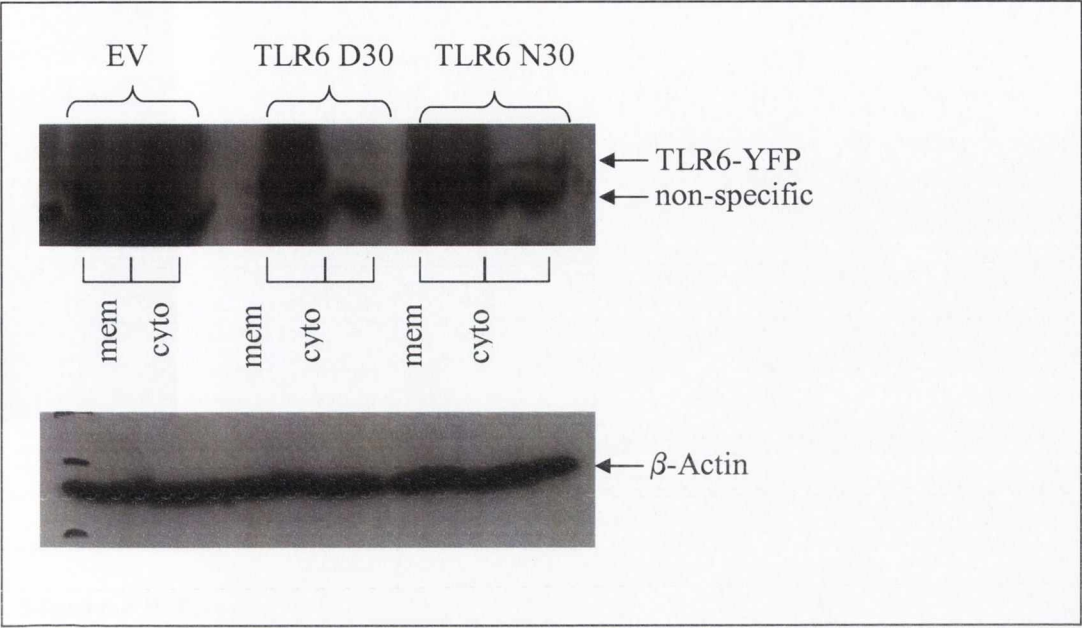


4.7 Signal peptide cleavage prediction of FLAG tagged TLR6 D30 and N30.

N-terminally FLAG tagged TLR6 was analysed using signal peptide cleavage prediction programme signalP. It was found that no cleavage of the N-terminal region of TLR6 occurs for either version of TLR6. A very low C score which determines the cleavage site and the absence of a Y and S score which are determined from the C score indicated that no apparent cleavage should occur.

Figure 4.8 Membrane fractionation revealed TLR6 N30 was found in both membrane and cytosolic fractions.

HEK 293T cells were seeded at 1×10^5 cells/ml into 10 cm dishes and were incubated at 37°C with 5% CO₂ overnight in DMEM. After 24 hours, the 10cm dishes were transiently transfected with 6µg EV, TLR6 D30-YFP or TLR6 N30-YFP and incubated for a further 24 hours. The cells were washed in 2ml cold PBS twice and were lysed in 400µl of membrane resuspension buffer. The cells were homogenised with a dounce homogeniser using 35 strokes. The lysed cells were centrifuged in an ultracentrifuge at 100,000g for 1 hour. The supernatant was removed from the pellet and 80µl sample buffer with DTT was added to the supernatant/cytosol fraction. The pellet was removed and 100µl sample buffer with DTT added to the pellet/membrane fraction. The samples were sonicated for 3 seconds and boiled for 5 minutes. The samples were resolved on a 12% SDS-PAGE gel using a constant current of 25mA per gel. Each protein gel was transferred to PVDF membrane for 2 hours at 150mA. The membrane was blocked in 5% Marvel in TBST for 1 hour at room temperature. The membrane was probed with anti GFP antibody 1:500 dilution in 5% Marvel in TBST and rolled over night at 4°C. TLR6-YFP is indicated by the black arrow. The membrane was re-probed for β -Actin loading control. The blot was developed by enhanced chemiluminescence. The results are representative of three experiments.



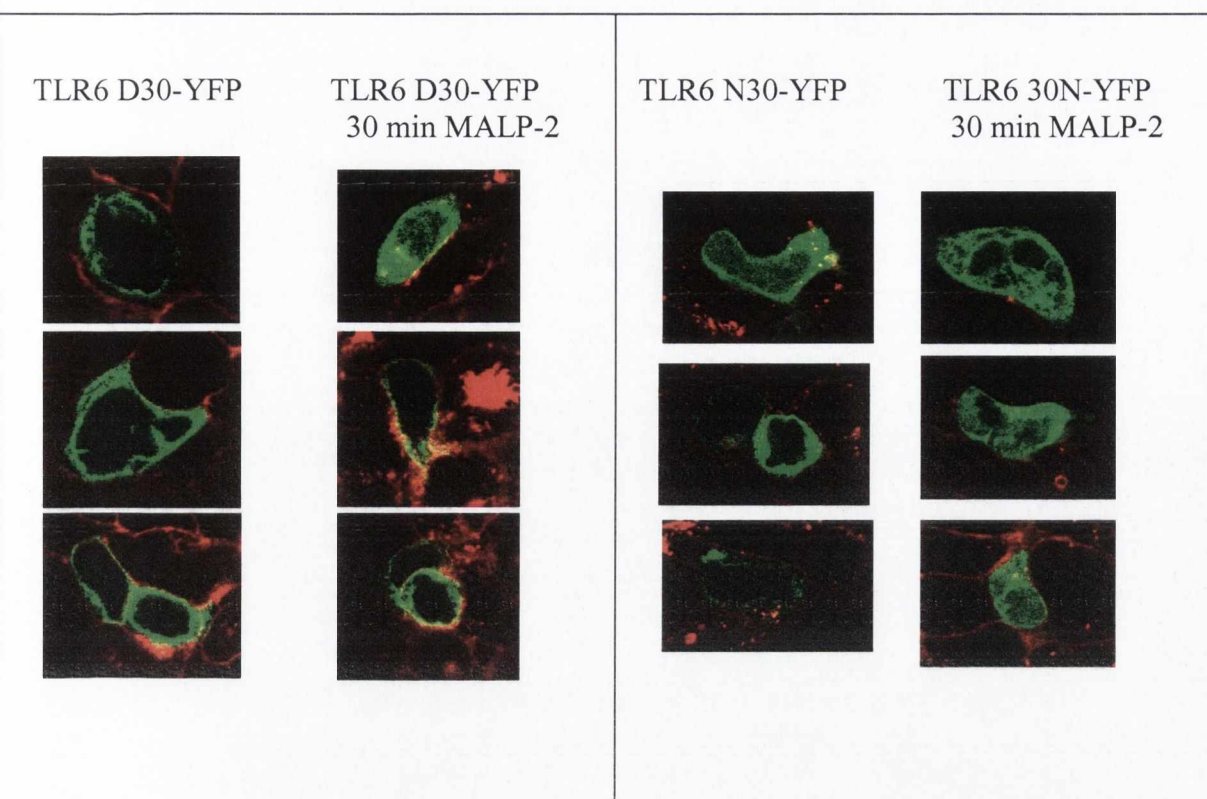
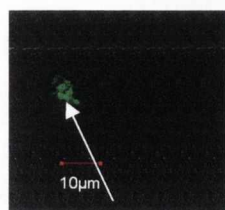


Figure 4.9 Confocal microscopy revealed TLR6 D30-YFP and TLR6 N30-YFP were found in the endomembrane system. TLR6 N30-YFP was found to be more diffuse.

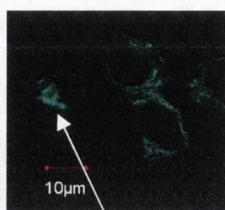
HEK 293T cells were seeded at 1×10^5 cells/ml in tissue culture dishes containing coverglass slides attached to the base. The cells were incubated over night at 37°C with 5% CO_2 . The following day the cells were transiently transfected with $2\mu\text{g}$ TLR6 D30-YFP and TLR6 N30-YFP. The cells were incubated for a further 24 hours and were analysed using a confocal microscope. Images of the cell were taken before stimulation and after 30 minute stimulation with 5nM MALP-2. TLR6 D30-YFP and TLR6 N30-YFP are displayed in green and the membrane is shown in red.

Figure 4.10 TLR6 D30-YFP co-localised with the endoplasmic reticulum.

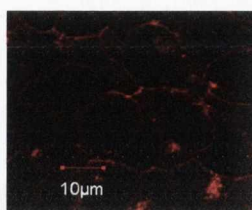
HEK 293T cells were seeded at 1×10^5 cells/ml in tissue culture dishes containing coverglass slides attached to the base. The cells were incubated over night at 37°C with 5% CO_2 . The following day the cells were transiently transfected with $2\mu\text{g}$ TLR6 D30-YFP, $2\mu\text{g}$ TLR6 N30-YFP and $0.25\mu\text{g}$ ER-CFP. The cells were incubated for a further 24 hours and were analysed using a confocal microscope. Images of the cell were taken before stimulation and after 30 minute stimulation with 5nM MALP-2. TLR6 D30-YFP is displayed in green, endoplasmic reticulum is displayed in cyan and the membrane is shown in red. Phase images are shown in gray. White arrows indicate the presence of TLR6-YFP and ER-CFP, arrows also indicate the presence of co-localisation.



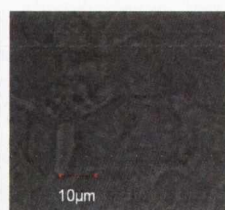
TLR6 D30-YFP



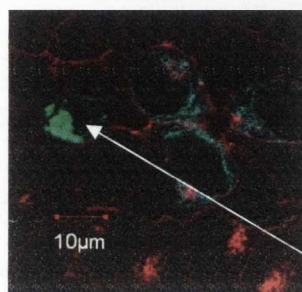
ER-CFP



membrane stain

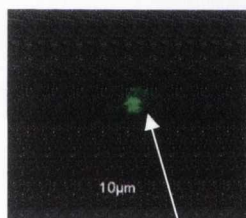


phase

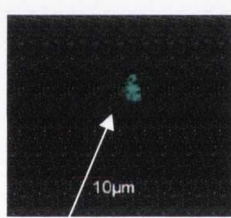


merge

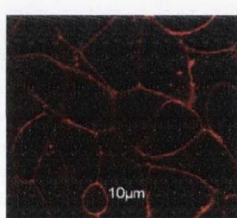
co-localisation



TLR6 N30-YFP



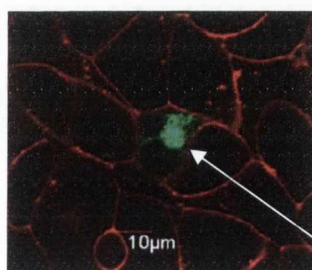
ER-CFP



membrane stain



phase



co-localisation

4.2.4 Investigating the role of TLR6 N30 in TLR functional assays

To address whether TLR6 N30 signalled similarly to wild type TLR6 D30, NF- κ B luciferase assays were carried out. HEK 293 cells were transfected with EV and increasing concentrations of TLR6 D30-YFP and TLR6 N30-YFP (figure 4.11). TLR6 wild type or variant did not up-regulate NF- κ B luciferase activity when over-expressed. As TLR6 and TLR2 operate as a heterodimer when responding to ligand, HEK 293 cells were transfected with TLR2-FLAG along with TLR6 D30-YFP and TLR6 N30-YFP. TLR2 alone highly activates the NF- κ B luciferase activity. An increase in luciferase activity was seen by adding over-expressed 10ng TLR6 D30-YFP over TLR2 activity (figure 4.12a), TLR6 N30 was unable to enhance the effect of TLR2. This experiment was also carried out with the addition of TLR2/6 ligand (4.12b); however no increase in activity was seen over untreated cells possibly due to saturation of activation of the NF- κ B luciferase reporter system.

4.2.5 Development of HEK 293T pDUO TLR2/6 stable cell lines

4.2.5.1 Establishing HEK 293T pDUO TLR2/6 D30 and HEK 293T pDUO TLR2/6 N30 stable cell lines

In order to achieve a greater understanding of the role of the TLR6 polymorphism N30 in TLR signalling it was important to have TLR2 present at a constant concentration as they both signal as a dimer upon ligand recognition. HEK 293T cells were stably transfected with pDUO TLR2/6 plasmid and pDUO TLR2/6 N30 plasmid which was obtained by site directed mutagenesis. The stably transfected cells were selected for by growing in the antibiotic blasticidin to which they

possessed a resistance gene to. The cells were analysed by FACS for the presence of TLR2 using a FITC conjugated antibody to human TLR2. The stable cells were compared to their parent cell HEK 293T cells (figure 4.13). As can be seen in figure 4.13 two peaks appear in the plot. In panels b and c region 2 (R2) show the presence of TLR2 on the cell surface of both the HEK 293T pDUO TLR2/6 D30 and the HEK 293T pDUO TLR2/6 N30 cells respectively. The mean fluorescent intervals (MFI) of region 2 (R2) of both D30 and N30 cells were obtained. The mean fluorescence interval (MFI) of D30 cells was 28.19 and the MFI of the N30 cells was 30.41 indicating that both pool have the same level of TLR2 present on the surface of the cells. The presence of the TLR2 indicates that the cells were transfected and that the pDUO plasmid is expressing TLR2 and hence should be expressing TLR6 as they are both on the same expression plasmid.

4.2.5.2 TLR signalling assays reveal that HEK 293T pDUO TLR2/6 N30 pool of stable cells is activated to a greater extent than TLR2/6 D30 cells in response to TLR2/6 ligand

Taking the cells from section 4.2.5.1, an NF- κ B luciferase assay was performed. HEK 293T, HEK 293T pDUO TLR2/6 D30 and HEK 293T pDUO TLR2/6 N30 cells were transiently transfected with 150ng and 75ng NF- κ B luciferase reporter plasmid and with 75 and 37ng TK renilla control plasmid. Each sample was normalized using an empty vector control. Figure 4.14 displays that none of the cells were capable of activating NF- κ B solely by being over-expressed. However when stimulated with 5nM and 10nM MALP-2 (TLR2/6 ligand), the HEK 293T pDUO TLR2/6 N30 cells responded 6 fold in the 150ng NF- κ B samples and 5 fold in the

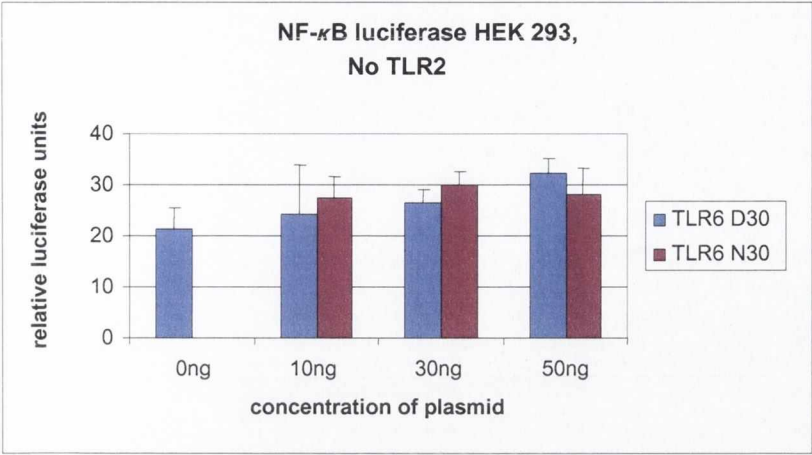


Figure 4.11 TLR6 alone did not activate NF- κ B

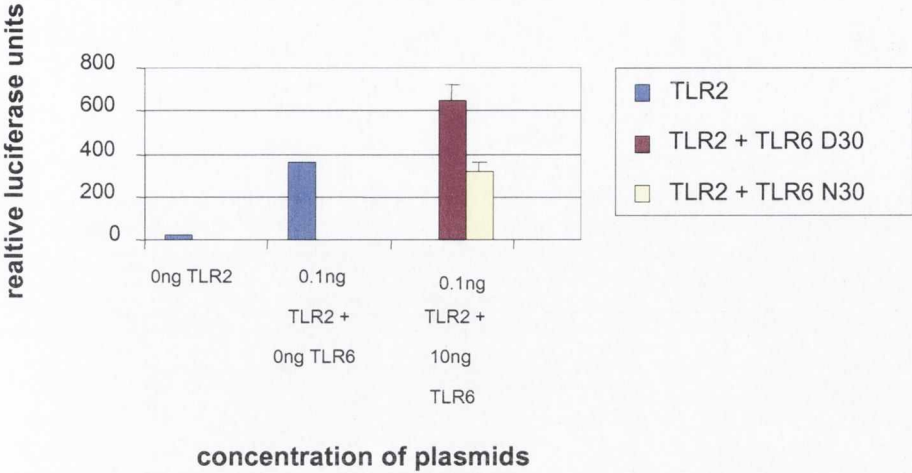
HEK 293 cells were seeded at 1×10^5 cells per 1ml into 24 well plates. After 24 hours, each well was transfected with at total of 300ng DNA. Each well was transfected with 150ng NF- κ B luciferase construct 60ng TK Renilla internal control construct, the remaining 90ng of DNA transfected into each well consisted of 20ng TLR2 co transfected with varying concentrations of TLR6 D30 of TLR6 N30. Each sample was normalized to 300ng DNA per well with empty vector.

The cells were incubated for 24 hours at 37°C at 5% CO₂. Subsequently some samples were treated with 200ng/ml FSL-1 for 5 hours. The samples were then lysed in 70 μ l passive lysis buffer and were vigorously shaken for 15 minutes. 30 μ l of each sample was placed into two 96well plates conducive for luminescent readings. 60 μ l of luciferase substrate solution was added to one plate and 60 μ l of colentraine was added to the second plate. Both plates were read using a luminometer and the readings were normalized.

Figure 4.12 TLR6 D30 increased NF- κ B luciferase activity when over-expressed with TLR2-FLAG. TLR6 N30 did not cause an increase in NF- κ B activity.

- (a) HEK 293T cells were seeded at 1×10^5 cells per 1ml into 24 well plates. After 24 hours, each well was transfected with a total of 300ng DNA. Each well was transfected with 150ng NF- κ B luciferase construct 60ng TK Renilla internal control construct, the remaining 90ng of DNA transfected into each well consisted of 0.1ng, TLR2-FLAG co-transfected with varying concentrations (0ng, 10ng) of TLR6 D30-YFP or TLR6 N30-YFP. Each sample was normalized to 300ng DNA per well with empty vector. The cells were incubated for 24 hours at 37°C at 5% CO₂.
- (b) A second set of samples were treated with 300ng/ml FSL-1 for 5 hours. The samples were then lysed in 70 μ l passive lysis buffer and were vigorously shaken for 15 minutes. 30 μ l of each sample was placed into two 96well plates conducive for luminescent readings. 60 μ l of luciferase substrate solution was added to one plate and 60 μ l of colentraine was added to the second plate. Both plates were read using a luminometer and the readings were normalized.

(a) **NF- κ B luciferase, HEK 293s, unstimulated**
0.1ng TLR2-FLAG



(b) **NF- κ B luciferase, HEK 293s + 0.1ng TLR2**
300ng/ml FSL-1

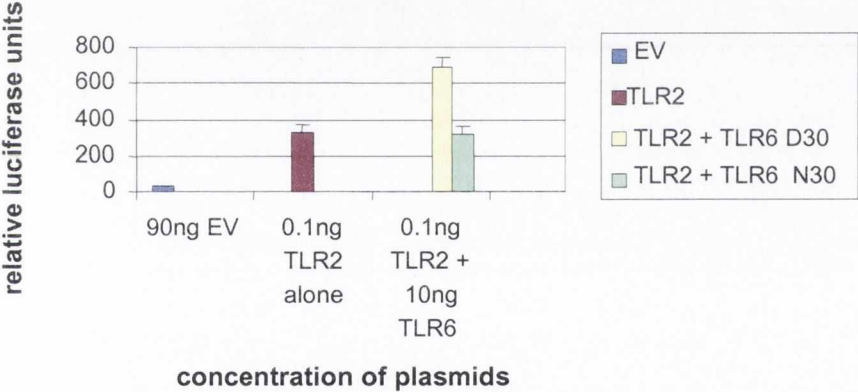
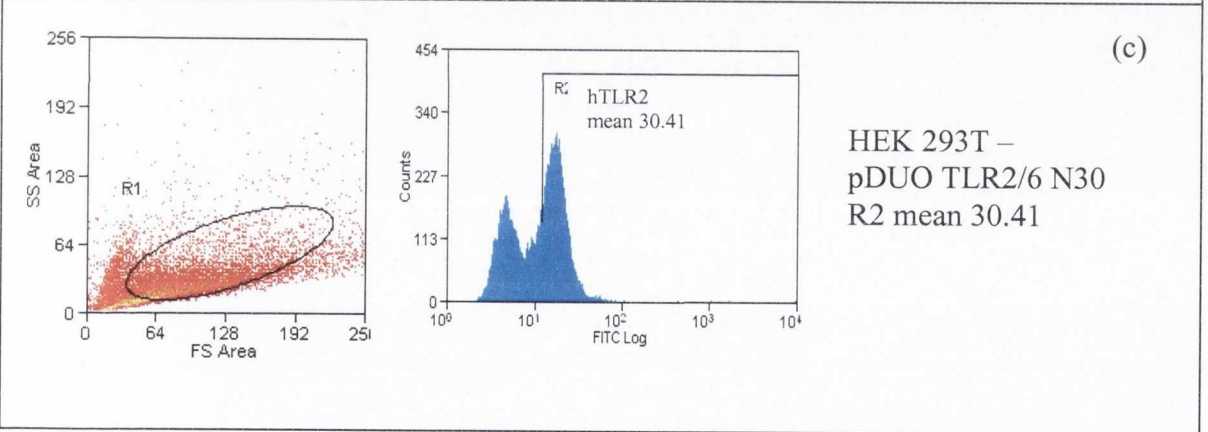
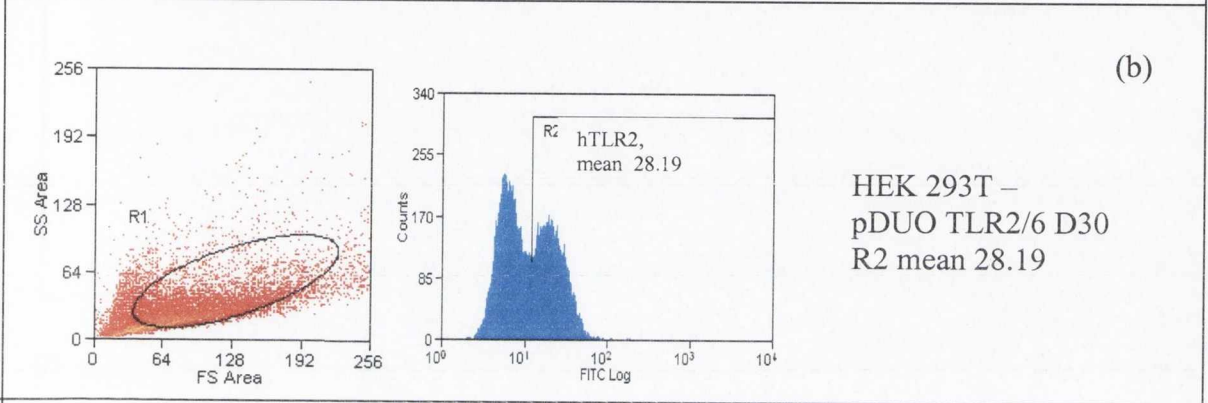
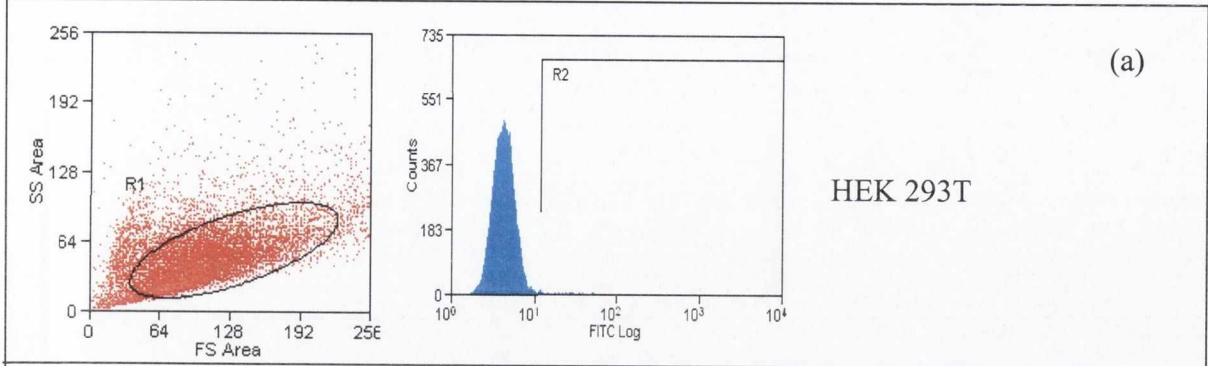


Figure 4.13 TLR2 was found on cell surface of stably transfected cells.

HEK 293T cells were transfected with pDUO TLR2/6 D30 from Invivogen and TLR2/6 N30 which was generated by site directed mutagenesis. The cells were incubated for three days during which they were passaged. An antibiotic killing curve was established to remove non-transfected cells. The cells were then grown for two weeks in 10 μ g/ml blasticidin in DMEM. HEK 293T, HEK 293T TLR2/6 D30 and HEK 293T TLR2/6 N30 cells were incubated on ice and were cell surface stained with anti-TLR2-FITC from Invivogen at 1:50 dilution (stock 0.1mg/ml, working concentration 0.2 μ g/100 μ l). The cells were analysed using a Cyan-ADP flow cytometer the same day.

Each panel a displays a forward versus side scatter plot of each cell type analysed, indicating the health of the cells and showing that a homogenous population of cells were analysed for the presence of TLR2.

- (a) Panel (a) displays HEK 293T cells cell surface stained for the presence of hTLR2. This plot shows no positive staining for the presence of hTLR2.
- (b) Panel (b) displays HEK 293T TLR2/6 D30 cells cell surface stained for the presence of hTLR2. The peak in R2 shows the presence of cell surface TLR2 and has a FITC log mean of 28.19.
- (c) Panel (c) displays HEK 293T TLR2/6 N30 cells cell surface stained for the presence of hTLR2. The peak in R2 shows the presence of cell surface TLR2 and has a FITC log mean of 30.41.



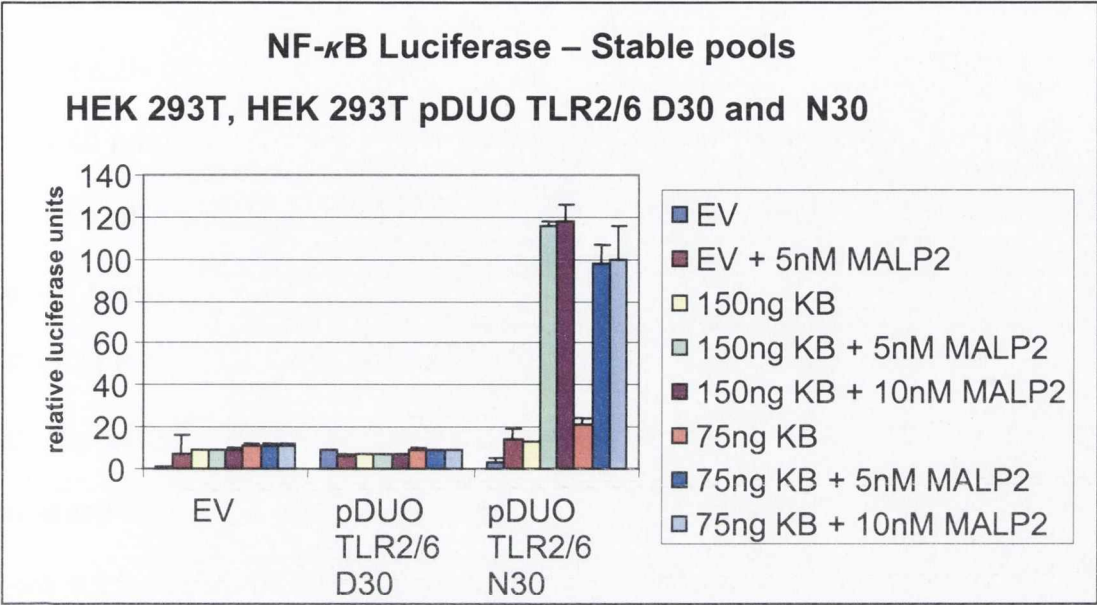


Figure 4.14 NF- κ B luciferase assay revealed HEK 293T pDUO TLR2/6 D30N cells strongly stimulated NF- κ B luciferase.

The HEK 293T stable cell lines were seeded at 1×10^5 cells per 1ml into 24 well plates. After 24 hours, each well was transfected with 150ng NF- κ B luciferase construct and 75ng TK Renilla internal control construct. The cells were also transfected with 75ng NF- κ B luciferase construct and 35ng TK renilla internal control, and 115ng empty vector control. The cells were incubated for 24 hours at 37°C at 5% CO₂. The cells were stimulated with 5nM or 10nM MALP-2 for 24 hours. The samples were then lysed in 70 μ l passive lysis buffer and were vigorously shaken for 15 minutes. Thirty microlitres of each sample was placed into two 96 well luminometer plates. Luciferase substrate solution (60 μ l) was added to one plate and 60 μ l of colentraine was added to the second plate. Both plates were read using a luminometer and the readings were normalized. This graph is representative of three experiments. Error bars show the standard deviation of three biological replicates.

75ng NF- κ B samples. The HEK 293T cells or the HEK 293T pDUO TLR2/6 D30 cells did not respond to the stimulus. The same pools of cells were analysed using Western blotting technique to investigate the extent of p38 phosphorylation. The pools of cells were seeded into 6 well plates and were stimulated with 5nM MALP-2 over a 60 minute time period. As can be seen in figure 4.15, HEK 293T cells were incapable of producing phosphorylated P38. HEK 293T pDUO TLR2/6 D30 cells slightly responded to the MALP-2 at the 30 minute time point while HEK 293T pDUO TLR2/6 N30 cells strongly responded to the stimulus at 15 and 30 minutes, with the effect diminishing at 45 and 60 minutes. A similar experiment was performed on the pools of cells (HEK 293T pDUO TLR2/6 D30 and N30) as in figure 4.15 the cells were stimulated with 5nM MALP-2 for 60 minutes. The samples were analysed for the degradation of I κ B α . I κ B α is an inhibitory protein which binds to the heterodimer NF- κ B to sequester it to the cytosol. Upon stimulation of the TLR2/6 pathway, I κ B kinase (IKK) proteins phosphorylate I κ B and target it for proteasomal degradation. Figure 4.16 looks at the potential of the stable cell lines to degrade I κ B α , HEK 293T cells were unable to degrade I κ B α similar to the pDUO TLR2/6 D30 pool of cells. HEK293T pDUO TLR2/6 N30 cells were capable of degrading I κ B α after 15 minutes stimulation with MALP-2 and the degradation continues up to the 60 minutes time point. A human IL-8 ELISA was carried out to determine whether the pools of cells were capable of producing the chemokine IL-8. HEK 293T cells that are transfected with TLRs have the potential to respond and produce IL-8 cytokine in response to the pathogen or ligand. HEK 293T cells stably transfected with pDUO TLR2/6 N30 are capable of responding to increasing doses of FSL-1 and MALP-2 ligand (figure 4.17). HEK 293T pDUO TLR2/6 D30 cells were also responded to FSL-1 however not to the same extent.

4.2.5.3 Re-sequencing stable cell line pools to ensure sequences are correct

Messenger RNA was extracted from HEK 293T cells, HEK 293T pDUO TLR2/6 D30 and N30 cell lines. A cDNA library was generated using random primers and using primers specific for human TLR6 a reverse transcriptase-PCR was carried out. TLR6 products were generated (figure 4.18) and the products were ligated into pGEM T Easy vector, the sequences were sent to Eurofins MWG for sequencing and all sequences are accurate (figure 4.19).

4.2.6 Generation of clonal cells

When it was established that the TLR6 sequences were correct, HEK 293T pDUO TLR2/6 D30 and N30 clonal cell lines were generated to establish homogeneity in the population of cells. The cells were counted and 100 cells were seeded into 20 ml of DMEM 10% FCS. 200µl of medium was placed into every well of a 96 well plate. The growth of each clone was observed closely so as to ensure that each set of clones originated from only one cell. After sufficient growth and expansion of the cells, they were analysed by flow cytometry for the presence of TLR2. A single peak demonstrated that one population of cells with the same level of TLR2. The mean fluorescence intervals (MFI) of TLR2 present on the surface of the clones is depicted in figure 4.20. The MFI of HEK 293T cells that have not been stably transfected but were incubated with TLR2-FITC is 4.2, this is compared to the MFIs of HEK 293T pDUO TLR2/6 D30 Clone C has an MFI of 16, N30 Clone D has an MFI of 31.2 while N30 Clone E has an MFI of 21.2. These readings suggest that TLR2 is expressed at different levels on the surface of the clone cells. Eight new clones were generated as previously described in this section they were labelled, clone 1 D30,

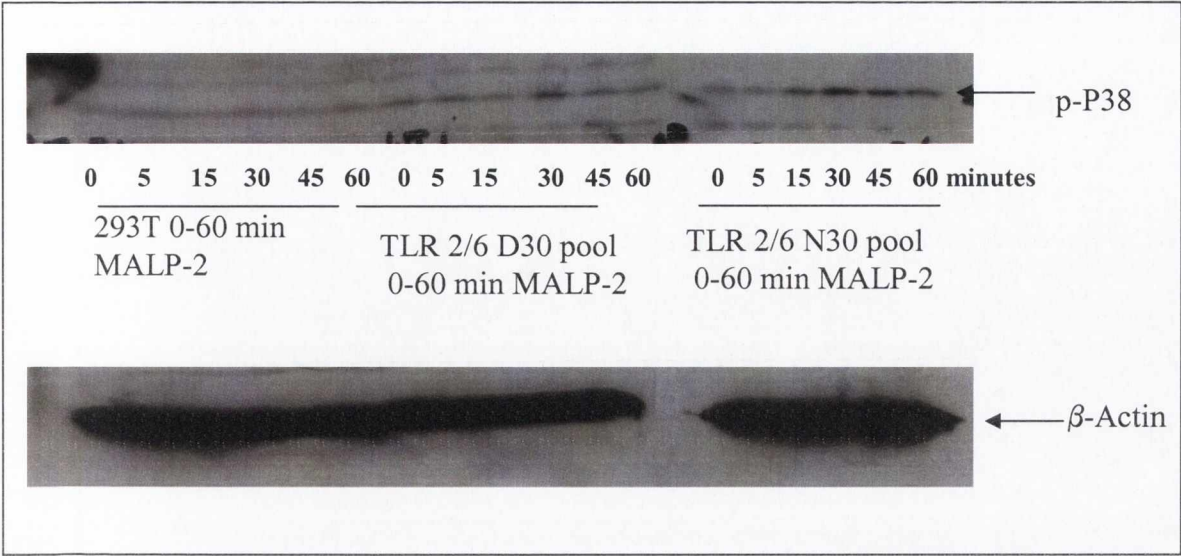


Figure 4.15 HEK 293T pDUO TLR2/6 N30 stable cell pool produced more p-p38 than the D30 stable pool.

HEK 293T, pDUO TLR2/6 D30 and pDUO TLR2/6 N30 stable pools were seeded into 6 well plates at 2×10^5 cells/ml. The cells were incubated at 37°C with 5% CO₂ for 24 hours. The cells were stimulated with 5nM MALP-2 over a 0-60 minute period. The cells were washed with 2ml cold PBS and harvested with 80μl sample buffer with DTT. The cells were scraped into 1.5ml eppendorf tubes and sonicated for 3 seconds. The samples were then boiled for 5 minutes. The samples were resolved on a 12% SDS-PAGE gel using a constant current of 25mA per gel. Each protein gel was transferred to PVDF membrane for 2 hours at 150mA. The membrane was blocked with 5% Marvel in TBST for 1 hour at room temperature. The membrane was probed with p-p38 antibody from Cell Signalling in 5% BSA-TBST overnight at 4°C. The membrane was re-probed for β-Actin loading control. The blot was developed by enhanced chemiluminescence. The results are representative of three experiments.

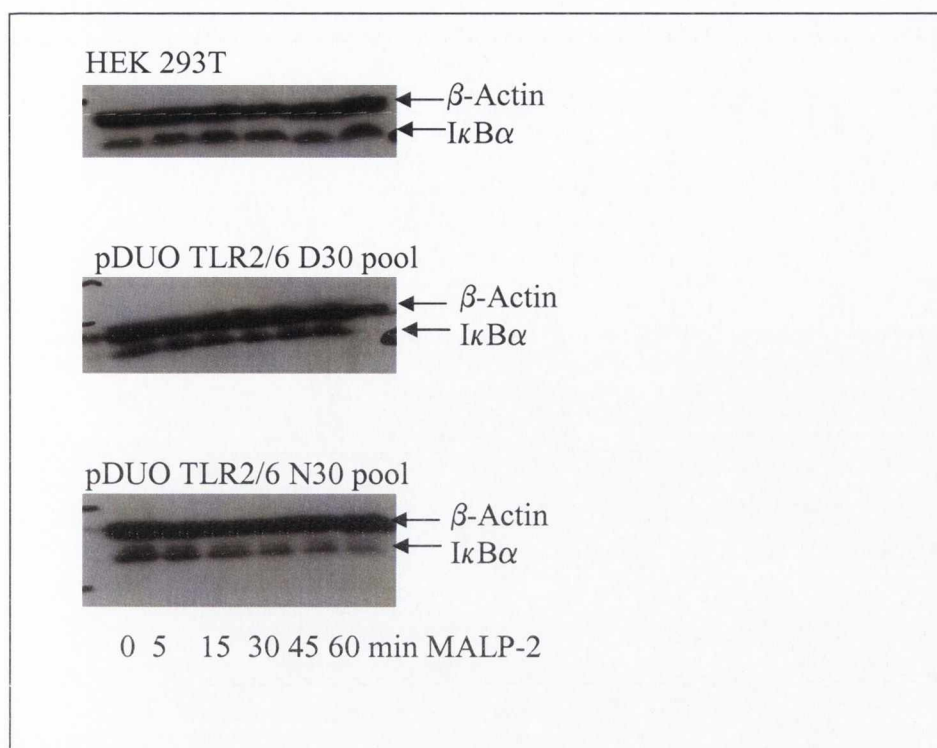


Figure 4.16 HEK 293T pDUO TLR2/6 N30 stable cell pool showed more IkBα degradation compared to the D30 stable pool

HEK 293T, pDUO TLR2/6 D30 and pDUO TLR2/6 N30 stable pools were seeded into 6 well plates at 2×10^5 cells/ml. The cells were incubated at 37°C with 5% CO_2 for 24 hours. The cells were stimulated with 5nM MALP-2 over a 0-60 minute time period. The cells were washed with 2ml cold PBS and harvested with 80 μl sample buffer 1M DTT. The cells were scraped into 1.5ml eppendorf tubes and sonicated for 3 seconds. The samples were then boiled for 5 minutes. The samples were resolved on a 12% SDS-PAGE gel using a constant current of 25mA per gel. Each protein gel was transferred to PVDF membrane for 2 hours at 150mA. The membrane was blocked with 5% Marvel in TBST for 1 hour at room temperature. The membrane was probed with IkBα antibody from Cell Signalling in 5% BSA-TBST overnight at 4°C . The membrane was re-probed for β-Actin loading control. The blot was developed by enhanced chemiluminescence. The results are representative of two experiments.

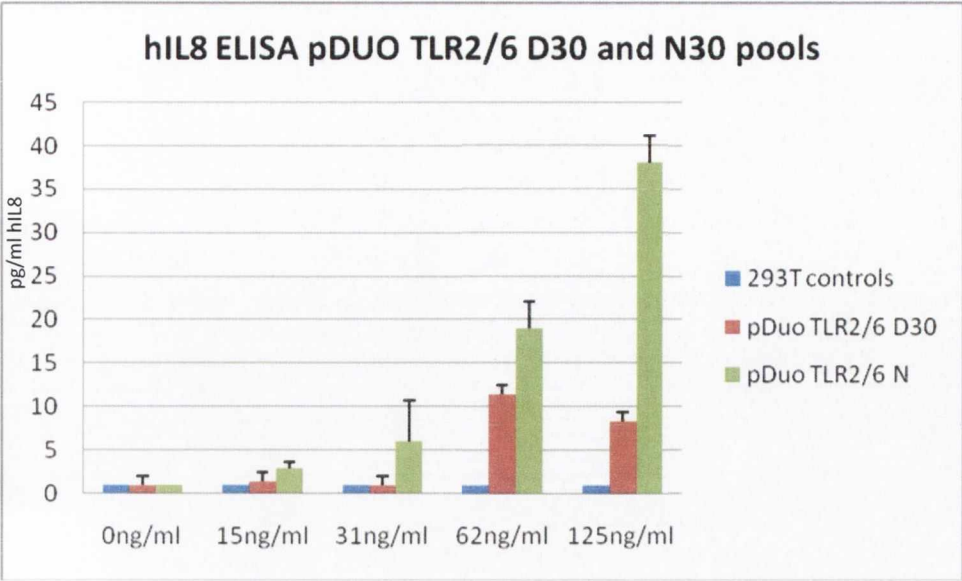


Figure 4.17 pDUO TLR2/6 N30 stables cells produced more hIL-8 than pDUO TLR2/6 D30 cells when stimulated with FSL-1

HEK 293T, pDUO TLR2/6 D30 and pDUO TLR2/6 N30 stable pools of cells were seeded into 96 well plates at 1×10^5 cells/ml in DMEM, the cells were incubated overnight at 37°C with 5% CO₂. After 24 hours, the cells were stimulated with 0-125ng/ml FSL-1. After 24 hours, the supernatant was removed from the cells and 100µl was plated onto hIL8 capture antibody coated ELISA plates. A sandwich ELISA for hIL8 was carried out, the plate was analysed using a plate reader at 450nm wavelength. Error bars show the standard deviation of three biological replicates.

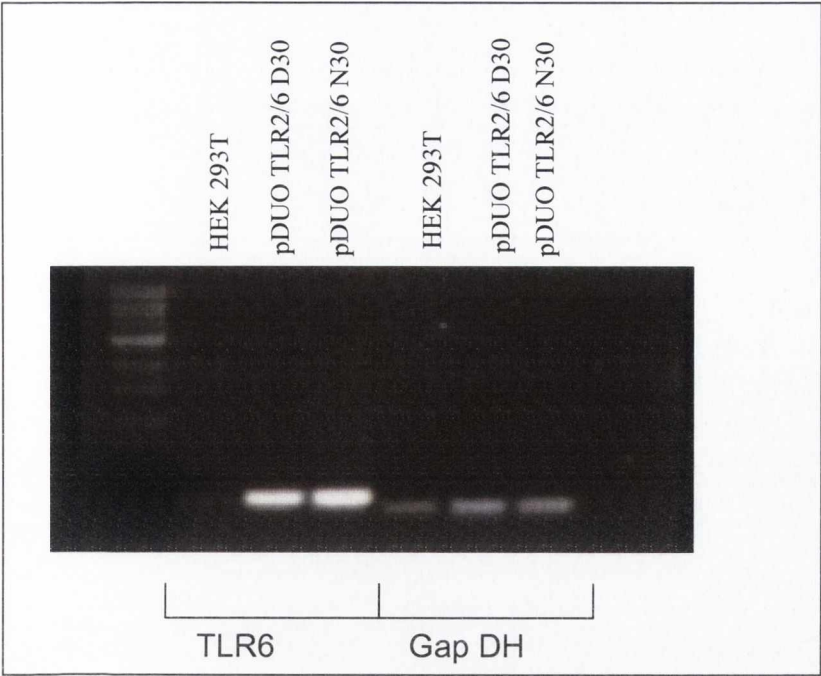


Figure 4.18 Stable cell lines were re-sequenced to ensure that the sequences were correct.

Messenger RNA (mRNA) was extracted from HEK 293T, HEK 293T pDUO TLR2/6 D30 and HEK 293T pDUO TLR2/6 N30 cell lines. A cDNA library was synthesized with random primers using the Improm II kit (Promega). Subsequently using TLR6 specific primers to the first 350 base pairs of TLR6 a reverse transcriptase polymerase chain reaction (RT-PCR) was carried out. GapDH primers were also used as a control. The PCR products were ligated into a pGEM T easy vector. They were then sequenced using T7 promoter.

CLUSTAL 2.0.11 multiple sequence alignment



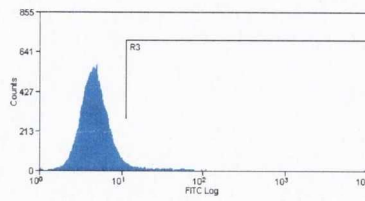
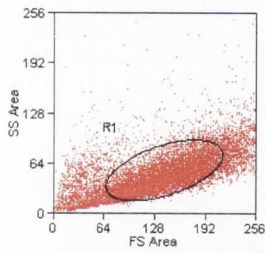
Figure 4.19 Sequences of TLR6 D30 and TLR6 N30 from MWG

PCR products were sent for sequencing to Eurofins MWG. They were aligned using ClustalW to human cDNA sequence of TLR6 obtained from NCBI. The sequences of both TLR6 D30 and TLR6 N30 were correct with the single nucleotide polymorphism present.

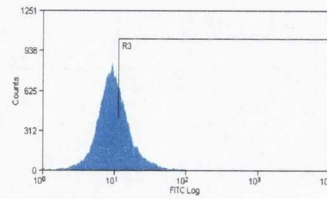
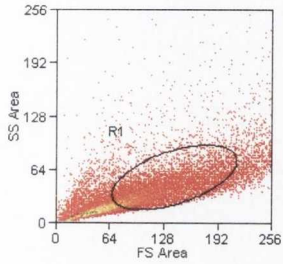
**Figure 4.20 pDUO TLR2/6 D30 and pDUO TLR2/6 N30 clones were cell
surface labelled with anti TLR2 FITC**

Clonal cells were generated from pDUO TLR2/6 D30 and N30 pools. The pools of cells were diluted so as to allow one cell to grow in a well of a 96 well plate. The cells grew over a period of two weeks and single cell clones were generated. The cells were continually grown in 10 μ g/ml blasticidin containing DMEM. The cells were counted and divided so as to have 1x10⁶ cells per sample. Each sample was blocked with 50% BSA-PBS and incubated with 1:50 dilution (0.2 μ g/100 μ l) of anti TLR2-FITC (Invivogen) for 20 minutes on ice. Each sample was washed in 3ml 2% BSA-PBS centrifuged at 1200rpm and re-suspended in 500 μ l sheath fluid. The samples were analysed using a Cyan ADP.

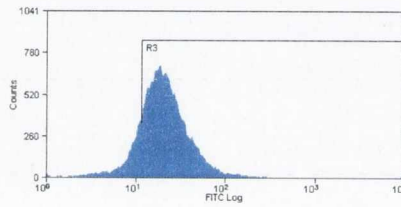
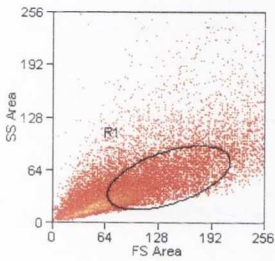
Each clone analysed was positive for TLR2, D30 clone C had a total mean fluorescence interval (MFI) of 16 compared to a reading of 4.2 for the HEK 293T control sample. Both clone D and clone E which were from the HEK 293T TLR2/6 N30 stable cell line had TLR2 present on the surface of the cells they had a total MFI of 31.2 and 21.2 respectively.



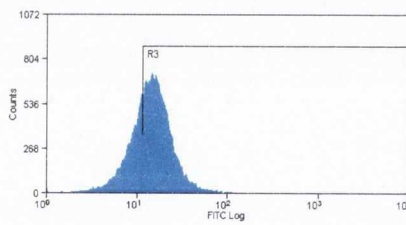
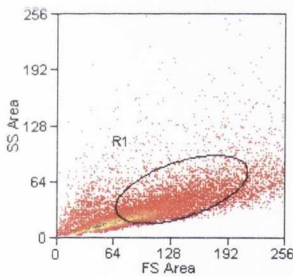
HEK 293T
Total mean 4.2



HEK 293T pDUO TLR2/6 D30
Clone C
36% positive
Total mean 16 MFI



HEK 293T pDUO TLR2/6 N30
Clone D
86% positive
Total mean 31.2 MFI



HEK 293T pDUO TLR2/6 N30
Clone E
71% positive
Total mean 21.2 MFI

clone 6 D30, clone 11 D30 clone 16 D30, clone 5 N30, clone 6 N30, clone 7 N30 and clone 9 N30. A p-38 phosphorylation assay was out on these cells to assess their ability to respond to TLR2/6 ligand MALP-2. N30 clones were capable of responding better to MALP-2 than the D30 clones (figure 4.21). A human IL-8 ELISA was performed to estimate the ability of the clones to produce IL-8 in response to FSL-1 (figure 4.22), the clones were compared to both the D30 and N30 pools. N30 clones were capable of responding better to the stimulus than the N30 and the D30 pools.

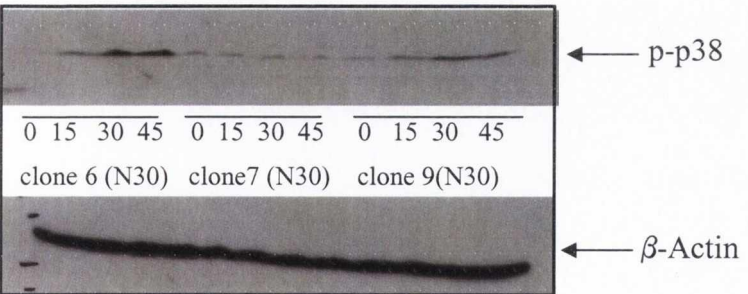
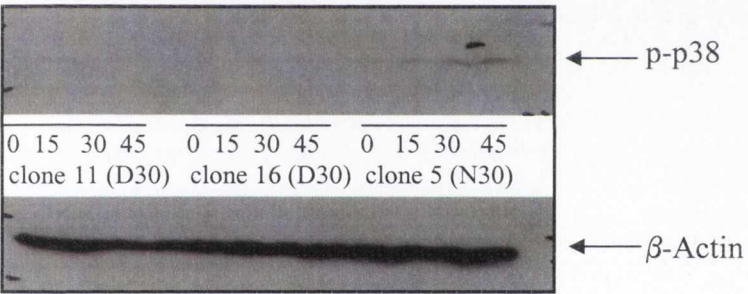
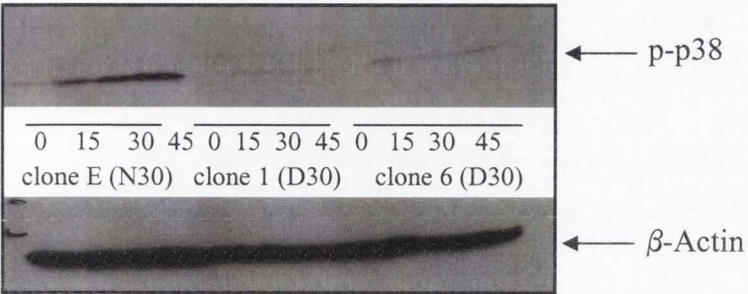
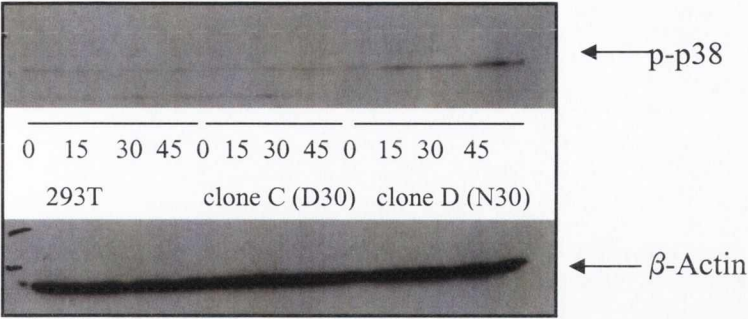
4.2.7 Detecting the presence of human TLR6 in the clonal cell population

A TLR6 antibody was obtained from Santa Cruz which detects the presence of human TLR6, the target epitope of the antibody is at the N-terminus of TLR6 and so it should be possible to detect the presence of cell surface TLR6 with this antibody. The clone cells HEK293T pDUO TLR2/6 D30 clones 1, 6 and 11 and cells HEK 293T TLR2/6 N30 clones 5,6 and 7 were all cell surface labelled with mouse monoclonal anti-human TLR6 and anti-murine-FITC. Results from figures 4.23 and 4.24 indicate that human TLR6 was not detected on the surface of these cells. Figure 4.23(b) displays an overlay plot to demonstrate that the MFI of HEK 293T cells compared to clone 6 D30 were the same. As the anti TLR6 was unable to detect of human TLR6 on the surface of the cells the cells were consequently permeabilized using a fix and perm kit from ADG. The cells were then labelled as before with monoclonal anti human TLR6 and secondary antibody anti-murine FITC. Permeabilized HEK 293T cells were compared to HEK 293T pDUO TLR2/6 D30 clones 1, 6, 11 and N30 clones 5,6 and 7 (figure 4.25 and figure 4.26). The antibody

detected the presence of cytoplasmic TLR6 in clones D30 6 and 11. Figure 4.25(b) displays an overlay of HEK 293T cells compared to the D30 clones 6 and 11, the shift in the green peak which displays the clones shows that TLR6 has been detected. The TLR6 antibody from Santa Cruz was unable to detect TLR6 in the mutant cells (figure 4.26), this could be due to a change in conformation in the TLR6 N30 stable cells shielding the antibody from binding to the epitope.

**Figure 4.21 pDUO TLR2/6 N30 clones showed activation of p38 when
stimulated with 5nM MALP-2**

Clonal cells were generated from pDUO TLR2/6 D30 and N30 pools. The pool of cells was diluted so as to allow one cell to grow in a well of a 96 well plate. The cells grew over a period of two weeks and single cell clones were generated. The cells were continually grown in 10 μ g/ml blasticidin containing DMEM. The cells were seeded into 24 well plates at 2x10⁵ cells/ml and incubated at 37°C with 5% CO₂ for 24 hours. The cells were stimulated with 5nM MALP-2 at 60, 30, 15 and 0 minute time points. The cells were washed in 2ml cold PBS and 50 μ l sample buffer with DTT was added to each sample. The samples were scraped into 1.5ml eppendorf tubes and were sonicated for 3 seconds and boiled for 5 minutes. The samples were resolved on a 12% SDS-PAGE gel using a constant current of 25mA per gel. Each protein gel was transferred to PVDF membrane for 2 hours at 150mA. The membrane was blocked with 5% Marvel in TBST for 1 hour at room temperature. The membrane was probed with p-p38 antibody from Cell Signalling in 5% BSA-TBST overnight at 4°C. The membrane was re-probed for β -Actin loading control. The blot was developed by enhanced chemiluminesence.



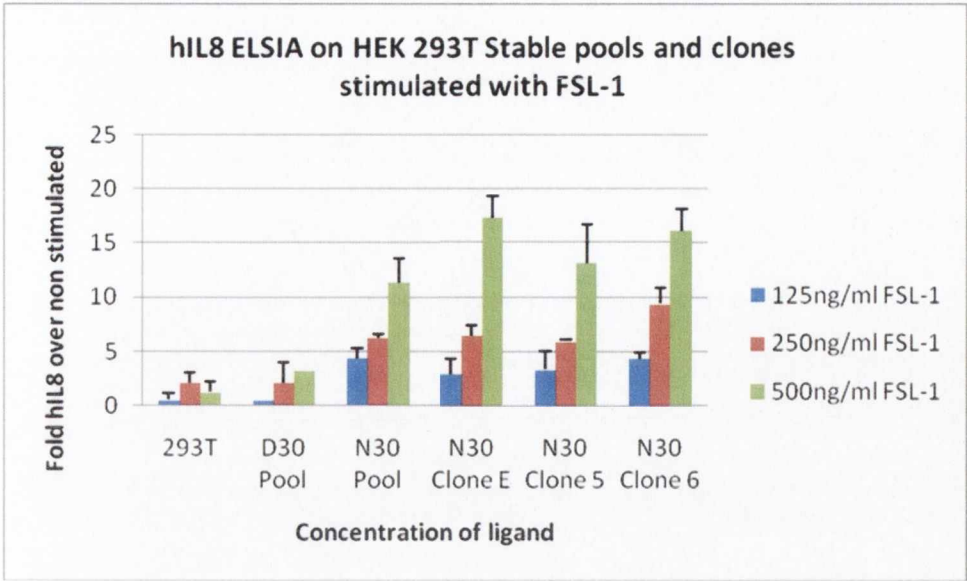


Figure 4.22 HEK 293T pDUO TLR2/6 stables and clones produced high concentrations of hIL-8

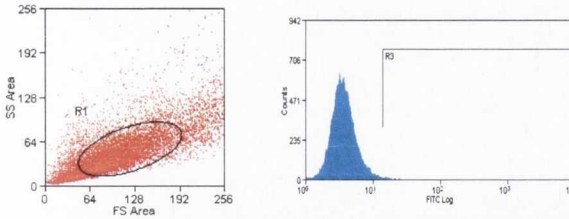
HEK 293T, pDUO TLR2/6 D30 and pDUO TLR2/6 N30 stable pools and N30 clonal cells were seeded into 96 well plates at 1×10^5 cells/ml in DMEM the cells were incubated overnight at 37°C with 5% CO₂. After 24 hours, the cells were stimulated with 0-500g/ml FSL-1. After 24 hours, the supernatant was removed from the cells and 100µl was plated onto ELISA plates coated with a hIL8 capture antibody. A sandwich ELISA was carried out for hIL8 and the plate was read using a plate reader at 450nm wavelength. These results represent fold production of hIL8 over non-stimulated controls. This experiment is representative of 2 experiments and error bars show the standard deviation of three biological replicates.

Figure 4.23 HEK 293T TLR2/6 D30 clones do not reveal the presence of TLR6 on the cell surface.

(A) FACS analysis was carried out on non-permeabilized HEK 293T TLR2/6 D30 Clones 1, 6 and 11. The cells were counted and 1×10^6 cells were used per sample. The cells were blocked with 50% BSA-PBS for 30 minutes at room temperature. The cells were then incubated on ice with 1:50 dilution ($0.2 \mu\text{g}/100 \mu\text{l}$) goat anti human TLR6 antibody (Santa Cruz). The cells were washed twice with 2 ml cold wash buffer (2% BSA-PBS) and secondary anti goat-FITC antibody was added to the cells at a 1:100 dilution. The cells were washed twice with 2ml of cold wash buffer and were re-suspended in $400 \mu\text{l}$ PBS. The cells were analysed on the same day using a Cyan ADP flow cytometer. The fluorescence of each clone was compared to HEK 293T cells. It was found that the non-permeabilized clones did not bind anti hTLR6 antibody.

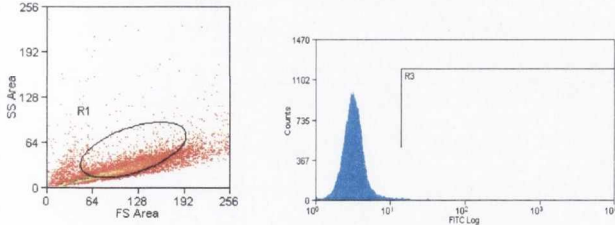
(B) An overlay plot depicts HEK 293T cells over-laid with HEK 293T TLR2/6 D30 clone 6 cells which have been labelled with antibody. These results are representative of two separate experiments.

A HEK 293T

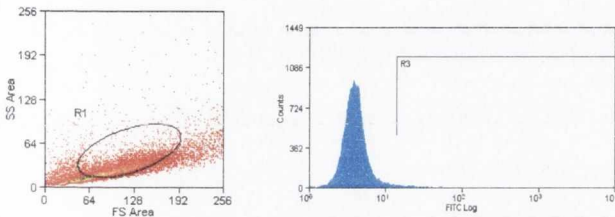


HEK 293T labelled with mouse anti human TLR6 and anti mouse FITC

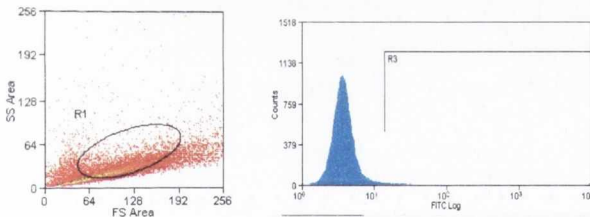
HEK 293T pDUO TLR2/6 D30 Clones



HEK 293T pDUO TLR2/6 clone 1 D30

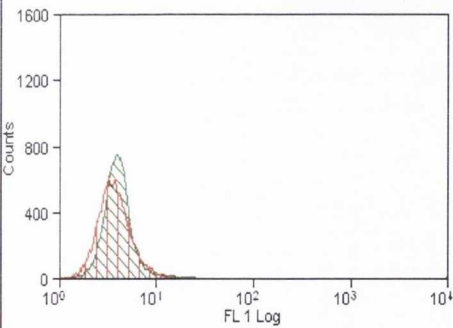


HEK 293T pDUO TLR2/6 clone 6 D30



HEK 293T pDUO TLR2/6 clone 11 D30

B



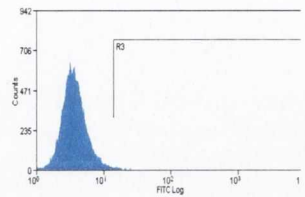
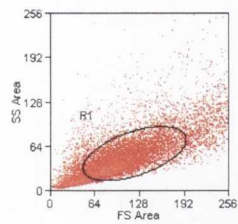
Overlay of HEK 293T plot and HEK pDUO TLR2/6 clone 6 D30 labelled with anti TLR6 and mouse- FITC

Figure 4.24 HEK 293T TLR2/6 N30 clones do not reveal the presence of TLR6 on the cell surface.

FACS analysis was carried out on non-permeabilized HEK 293T TLR2/6 N30 clones 5, 6 and 7. The cells were counted and 1×10^6 cells were used per sample. The cells were blocked with 50% BSA-PBS for 30 minutes at room temperature. The cells were then incubated on ice with 1:50 dilution ($0.2 \mu\text{g}/100 \mu\text{l}$) goat anti hTLR6 antibody (Santa Cruz). The cells were washed twice with 2 ml cold wash buffer (2% BSA-PBS) and secondary anti goat-FITC antibody was added to the cells at a 1:100 dilution. The cells were washed twice with 2ml of cold wash buffer and were re-suspended in $400 \mu\text{l}$ PBS. The cells were analysed on the same day using a Cyan ADP flow cytometer. The fluorescence of each clone was compared to HEK 293T cells. It was revealed that non-permeabilized HEK 293 TLR2/6 N30 clones do not bind anti-hTLR6.

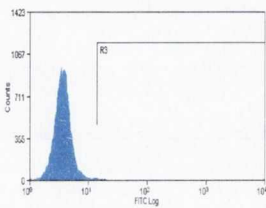
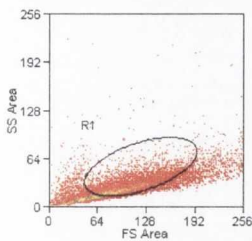
These results are representative of two separate experiments.

HEK 293T

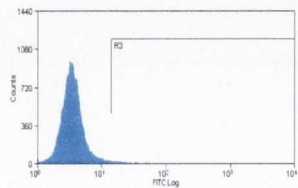
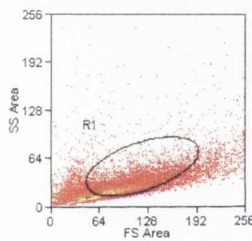


HEK 293T labelled with
mouse anti human TLR6
and anti mouse FITC

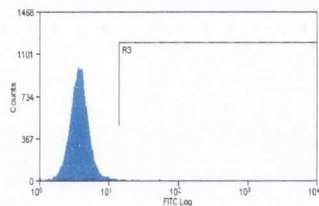
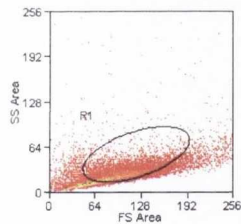
HEK 293T pDUO TLR2/6 N30 Clones



HEK 293T TLR2/6
clone 5 N30



HEK 293T TLR2/6
clone 6 N30



HEK 293T TLR2/6
clone 7 N30

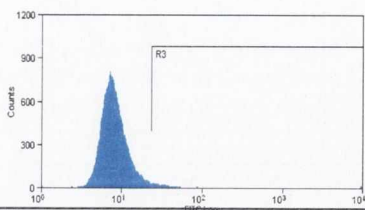
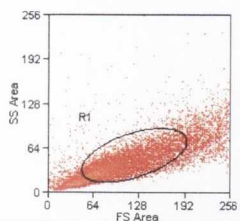
**Figure 4.25 N terminal antibody of human TLR6 stained cell permeabilized
TLR2/6 D30 clones.**

(A) FACS analysis was carried out on permeabilized HEK 293T TLR2/6 D30 clones 1, 6 and 11. The cells were counted and 1×10^6 cells were used per sample. The cells were blocked with 50% BSA-PBS for 30 minutes at room temperature. The samples were fixed and permeabilized at room temperature using a fix and perm kit from ADG (An Der Grub). The cells were then incubated on ice with 1:50 dilution of monoclonal anti human TLR6 antibody (Santa Cruz). The cells were washed twice with 2 ml cold wash buffer (2% BSA-PBS) and secondary anti murine-FITC antibody was added to the cells at a 1:100 dilution. The cells were washed twice with 2ml of cold wash buffer and were re-suspended in 400 μ l PBS. Samples were analysed on the same day using a Cyan ADP flow cytometer.

(B) An overlay plot depicts HEK 293T cells over-laid with HEK 293T WT TLR2/6 clone 6 cells and clone 11. The plots show that clone 6 D30 and clone 11 D30 stained positive for the presence of intracellular hTLR6. These results are representative of two separate experiments.

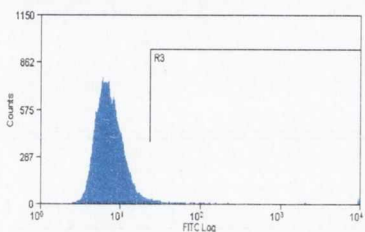
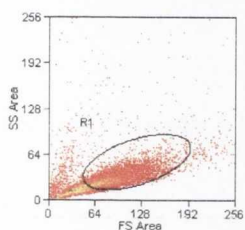
A

HEK 293T

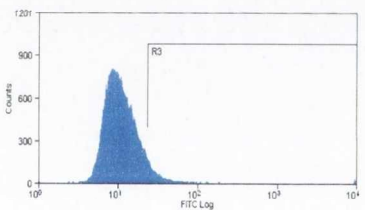
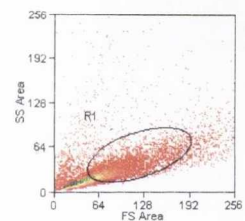


HEK 293T

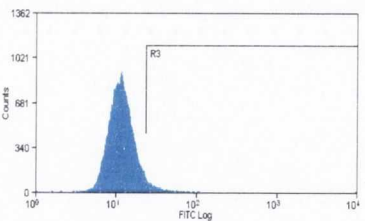
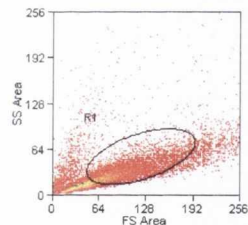
HEK 293T pDUO TLR2/6 D30 Clones



HEK 293T TLR2/6
Clone 1 D30

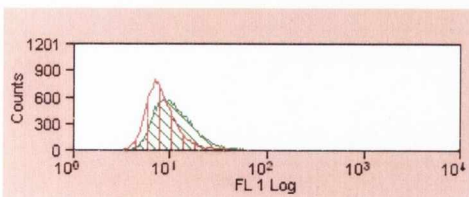


HEK 293T TLR2/6
Clone 6 D30

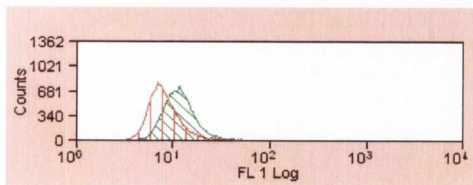


HEK 293T TLR2/6
Clone 11 D30

B



Overlay 293T red
Clone 6 D30 green



Overlay 293T red
Clone 11 D30 green

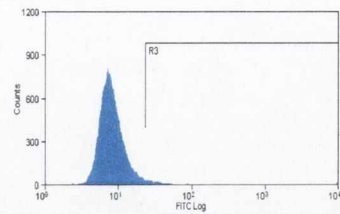
Figure 4.26 N terminal antibody of human TLR6 did not stain cell permeabilized TLR2/6 N30 clones.

(A) FACS analysis was carried out on permeabilized HEK 293T TLR2/6 N30 clones 5, 6 and 7. The cells were counted and 1×10^6 cells were used per sample. The cells were blocked with 50% BSA-PBS for 30 minutes at room temperature. The samples were fixed and permeabilized at room temperature using a fix and perm kit from ADG (An Der Grub). The cells were then incubated on ice with 1:50 dilution of goat anti human TLR6 antibody (Santa Cruz). The cells were washed twice with 2 ml cold wash buffer (2% BSA PBS) and secondary anti goat-FITC antibody was added to the cells at a 1:100 dilution. The cells were washed twice with 2ml of cold wash buffer and were re-suspended in 400 μ l PBS. Samples were analysed on the same day using a Cyan ADP flow cytometer.

(B) An overlay plot depicts HEK 293T cells over-laid with TLR2/6 N30 clones 5, 6 and 7, all of which have been labelled with primary and secondary antibody. The plot shows that the TLR6 N30 clones were not positively stained for the presence of human TLR6. These results are representative of two separate experiments.

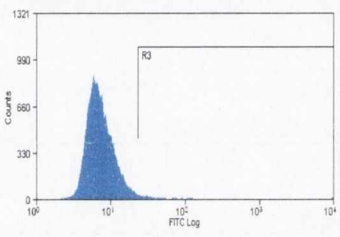
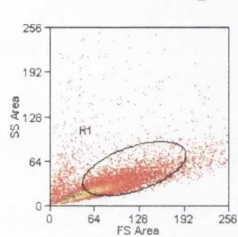
A

HEK 293T

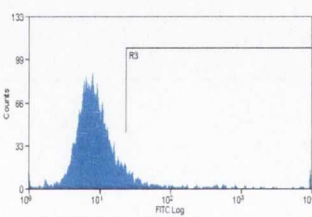
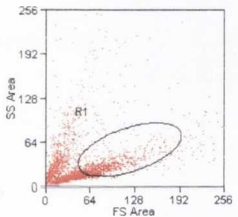


HEK 293T

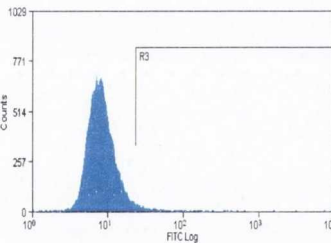
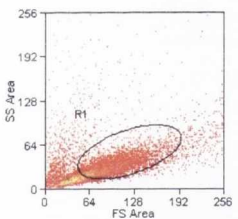
HEK 293T pDUO TLR2/6 N30 Clones



HEK 293T TLR2/6
N30 Clone 5

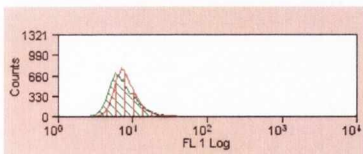


HEK 293T TLR2/6
N30 Clone 6

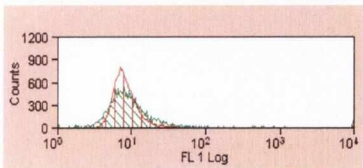


HEK 293T TLR2/6
N30 Clone 7

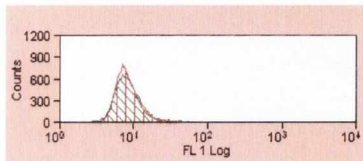
B



Overlay HEK 293T red
Clone 5 N30 green



Overlay HEK 293T red
Clone 6 N30 green



Overlay HEK 293T red
Clone 7 N30 green

4.3 Discussion

TLR6 forms a heterodimeric partnership with TLR2, it is required for recognition of bacterial components of *S. pneumoniae*. Professor Adrian Hill and colleagues found a link between a TLR6 polymorphism and susceptibility to IPD. It was found that children in Kenya had a six fold increase in susceptibility of invasive pneumococcal disease (IPD) compared to wild type controls when they possessed the TLR6 polymorphism D30N. The study was also carried out in a UK population in individuals with IPD and the same finding was confirmed.

Previous studies investigating the role of rare polymorphisms in TLRs 1, 6 and 10 revealed that rare polymorphisms of TLR6 contribute greatly to susceptibility to disease. Polymorphisms L251end, Thr255Ile and Asn690Thr of TLR6 were found to mediate reduced signalling towards pro-inflammatory NF- κ B. It was postulated that the rare polymorphisms were maintained at a low level due to natural selection (Ma et al., 2007).

The TLR6 D30N polymorphism was found to be located at position -2 of the signal peptide sequence of TLR6. Previous studies on the signal peptide cleavage sites of proteins revealed that the amino acids at positions -1 and -3 must be small and neutral for cleavage to occur correctly. The amino acid of interest in this project was situated between these amino acids and so it was thought that the N30 amino acid may affect cleavage of the N terminal signal peptide sequence. The secondary structure of TLR6 was found using PSIPRED an online secondary structure prediction programme. The secondary structure of TLR6 D30 was compared to TLR6 N30 and it was found that the hydrophobic alpha helix that makes up the core of the signal peptide was extended in the TLR6 N30 secondary structure. Figure

4.27 depicts a cartoon of TLR6 D30 and TLR6 N30 based on 2D structural modelling. An alpha helix shown as a green coil is extended in the TLR6 N30 structure. It extends further into the vicinity of where the signal peptidase cleaves the protein indicating that there may be a difference in the capacity of the signal peptidase to cleave the TLR6 N30 compared to the TLR6 D30. Signal sequences or signal peptides are located on the N-terminal region of the amino acid chain and are cleaved off while the protein is translocated through the membrane. Signal peptides play a central role in targeting and translocation of nearly all secreted proteins and integral membrane proteins (Bruch et al., 1989). They generally follow the same consensus; containing three structurally and functionally distinct regions. The first is the N-terminal positively charged n-region, the second is a central hydrophobic h-region and thirdly a neutral but polar c-region (figure 4.28) (Claros et al., 1997). A common feature of signal peptides follows the (-3, -1) rule, which specifies that the amino acids at position -1 and -3 relative to the cleavage site must be small and neutral for cleavage to occur correctly (von Heijne, 1983).

Subsequent to finding that there was a difference in signal peptide structure, the cellular localisation of TLR6 D30 compared to TLR6 N30 was investigated. Two forms of TLR6 N30 were generated, a FLAG tagged form and a YFP tagged form. It was found that the FLAG tag was situated at the N-terminus of the mature polypeptide, more specifically it lay in the signal peptide region. When inserted into the signal peptide cleavage site prediction programme it was found that FLAG-TLR6 D30 and N30 forms did not reveal any putative cleavage site. This finding was confirmed by Western-blotting as the N-terminal tag was not cleaved off during maturation. This indicates that the signal peptide cleavage does not occur without the presence of the signal peptide, however the TLR6 was found in the membrane

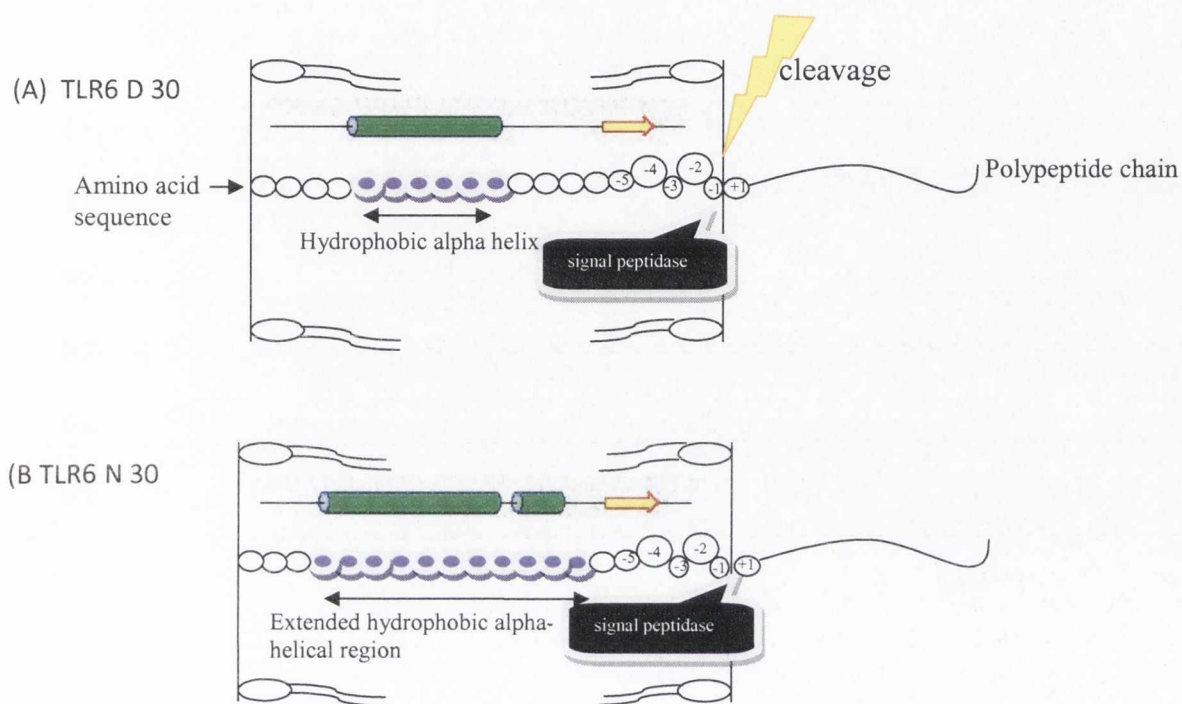


Figure 4.27 The alpha helical region of the signal peptide differs for each form of TLR6

The secondary structure for TLR6 D30 (A) and TLR6 N30 was found using signalP secondary structure prediction programme. The hydrophobic core (green barrel) of TLR6 N30 is longer and has an extra length of alpha helix compared to TLR6 D30. The signal peptidase cleaves the signal peptide between amino acids -1 and +1. The D30 amino acid is situated at position -2 and could influence the secondary structure of the N-terminal signal peptide.

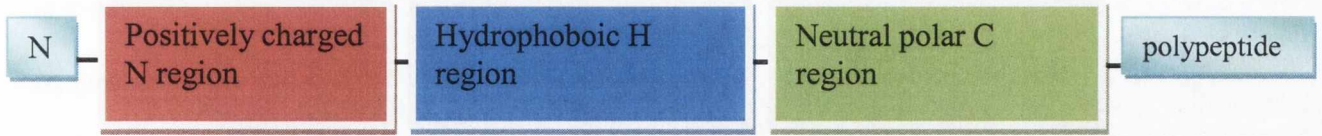


Figure 4.28 The signal sequence of a protein

The signal sequence of a protein is found at the N terminal end, it is approximately 20 amino acids long and it generally consists of a positively charged N region, a hydrophobic H region at the core and a neutral polar C region. The signal sequence is cleaved after the C region leaving the remainder of the polypeptide to fold into its 3D structure.

fraction suggesting that possibly other possible mechanisms which direct the protein to the membrane system. One could extrapolate from this finding that the signal peptide plays a smaller role in directing the protein to the membrane. As the FLAG tag was found to be situated in the signal sequence of the TLR6 and so this form was not used in further experiments. TLR6-YFP D30 and TLR6-YFP N30 were used in membrane fraction assays. It was found that when separating membrane fractions from cytosolic fractions TLR6 D30 was found to be located in the membrane fraction. The membrane fractions consist of the plasma membrane and the endomembrane system. The TLR6 N30 was found to be located in the membrane and the cytosolic fractions.

Confocal analysis was also carried out to attempt to visualise the sub-cellular localisation of the TLR6 D30-YFP and TLR6 N30-YFP within HEK 293T cells. It was found that the proteins were both found on the ER where the proteins were co-localised with ER-CFP marker. Both proteins were found on the ER however the TLR6 N30-YFP protein was also found diffused throughout the cytosol. In 1983 von Heine stated that the amino acids at position -1 to -5 confer signal processing specificity (von Heijne, 1983). The TLR6 polymorphism in question in this project lies within this region at -2 and so may play an important role in the signal processing. Nascent proteins are synthesised by ribosomes (figure 4.29), the ribosomes are synthesized in the nucleus but are transported to the cytoplasm to synthesize proteins. The ribosome/signal peptide complex must recognise the signal peptide recognition protein (SRP) and then be translocated to the SRP receptor on the ER where it binds to the translocon. Before binding to the translocon the SRP/ribosome/signal peptide must successfully remove the Sec61 complex which

acts as a plug in the translocon pore. This is a multi-step process which is highly regulated to minimise the translocation of disingenuous proteins that do not have fully functional signal peptide sequences. The Sec61 complex also positions the nascent chain in preparation of subsequent events. The N terminus of the signal peptide is facing the cytosol and the mature portion of the nascent chain is inserted into the aqueous pore of the Sec61 complex. When the nascent polypeptide reaches a critical length and now the ribosome is bound more tightly to the Sec61p complex. At this point the sequence comes in contact with the translocating chain associating membrane (TRAM) protein (Gorlich et al., 1992). The nascent chain is now committed to translocation.

Initial steps leading to recognition and insertion of the nascent chain into the membrane are likely based primarily on protein-protein interactions (Mothes et al., 1998). They found that all residues of the signal peptide were found in contact with the Sec61 α complex, and that TRAM is in contact with the N terminus of the signal sequence and also certain residues of the hydrophobic core. The insertion into the translocon is followed by signal sequence cleavage, transmembrane domain insertion into the lipid bilayer and translocation all occurs upon continued elongation of the polypeptide chain. It has been found that alterations in translocon charge can influence the orientation that the signal peptide takes inside the pore. In this case a change a charge of the polypeptide chain may also influence the orientation, as the polypeptide and translocon are known to interact at all points (Goder et al., 2004). It is also important that when in the translocon the protein must form looped structure to enter the ER, if it does not form a looped structure it enters cytosol (Hegde and Kang, 2008).

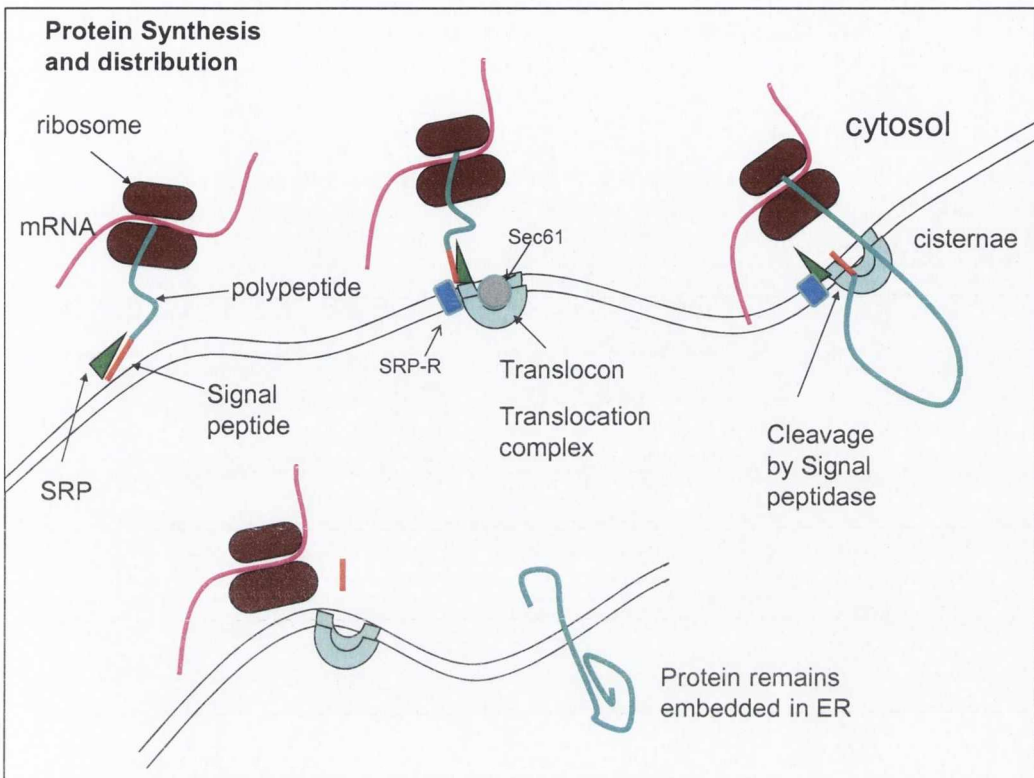


Figure 4.29 Polypeptide synthesis, translocation and membrane insertion

The synthesis of proteins is carried out by a 60S and a 40S ribosomal subunits they join together on an mRNA molecule to form the 80S ribosome. The ribosomal subunits consist of ribosomal proteins and ribosomal RNAs (rRNAs) they are assembled in the nucleus and are transported to the cytoplasm where they perform protein synthesis. The N-terminal signal peptide sequence is processed first; proteins destined to be membrane bound are targeted to the endoplasmic reticulum membrane. A sequence recognition particle (SRP) binds to the hydrophobic core of the signal sequence. The SRP bound signal sequence is then recognised by the SRP receptor where the signal sequence is transferred to the translocon. The signal sequence must interact with the Sec61 complex which forms a channel and mediates a ‘proof-reading’ step preventing translocation of inappropriate proteins lacking functional signal sequences. Once inserted into the translocon polypeptide elongation occurs, the signal sequence becomes cleaved by the signal peptide protease and the transmembrane domain becomes inserted into the lipid bilayer.

The fraction of TLR6 N30 that is observed to be in the cytoplasm may be due to an inability for the ribosome/signal peptide/SRP to translocate through the membrane. The translocation of TLR6 N30 may be inefficient with some of the TLR6 entering the membrane and some of it escaping to the cytosol.

Functional assays were carried out to investigate the potential of the TLR6 N30 variant to respond to its ligand and to determine whether the variant could signal to pro-inflammatory transcription factor NF- κ B. HEK 293 cells were transfected with empty vector, TLR D30-YFP and TLR6 N30-YFP along with NF- κ B luciferase construct. The findings revealed that TLR6 alone could not activate NF- κ B luciferase with or without the presence of ligand. To remedy this, the cells were transfected with TLR2-FLAG and TLR6 N30 and D30. It was found that over-expressed TLR6 D30 was capable of activating NF- κ B whereas the TLR6 N30 variant was not. A similar experiment was carried out by Ma and colleagues in 2007 (Ma et al., 2007) where they investigated the ability of rare TLR6 polymorphisms to activate NF- κ B. Similarly TLR6 L251end, Thr255Ile and Asn690Thr polymorphisms had a reduced ability to activate NF- κ B.

To extend further the knowledge of TLR6 D30 compared to TLR6 N30 stable cell lines were generated using pDUO-hTLR2/TLR6 a co-expressing vector from Invivogen. The pDUO TLR2/TLR6 D30 vector was mutated using site directed mutagenesis to generate pDUO TLR2/6 N30. The constructs were stably transfected into HEK 293T cells generating two different pools. The cells were analysed by FACS analysis for the presence of cell surface TLR2. TLR2 was found to be cell surface expressed in both pools, the pDUO TLR2/6 D30 pool had a mean

fluorescence interval (MFI) of 28.10 and the pDUO TLR2/6 N30 had an MFI of 30.41 indicating that approximately the same level of TLR2 was present on the surface of the cells. As the vector used co-expressed TLR2 and TLR6 a conclusion was drawn that TLR6 was also expressed at the protein level.

Functional TLR signalling assays were then performed on the pools of cells to assess the signalling capabilities of both cell lines. An NF- κ B luciferase assay was performed by seeding the cells into 24 well plates and transfecting the cells with 150ng and 75ng NF- κ B luciferase construct with 75ng and 37ng TK Renilla control vector. The pools of cells were compared to HEK 293T cells alone, each cell line was normalized with empty vector control. The cells were treated with and without 5nM MALP-2 for 24 hours. Surprisingly the HEK 293T pDUO TLR2/6 D30 cells did not activate NF- κ B similar to the HEK 293T empty vector controls, the HEK 293T TLR2/6 N30 pool of cells showed a six fold activation of NF- κ B. The samples transfected with 75ng NF- κ B luciferase showed a five fold response. A phospho-P38 (p-P38) assay was also performed on these cells to assess the ability of the cells to signal down the MAPK pathway. HEK 293T, HEK 293T pDUO TLR2/6 D30 and N30 cells were stimulated with 5nM MALP-2. The cells were harvested and cellular lysate samples were immuno-blotted for the presence of p-P38. The results showed that HEK-293T cells do not respond to 5nM MALP-2 stimulation. TLR WT 2/6 (D30) pool of cells responded slightly to the presence of the TLR2/6 ligand at 30 minutes stimulation however the HEK 293T pDUO TLR2/6 N30 cells showed a stronger phosphorylation of P38 with phosphorylation occurring at 15, 30 and 45 minutes. Another component of TLR2/6 signalling is the degradation of I κ B α , I κ B α is degraded to allow the release of NF- κ B into the nucleus. The pools of cells were analysed for their potential to degrade I κ B α . I κ B α degradation was observed in the

HEK 293T pDUO TLR2/6 N30 pool of cells after 30 minutes stimulation however no degradation of I κ B α was observed for the HEK 293T or HEK 293T TLR2/6 D30 pool of cells. The chemokine IL-8 is released by HEK 293 TLR2 cells in response to its ligand, the stable cell line pools were stimulated with FSL-1, a TLR2/6 ligand. In response to increasing doses the HEK 293T cells did not produce any IL-8, the HEK 293T TLR2/6 D30 cells did produce some hIL8, up to 10pg/ml, however the HEK 293T pDUO TLR2/6 N30 cells showed a much higher increase in hIL8 production, up to 35pg/ml, in response to the increasing doses of FSL-1. After receiving the results that the HEK 293T TLR2/6 N30 cells responded in such a fashion to TLR2/6 ligand the vectors were re-sequenced to ensure that the sequences were correct, to rule out any other explanation for the high response to TLR2/6 ligands. The sequences were sent to Eurofins MWG, and the sequences were obtained, it was found that the sequences were correct, confirming that the pDUO TLR2/6 N30 cells were highly responsive to TLR2/6 ligand.

After confirmation that the sequences were indeed correct, clonal cells were then generated from each pool. Single cell clones from each pool were expanded, and after sufficient growth the cells were analysed again by flow cytometry for the presence of cell surface TLR2. The presence of cell surface TLR2 was confirmed and functional signalling assays were carried out. The cells were stimulated with 5nM MALP-2. After testing a range of clones the general finding was that the HEK 293T pDUO TLR2/6 N30 clones responded better to the addition of ligand than the HEK 293T pDUO TLR2/6 D30 clones. Combining the evidence from the stable pools and the clonal cells it has been found that the TLR6 N30 form responds far better to ligand and produced a much larger pro-inflammatory response than the TLR6 D30 form. The initial finding that started this project found that individuals

that possessed the TLR6 variant had a seven fold increase in susceptibility to IPD, which can manifest as severe sepsis in infected patients; a phenotype that is consistent with the data from the stable cells, which are hyper-responsive to TLR2 ligand.

The issue of where the TLR6 receptor was located in these cells had not yet been solved as a TLR6 specific antibody remained illusive. A number of antibodies were tested and eventually an antibody from Santa Cruz was able to detect the presence of TLR6. The TLR6 antibody is targeted to an epitope at the N-terminal region of TLR6. It was thought that by testing the capabilities of both forms of TLR6 to access the plasma membrane it would give further insight into the receptors ability to signal. Cell surface and intracellular analysis of the stable cell line clones was carried out. HEK 293T pDUO TLR2/6 D30 clones 1,6 and 11 and pDUO TLR2/6 N30 5,6 and 7 cells were examined for the presence of TLR6. The cells were counted, and were subsequently cell surface and intracellularly analysed. Results from cell surface studies indicate that TLR6 was not detected on the cell surface of any of the clones. However when the cells were permeabilized the presence of TLR6 was detected in the HEK 293T TLR2/6 D30 clones. The same experiment carried out on the HEK 293T TLR2/6 N30 cells found that the antibody did not bind to the TLR6. It is thought that TLR6 is present in these cells as it was previously identified in this project at the cDNA level. As the antibody is targeted to a N-terminal epitope tag the inability of the hTLR6 antibody to bind to the variant form could be as a result of incorrect conformation of the variant TLR6.

The aberrant localisation of the variant TLR6 could suggest that the increase in signalling in the TLR6 N30 cells could be as a result of mis-directed protein targeting. TLR6 that is not targeted to the endomembrane system and is found

dispersed through the cell could hinder the correct down-regulation of the signal. After any TLR signalling occurs, negative regulators are activated to dampen down the response to avoid abnormal signalling which could lead to the development of deregulated inflammation.

This novel study reveals an important link between a TLR6 variant with the progression of infectious disease. The information obtained in this study provides a link between genetic association studies and biologically significant inflammatory signalling events, suggesting that TLR6 may potentially be more relevant to the progression of infectious disease than it may first appear.

Chapter Five

Final discussion and future perspectives

Chapter 5

5.1 Discussion

The health of a human is influenced by genotype, immune system, environment and age. This project focuses on our genotype and its influence on the innate immune system. A clearer understanding of evolutionary genetics and its role in the progression of disease has important implications in diagnosing, understanding and treating medical conditions such as infectious disease.

The TLR family of PRRs are widely known to activate the innate immune signalling system, which underpins the body's defence against invading pathogens. Both TLR6 and the TLR adaptor Mal play an important role in mediating the pro-inflammatory signal towards transcription factors which mediate the transcription of pro-inflammatory genes. Without which the potential for disease development is inevitable. Investigating the signalling potential of TLR polymorphisms has yielded a great deal of knowledge about the role that the TLRs play in disease. TLR4 polymorphisms Asp299Gly and Thr399Ile are associated with LPS hypo-responsiveness (Arbour et al., 2000), and these polymorphisms have been found to predisposed individuals to increased gram negative infections (Agnese et al., 2002). It has been found that a person infected with TB may not always suffer the symptoms of the disease; the bacterium can lie dormant and act as an asymptomatic latent TB infection. However one in ten latent infections will progress to active TB disease which if left untreated will kill more than half its victims. One possible explanation for this is due to the polymorphisms in the innate immune system causing individuals to react differently to the presence of the bacterium.

This study was developed from the initial finding that polymorphisms in Mal and TLR6 were associated with infectious disease by Professor Adrian Hill. The Mal polymorphism was found to be associated with protection from malaria, TB, IPD and bacteraemia while the TLR6 polymorphism was found to be associated with IPD and the development of septicaemia.

Following the disease association of the S180L polymorphism in Mal, a functional reason for the protection afforded by the polymorphism to individual from infectious diseases was endeavoured to be found. It was found that the serine to leucine variant effected reduced signalling upon stimulation with TLR2 ligands and that the leucine form of Mal was unable to bind to TLR2 hindering downstream signalling. The hypothesis behind this finding was that heterozygous individuals infected with disease potentiated a weaker response to the presence of the pathogen and therefore did not suffer the symptoms of the disease to the same extreme. In heterozygous individuals one copy of the Mal Ser180 form is also present and so they would have the ability to signal to some extent and so the presence of the pathogen would be identified and dealt with, be it without the harmful effects of an over active immune system response (Khor et al., 2007).

In 2006 the serine to leucine 180 polymorphism was also identified by Aderem and colleagues to be associated with meningeal TB, however the number of individuals genotyped was found to be insufficient and the association with disease was found to be insignificant (Hawn et al., 2006). Aderem and colleagues did however find a SNP C558T (Ala186Ala) a synonymous SNP in Mal was associated with reduced IL-6 production which is associated with disease susceptibility.

Differing reports have been published with either agree or disagree with the findings by Khor et al., this is something that has occurred with many polymorphisms that have been studied over the years. Variation in age, ethnicity of populations, and the number of individuals studied have all contributed to different findings within recent years. Discrepant findings can be attributed to heterogeneity of association in different populations. However two other studies have found disease associations with Mal L810 further emphasising the importance of Mal for host defense.

The second part of this project examined at the susceptibility of a TLR6 variant to cause a seven fold increase in developing IPD when infected with *S. pneumoniae*. Most individuals harbour harmless *S. pneumoniae* in the upper mucosal surface of the upper respiratory tract however an invasive infection can become established by the disruption of the respiratory epithelial membrane. Signalling through TLR2 has been found to cause the up-regulation of a pro-inflammatory response which has been found to decrease the integrity of the trans-epithelial barrier and increase bacterial migration across the epithelium (Beisswenger et al., 2007).

The question has arisen as to why variants arise in a population that give rise to susceptibility to disease, and why if natural selection is so important do we still suffer from fever, get cancer and diabetes? Many attempts have been made to answer this and it is suggested that some genes that may give rise to a susceptibility to disease may have larger benefits in the long run and that the benefits that they provide outweigh the costs that they may inflict in some cases. In the case of Mal a decrease in inflammatory signalling may seem detrimental while a slight decrease in

the production of inflammatory cytokines will protect from fever in the case of malaria and the development of septicemia in IPD. In the case of TLR6 the over-activation of signalling occurring in the variant TLR6 is only found in a very small number of cases, however, individuals who have the mutation have a seven fold increase in susceptibility to disease. Many cases of mis-localised proteins occur during the progression of evolution. It has been found that deregulated translocation of proteins has been used to a cell's advantage. In some cases evolution has exploited a signal sequences ability to mis-localise some proteins for the functional benefit of the cell. In *Chlamydomonas reinhardtii* a PDI (protein disulfide isomerase) homologue RB60 serves independent functions in two distinct locations. The protein is dual localized due to inefficient co-translational translocation into the ER and to chloroplasts (Levitan et al., 2005). On an adverse side of dual localisation, proteins that are mis-localised can cause disease. For example, Alzheimer's precursor protein becomes targeted to the mitochondria and can initiate apoptosis (Devi et al., 2006), secondly, apolipoprotein E interacting with cytoskeletal components to cause the formation of neurofibrillary tangles (Huang et al., 2001). However, these mechanisms have been discounted due to the absence of a mechanism which explains how a signal containing proteins could be found in the cytosol. Inefficient signal sequences have been known to mis-localize proteins, it has been shown that mis-localised protein can have beneficial effects and be used by the body, in a process known as 'dual targeting'. Proteins can have two different functions in different locations. This may work to explain why dual targeting has occurred in nature, in some cases it is beneficial however in the case of the TLR6 N30 mutant the variant has been found to be pathogenic.

It is known that bacteraemia is a frequent occurrence as bacteria can enter the blood stream on a daily basis, through vigorous tooth brushing, infected catheterisation, or a dirty wound. On most occasions bacteria in the blood is quickly resolved without incidence, however on rare occasions patients suffer from an exacerbation of effects, developing sepsis and possibly septic shock and no explanation can be offered for these situations. Usually incidents such as this can be explained by multi-drug resistant microbes present in the bloodstream; however this may not always be the case. The overactive role of the TLR6 variant identified in this project may go some way towards explaining these situations.

By combining the knowledge that is being found in the human genome with biological information about the immune system will help to create a greater knowledge basis for medical geneticists in the future. In the future the development of a personalized medical treatment system could unfold. A personal treatment system could be tailored to the genotype of each individual. Different polymorphisms in different genes could indicate a susceptibility to certain diseases and the doctor could offer medical treatment to avoid the development of this disease, the individual could also tailor their lifestyle by altering their diet or exercising to avoid the onset of particular diseases to which they may be predisposed. A second area where information about an individual's genome is useful is in the area of pharmacogenetics. When a patient responds differently to the same dose of a drug, the variation in responses to drugs can vary enormously and information about the individual's genome could reduce some of the associated ambiguity.

It would be interesting to establish if the cytokine levels in the serum of patients reflected the results found in the stable cell lines, in that the production of cytokine

levels in the case of the TLR6 N30 variant would be higher than that of the TLR6 D30 wild type form. Also, it would be interesting to determine if this TLR6 N30 polymorphism played a role in susceptibility to other diseases. As the variant form of TLR6 produces a very high inflammatory response it could be extended that the variant may play a large role in the development of other infectious diseases caused by *S. aureus*, *L. monocytogenes* and *M. tuberculosis*. Further analysis of the structure of the TLR6 D30 and TLR2 compared to TLR6 N30 and TLR2 heterodimer by crystallisation studies would give further insight into the binding of the two TLRs and whether the signal peptide region of the TLR6 N30 form influenced the binding capacity of the heterodimeric pair. By carrying out mass spectrometry it could be established whether the signal peptide of the TLR6 N30 form is cleaved off by signal peptide proteases. By carrying out trypsin digestions it could be determined whether the peptide is present in the wild type D30 form of the protein compared to the variant. Further experiments could also be carried out on the HEK 293T pDUO TLR2/6 clonal cells, by stimulating the TLR2/6 D30 and TLR2/6 N30 clonal cells to see if the presence of ligand altered the localisation of the TLR6. Furthermore, it would be interesting to carry out membrane fractionation assays on the stable cell lines to determine the localisation of TLR6 in these cells, as there would be a higher concentration of protein present compared to the transiently transfected samples. A greater understanding of the localisation of the two proteins would be determined.

In conclusion, this novel study firmly establishes that variants within the TLR signalling pathway influence the pathogenesis of infectious disease, the adaptor protein Mal-L180 variant plays a role in protection from disease by dampening the immune response to invading pathogens while the TLR6 variant causes susceptibility

to disease by causing an over-exaggerated response to ligand establishing a susceptibility to IPD and the development of septicaemia. Utilising information obtained from genetic association studies and linking it with biologically significant inflammatory signalling effects has provided a possible mechanistic explanation for the development of common infectious diseases. This thesis contributes to the validation that that TLR signalling pathways are important in the pathogenesis of disease – to which new therapeutic targets for infectious and inflammatory diseases could be generated.

Chapter Six

References

Chapter 6

References

- ADEREM, A. & UNDERHILL, D. M. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*, 17, 593-623.
- AGNESE, D. M., CALVANO, J. E., HAHM, S. J., COYLE, S. M., CORBETT, S. A., CALVANO, S. E. & LOWRY, S. F. 2002. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J Infect Dis*, 186, 1522-5.
- AKIRA, S., YAMAMOTO, M. & TAKEDA, K. 2003. Role of adapters in Toll-like receptor signalling. *Biochem Soc Trans*, 31, 637-42.
- ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, 413, 732-8.
- ALLISON, A. C. 1954. Protection afforded by sickle-cell trait against subtertian malarial infection. *Br Med J*, 1, 290-4.
- APT, A. S. & KONDRAT'EVA, T. K. 2008. [Tuberculosis: pathogenesis, immune responses and genetics of the host]. *Mol Biol (Mosk)*, 42, 880-90.
- ARBOUR, N. C., LORENZ, E., SCHUTTE, B. C., ZABNER, J., KLINE, J. N., JONES, M., FREES, K., WATT, J. L. & SCHWARTZ, D. A. 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet*, 25, 187-91.
- BEISSWENGER, C., COYNE, C. B., SHCHEPETOV, M. & WEISER, J. N. 2007. Role of p38 MAP kinase and transforming growth factor-beta signaling in transepithelial migration of invasive bacterial pathogens. *J Biol Chem*, 282, 28700-8.
- BELL, J. K., BOTOS, I., HALL, P. R., ASKINS, J., SHILOACH, J., SEGAL, D. M. & DAVIES, D. R. 2005. The molecular structure of the Toll-like receptor 3 ligand-binding domain. *Proc Natl Acad Sci U S A*, 102, 10976-80.
- BOPST, M., HAAS, C., CAR, B. & EUGSTER, H. P. 1998. The combined inactivation of tumor necrosis factor and interleukin-6 prevents induction of the major acute phase proteins by endotoxin. *Eur J Immunol*, 28, 4130-7.
- BRANDTZAEG, P. & VAN DEUREN, M. 2002. Current concepts in the role of the host response in *Neisseria meningitidis* septic shock. *Curr Opin Infect Dis*, 15, 247-52.
- BRIGHTBILL, H. D., LIBRATY, D. H., KRUTZIK, S. R., YANG, R. B., BELISLE, J. T., BLEHARSKI, J. R., MAITLAND, M., NORGARD, M. V., PLEVY, S. E., SMALE, S. T., BRENNAN, P. J., BLOOM, B. R., GODOWSKI, P. J. & MODLIN, R. L. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science*, 285, 732-6.
- BRUCH, M. D., MCKNIGHT, C. J. & GIERASCH, L. M. 1989. Helix formation and stability in a signal sequence. *Biochemistry*, 28, 8554-61.
- BUTLER, M. P., HANLY, J. A. & MOYNAGH, P. N. 2005. Pellino3 is a novel upstream regulator of p38 MAPK and activates CREB in a p38-dependent manner. *J Biol Chem*, 280, 27759-68.
- BUTLER, M. P., HANLY, J. A. & MOYNAGH, P. N. 2007. Kinase-active interleukin-1 receptor-associated kinases promote polyubiquitination and degradation of the Pellino family: direct evidence for PELLINO proteins being ubiquitin-protein isopeptide ligases. *J Biol Chem*, 282, 29729-37.
- CAMPBELL, K. D. 2007. Geographic patterns of haplogroup R1b in the british isles. *Journal of Genetic Genealogy*, 3, 1-13.

- CARPENTER, S. & O'NEILL, L. A. 2009. Recent insights into the structure of Toll-like receptors and post-translational modifications of their associated signalling proteins. *Biochem J*, 422, 1-10.
- CARTY, M., GOODBODY, R., SCHRODER, M., STACK, J., MOYNAGH, P. N. & BOWIE, A. G. 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat Immunol*, 7, 1074-81.
- CASTIBLANCO, J., VARELA, D. C., CASTANO-RODRIGUEZ, N., ROJAS-VILLARRAGA, A., HINCAPIE, M. E. & ANAYA, J. M. 2008. TIRAP (MAL) S180L polymorphism is a common protective factor against developing tuberculosis and systemic lupus erythematosus. *Infect Genet Evol*, 8, 541-4.
- 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. & INOHARA, N. 2003a. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol*, 4, 702-7.
- CHAMAILLARD, M., PHILPOTT, D., GIRARDIN, S. E., ZOUALI, H., LESAGE, S., CHAREYRE, F., BUI, T. H., GIOVANNINI, M., ZAEHRINGER, U., PENARD-LACRONIQUE, V., SANSONETTI, P. J., HUGOT, J. P. & THOMAS, G. 2003b. Gene-environment interaction modulated by allelic heterogeneity in inflammatory diseases. *Proc Natl Acad Sci U S A*, 100, 3455-60.
- CHAPMAN, S. J., KHOR, C. C., VANNBERG, F. O., FRODSHAM, A., WALLEY, A., MASKELL, N. A., DAVIES, C. W., SEGAL, S., MOORE, C. E., GILLESPIE, S. H., DENNY, P., DAY, N. P., CROOK, D. W., DAVIES, R. J. & HILL, A. V. 2007. IkappaB genetic polymorphisms and invasive pneumococcal disease. *Am J Respir Crit Care Med*, 176, 181-7.
- CHEN, Y. C., GIOVANNUCCI, E., KRAFT, P., LAZARUS, R. & HUNTER, D. J. 2007. Association between Toll-like receptor gene cluster (TLR6, TLR1, and TLR10) and prostate cancer. *Cancer Epidemiol Biomarkers Prev*, 16, 1982-9.
- CHEN, Z. J. 2005. Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol*, 7, 758-65.
- CLAROS, M. G., BRUNAK, S. & VON HEIJNE, G. 1997. Prediction of N-terminal protein sorting signals. *Curr Opin Struct Biol*, 7, 394-8.
- COBAN, C., ISHII, K. J., KAWAI, T., HEMMI, H., SATO, S., UEMATSU, S., YAMAMOTO, M., TAKEUCHI, O., ITAGAKI, S., KUMAR, N., HORII, T. & AKIRA, S. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J Exp Med*, 201, 19-25.
- COX, F. E. 2002. History of human parasitology. *Clin Microbiol Rev*, 15, 595-612.
- DAVILA, S., HIBBERD, M. L., HARI DASS, R., WONG, H. E., SAHIRATMADJA, E., BONNARD, C., ALISJAHBANA, B., SZESZKO, J. S., BALABANOVA, Y., DROBNIOWSKI, F., VAN CREVEL, R., VAN DE VOSSE, E., NEJENTSEV, S., OTTENHOFF, T. H. & SEIELSTAD, M. 2008. Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. *PLoS Genet*, 4, e1000218.
- DEMIRCI, F. Y., MANZI, S., RAMSEY-GOLDMAN, R., KENNEY, M., SHAW, P. S., DUNLOP-THOMAS, C. M., KAO, A. H., RHEW, E. Y., BONTEMPO, F., KAMMERER, C. & KAMBOH, M. I. 2007. Association study of Toll-like receptor 5 (TLR5) and Toll-like receptor 9 (TLR9) polymorphisms in systemic lupus erythematosus. *J Rheumatol*, 34, 1708-11.
- DEVI, L., PRABHU, B. M., GALATI, D. F., AVADHANI, N. G. & ANANDATHEERTHAVARADA, H. K. 2006. Accumulation of amyloid precursor protein in the mitochondrial import channels of human Alzheimer's disease brain is associated with mitochondrial dysfunction. *J Neurosci*, 26, 9057-68.
- DUNNE, A., EJDEBACK, M., LUDIDI, P. L., O'NEILL, L. A. & GAY, N. J. 2003. Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88. *J Biol Chem*, 278, 41443-51.

- ENGWERDA, C. R., MYNOTT, T. L., SAWHNEY, S., DE SOUZA, J. B., BICKLE, Q. D. & KAYE, P. M. 2002. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. *J Exp Med*, 195, 1371-7.
- ERRIDGE, C., STEWART, J. & POXTON, I. R. 2003. Monocytes heterozygous for the Asp299Gly and Thr399Ile mutations in the Toll-like receptor 4 gene show no deficit in lipopolysaccharide signalling. *J Exp Med*, 197, 1787-91.
- FERRARI, G., LANGEN, H., NAITO, M. & PIETERS, J. 1999. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell*, 97, 435-47.
- FERWERDA, B., ALONSO, S., BANAHAN, K., MCCALL, M. B., GIAMARELLOS-BOURBOULIS, E. J., RAMAKERS, B. P., MOUKTAROU, M., FAIN, P. R., IZAGIRRE, N., SYAFRUDDIN, D., CRISTEA, T., MOCKENHAUPT, F. P., TROYE-BLOMBERG, M., KUMPF, O., MAIGA, B., DOLO, A., DOUMBO, O., SUNDARESAN, S., BEDU-ADDO, G., VAN CREVEL, R., HAMANN, L., OH, D. Y., SCHUMANN, R. R., JOOSTEN, L. A., DE LA RUA, C., SAUERWEIN, R., DRENTH, J. P., KULLBERG, B. J., VAN DER VEN, A. J., HILL, A. V., PICKKERS, P., VAN DER MEER, J. W., O'NEILL, L. A. & NETEA, M. G. 2009. Functional and genetic evidence that the Mal/TIRAP allele variant 180L has been selected by providing protection against septic shock. *Proc Natl Acad Sci U S A*, 106, 10272-7.
- 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. & O'NEILL, L. A. 2001. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature*, 413, 78-83.
- FITZGERALD, K. A., ROWE, D. C., BARNES, B. J., CAFFREY, D. R., VISINTIN, A., LATZ, E., MONKS, B., PITHA, P. M. & GOLENOCK, D. T. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med*, 198, 1043-55.
- FRANK, M. M. & FRIES, L. F. 1991. The role of complement in inflammation and phagocytosis. *Immunol Today*, 12, 322-6.
- FREMOND, C. M., TOGBE, D., DOZ, E., ROSE, S., VASSEUR, V., MAILLET, I., JACOBS, M., RYFFEL, B. & QUESNIAUX, V. F. 2007. IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to Mycobacterium tuberculosis infection. *J Immunol*, 179, 1178-89.
- FULLER, G. L., WILLIAMS, J. A., TOMLINSON, M. G., EBLE, J. A., HANNA, S. L., POHLMANN, S., SUZUKI-INOUE, K., OZAKI, Y., WATSON, S. P. & PEARCE, A. C. 2007. The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. *J Biol Chem*, 282, 12397-409.
- GANCZAKOWSKI, M., TOWN, M., BOWDEN, D. K., VULLIAMY, T. J., KANEKO, A., CLEGG, J. B., WEATHERALL, D. J. & LUZZATTO, L. 1995. Multiple glucose 6-phosphate dehydrogenase-deficient variants correlate with malaria endemicity in the Vanuatu archipelago (southwestern Pacific). *Am J Hum Genet*, 56, 294-301.
- GARCIA-SASTRE, A. 2002. Mechanisms of inhibition of the host interferon alpha/beta-mediated antiviral responses by viruses. *Microbes Infect*, 4, 647-55.
- GAY, N. J. & GANGLOFF, M. 2007. Structure and function of Toll receptors and their ligands. *Annu Rev Biochem*, 76, 141-65.
- GEIJTENBEK, T. B., VAN VLIET, S. J., ENGERING, A., T HART, B. A. & VAN KOOYK, Y. 2004. Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu Rev Immunol*, 22, 33-54.
- GEORGES, H., LEROY, O., VANDENBUSSCHE, C., GUERY, B., ALFANDARI, S., TRONCHON, L. & BEAUCAIRE, G. 1999. Epidemiological features and prognosis of severe community-acquired pneumococcal pneumonia. *Intensive Care Med*, 25, 198-206.
- GEWIRTZ, A. T. 2007. TLRs in the Gut. III. Immune responses to flagellin in Crohn's disease: good, bad, or irrelevant? *Am J Physiol Gastrointest Liver Physiol*, 292, G706-10.

- GHOSH, S. & HAYDEN, M. S. 2008. New regulators of NF-kappaB in inflammation. *Nat Rev Immunol*, 8, 837-48.
- GIBBONS, R. J. 1992. Infectious agents must overcome innate host defenses to establish a focus of infection. *Infectious diseases*.
- GILMORE, T. D. 2006. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene*, 25, 6680-4.
- GIRARDIN, S. E., BONECA, I. G., CARNEIRO, L. A., ANTIGNAC, A., JEHANNO, M., VIALA, J., TEDIN, K., TAHA, M. K., LABIGNE, A., ZHRINGER, U., COYLE, A. J., DISTEFANO, P. S., BERTIN, J., SANSONETTI, P. J. & PHILPOTT, D. J. 2003a. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science*, 300, 1584-7.
- GIRARDIN, S. E., BONECA, I. G., VIALA, J., CHAMAILLARD, M., LABIGNE, A., THOMAS, G., PHILPOTT, D. J. & SANSONETTI, P. J. 2003b. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem*, 278, 8869-72.
- GIRARDIN, S. E., TRAVASSOS, L. H., HERVE, M., BLANOT, D., BONECA, I. G., PHILPOTT, D. J., SANSONETTI, P. J. & MENGIN-LECREULX, D. 2003c. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J Biol Chem*, 278, 41702-8.
- GODER, V., JUNNE, T. & SPIESS, M. 2004. Sec61p contributes to signal sequence orientation according to the positive-inside rule. *Mol Biol Cell*, 15, 1470-8.
- GOOD, M. F. & DOOLAN, D. L. 1999. Immune effector mechanisms in malaria. *Curr Opin Immunol*, 11, 412-9.
- GORLICH, D., HARTMANN, E., PREHN, S. & RAPOPORT, T. A. 1992. A protein of the endoplasmic reticulum involved early in polypeptide translocation. *Nature*, 357, 47-52.
- GRAY, P., DUNNE, A., BRIKOS, C., JEFFERIES, C. A., DOYLE, S. L. & O'NEILL, L. A. 2006. MyD88 adapter-like (Mal) is phosphorylated by Bruton's tyrosine kinase during TLR2 and TLR4 signal transduction. *J Biol Chem*, 281, 10489-95.
- GRINGHUIS, S. I., DEN DUNNEN, J., LITJENS, M., VAN HET HOF, B., VAN KOOYK, Y. & GEIJTENBEEK, T. B. 2007. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity*, 26, 605-16.
- GROSS, O., GEWIES, A., FINGER, K., SCHAFER, M., SPARWASSER, T., PESCHEL, C., FORSTER, I. & RULAND, J. 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature*, 442, 651-6.
- GUTIERREZ, O., PIPAON, C., INOHARA, N., FONTALBA, A., OGURA, Y., PROSPER, F., NUNEZ, G. & FERNANDEZ-LUNA, J. L. 2002. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J Biol Chem*, 277, 41701-5.
- HAWN, T. R., DUNSTAN, S. J., THWAITES, G. E., SIMMONS, C. P., THUONG, N. T., LAN, N. T., QUY, H. T., CHAU, T. T., HIEU, N. T., RODRIGUES, S., JANER, M., ZHAO, L. P., HIEN, T. T., FARRAR, J. J. & ADEREM, A. 2006. A polymorphism in Toll-interleukin 1 receptor domain containing adaptor protein is associated with susceptibility to meningeal tuberculosis. *J Infect Dis*, 194, 1127-34.
- HAWN, T. R., VERBON, A., LETTINGA, K. D., ZHAO, L. P., LI, S. S., LAWS, R. J., SKERRETT, S. J., BEUTLER, B., SCHROEDER, L., NACHMAN, A., OZINSKY, A., SMITH, K. D. & ADEREM, A. 2003. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J Exp Med*, 198, 1563-72.
- HAYASHI, F., SMITH, K. D., OZINSKY, A., HAWN, T. R., YI, E. C., GOODLETT, D. R., ENG, J. K., AKIRA, S., UNDERHILL, D. M. & ADEREM, A. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, 410, 1099-103.

- HEGDE, R. S. & KANG, S. W. 2008. The concept of translocational regulation. *J Cell Biol*, 182, 225-32.
- HEGDE, R. S., KANG, S.W. 2008. The concept of translocational regulation. *The journal of cell biology*, 182.
- HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H. & BAUER, S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science*, 303, 1526-9.
- HEMMI, H., KAISHO, T., TAKEUCHI, O., SATO, S., SANJO, H., HOSHINO, K., HORIUCHI, T., TOMIZAWA, H., TAKEDA, K. & AKIRA, S. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol*, 3, 196-200.
- HEMMI, H., TAKEUCHI, O., KAWAI, T., KAISHO, T., SATO, S., SANJO, H., MATSUMOTO, M., HOSHINO, K., WAGNER, H., TAKEDA, K. & AKIRA, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*, 408, 740-5.
- HIDAKA, F., MATSUO, S., MUTA, T., TAKESHIGE, K., MIZUKAMI, T. & NUNOI, H. 2006. A missense mutation of the Toll-like receptor 3 gene in a patient with influenza-associated encephalopathy. *Clin Immunol*, 119, 188-94.
- HISAMATSU, T., SUZUKI, M., REINECKER, H. C., NADEAU, W. J., MCCORMICK, B. A. & PODOLSKY, D. K. 2003. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology*, 124, 993-1000.
- HOSHINO, K., TAKEUCHI, O., KAWAI, T., SANJO, H., OGAWA, T., TAKEDA, Y., TAKEDA, K. & AKIRA, S. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol*, 162, 3749-52.
- HUANG, Y., LIU, X. Q., WYSS-CORAY, T., BRECHT, W. J., SANAN, D. A. & MAHLEY, R. W. 2001. Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. *Proc Natl Acad Sci U S A*, 98, 8838-43.
- INOHARA, CHAMAILLARD, MCDONALD, C. & NUNEZ, G. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem*, 74, 355-83.
- INOHARA, N., KOSEKI, T., DEL PESO, L., HU, Y., YEE, C., CHEN, S., CARRIO, R., MERINO, J., LIU, D., NI, J. & NUNEZ, G. 1999. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem*, 274, 14560-7.
- INOHARA, N., OGURA, Y., FONTALBA, A., GUTIERREZ, O., PONS, F., CRESPO, J., FUKASE, K., INAMURA, S., KUSUMOTO, S., HASHIMOTO, M., FOSTER, S. J., MORAN, A. P., FERNANDEZ-LUNA, J. L. & NUNEZ, G. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem*, 278, 5509-12.
- JIANG, Z., GEORGEL, P., DU, X., SHAMEL, L., SOVATH, S., MUDD, S., HUBER, M., KALIS, C., KECK, S., GALANOS, C., FREUDENBERG, M. & BEUTLER, B. 2005. CD14 is required for MyD88-independent LPS signaling. *Nat Immunol*, 6, 565-70.
- JIANG, Z., JOHNSON, H. J., NIE, H., QIN, J., BIRD, T. A. & LI, X. 2003. Pellino 1 is required for interleukin-1 (IL-1)-mediated signaling through its interaction with the IL-1 receptor-associated kinase 4 (IRAK4)-IRAK-tumor necrosis factor receptor-associated factor 6 (TRAF6) complex. *J Biol Chem*, 278, 10952-6.
- JIN, M. S. & LEE, J. O. 2008. Structures of the toll-like receptor family and its ligand complexes. *Immunity*, 29, 182-91.
- JOHNSON, C. M., LYLE, E. A., OMUETI, K. O., STEPENSKY, V. A., YEGIN, O., ALPSOY, E., HAMANN, L., SCHUMANN, R. R. & TAPPING, R. I. 2007. Cutting edge: A common

- polymorphism impairs cell surface trafficking and functional responses of TLR1 but protects against leprosy. *J Immunol*, 178, 7520-4.
- KAGAN, J. C. & MEDZHITOV, R. 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell*, 125, 943-55.
- KAGAN, J. C., SU, T., HORNG, T., CHOW, A., AKIRA, S. & MEDZHITOV, R. 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol*, 9, 361-8.
- KAJAVA, A. V. 1998. Structural diversity of leucine-rich repeat proteins. *J Mol Biol*, 277, 519-27.
- KANG, T. J. & CHAE, G. T. 2001. Detection of Toll-like receptor 2 (TLR2) mutation in the lepromatous leprosy patients. *FEMS Immunol Med Microbiol*, 31, 53-8.
- KARIN, M. & GRETEN, F. R. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol*, 5, 749-59.
- KAWAGOE, T., SATO, S., MATSUSHITA, K., KATO, H., MATSUI, K., KUMAGAI, Y., SAITOH, T., KAWAI, T., TAKEUCHI, O. & AKIRA, S. 2008. Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. *Nat Immunol*, 9, 684-91.
- KAWAI, T. & AKIRA, S. 2008. Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci*, 1143, 1-20.
- KENNY, E. F., TALBOT, S., GONG, M., GOLENBOCK, D. T., BRYANT, C. E. & O'NEILL, L. A. 2009. MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. *J Immunol*, 183, 3642-51.
- KHOR, C. C., CHAPMAN, S. J., VANNBERG, F. O., DUNNE, A., MURPHY, C., LING, E. Y., FRODSHAM, A. J., WALLEY, A. J., KYRIELEIS, O., KHAN, A., AUCAN, C., SEGAL, S., MOORE, C. E., KNOX, K., CAMPBELL, S. J., LIENHARDT, C., SCOTT, A., AABY, P., SOW, O. Y., GRIGNANI, R. T., SILLAH, J., SIRUGO, G., PESHU, N., WILLIAMS, T. N., MAITLAND, K., DAVIES, R. J., KWIATKOWSKI, D. P., DAY, N. P., YALA, D., CROOK, D. W., MARSH, K., BERKLEY, J. A., O'NEILL, L. A. & HILL, A. V. 2007. A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. *Nat Genet*, 39, 523-8.
- KIECHL, S., LORENZ, E., REINDL, M., WIEDERMANN, C. J., OBERHOLLENZER, F., BONORA, E., WILLEIT, J. & SCHWARTZ, D. A. 2002. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med*, 347, 185-92.
- KIM, J., OCHOA, M. T., KRUTZIK, S. R., TAKEUCHI, O., UEMATSU, S., LEGASPI, A. J., BRIGHTBILL, H. D., HOLLAND, D., CUNLIFFE, W. J., AKIRA, S., SIELING, P. A., GODOWSKI, P. J. & MODLIN, R. L. 2002. Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses. *J Immunol*, 169, 1535-41.
- KOBAYASHI, K. S., CHAMAILLARD, M., OGURA, Y., HENEGARIU, O., INOHARA, N., NUNEZ, G. & FLAVELL, R. A. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*, 307, 731-4.
- KOBAYASHI, T., TAKAESU, G. & YOSHIMURA, A. 2006. Mal-function of TLRs by SOCS. *Nat Immunol*, 7, 123-4.
- KOBE, B. & KAJAVA, A. V. 2001. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol*, 11, 725-32.
- KRISANAPRAKORNKIT, S., KIMBALL, J. R., WEINBERG, A., DARVEAU, R. P., BAINBRIDGE, B. W. & DALE, B. A. 2000. Inducible expression of human beta-defensin 2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun*, 68, 2907-15.
- KRISHNASWAMY, G., KELLEY, J., YERRA, L., SMITH, J. K. & CHI, D. S. 1999. Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. *J Interferon Cytokine Res*, 19, 91-104.

- KRONBORG, G., WEIS, N., MADSEN, H. O., PEDERSEN, S. S., WEJSE, C., NIELSEN, H., SKINHOJ, P. & GARRED, P. 2002. Variant mannose-binding lectin alleles are not associated with susceptibility to or outcome of invasive pneumococcal infection in randomly included patients. *J Infect Dis*, 185, 1517-20.
- KU, C. L., PICARD, C., ERDOS, M., JEURISSEN, A., BUSTAMANTE, J., PUEL, A., VON BERNUTH, H., FILIPE-SANTOS, O., CHANG, H. H., LAWRENCE, T., RAES, M., MARODI, L., BOSSUYT, X. & CASANOVA, J. L. 2007. IRAK4 and NEMO mutations in otherwise healthy children with recurrent invasive pneumococcal disease. *J Med Genet*, 44, 16-23.
- KUNO, K., OKAMOTO, S., HIROSE, K., MURAKAMI, S. & MATSUSHIMA, K. 1993. Structure and function of the intracellular portion of the mouse interleukin 1 receptor (type I). Determining the essential region for transducing signals to activate the interleukin 8 gene. *J Biol Chem*, 268, 13510-8.
- KWIATKOWSKI, D. P. 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. *Am J Hum Genet*, 77, 171-92.
- LARANGE, A., ANTONIOS, D., PALLARDY, M. & Kerdine-Romer, S. 2009. TLR7 and TLR8 agonists trigger different signaling pathways for human dendritic cell maturation. *J Leukoc Biol*, 85, 673-83.
- LAZARUS, R., RABY, B. A., LANGE, C., SILVERMAN, E. K., KWIATKOWSKI, D. J., VERCELLI, D., KLIMECKI, W. J., MARTINEZ, F. D. & WEISS, S. T. 2004. TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am J Respir Crit Care Med*, 170, 594-600.
- LEE, J., TATTOLI, I., WOJTAŁ, K. A., VAVRICKA, S. R., PHILPOTT, D. J. & GIRARDIN, S. E. 2009. pH-dependent internalization of muramyl peptides from early endosomes enables Nod1 and Nod2 signaling. *J Biol Chem*, 284, 23818-29.
- LEVITAN, A., TREBITSH, T., KISS, V., PEREG, Y., DANGOOR, I. & DANON, A. 2005. Dual targeting of the protein disulfide isomerase RB60 to the chloroplast and the endoplasmic reticulum. *Proc Natl Acad Sci U S A*, 102, 6225-30.
- LIN, R., HEYLBROECK, C., PITHA, P. M. & HISCOTT, J. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol*, 18, 2986-96.
- LIN, R., MAMANE, Y. & HISCOTT, J. 1999. Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. *Mol Cell Biol*, 19, 2465-74.
- LORENZ, E., MIRA, J. P., CORNISH, K. L., ARBOUR, N. C. & SCHWARTZ, D. A. 2000. A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. *Infect Immun*, 68, 6398-401.
- LOWENSTEIN, C. & MATSUSHITA, K. 2004. The acute phase response and atherosclerosis. *Drug discovery today: disease mechanisms*, 7.
- LUND, J., SATO, A., AKIRA, S., MEDZHITOV, R. & IWASAKI, A. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med*, 198, 513-20.
- LUZZATTO, L., USANGA, F. A. & REDDY, S. 1969. Glucose-6-phosphate dehydrogenase deficient red cells: resistance to infection by malarial parasites. *Science*, 164, 839-42.
- MA, X., LIU, Y., GOWEN, B. B., GRAVISS, E. A., CLARK, A. G. & MUSSER, J. M. 2007. Full-exon resequencing reveals toll-like receptor variants contribute to human susceptibility to tuberculosis disease. *PLoS One*, 2, e1318.
- MADSEN, H. O., SATZ, M. L., HOGH, B., SVEJGAARD, A. & GARRED, P. 1998. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. *J Immunol*, 161, 3169-75.

- MAEDA, S., HSU, L. C., LIU, H., BANKSTON, L. A., IIMURA, M., KAGNOFF, M. F., ECKMANN, L. & KARIN, M. 2005. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science*, 307, 734-8.
- MANSELL, A., BRINT, E., GOULD, J. A., O'NEILL, L. A. & HERTZOG, P. J. 2004. Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)-6 to mediate NF-kappaB activation by toll-like receptor (TLR)-2 and TLR4. *J Biol Chem*, 279, 37227-30.
- MANSELL, A., SMITH, R., DOYLE, S. L., GRAY, P., FENNER, J. E., CRACK, P. J., NICHOLSON, S. E., HILTON, D. J., O'NEILL, L. A. & HERTZOG, P. J. 2006. Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat Immunol*, 7, 148-55.
- MATSUSHIMA, N., TANAKA, T., ENKHBAYAR, P., MIKAMI, T., TAGA, M., YAMADA, K. & KUROKI, Y. 2007. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. *BMC Genomics*, 8, 124.
- MEANS, T. K., LIEN, E., YOSHIMURA, A., WANG, S., GOLENBOCK, D. T. & FENTON, M. J. 1999. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J Immunol*, 163, 6748-55.
- MEISTER, J. 2000. Community-acquired pneumonia: a case study. *Clin Excell Nurse Pract*, 4, 13-7.
- MIGGIN, S. M., PALSSON-MCDERMOTT, E., DUNNE, A., JEFFERIES, C., PINTEAUX, E., BANAHAN, K., MURPHY, C., MOYNAGH, P., YAMAMOTO, M., AKIRA, S., ROTHWELL, N., GOLENBOCK, D., FITZGERALD, K. A. & O'NEILL, L. A. 2007. NF-kappaB activation by the Toll-IL-1 receptor domain protein MyD88 adapter-like is regulated by caspase-1. *Proc Natl Acad Sci U S A*, 104, 3372-7.
- MINORETTI, P., GAZZARUSO, C., VITO, C. D., EMANUELE, E., BIANCHI, M., COEN, E., REINO, M. & GEROLDI, D. 2006. Effect of the functional toll-like receptor 4 Asp299Gly polymorphism on susceptibility to late-onset Alzheimer's disease. *Neurosci Lett*, 391, 147-9.
- MOENS, L., VERHAEGEN, J., PIERIK, M., VERMEIRE, S., DE BOECK, K., PEETERMANS, W. E. & BOSSUYT, X. 2007. Toll-like receptor 2 and Toll-like receptor 4 polymorphisms in invasive pneumococcal disease. *Microbes Infect*, 9, 15-20.
- MOORE, C. E., SEGAL, S., BERENDT, A. R., HILL, A. V. & DAY, N. P. 2004. Lack of association between Toll-like receptor 2 polymorphisms and susceptibility to severe disease caused by *Staphylococcus aureus*. *Clin Diagn Lab Immunol*, 11, 1194-7.
- MOTHES, W., JUNGNIKKEL, B., BRUNNER, J. & RAPOPORT, T. A. 1998. Signal sequence recognition in cotranslational translocation by protein components of the endoplasmic reticulum membrane. *J Cell Biol*, 142, 355-64.
- MOYNAGH, P. N. 2009. The Pellino family: IRAK E3 ligases with emerging roles in innate immune signalling. *Trends Immunol*, 30, 33-42.
- NAGPAL, K., PLANTINGA, T. S., WONG, J., MONKS, B. G., GAY, N. J., NETEA, M. G., FITZGERALD, K. A. & GOLENBOCK, D. T. 2009. A TIR domain variant of MyD88 adapter-like (Mal)/TIRAP results in loss of MyD88 binding and reduced TLR2/TLR4 signaling. *J Biol Chem*, 284, 25742-8.
- NAKAO, Y., FUNAMI, K., KIKKAWA, S., TANIGUCHI, M., NISHIGUCHI, M., FUKUMORI, Y., SEYA, T. & MATSUMOTO, M. 2005. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. *J Immunol*, 174, 1566-73.
- NIELSEN, H., ENGELBRECHT, J., BRUNAK, S. & VON HEIJNE, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng*, 10, 1-6.
- NISHIYA, T., KAJITA, E., HORINOUCHE, T., NISHIMOTO, A. & MIWA, S. 2007. Distinct roles of TIR and non-TIR regions in the subcellular localization and signaling properties of MyD88. *FEBS Lett*, 581, 3223-9.

- NUNEZ MIGUEL, R., WONG, J., WESTOLL, J. F., BROOKS, H. J., O'NEILL, L. A., GAY, N. J., BRYANT, C. E. & MONIE, T. P. 2007. A dimer of the Toll-like receptor 4 cytoplasmic domain provides a specific scaffold for the recruitment of signalling adaptor proteins. *PLoS One*, 2, e788.
- NYMAN, T., STENMARK, P., FLODIN, S., JOHANSSON, I., HAMMARSTROM, M. & NORDLUND, P. 2008. The crystal structure of the human toll-like receptor 10 cytoplasmic domain reveals a putative signaling dimer. *J Biol Chem*, 283, 11861-5.
- O'BRIEN, K. L., WOLFSON, L. J., WATT, J. P., HENKLE, E., DELORIA-KNOLL, M., MCCALL, N., LEE, E., MULHOLLAND, K., LEVINE, O. S. & CHERIAN, T. 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet*, 374, 893-902.
- O'NEILL, L. 2000. The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. *Biochem Soc Trans*, 28, 557-63.
- OGUS, A. C., YOLDAS, B., OZDEMIR, T., UGUZ, A., OLCEN, S., KESER, I., COSKUN, M., CILLI, A. & YEGIN, O. 2004. The Arg753Gln polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur Respir J*, 23, 219-23.
- OMUETI, K. O., MAZUR, D. J., THOMPSON, K. S., LYLE, E. A. & TAPPING, R. I. 2007. The polymorphism P315L of human toll-like receptor 1 impairs innate immune sensing of microbial cell wall components. *J Immunol*, 178, 6387-94.
- OZINSKY, A., UNDERHILL, D. M., FONTENOT, J. D., HAJJAR, A. M., SMITH, K. D., WILSON, C. B., SCHROEDER, L. & ADEREM, A. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A*, 97, 13766-71.
- PALSSON-MCDERMOTT, E. M., DOYLE, S. L., MCGETTRICK, A. F., HARDY, M., HUSEBYE, H., BANAHAN, K., GONG, M., GOLENBOCK, D., ESPEVIK, T. & O'NEILL, L. A. 2009. TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway. *Nat Immunol*, 10, 579-86.
- PARK, B., BRINKMANN, M. M., SPOONER, E., LEE, C. C., KIM, Y. M. & PLOEGH, H. L. 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nat Immunol*, 9, 1407-14.
- PARK, B. S., SONG, D. H., KIM, H. M., CHOI, B. S., LEE, H. & LEE, J. O. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*, 458, 1191-5.
- PAYTON, A., PAYNE, D., MANKHAMBO, L. A., BANDA, D. L., HART, C. A., OLLIER, W. E. & CARROL, E. D. 2009. Nitric oxide synthase 2A (NOS2A) polymorphisms are not associated with invasive pneumococcal disease. *BMC Med Genet*, 10, 28.
- PIAO, W., SONG, C., CHEN, H., WAHL, L. M., FITZGERALD, K. A., O'NEILL, L. A. & MEDVEDEV, A. E. 2008. Tyrosine phosphorylation of MyD88 adapter-like (Mal) is critical for signal transduction and blocked in endotoxin tolerance. *J Biol Chem*, 283, 3109-19.
- 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. & BEUTLER, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science*, 282, 2085-8.
- QIN, B. Y., LIU, C., LAM, S. S., SRINATH, H., DELSTON, R., CORREIA, J. J., DERYNCK, R. & LIN, K. 2003. Crystal structure of IRF-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation. *Nat Struct Biol*, 10, 913-21.
- RAMADORI, G. & CHRIST, B. 1999. Cytokines and the hepatic acute-phase response. *Semin Liver Dis*, 19, 141-55.
- REARDON, C. & MAK, T. W. 2009. cIAP proteins: keystones in NOD receptor signal transduction. *Immunity*, 30, 755-6.
- REILING, N., EHLERS, S. & HOLSCHER, C. 2008. MyDths and un-TOLled truths: sensor, instructive and effector immunity to tuberculosis. *Immunol Lett*, 116, 15-23.

- RIGATO, O., UJVARI, S., CASTELO, A. & SALOMAO, R. 1996. Tumor necrosis factor alpha (TNF-alpha) and sepsis: evidence for a role in host defense. *Infection*, 24, 314-8.
- ROCK, F. L., HARDIMAN, G., TIMANS, J. C., KASTELEIN, R. A. & BAZAN, J. F. 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci U S A*, 95, 588-93.
- ROY, S., KNOX, K., SEGAL, S., GRIFFITHS, D., MOORE, C. E., WELSH, K. I., SMARASON, A., DAY, N. P., MCPHEAT, W. L., CROOK, D. W. & HILL, A. V. 2002. MBL genotype and risk of invasive pneumococcal disease: a case-control study. *Lancet*, 359, 1569-73.
- SCHAAF, B. M., BOEHMKE, F., ESNAASHARI, H., SEITZER, U., KOTHE, H., MAASS, M., ZABEL, P. & DALHOFF, K. 2003. Pneumococcal septic shock is associated with the interleukin-10-1082 gene promoter polymorphism. *Am J Respir Crit Care Med*, 168, 476-80.
- SCHERER, D. C., BROCKMAN, J. A., CHEN, Z., MANIATIS, T. & BALLARD, D. W. 1995. Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. *Proc Natl Acad Sci U S A*, 92, 11259-63.
- SCHOFIELD, L. & HACKETT, F. 1993. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. *J Exp Med*, 177, 145-53.
- SCHOTT, E., WITT, H., NEUMANN, K., BERGK, A., HALANGK, J., WEICH, V., MULLER, T., PUHL, G., WIEDENMANN, B. & BERG, T. 2008. Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. *J Viral Hepat*, 15, 71-8.
- SCHOTT, E., WITT, H., NEUMANN, K., TAUBE, S., OH, D. Y., SCHREIER, E., VIERICH, S., PUHL, G., BERGK, A., HALANGK, J., WEICH, V., WIEDENMANN, B. & BERG, T. 2007. A Toll-like receptor 7 single nucleotide polymorphism protects from advanced inflammation and fibrosis in male patients with chronic HCV-infection. *J Hepatol*, 47, 203-11.
- SCHRODER, N. W., DITERICH, I., ZINKE, A., ECKERT, J., DRAING, C., VON BAEHR, V., HASSLER, D., PRIEM, S., HAHN, K., MICHELSEN, K. S., HARTUNG, T., BURMESTER, G. R., GOBEL, U. B., HERMANN, C. & SCHUMANN, R. R. 2005. Heterozygous Arg753Gln polymorphism of human TLR-2 impairs immune activation by *Borrelia burgdorferi* and protects from late stage Lyme disease. *J Immunol*, 175, 2534-40.
- SCHWANDNER, R., DZIARSKI, R., WESCHE, H., ROTHE, M. & KIRSCHNING, C. J. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem*, 274, 17406-9.
- SERGHIDES, L., SMITH, T. G., PATEL, S. N. & KAIN, K. C. 2003. CD36 and malaria: friends or foes? *Trends Parasitol*, 19, 461-9.
- SETH, R. B., SUN, L., EA, C. K. & CHEN, Z. J. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*, 122, 669-82.
- SHAULIAN, E. & KARIN, M. 2002. AP-1 as a regulator of cell life and death. *Nat Cell Biol*, 4, E131-6.
- STARY, G., BANGERT, C., TAUBER, M., STROHAL, R., KOPP, T. & STINGL, G. 2007. Tumoricidal activity of TLR7/8-activated inflammatory dendritic cells. *J Exp Med*, 204, 1441-51.
- STEINER, D. F., DOCHERTY, K. & CARROLL, R. 1984. Golgi/granule processing of peptide hormone and neuropeptide precursors: a minireview. *J Cell Biochem*, 24, 121-30.
- STEINER, D. F., QUINN, P. S., CHAN, S. J., MARSH, J. & TAGER, H. S. 1980. Processing mechanisms in the biosynthesis of proteins. *Ann N Y Acad Sci*, 343, 1-16.
- SUN, J., WIKLUND, F., ZHENG, S. L., CHANG, B., BALTER, K., LI, L., JOHANSSON, J. E., LI, G., ADAMI, H. O., LIU, W., TOLIN, A., TURNER, A. R., MEYERS, D. A., ISAACS, W. B., XU, J. & GRONBERG, H. 2005. Sequence variants in Toll-like receptor gene cluster (TLR6-TLR1-TLR10) and prostate cancer risk. *J Natl Cancer Inst*, 97, 525-32.

- SUN, L. & CHEN, Z. J. 2004. The novel functions of ubiquitination in signaling. *Curr Opin Cell Biol*, 16, 119-26.
- SVANBORG, C., GODALY, G. & HEDLUND, M. 1999. Cytokine responses during mucosal infections: role in disease pathogenesis and host defence. *Curr Opin Microbiol*, 2, 99-105.
- TABEL, Y., BERDELI, A. & MIR, S. 2007. Association of TLR2 gene Arg753Gln polymorphism with urinary tract infection in children. *Int J Immunogenet*, 34, 399-405.
- TACHADO, S. D., GEROLD, P., SCHWARZ, R., NOVAKOVIC, S., MCCONVILLE, M. & SCHOFIELD, L. 1997. Signal transduction in macrophages by glycosylphosphatidylinositols of *Plasmodium*, *Trypanosoma*, and *Leishmania*: activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. *Proc Natl Acad Sci U S A*, 94, 4022-7.
- TAKEUCHI, O., KAWAI, T., SANJO, H., COPELAND, N. G., GILBERT, D. J., JENKINS, N. A., TAKEDA, K. & AKIRA, S. 1999. TLR6: A novel member of an expanding toll-like receptor family. *Gene*, 231, 59-65.
- TAKEUCHI, O., KAWAI, T., SANJO, H., COPELAND, N.G., GILBERT, D.J., JENKINS, N.A., TAKEDA, K., AND S. AKIRA 1999. TLR6: A novel member of an expanding Toll-like receptor family. *Gene*, 231, 59-65.
- TAKEUCHI, O., SATO, S., HORIUCHI, T., HOSHINO, K., TAKEDA, K., DONG, Z., MODLIN, R. L. & AKIRA, S. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol*, 169, 10-4.
- TAO, K., FUJII, M., TSUKUMO, S., MAEKAWA, Y., KISHIHARA, K., KIMOTO, Y., HORIUCHI, T., HISAEDA, H., AKIRA, S., KAGAMI, S. & YASUTOMO, K. 2007. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. *Ann Rheum Dis*, 66, 905-9.
- TRIAANTAFILOU, M., GAMPER, F. G., HASTON, R. M., MOURATIS, M. A., MORATH, S., HARTUNG, T. & TRIANTAFILOU, K. 2006. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *J Biol Chem*, 281, 31002-11.
- TSCHOPP, J., MARTINON, F. & BURNS, K. 2003. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol*, 4, 95-104.
- TULIC, M. K., HURRELBRINK, R. J., PRELE, C. M., LAING, I. A., UPHAM, J. W., LE SOUEF, P., SLY, P. D. & HOLT, P. G. 2007. TLR4 polymorphisms mediate impaired responses to respiratory syncytial virus and lipopolysaccharide. *J Immunol*, 179, 132-40.
- TURNER, M. W. 1998. Mannose-binding lectin (MBL) in health and disease. *Immunobiology*, 199, 327-39.
- VAN VLIET, S. J., DEN DUNNEN, J., GRINGHUIS, S. I., GEIJTENBEEK, T. B. & VAN KOOYK, Y. 2007. Innate signaling and regulation of Dendritic cell immunity. *Curr Opin Immunol*, 19, 435-40.
- VIALA, J., CHAPUT, C., BONECA, I. G., CARDONA, A., GIRARDIN, S. E., MORAN, A. P., ATHMAN, R., MEMET, S., HUERRE, M. R., COYLE, A. J., DISTEFANO, P. S., SANSONETTI, P. J., LABIGNE, A., BERTIN, J., PHILPOTT, D. J. & FERRERO, R. L. 2004. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol*, 5, 1166-74.
- VON HEIJNE, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem*, 133, 17-21.
- WANG, Y., ZHANG, H. X., SUN, Y. P., LIU, Z. X., LIU, X. S., WANG, L., LU, S. Y., KONG, H., LIU, Q. L., LI, X. H., LU, Z. Y., CHEN, S. J., CHEN, Z., BAO, S. S., DAI, W. & WANG, Z. G. 2007. Rlg-I^{-/-} mice develop colitis associated with downregulation of G alpha i2. *Cell Res*, 17, 858-68.

- WEST, A. P., KOBLANSKY, A. A. & GHOSH, S. 2006. Recognition and signaling by toll-like receptors. *Annu Rev Cell Dev Biol*, 22, 409-37.
- WOEHRLE, T., DU, W., GOETZ, A., HSU, H. Y., JOOS, T. O., WEISS, M., BAUER, U., BRUECKNER, U. B. & MARION SCHNEIDER, E. 2008. Pathogen specific cytokine release reveals an effect of TLR2 Arg753Gln during Candida sepsis in humans. *Cytokine*, 41, 322-9.
- XU, Y., TAO, X., SHEN, B., HORNG, T., MEDZHITOV, R., MANLEY, J. L. & TONG, L. 2000. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature*, 408, 111-5.
- YONEYAMA, M., SUHARA, W., FUKUHARA, Y., FUKUDA, M., NISHIDA, E. & FUJITA, T. 1998. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *Embo J*, 17, 1087-95.
- ZEE, R. Y., HEGENER, H. H., GOULD, J. & RIDKER, P. M. 2005. Toll-like receptor 4 Asp299Gly gene polymorphism and risk of atherothrombosis. *Stroke*, 36, 154-7.

Chapter Seven

Record of publication

Chapter 7

Record of publication

Khor., C.C., Chapman, S.J., Vannberg, F.O., Dunne, A., Murphy, C., Ling, E.Y., Frodsham, A.J., Walley., A.J., Kyrieleis, O., Khan, A., Aucan, C., Segal, S., Moore, C.E., Knox, K., Campbell, S.J., Lienhardt, C., Scott, A., Asby, P., Sow, O.Y., Grignani, R.Y., Williams, T.N., Davies, R.J., Day, N.P., Crook, D.W., Marsh, K., Berkley, J.A., O'Neill, L.A. and hill, A.V.S. A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. *Nature Genetics* (2007), **39**(4), 523-8

Miggin, S. M., Palsson-McDermott, E., Dunne, A., Jeffries, C., Pinteaux, E., Banahan, K., Murphy, C., Moynagh, P., Yamamoto, M., Akira, S., Rothwell, N., Golenbock, D., Fitzgerald, K.A., and O'Neill, L.A.J. (2007). "NF-kappaB activation by TOLL-IL-1 receptor domain protein MyD88 adapter like is regulated by Caspase-1." *PNAS* **104**(9): 3372-3377.

Susan Carpenter , Thaddeus Carlson , Jerome Dellacasagrande , Amaya Garcia , Sharon Gibbons, Paul Hertzog, Anthony Lyons, Lih-Ling Lin, Marina Lynch, Tom Monie, Caroline Murphy, Katherine Seidl, Christine Wells, Aisling Dunne, Luke O'Neill (corr-auth). TRIL, a functional component of the TLR4 signaling complex, highly expressed in brain. *Journal of Immunology* (2009).

Fredrik O Vannberg, Chicea C. Khor, Stephen J. Chapman, Caroline Murphy, Anna Rautanen, Sunny H. Wong, Andrew J. Walley, Shelley Segal, Catrin E. Moore, Kyle Knox, Denise L.M. Goh, Anthony Scott, Nobert Peschu, Thomas N. Williams, Robert J. O. Davies, Derrick W. Crook, Kevin Marsh, James A. Berkley, Luke A.J. O'Neill, Adrian V.S. Hill. A rare variant of TLR6 abrogates function and associates with 6-fold increased risk of invasive pneumococcal disease. Paper is currently in preparation for submission to a scientific journal.

E.C. Lavelle, L.A.J. O'Neill, E. Creagh and C.Murphy. The role of TLRs, NLRs and RLRs in mucosal innate immunity and homeostasis. Accepted for publication in *Mucosal Immunology*.

Caroline Murphy and Luke A.J. O'Neill. Negative regulators of NF- κ B activation and type I interferon pathways. Currently in submission as a book chapter for book titled, "Innate and Adaptive Immune Regulation and Cancer Immunotherapy", Springer-Cancer