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Characterisation of the Novel Leucine Rich Repeat containing protein KIAA0644

Thesis submitted to the

University of Dublin

For the

Degree of Doctor of Philosophy

By

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August 2008

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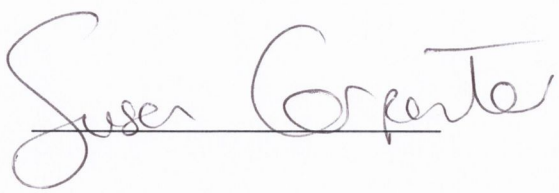
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Susan Carpenter

This is the page I have waited four years to write! I knew once I reached this page it was all finally complete. It has been a fantastic journey and there are a number of people who have helped along the way. First and foremost I have to thank my boss Prof Luke O'Neill. You have given me so much support and advice I will be forever indebted to you. I had the best four years working in the lab and some brilliant nights out so thank you for making my PHD such an enjoyable experience! Thanks to my co-supervisor Dr Aisling Dunne for her eternal patience and encouragement. Thanks also to all the members of the LON lab, Fred, Kathy, Emma, Cait, Sarah, Caroline M, Anne, Eva, Clare, Elaine, Liz, Andrea, Christine and Sinead.

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AMIGO	Amphoterin-induced gene and ORF-1
bp	base pair
BMDM	Bone Marrow Derived Macrophage
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CpG	Cytosine phosphate guanine
CREB	Ca2+/cAMP response element binding protein
DC	Dendritic cell
DD	Death domain
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ehtylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FCS	Fetal calf serum
FLRT	Fibronectin-leucine-rich transmembrane
HEK	Human Embryonic Kidney
HSV-1	Herpes Simplex Virus-1
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of κB

IKK	IκB kinase
IL	Interleukin
IL-1R	IL-1 receptor
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
LB broth	Luria-Bertani broth
LBP	LPS binding protein
LINGO	LRR and Ig domain containing Nogo receptor interacting protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
MALP-2	Macrophage-activating lipoprotein-2kDa
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
Mal	MyD88 adaptor like
Mda5	Melanoma-differentiation-associated gene 5
MEF	Mouse embryonic fibroblast
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
NGL-1	Netrin-G1 ligand

NgR1	nucleotide-binding domain leucine-rich repeat containing protein 1
NLRR	neuronal leucine rich repeat family
NOD	nucleotide-binding domain
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pattern associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
P(I:C)	polyinosine-polycytidilic acid
PMSF	Phenylmethylsuphonylflouride
PRR	Pathogen recognition receptor
PVDF	Polyvinylidene diflouride
RIG-I	Retinoic acid inducible gene-I
RNA	Ribonucleic acid
RLR	RIG like receptor family
SARM	sterile-α and HEAT/Armadillo motifs containing protein
SDS	Sodium Dodecyl Sulphate
SOCs-1	Suppressor of cytokine signalling 1
TAB	TAK1 binding protein
TAK	TGFβ-activated kinase
TANK	TRAF family member associated NFκB activator
TBE	Tris borate EDTA

TBK1	Tank binding kinase -1
TEMED	N, N, N', N', - Tetramethylethylenediamine
TICAM-1	TIR domain containing adaptor-1
TICAM-2	TIR containing adaptor molecule-2
TIR	Toll-IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
Tollip	Toll/IL1R interacting protein
TRIF	TIR domain-containing adaptor inducing IFN-beta
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adaptor protein
WNV	West Nile Virus

Abstract

This thesis reports the existence of a novel leucine rich repeat containing protein annotated as KIAA0644 in the Kazusa DNA Research Institute database (www.Kazusa.or.jp), which was discovered in a study for proteins induced by Lipopolysaccaharide (LPS). This protein is expressed and highly conserved across species. It contains thirteen leucine rich repeats, a signal sequence, a fibronectin domain and a single spanning transmembrane region. It shows sequence similarity to Toll-like receptor, Nogo receptor and the Fibronectin leucine-rich transmembrane families. KIAA0644 is highly expressed in the brain, lung and ovary. RT-PCR results demonstrate that at the mRNA level KIAA0644 is induced by Lipopolysaccaharide (LPS). An antibody to the C terminus of the protein was raised and expression of KIAA0644 in the brain of human tissue samples was confirmed at the protein level. The protein was induced by LPS in the brain derived cell lines U373 and 1321-N1. KIAA0644 was also shown to be induced by LPS in the monocytic cell line THP1, in primary human peripheral blood mononuclear cells (PBMC), primary murine bone marrow derived macrophages (BMDM) and also in primary murine mixed glial cells.

Further investigation into the localisation of the protein revealed that endogenous KIAA0644 is mainly present in the cytosol whilst over-expressed KIAA0644 localises to the membrane fraction. Confocal imaging of KIAA0644 in A172 and U373/CD14 cells revealed the presence of KIAA0644 in undefined vescicles throughout the cytosol of the cell.

Biological assays were carried out to try and determine a function for this novel protein. siRNA experiments were carried out on U373/CD14 cells, THP1 cells and

human PBMCs. siRNAs specific to KIAA0644 were obtained and shown to reduce KIAA0644 expression at both the RNA and protein level. When KIAA0644 expression is knocked down in cells there is no IκB degradation or phosphorylation of p38 in response to LPS stimulation. There is also a decrease in proinflammatory cytokine release from cells in response to LPS. Further biological assays indicate that over-expression of KIAA0644 can enhance LPS signalling. KIAA0644 can bind LPS and physical interaction between KIAA0644 and TLR4 and KIAA0644 and CD14 was observed. The interaction between KIAA0644 and TLR4 increases upon LPS stimulation and the interaction between KIAA0644 and CD14 decreases post LPS stimulation.

This study therefore demonstrates that KIAA0644 is a functional protein which may be acting as a co-receptor within the LPS signalling pathway.

Chapter One

Introduction

Chapter 1 Introduction

1.1 The immune system

The immune system has evolved to protect the body against invading pathogens. The mammalian immune system consists of both innate and adaptive immunity. The innate immune system is the first line of defence and it is also responsible for priming the adaptive system to eliminate pathogens, leading to immunological memory. Innate immune recognition refers to all forms of self/non self discrimination that is not mediated by antigen receptors (Janeway *et al.*, 1992). Vertebrates are unique within the animal kingdom since they have evolved adaptive immune systems. Within adaptive immunity microbes are recognised by antigen receptors on cells. These cells then become memory cells capable of retaining memory to the exposed antigen and therefore responding more rapidly if encountered again.

1.1.1 Innate Immunity

The innate immune system is the dominant immune system found in all plants, insects, fungi and in primitive multicellular organisms. It is the primary line of defence protecting us from a wide range of pathogens. It has evolved to recognise characteristic conserved structures present on microbes also known as pattern associated molecular patterns (PAMPs). These molecular patterns tend to evolve slowly. They are found embedded within structures that are essential to the microbes' survival. PAMPs are usually conserved within whole classes of bacteria, viruses or fungi. The innate immune system relies on germline encoded preformed receptors also known as pattern recognition receptors (PRRs) to recognise these infectious agents (Janeway *et al.*, 1992). PRRs are expressed on the cell surface, within intracellular compartments and some are also present in a secreted form. Once PRRs

are triggered by a PAMP it leads to a signalling cascade which culminates in an inflammatory response inducing the production of a host of cytokines and chemokines necessary to return the body to a state of homeostasis. It is through the recognition of PAMPs that the innate immune system is capable of discriminating between self and non self.

1.2 The IL-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily

The novel protein investigated in this thesis is KIAA0644. Preliminary data on this protein suggested that it may belong to the Toll like receptor (TLR) superfamily, therefore I will now describe the TLR system and its role in the innate immune system. TLRs function as key regulators of both innate and adaptive immunity. TLRs are germline-encoded type I transmembrane receptors which are expressed on numerous cell types including macrophages and dendritic cells. They are PRRs, recognising PAMPs which are unique to microbes and essential for their survival (Janeway, 1992).

The term Toll was originally coined for a cell surface receptor governing dorsol/ventral orientation in the *Drosophila* embryo (Stein *et al.*, 1991). Toll was found to display remarkable sequence similarity in its intracellular domain to that of a mammalian receptor for the cytokine interleukin-1 (IL-1), termed the Type 1 IL-1 receptor (IL-1RI) (Gay, N *et al.,* 1991). Like the IL-1R1, Toll was then found to have a role in immune defence specifically against the invading fungus *Aspergillus Fumigatus* in *Drosophila* (Lemaitre, B *et al.*, 1996). The intracellular signalling pathways of IL-1R1 and Toll were found to share common signalling intermediates with both receptors leading to the activation of the Rel family of transcription factors,

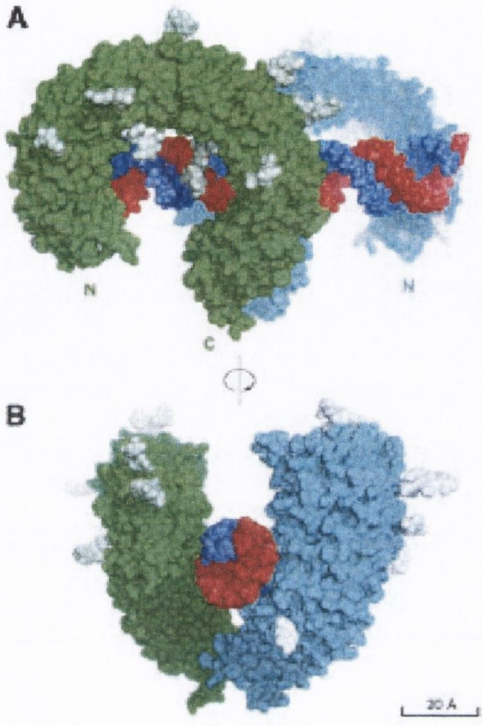
such as nuclear factor κB (NFκB) in mammals and Dif in *Drosophila* (Imler *et al.*, 2001). Although both Toll and IL-R1 share a conserved intracellular domain now termed the Toll/IL-1R/Resistance (TIR) domain, they differ extracellularly. IL-1R1 possesses immunoglobulin domains, whereas Toll comprises of extracellular leucine rich repeat domains.

Following the observation of the similarity shared between Toll and IL-1R1 a human homologue to *Drosophila* Toll was identified one year later (Medzhitov *et al.*, 1997). Human Toll or hToll is now termed TLR4. To date a total of thirteen TLRs have been identified, ten of which are expressed in humans. TLRs 1-9 are common between mice and human, however murine TLR8 appears to be non-functional. TLR10 is only functional in humans and TLR11, 12 and 13 are only expressed in mice (Dunne *et al.*, 2003, Takeda *et al.*, 2003, Zhang et al., 2004). Ligands have been described for all TLRs except TLR10, TLR12 and TLR13.

TLRs belong to a wider superfamily called the IL-1R/TLR superfamily. All members of the superfamily possess a cytoplasmic TIR domain which is essential for signalling. The superfamily can be divided into three groups: those members possessing extracellular immunoglobulin domains such as the IL-1 and IL-18 receptors, the TLR sub group which have extracellular leucine rich repeat domains and finally the adaptor sub group including MyD88, MAL, TRIF and TRAM which are cytoplasmic and do not have an extracellular region (Akira *et al.*, 2000, Dunne *et al.*, 2003).

1.3 TLRs

As stated above TLRs are a family of type 1 transmembrane receptors. They are characterized by the presence of an extracellular leucine rich repeat domain (LRR) and an intracellular Toll/IL-1 receptor (TIR) domain (Hashimoto *et al.,* 1988, Medzhitov *et al.,* 1997, Rock *et al.,* 1998.) LRRs are found on a diverse number of proteins and are involved in ligand recognition and signal transduction (Kobe *et al.,* 1995). The consensus motif used to identify LRRs within a protein sequence is LXXLXLXXNXLXXLXXXXXXXXLXX, in which X is any amino acid. The LRR domain is separated from the transmembrane region by a LRR carboxy-terminal domain. The crystal structure of the human TLR3 Ectodomain has been obtained by two labs independently (Choe *et al.,* 2005, Bell *et al.*, 2005) (Fig 1.1). Both results show the structure at 2.1 angstroms shows a large horseshoe- shaped solenoid assembled from 23 leucine rich repeats. This structure shows that TLR3 is mostly masked by carbohydrate but there is one face which is glycosylation free and it was predicted that this is the region to which the ligand to TLR3, double stranded RNA binds. More recent results have helped confirm this hypothesis. Two studies have been carried out on the ectodomain of TLR3, one study worked on the murine form whereas the latter study was on the human TLR3 ECD (Liu *et al.*, 2008, Leonard *et al.,* 2008). Both results indicate that in solution TLR3 ECD is monomeric however when binding to dsRNA it dimerises. Each TLR3 ECD binds the dsRNA at the C terminal and also at the N terminal of the TLR3 structure. The mTLR3 ECD has been shown to bind to the sugar-phosphate backbone of the dsRNA which helps to examine why the receptor does not bind to specific nucleotide sequences. TLR3 ECD only binds dsRNA at an acidic ph of 6.5 which is explained by its endosomal location within cells.



(Liu *et al.*, 2008)

Figure 1.1 Structural representation of the ectodomain of TLR3 binding to dsRNA. The 23 leucine rich repeats present in the ectodomain of TLR3 forms a horseshoe shaped solenoid. The TLR3 ectodomains (green and cyan) form a dimer on the double stranded RNA (blue and red). Part (A) shows how the dsRNA binds at the N and C terminal of the TLR3 ECDs. Part (B) indicates how the C termini of the dimers come together within the complex.

As mentioned previously, members of the TLR family all contain a cytosolic TIR domain. The TIR domain portion of the TLR is required for intracellular signalling. This domain spans about 200 amino acids, with varying degrees of sequence similarity among family members. Three particular boxes can be identified which are highly conserved among family members. Box 1 is considered the signature sequence of the family whereas boxes 2 and 3 contain amino acids critical for signalling (Fig 1.2). The crystal structure of the TIR domains of TLR1 and TLR2 has revealed a core structural element centred around box 2 (Xu *et al.,* 2000.) (Fig 1.3). This region, termed the BB loop, forms an exposed surface patch and contains a critical proline or arginine residue. These amino acids are located at the tip of the loop and are thought to form a point of contact with downstream signalling components. Recently the crystal structure of the TIR domain of TLR10 has been published (Nyman *et al.*, 2008). The 2.2A crystal structure indicates a symmetric dimer within an asymmetric unit. The BB loop is shown to form a tight interaction at the surface dimer interaction and the DD loop is also present in this region. A pocket where potential peptides could bind was revealed, this pocket was made up of some of the BB loop and also an alphaC-helix. This structure helps to explain how blocking peptides to the BB loop function. Once a blocking peptide is added it will bind within the BB loop region and inhibit activation of the signalling cascade.

1.4 Natural TLR ligands

1.4.1 TLR4

TLR4 was the first mammalian toll to be identified and characterized (Medzhitov *et al.,* 1997). TLR4 is the receptor for lipopolysaccharide (LPS) (Fig 1.4) from gram

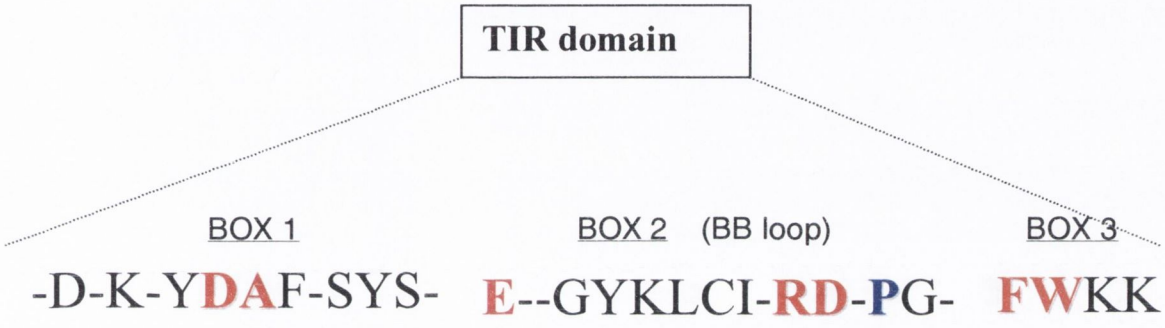
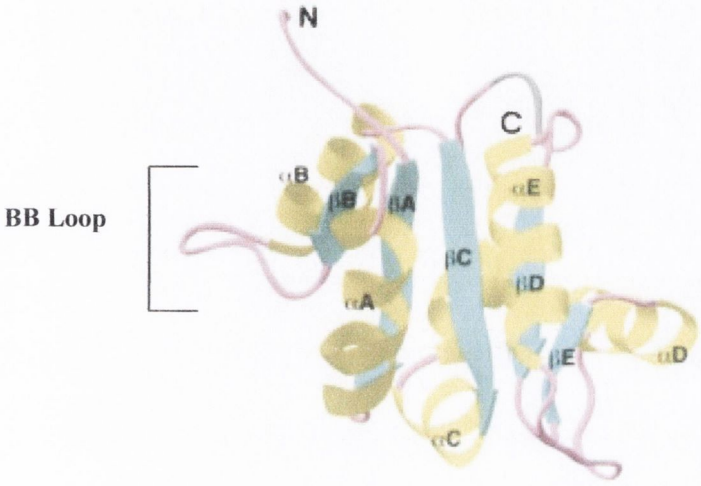


Figure 1.2 Components of the TIR domain. The TIR domain is made up of three boxes which all contain amino acids critical for signalling. The most highly conserved amino acids are highlighted in red. The proline in box 2 (in blue), is highly conserved in TLRs. When this is mutated to a histidine in TLR4 mice become hyopresponsive to LPS (Poltorak *et al.,* 1998).



Xu, Y. et al. (2000) Nature 408(6808), 111-115.

Figure 1.3 Crystal structure of the TIR domain. This structure reveals a central five stranded parallel beta-sheet which is surrounded by five helices on both sides. Conserved residues are located in the hydrophobic core. Different TIR domains can contain large insertions or deletions in several loop regions, therefore the size of TIR domains can vary (Xu *et al.,* 2000). The BB loop protrudes from a conserved patch. The conserved proline residue is situated at the tip of the BB loop and when this is mutated to a histidine in C3H/HeJ mice they are rendered hyporesponive to LPS (Poltorak *et al.,* 1998).

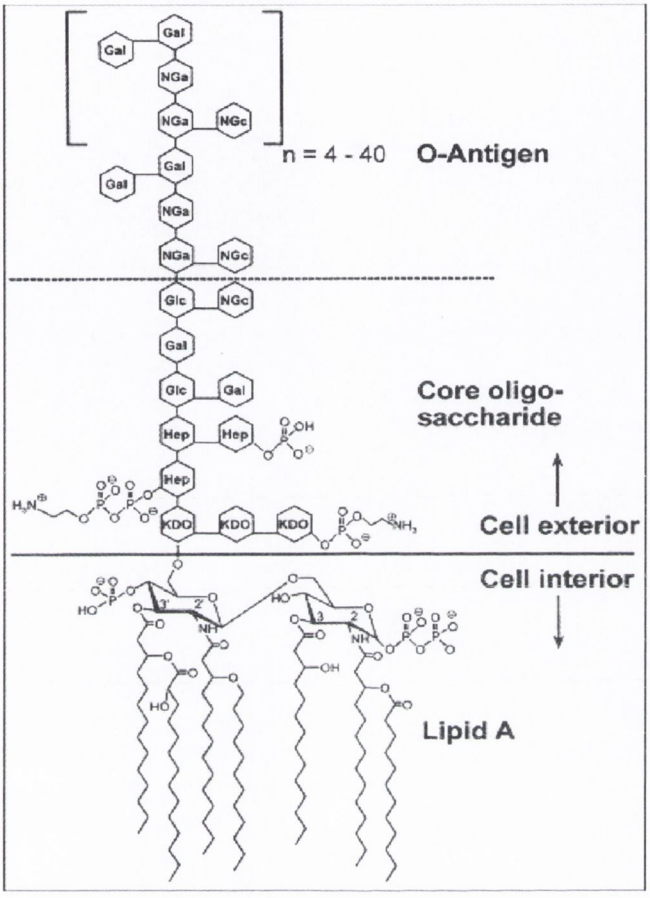
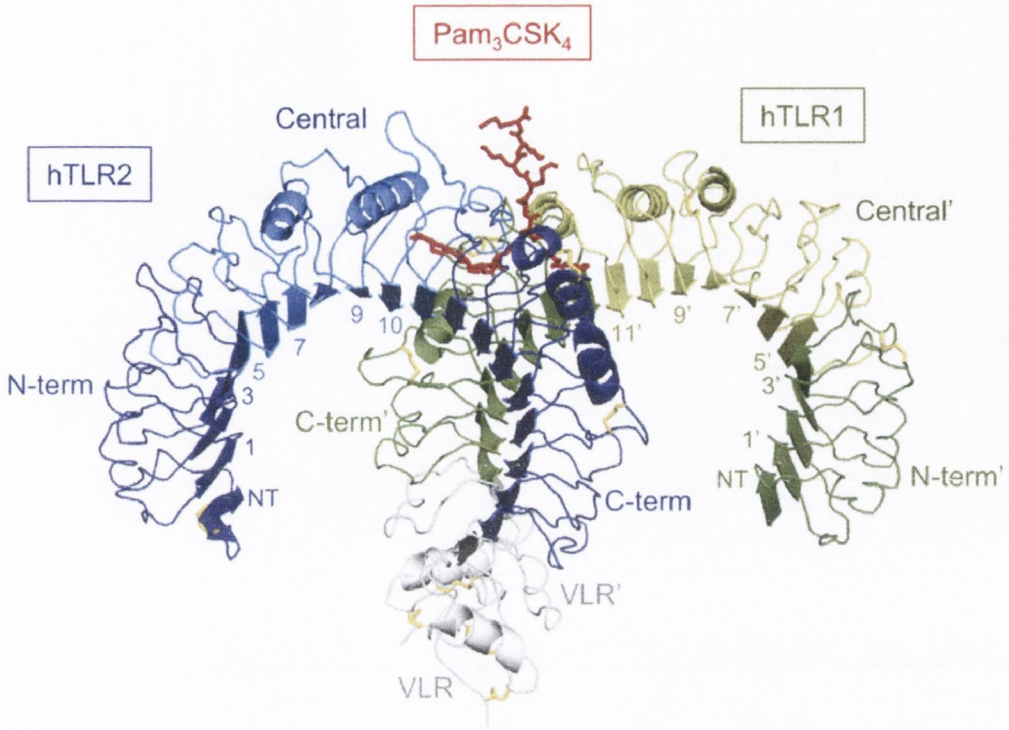


Figure 1.4 Structure of LPS. LPS consists of three parts, the O-antigen side chain, the core oligosaccharide and the Lipid A protein. The presence or absence of the O side chains determines whether LPS is rough or smooth. The lipid A region is the endotoxic component of LPS. It is made up of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 to 7 saturated fatty acids attached. The core oligosaccharide is attached to the 6th position of one NAG. IT consists of a short chain of sugars. There are two unusual sugars present, heptose and 2-keto-3-deoxyoctonoic acid (KDO). The O antigen is attached to the core polysaccharide. It consists of 3 to 5 sugars and the individual chains can vary in length from 4 to 40.



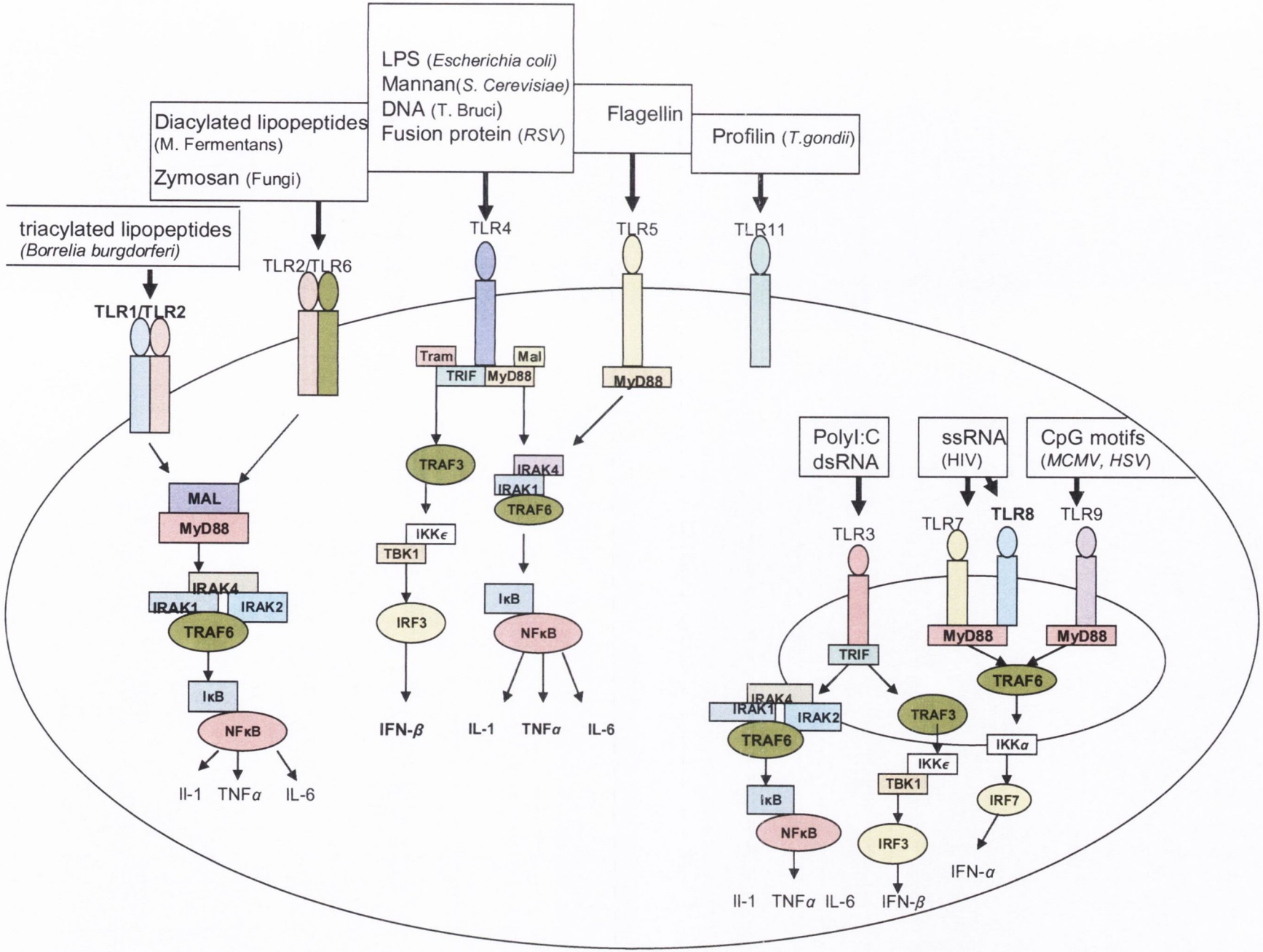
(Jin *et al.*, 2007)

Figure 1.5 Structure of the TLR1/TLR2:Pam_3Cys_4 complex. Binding of Pam_3Cys_4 causes dimerisation of the TLR1 and TLR2 ectodomains. The C termini of the TLRs converge and result in the formation of this 'M' shapped structure. It is hypothesised that this coming together of the C termini result in the heterodimerisation of the TIR domains and then recruit the adaptor proteins and lead to down stream signalling.

negative bacteria such as *Escherichia coli* and *Salmonella Minnesoto* (Poltorak *et al.*, 1998, Qureshi *et al.*, 1999). LPS is an essential component of the outer membrane of gram negative bacteria. It mainly functions as a permeability barrier to protect the bacteria. LPS is made up of polysaccharide (O) side chains, a core polysaccharide and a lipid A portion. The lipid A portion contains the hydrophobic membrane anchoring region and most importantly it is the toxic portion of LPS. Lipid A tends to be highly conserved across all gram negative bacteria. The core polysaccharide is associated with immunogenicity and is generally common to all members of a particular genus of bacterial. However they can vary structurally between different genera, for example *Salmonella* and *Escherichia* have similar but not identical cores. The O polysaccharide side chain can vary between different bacterial strains. The presence or absence of the O side chains are what determine whether LPS is considered rough or smooth. When O side chains are present LPS is considered smooth, whereas absence of O side chain as a result of mutation would make the LPS rough. Loss of the O antigen within bacterial strains can lead to a loss in virulence. Rough mutants can be more susceptible to antibiotics and bile salts. It is thought that smooth LPS is more resistant to phagocytosis than rough LPS and smooth antigens can more readily adhere to certain tissue types. The O polysaccharide is also needed for antigenic variation within a strain of bacteria, therefore allowing the bacteria increased opportunity to infect the host and evade the immune system.

TLR4 requires co-receptors to respond to LPS. These include CD14 which is a glycosylphosphatidyl inositol (GPI) anchored glycoprotein (Wright *et al.*, 1990) and MD2 (Figure 1.6). LPS binds to LPS binding protein in serum which transfers LPS monomers to CD14 which in turn concentrates the LPS to allow binding to

Figure 1.6 The signalling pathways involved in TLR responses to various ligands. TLRs respond to a host of ligands from various bacteria, fungi and viruses. TLR1/2 respond to triacylated lipopeptides, TLR2/6 respond to diacylated lipopeptides. TLR4 is the receptor for gram negative LPS. TLR5 recognises flagellin and TLR11 responds to profilin. TLR3, 7, 8 and 9 are all found inside the cell, on endosomal membranes. TLR3 responds to dsRNA, TLR7 and TLR8 respond to ssRNA and TLR9 respond to CpG motifs. TLRs use different adaptor proteins to activate a number of signaling pathways. TLR2 and TLR4 use Mal and MyD88 to activate NFκB. Once the adaptors are in place, IRAK 1, IRAK2, IRAK4 and TRAF6 are recruited. TRAF6 and IRAK1 dissociate from the complex and interact with TAK1, and TAB1 and TAB2. TAK1 and TAB2 become phosphorylated, TRAF6, TAK1, TAB1 and 2 dissociates from the membrane to the cytosol and IRAK1 gets degraded. TAK1 activates the IκB complex; this in turn phosphorylates IκB releasing NFκB which translocates to the nucleus.TLR3 and TLR4 use TRIF to activate IRF3, while TLR7/8 and 9 use MyD88. TRAF3 IKKϵ and TBK are recruited and they activate IRF3, which induces the IFN-β promoter to increase expression of IFN inducible genes. TLR3 is also capable of activating NFκB via an interaction between TRIF and RIP-1.


LPS (Escherichia coli)
Mannan(S. Cerevisiae)
DNA (T. Bruci)
Fusion protein (RSV)
Diacylated lipopeptides
(M. Fermentans)
Zymosan (Fungi)
Flagellin
Profilin (T.gondii)
triacylated lipopeptides
(Borrelia burgdorferi)
TLR4
TLR5
TLR11
TLR2/TLR6
TLR1/TLR2
Tram
Mal
TRIF
MyD88
MyD88
TRAF3
IRAK4
IRAK1
TRAF6
IKKε
TBK1
IRF3
IκB
NFκB
IFN-β
IL-1
TNFα
IL-6
MAL
MyD88
IRAK4
IRAK1
IRAK2
TRAF6
IκB
NFκB
Il-1
TNFα
IL-6
Poly I:C
dsRNA
ssRNA
(HIV)
CpG motifs
(MCMV, HSV)
TLR3
TLR7
TLR8
TLR9
TRIF
MyD88
MyD88
TRAF6
IRAK4
IRAK1
IRAK2
TRAF6
TRAF3
IKKα
IKKε
TBK1
IRF7
IκB
NFκB
IRF3
IFN-α
Il-1
TNFα
IL-6
IFN-β

TLR4/MD2 complex (Takeda *et al.*, 2003). Once this pathway is triggered it leads to the activation of the transcription factor NFκB which regulates the transcription of proinflammatory cytokines such as TNF-α, IL-1 and IL-6 (Fig1.6). It can also activate many members of the mitogen activated proteins kinase family (MAPK) notably p38 and JUN N-terminal kinase (JNK). These kinases are involved in the transcription of genes and they also regulate mRNA stability. The initial evidence proving that TLR4 is the receptor for LPS came from a mutant mouse strain C3H/HeJ, which is hyporesponsive to LPS challenge (Table 1.1). These mice have a mutation in their TLR4 gene. This results in the highly conserved proline within the BB loop region being substituted for a histidine (Poltorak *et al.*, 1998). Cells from mice deficient in TLR4 have also been shown to be hyporesponsive to LPS (Hoshino *et al.*, 1999). The TLR4 mutated strain C3H/HeJ is highly susceptible to infection by a number of gram negative bacteria including *Salmonella typhimurium and Nesseria meningitis* (Cooke *et al.*, 2004)

TLR4 is also capable of responding to components of fungal pathogens such as mannans from *Saccharomyces cerevisiae* and *C. albicans* and glucuronoxylomannan from *C. neoformans* (Shoham *et al.*, 2001) (Netea *et al.*, 2004) although these components are poorly described chemically (Table 1.1). Other TLR4 ligands include taxol (Perera *et al.*, 2001) and the fusion protein from respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000).

1.4.2 TLR1/2/6

TLR2 appears to be capable of recognising a large spectrum of microbes. This is partly due to its ability to form heterodimers with TLR1 and TLR6. TLR2 responds to

TLR mice	Ligand	Pathogen	Phenotype of knockout
TLR4			
	LPS	Gram negative bacteria (*Escherichia coli,*	Hyporesponsive ↓ Proinflammatory cytokines
		Salmonella minnesota)	(TNFα, IL-6, IL-1) ↓ Proliferation of splenic B cells
		Salmonella typhimurium *Nesseria meningitis*	↓ Survival
TLR2		Gram positive bacteria *Staphylococcus aureus* *Streptococcus pneumoniae*	↓ Survival
TLR1/2			
	Triacylated Lipoproteins	Mycobacteria, meningococci	↓ Cytokine production
TLR2/6			
	Diacylated Lipoproteins	Mycoplasma	↓ Cytokine production
TLR3			
	dsRNA		↓ Cytokine production
		West Nile Virus	↑ Resistance
TLR5			
	Flagellin	*Salmonella typhimurium*	No obvious phenotype due to TLR redundancy
		Pseudomonas aeruginosa	
TLR7/8			
	ssRNA	*HIV*	↓ Cytokine release from DCs
		Denge virus *Influenza*	CD40 not upregulated
TLR9			
	CpG DNA	*MCMV* *HSV*	↓ DC maturation ↓ Splenocyte proliferation
			↓ Inflammatory cytokines
TLR11			
	Profilin	*Toxoplasma gondii*	
		Uropathogenic bacteria	↓ Survival

Table 1.1 Examples of TLR ligands, the pathogens from which they are derived and the phenotype displayed by TLR knock-out studies.

lipoproteins and lipopeptides from a wide range of pathogens including *Borrelia burgdorferi, Treponema pallidun* and *Mycoplasma fermentans (*Khor *et al.,* 2007*)* (Table 1.1) . Lipoproteins present in bacteria are triacylated and are recognized by TLR1/TLR2. This was proven when macrophages deficient in TLR1 showed impaired cytokine production when challenged with triacylated lipopeptides (Takeuchi *et al.*, 2002) (Table 1.1). Recently the crystal structure for the TLR1/TLR2 heterodimer has been described (Jin M *et al.*, 2007) (Fig 1.5). In this study they show that binding of the tri-acylated lipopeptide Pam_3Csk_4 to the TLR1 and TLR2 ectodomains induces the formation of an 'M' shaped structure. Two of the ligands lipid chains insert into a pocket in TLR2 while the remaining lipid chain inserts into TLR1. It is the two C termini of the TLRs converging that result in the 'M' shapped structure. It is proposed that the converging of the C termini of the TLRs initiates the heterodimerisation of the intracellular TIR domains thus activating downstream signalling cascades.

TLR2/TLR6 heterodimer respond to diacylated lipopeptides and can also respond to Zymosan, a component of the cell membrane of fungi (Kataoka *et al.*, 2002), (Underhill *et al.*, 1999). TLR2 deficient cells confirmed that TLR2 can act as a receptor for components of gram positive bacteria as they were highly susceptible to infection with *Staphylococcus aureus and Streptococcus pneumoniae* (Echchannaoui *et al.*, 2002), Takeuchi *et al.*, 2002) (Table 1.1).TLR2 and TLR6 knockout mice infected with *Mycobacterium tuberculosis* showed decreased TNFα, IL-1β and TGF-β mRNA levels. However they had increased levels of IL-4 and IL-6 (Sugawara *et al.*, 2003). Although TLR2 knockout mice have been shown to be more susceptible to high aerosol doses of *Mycobacterium tuberculosis* they can still respond normally to a

low dose challenge. This result would suggest that the function of TLRs is crucial to contain high levels of microbial infection (Trinchieri *et al.*, 2007).

Other TLR2 ligands include lipotechoic acid from gram positive bacteria, lipoarabinomannan from mycobacterial cell walls, porins present in *Nesseria* and GPI anchors and glycoinositolphopholipids from *Trypanosoma cruzi* (West *et al.*, 2006).

1.4.3 TLR3

Double stranded RNA (dsRNA) is produced by viruses during their life cycle and it is the PAMP recognized by TLR3. As mentioned previously the mechanism by which TLR3 binds to dsRNA has been described (Liu *et al.*, 2008, Leonard *et al.*, 2008). TLR3 deficient cells show reduced responses to the synthetic dsRNA ligand polyinosine-polycytidylic acid (PolyI:C) (Alexopoulou *et al.*, 2001). TLR3 signals lead to the activation of NFκB and type I interferons (IFNs) (Fig 1.6). Although TLR3 recognises a viral PAMP, evidence to date suggests that TLR3 is not required for initial detection of viruses in all cell types. TLR3 deficient cells are not more susceptible to infection by *mouse cytomegalovirus* (MCMV), *lymphocytic choriomeningitis virus* (LCMV) or *reovirus* (Edelmann *et al.*, 2004) (Table 1.1). Plasmacytoid dendritic cells specifically use TLR3 to produce type 1 IFNs in response to dsRNA (Kato *et al.*, 2005). Recent studies have shown that melanoma-differentiation-associated gene 5 (Mda5) and retinoic-acid-inducible protein I (RIG-I) are capable of recognizing dsRNA in a range of cell types (Andrejeva *et al.*, 2004, Yoneyama *et al.*, 2004). TLR3 is predominantly expressed in the brain, heart lung and muscle. Within the brain it has been suggested that TLR3 is involved in mediating the entry of West Nile virus across the blood brain barrier which can lead to lethal

encephalitis (Wang *et al.*, 2004). This was demonstrated in TLR3 $^{-/-}$ mice, which showed greater resistance to West Nile virus infection.

1.4.4 TLR5

TLR5 is predominantly expressed on epithelial cells, monocytes and immature cells. It is localized to the basolateral surface of intestinal epithelial cells where it is capable of recognizing flagellin from bacteria which have invaded the epithelia (Gewirtz *et al.*, 2001) (figure 1.6). Flagellin is a 55kd monomeric component of bacterial flagella which are used for motility (Hayashi *et al.*, 2001) (Fig 1.6). Salmonella possess two flagellin proteins FliC and FljB, which are capable of activating NFκB dependant genes (Simon *et al.*,, 2007). A study on TLR5 knockout mice highlighted redundancy within the TLRs. It appears that TLR4 can function to induce antimicrobial responses in TLR5 deficient mice challenged by *Salmonella typhimurium and Pseudomonas aeruginosa* (Feuillet *et al.*, 2006). A recent study has shown that some bacteria are capable of evading recognition by TLR5 by possessing flagellin which has no immunostimulatory properties; these include Helicobacter pylori and Campylobacter jejuni. TLR5 knockout mice have been shown to be susceptible to Escherichia coli induced urinary tract infection (Nissen et al., 2007) (Table 1.1). This is the first evidence to implicate TLR5 in host protection in the urinary tract.

1.4.5 TLR7/8

TLR7 and TLR8 are structurally quite similar and are both localized to endosomal compartments. TLR8 is non-functional in mice. Murine TLR7 and human TLR8 both respond to a range of synthetic anti-viral compounds including resiquimod (R848) and loxoribine (Hemmi *et al.*, 2002) (Fig 1.6). It has recently been shown that TLR7

and TLR8 respond to guanosine (G) and uridine (U) rich single stranded RNA (ssRNA) from the human immunodeficiency virus- 1 (HIV-1) (Heil *et al.*, 2004). TLR7 and TLR8 respond to ssRNA viruses such as *Dengue* and *Influenza.* TLR7 deficient dendritic cells showed impaired responses to ssRNA and failed to release IFN-α or TNFα (Lund *et al.*, 2004) (Table 1.1).

1.4.6 TLR9

Studies carried out on TLR9 deficient mice have suggested that unmethylated CpG is the natural ligand for TLR9 (Hemmi *et al.*, 2000) (Fig 1.6). TLR9 deficient mice did not produce any inflammatory cytokines in response to CpG, their splenocytes failed to proliferate and there was no maturation of dendritic cells (Table 1.1). TLR9 like TLR7 and TLR8 is localized to endosomal compartments which may allow them to discriminate between self and non self, as host DNA and RNA tend not to enter into endosomal compartments (Barton *et al.*, 2006). TLR9 has been shown to be able to respond to murine cytomegalovirus (MCMV) and herpes simplex virus, presumably through the presence of unmethylated CpG within these viral genomes (Krug *et al.*, 2004). It was reported that TLR9 can respond to hemozoin from *Plasmodium falciparum* (Coban *et al.*, 2005), however more recent data argues that it is malarial DNA bound to the hemozoin which activates TLR9 (Parroche *et al.*, 2007). Recently it has been reported that the DNA sugar backbone 2'deoxyribose is critical for TLR9 activation and not CpG motifs (Haas *et al.*, 2008). They show that the natural phosphodiester (PD) DNA sugar backbone can act as a basal TLR9 agonist. When bases are added it greatly increases the activation of TLR9. They also show that most studies previously carried out indicating that it is only the CpG motifs that activate TLR9 were carried out using phosphorothioate (PS)-modified DNA with CpG motifs

attached. They could show that PS-modified DNA alone actually acts as an antagonist to TLR9, however once the CpG is attached it becomes an agonist. This paper has radically changed the views of TLR9 signalling. They clearly show that it is not the unmethylated CpG motifs that allow TLR9 to discriminate between 'self' and 'non-self' DNA, instead it is the localisation of TLR9 to endosomal compartments which prevents it was responding to self DNA.

1.4.7 TLR10

To date there is no known ligand for TLR10. TLR10 appears most homologous in sequence to TLR1 and TLR6. The crystal structure for TLR10 has been published recently (Nyman *et al.*, 2008). The structure revealed a 2.2A crystal structure which indicates a symmetric dimmer within an asymmetric unit. The BB loop is shown to form a tight interaction at the surface dimer interaction and the DD loop is also present in this region. A pocket where potential peptides could bind was revealed, this pocket was made up of part of the BB loop and also an alphaC-helix.

1.4.8 TLR11

TLR11 is only functional in mice. This is due to the presence of a stop codon in the coding region of human TLR11 gene (Zhang *et al.*, 2004). TLR11 knockout mice are highly susceptible to infection by uropathogenic *Escherichia coli* (Table 1.1). TLR11 also responds to profilin-like proteins from Toxoplasma gondii (Yarovinsky et al., 2005) (Fig 1.6). The recent finding that TLR5 also has a function within the urinary tract is very important as it could be functioning in humans as the key receptor to mount host responses to uropathogenic Escherichia coli as TLR11 is non-functional.

1.5 TLR Localisation

TLRs are present in many cell types and are distributed throughout the body. TLRs are more predominantly expressed in certain regions of the body to ensure that they are expressed at the most suitable site to fight infection. It may also be a mechanism to help the immune system discriminate between commensal and pathogenic bacteria. The table 1.2 shows the regions of the body where the TLRs are mainly expressed (Dunne *et al.*, 2003).

TLR2, TLR4, TLR1, TLR6 and TLR5 are all plasma membrane localized TLRs. These TLRs mainly recognize cell wall components. TLR3, TLR7, TLR8 and TLR9 are all intracellular, mainly localised to endosomal compartments where they all recognize nucleic acid structures. Therefore the location of these receptors is significant for the function of each (Takeda *et al.*, 2005).

Although TLR2 is predominatly expressed on the plasma membrane, there are forms of it that are cytosolic. Soluble TLR2 has reported to have been detected naturally in human plasma and breast milk (Lebouder *et al.*, 2003). sTLR2 is believed to be the result of posttranslational modification of TLR2 protein present in intracellular compartments. sTLR2 appears to be capable of modulating cell activity in response to lipopeptides. Having different forms of TLR2 may help broaden the spectra of bacterial components it is exposed and responds to.

TLR5 is expressed on the basolateral side the intestinal epithelium. The location of this TLR helps it to recognise its ligand, flagellin (Gewirtz *et al.*, 2001). It also gives

TLR1	Ubiquitously expressed
TLR2	Brain, heart, lungs, muscle
TLR3	Brain, heart, lung, muscle, pancreas, placenta, dendritic cells
TLR4	Placenta, peripheral blood, Leukocytes
TLR5	Ovaries, monocytes
TLR6	Spleen, thymus, ovary, lungs
TLR7	Plasmacytoid dendritic cells
TLR8	Myeloid dendritic cells
TLR9	B cells
TLR10	B cells

Table 1.2 Regions of the body where TLRs are predominantly expressed. TLRs are located on numerous cells throughout the body. This table highlights the areas in which they are expressed at a higher level. (Adapted from Dunne *et al.*, 2003)

an insight into how TLRs distinguish between commensal and pathogenic bacteria, as only pathogens which cross the epithelial barrier alert the TLR to a danger signal.

1.6 TLR signalling

Since the TLRs belong to the IL-1 receptor (IL-1R)/TLR superfamily it is not surprising to find that the signalling pathways activated by the TLRs are similar to the downstream signalling events activated by IL-1 or IL-18 receptor complexes (O'Neill and Dinarello 2000). There are two main pathways activated by the TLRs. The first pathway involves the activation of the transcription factor NFκB, which regulates the transcription of many proinflammatory genes. The second pathway leads to the activation of MAP kinases and Jun amino-terminal kinase (JNK), (McGettrick *et al.*, 2004). These are both involved in the transcription of genes and they also regulate mRNA stability. There are four adaptor proteins used by the TLRs which lead to the activation of these pathways. Some of these proteins are used by all the TLRs and others are more specific to certain TLRs.

1.6.1 TLR signalling via the MyD88-dependent pathway

Myeloid differentiation factor 88 (MyD88) was the first adaptor protein identified and it is used by all TLRs except TLR3, since PolyI:C activation of TLR3 was normal in MyD88 knockout cells (Alexopoulou, Holt *et al.*, 2001). Like TLRs, MyD88 possesses a TIR domain and it is believed that it interacts with TLRs through a TIR-TIR interaction. MyD88 also possesses an N-terminal death domain which recruits Interleukin 1 receptor associated kinase 4 (IRAK-4) which in turn phosphorylates Interleukin 1 receptor associated kinase 1 (IRAK1) (Suzuki *et al.*, 2002, Li *et al*, 2002). IRAK1 once phosphorylated can associate with tumor necrosis factor (TNF)

receptor activated factior 6 (TRAF6). Both proteins leave the receptor complex and interact with TGF- β activated kinase 1 (TAK1) and two TAK1 binding proteins TAB1, TAB2 (Jiang *et al.,* 2002). Both TRAF6 and TAK1 undergo a number of ubiquitination reactions and the activation of the TAK1/TAB1/TAB2 complex depends on this polyubquitination of TRAF6 (Wang *et al.*, 2000) TAK1 and TAB2 become phosphorylated which initiates the dissociation of the TRAF6/TAK1/TAB1/TAB2 complex from the membrane to the cytosol (Qian *et al,*. 2001) TAK1 is then active and is capable of activating the IκB kinase (IKK) complex (Wang *et al* 2001). NFκB is held in an inactive form in the cytoplasm by IκBα. IκBα is phosphorylated and then ubiquitinated at Lys 21 and Lys22 which targets it for degradation by the 26S proteasome. This process frees NFκB to translocate to the nucleus and induce expression of proinflammatory cytokines (figure 1.5) (Mercurio *et al.*, 1997; Regnier *et al.,* 1997; Woronicz *et al.,* 1997; Zandi *et al.*, 1997; Karin and Ben-Neriah 2000; Chen *et al.*, 1995). In order to prevent over activation of the NFκB pathway IκBα is quickly resynthesised (Krappmann and Scheidereit 1997). TAK1 is also capable of phosphorylating MKK6 and 7 which leads to the activation of p38 and JNK (Wang *et al.,* 2001).

IRAK-4 is needed for the recruitment of IRAK-1 to the receptor complex. However the mechanistic role for IRAK-4 as a kinase in the pathway has remained elusive until recently. Many contradictory papers have been published on IRAK-4's kinase function. Qin *et al* showed that the IRAK-4 kinase inactive mutant had the same ability as wild type IRAK-4 to restore IL-1 mediated signalling in IRAK-4 deficient cells, whereas Lye *et al* showed that the kinase activity of IRAK-4 is essential for IL-1 signalling (Qin *et al.*, 2004, Lye *et al.*, 2004). More recently Koziczak-Holbro *et al*

made IRAK-4 kinase dead knockin mice. These mice failed to respond to IL-1, TLR4 or TLR7 ligands therefore demonstrating that the kinase activity of IRAK-4 is essential for the activation of the signalling cascades (Koziczak-Holbro *et al.*, 2007).

IRAK-1 is an essential component of the IL-1 signalling pathway. However IRAK-1 deficient macrophages are capable of responding to LPS albeit with less cytokine production in comparison to wild type. It was always thought that there must be another IRAK capable of compensating for IRAK1. Up until recently it was only hypothesised that this could be IRAK-2, this has now been confirmed (Keating *et al., 2007,* Kawagoe *et al.*, 2008). It had been thought that IRAK-2 was catalytically inactive however Keating *et al* and Kawagoe *et al* have shown that this is not the case. Keating *et al* show that IRAK-2 is involved in a number of TLR signalling pathways which lead to the activation of NFκB. They show through the use of reporter gene assays and knockdown techniques that IRAK 2 is involved in the activation of TRAF6 ubquitination and subsequent activation of NFκB. Kawagoe *et al* show that IRAK-2 deficient cells display normal MAPK and early NFκB responses which they believe to be due to the presence of IRAK-1.They believe that IRAK-2 is activated for longer than IRAK-1 and is therefore more critical for late stage NFκB responses. They show that IRAK-1 and IRAK-2 double knockouts are resistant to LPS-induced toxic shock and they produce very low levels of IL-6 in response to IL-1 stimulation. These papers indicated that both kinases work cooperatively to ensure an optimal immune response is initiated upon activation.

The second adaptor protein to be identified was MAL (MyD88 adaptor like, also known as TIRAP) (Fitzgerald *et al.,* 2001; Horng *et al.,* 2001). Mal was identified

through database searches identifiying molecules similar to MyD88. Mal was initially shown to have a role in TLR4 signalling when over-expression of the dominant negative form of the protein inhibited NFκB following LPS stimulation but not following IL-1 or IL-18 (Fitzgerald *et* al., 2001). Mal has been shown to have a role in TLR4 and TLR2 signalling leading to NFκB activation (figure1.5). This was confirmed in Mal knockout mice which showed impaired responses to TLR2 and TLR4 ligands (Yamamoto *et al.,* 2002, Horng *et al.,* 2002). Mal has been reported to undergo tyrosine phosphorylation by Brutons tyrosine kinase (BTK) following activation of the TLR4 and TLR2 signalling pathways. This was demonstrated by used of the BTK inhibitor LFM-A13 which blocked tyrosine Phosphorylation of MAL following LPS and lipopetide-2 stimulation (Gray *et al*, 2006). Mal has been reported to be regulated by suppressor of cytokine signalling 1 (SOCS-1). SOCS-1 acts as a negative regulator of TLR signalling and it has been shown to cause Mal degradation following TLR4 and TLR2 stimualtion (Mansell *et al*, 2006). Mal does not get degraded in SOCS-1 deficient macrophages therefore highlighting the importance of this protein in recycling Mal and preventing excessive activation of the TLR pathway. NFκB activation by Mal in response to TLR2 and TLR4 ligands has been reported to be regulated by caspase -1. The two proteins were shown to interact and caspase-1 appears to cleave Mal (Miggins *et al.*, 2007). Miggins *et al* could show that a mutant form of Mal was unable to get cleaved by caspase-1. This acted as a dominant negative on TLR2 and TLR4 signalling, indicating that the cleavage of Mal is essential to the activation of the downstream signalling pathways. Recently the fundamental role of Mal within the TLR signalling pathway has been revealed, and that is to act as a bridging adaptor to facilitate the movement of MyD88 to TLR4 to initiate signalling (Kagan *et al.,* 2006). Mal has been shown to possess a

phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain which allows it to move to the plasma membrane where it then brings MyD88 in contact with TLR4 and TLR2 hence initiating downstream signalling.

Studies carried out on MyD88 knockout mice showed that they were unable to produce inflammatory cytokines in response to TLR4, TLR2, TLR7 or TLR9 ligands. Although the MyD88 knockout mice were unable to produce cytokines in response to LPS stimulation they were capable of activating NFκB with delayed kinetics compared to wild type mice (Kawai *et al.*, 1999). These findings suggested that there is a MyD88-independent component on the TLR4 signalling pathway. It was subsequently found that IRF3 was activated and homodimerisation of IRF3 occurred in a MyD88 and Mal-independent manner in response to LPS (Kawai *et al.*, 1999; Yamamoto *et al.*, 2002;). LPS induction of IFN-inducible genes such as IP-10, GARG-16 and IRG-1 was equivalent in wild type, MyD88 and Mal knockout cells. However in order to fully understand what was responsible for the MyD88 independent responses, a MyD88 and Mal double knockout was generated. IFN-inducible genes were induced in response to LPS in the double knockout macrophages therefore suggesting the existence of another adaptor molecule on the TLR4 pathway which is independent of MyD88. The TLR3 pathway was also shown to activate IRF3 and induce IFNα/β in a MyD88 independent manner, which again suggested that another adaptor must be present that allows for the activation of IRF3 on both the TLR4 and TLR3 signalling pathways (Yamamoto *et al.*, 2002).

1.6.2 MyD88 independent/TRIF dependent TLR signalling

MyD88 is the essential adaptor molecule required for signalling by all the TLRs with the exception of TLR3 and part of the TLR4 signalling cascade. TIR domain-containing adaptor inducing IFN-beta (TRIF) was the third adaptor molecule to be identified (Yamamoto *et al*, 2002). The IRF3 activation and subsequent induction of IFN-inducible genes in response to LPS was shown to require TRIF and a fourth adaptor TRIF-related adaptor protein (TRAM). Overexpression of TRIF activated NFκB and the IFN-β promoter (Yamamoto *et al*, 2002). It has also been shown that the noncanonical IKKs,IKKϵ, Tank binding kinase -1 (TBK1), interact with both TRIF and TRAM mediating the activation of IRF3 and the induction of the IFNβ promoter (Fitzgerald *et al.*, 2001; Sharma *et al.*, 2003). The initial evidence that TRIF was involved in the TLR3 pathway showed that a dominant negative version of TRIF inhibited TLR3-dependent activation of NFκB and IFNβ promoters (Yamamoto *et al*, 2002). TRIF has been shown to bind to TLR3 and recruit TRAF6 directly via a TRAF6 binding motif present in the N-terminal domain. IRAK4 and IRAK2 are recruited. TAK1 becomes activated which ultimately leads to NFκB activation (Sato *et al*, 2003; Jiang *et al.*, 2004, Keating *et al.*, 2007) (Fig 1.5).

TRIF has also been shown to activate NFκB by an alternative pathway. A RIP homotypic interaction motif (RHIM) was identified in the C terminus of TRIF. This motif was shown to bind via the RHIM motifs to a receptor interacting protein (RIP1) (Meylan *et al.*, 2004). They showed that a dominant negative form of RIP1 was capable of inhibiting TRIF dependent NFκB activation. Studies on TLR3 knockout mice showed impaired TLR3 dependent NFκB activation indicating that RIP1 is also involved in TRIF dependent NFκB activation

Studies were carried out on TRIF/TRAM double knockout mice. These mice were capable of responding to TLR2, TLR7 and TLR9 ligands (Yamamoto *et al.*, 2003). Activation of the MyD88 dependent pathway in response to LPS was unaffected. However LPS induced cytokine production, which was believed to be a MyD88 dependent event, was affected in these mice (Yamamoto *et al.*, 2003).

It has been shown that the activation of NFκB in a MyD88 dependent manner occurs earlier than the MyD88 independent activation of NFκB. A study carried out by Covert *et al*, explains how this occurs (Covert *et al.*, 2005). They suggest that the reason for the time delay in the activation of NFκB in a MyD88 independent manner is due to the pathway requiring protein synthesis to occur. They showed that LPS-stimulated MyD88-deficient cells which were treated with the protein synthesis inhibitor cyclohexamide were unable to activate NFκB. They noted that TNFα transcripts were increased in MyD88 deficient cells stimulated with LPS and when they pretreated these cells with a soluble TNF receptor it blocked LPS induced NFκB activation. This result suggested that the TRIF dependent pathway activated TNFα production and secretion in an NFκB-independent manner and this production of TNFα is required to bind to its receptor and activate NFκB. They have also shown that it is IRF3 which is responsible for the activation of TNFα. Another study carried out by Werner *et al* also suggested that TNFα plays a role in LPS induced NFκB activation (Werner *et al.*, 2005). All these results explain the delayed MyD88-independent NFκB activation and indicate that the combination of both pathways allow for the most efficient responses to LPS stimulation.

The family of serine/threonine kinases termed the mitogen activated protein kinases (MAPKs) are involved in the TLR signalling pathways. There are three groups of MAPKs associated with TLR signalling, they are the extracellular signal regulated kinases (ERKs), the c-Jun N terminal kinase (JNK) and p38 MAPK. MAP kinase kinases (MAPKK) are responsible for the Phosphorylation and subsequent activation of MAPKs. It has been shown that LPS activate ERK1/2 via the Ras/Raf-1/MEK pathway, JNK is activated via the MEKK1/MEK4 pathway and p38 via MEK3 activation (Swantek *et al.*, 1997; Van der Bruggen *et al.*, 1999; Delgado *et al.*, 2000). The adaptor molecules, MyD88 and TRIF have been shown to activate the MAPKs through the interaction of TRAF6 followed by the activation of TAK1 and MKK6 which leads to the activation of NFκB, JNK and p38 (Wang *et al.*, 2001).

1.6.3 Function of the fifth adaptor protein SARM

A fifth adaptor sterile-α and HEAT/Armadillo motifs containing protein (SARM) has been identified (O'Neill *et al.*, 2003). SARM has been reported to act as a negative regulator of TRIF dependent signalling in TLRs (Carty *et al.*, 2006). Carty *et al* demonstrate that SARM and TRIF interact and studies carried out using RNAi showed that knockdown of the protein led to enhanced TRIF dependent cytokine production. These results suggest that SARM is acting as a negative regulator of TRIF signalling. Recently contradictory results have emerged regarding the function of this adaptor. Another paper has shown SARM to have activating functions. Kim *et al* show SARM to be a brain enriched protein mainly expressed on neurons where it functions in cell death. Studies carried out on SARM knockout mice show contradicting results to those obtained by Carty *et al.* Kim *et al* suggest that SARM knockout cells respond to microbial products at comparable levels to wild type cells.

These results would indicate that SARM does not play a role within TLR signalling, They show that SARM knockout mice are resistant to stress induced neuronal death and therefore the function of this adaptor is in mediating neuronal cell death. However there is a possibility that there are human mouse differences with regard to SARM function. All experiments carried out by Carty *et al.*, used human cell lines and primary cells and perhaps SARM does play a function as a negative regulator of TLR signalling in vitro in human cells.

1.7 Co-Receptors in TLR signalling

TLRs are capable of recognising a wide variety of PAMPs. One TLR is capable of recognising structurally different ligands and it is believed this is due to the use of co-receptors. TLR4 is the PRR for LPS, however in order for TLR4 to recognize LPS it is required to have a number of accessory proteins, or co-receptors.

1.7.1 CD14/LBP

LPS binding protein (LBP) is a blood born acute phase protein which is produced in the liver and lungs (Schumann *et al.*, 1990). The function of LBP is to transfers LPS monomers to another co-receptor CD14 (Yu *et al.*, 1996) (Fig 1.7). LBP binds to the lipid A portion of LPS. The evidence for the function of LBP became clear from the work carried out on the LBP knockout mice. They showed that LBP is capable of enhancing the sensitivity to LPS by approximately 300 fold (Wurfel *et al.*, 1997). The LBP knockout mice were more susceptible to infection by ordinarily non-pathogenic gram negative bacteria (Jack *et al.*, 1997).

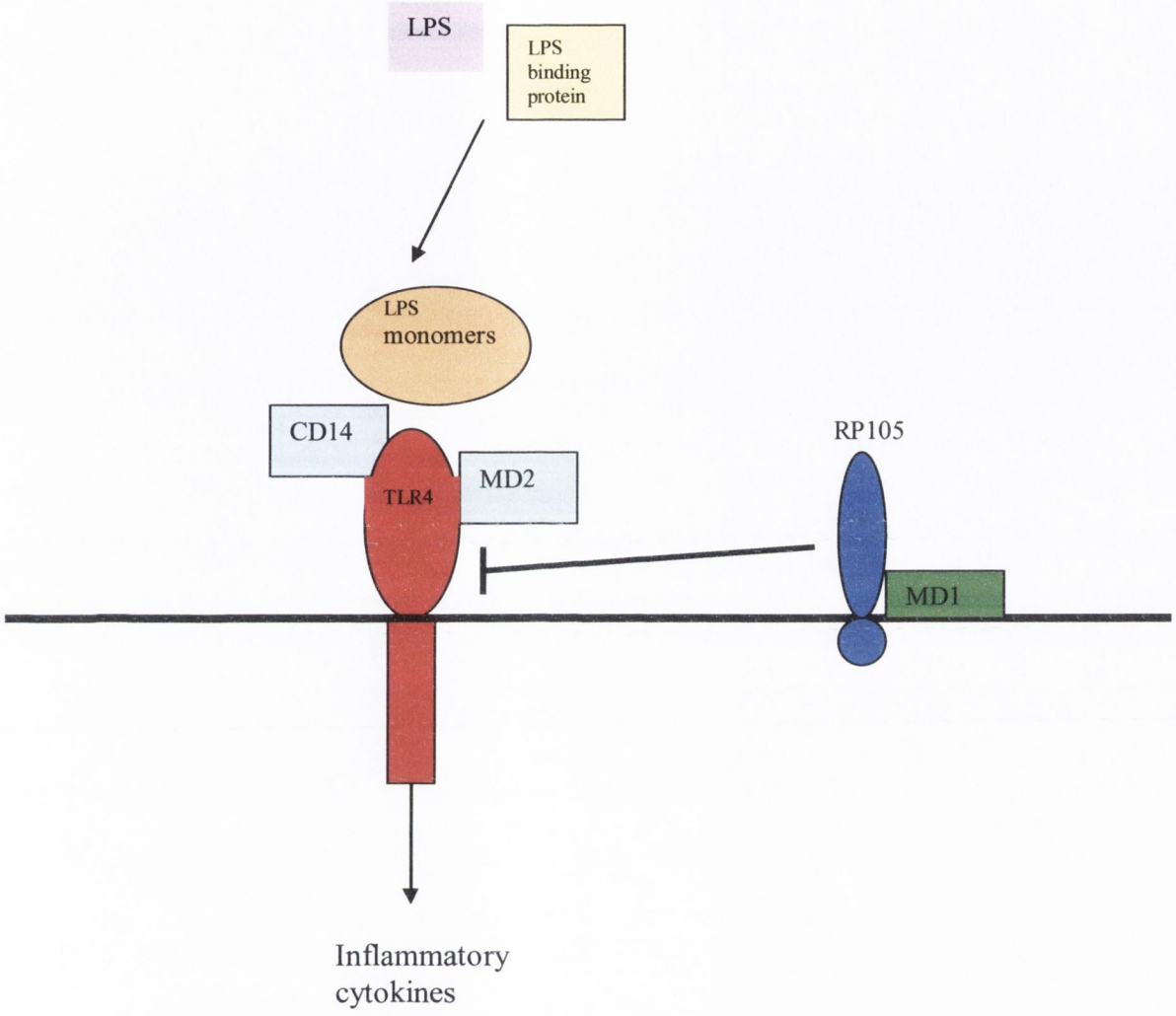


Figure 1.7 Co-receptors in TLR4 signalling. LPS binds to LPS binding protein, which transfers LPS monomers to CD14. CD14 acts as a co receptor for TLR4. MD2 is another accessory protein which aids TLR4s recognition of LPS and therefore initiates the signalling pathway. All pathways in the innate immune system must be tightly regulated to protect the host. RP105 acts as an endogenous inhibitor of TLR4, it can therefore turn off the TLR4 signalling pathway when required by the host.

CD14 is a 50 kDa, LRR containing glycoprotein, that can be secreted into the serum or it can be expressed as a glycophosphoinositol (GPI) linked protein on the surface of cells (Wright *et al.,* 1990). There have been more than 1500 publications on the role of CD14 in LPS signalling. Although CD14 deficient cells are insensitive to LPS (Haziot *et al.*, 1996, Moore *et al.*, 2000), it appears that CD14 has an important but not an essential role in LPS signalling. The presence of CD14 can greatly increase the LPS response in cells. Since CD14 is a GPI anchored protein it does not have the ability to signal within a cell. Therefore CD14 binds monomeric LPS and transfers it to the TLR4/MD2 signalling complex which can then initiate a downstream signalling cascade (Fig 1.7). CD14 has been reported to be required for responses to smooth LPS only and not to rough LPS (Jiang *et al.*, 2005). It has been suggested that CD14 is required for the MyD88 dependent pathway in response to smooth LPS, however CD14 is needed for both MyD88 dependent and independent pathways in response to rough LPS. This result indicated that TLR4/MD2 can bind to rough but not smooth LPS in the absence of CD14.

1.7.2 MD2

MD2 is another accessory protein that is required by TLR4 for LPS recognition (Fig 1.7). MD2 is an 18-25 kDa protein that does not possess a transmembrane domain. It exists mainly as a soluble protein except when it associates with the ectodomain of TLR4 at the cell surface (Visintin *et al.*, 2001, Medzhitov *et al.,* 2001). Many studies have shown that MD2 can exist as a monomeric protein and also as a polymeric protein. Only the monomeric form of the protein appears to be capable of binding to LPS and TLR4 (Re *et al.*, 2002). This has been shown to be the case in the published structure of the TLR4/MD2 complex (Kim *et al.*, 2007). They have proposed models

for murine TLR4/MD2 and human TLR4/MD2. Within these models they show that monomeric MD2 binds to each TLR4 receptor and they confirm that it is MD2 that binds to LPS. They show that the MD2 structure is made up of a β cup fold and two parallel β sheets which come apart along one side of the protein and expose a hydrophobic core capable of binding LPS. TLR4 and MD2 have been shown to interact through charge enhanced hydrogen bonds. They crystallised and obtained the structure of MD2 and TLR4 binding to the LPS antagonist Eritoran and they speculate that this would best represent the binding of LPS to the TLR4/MD2 complex since Eritoran can compete with LPS for binding to MD2.

MD2 has been shown to mainly associate with TLR4 in the endoplasmic reticulum and the endoplasmic reticulum chaperon protein gp96 is needed for TLR4 and MD2 to interact (Randow *et al.*, 2001). The presence of MD2 allows glycosylation of TLR4 to occur which is essential for the cell surface expression of TLR4. Although the majority of TLR4/MD2 is found to be expressed in the golgi (Nagai Y et al., 2002), it is still unclear how TLR4/MD2 traffics between the golgi and the cell surface. MD2 is ubiquitously expressed on a number of tissues, however interestingly it is not expressed on intestinal or airway epithelial cells (Abreu *et al.*, 2002, Jia *et al.*, 2004). It is hypothesised that the reason for this lack of expression is due to TLR4/MD2 complexes inability to discriminate between pathogenic and commensal bacteria. Since airway and intestinal cells are constantly in contact with commensal bacteria it would be dangerous to have TLR4/MD2 in a constant state of activation leading to a build up of proinflammatory cytokines.

1.7.3 RP105

RP105 is another receptor believed to be involved in TLR4 signalling and has been shown to act as an endogenous inhibitor of TLR4 (Divanovic *et al.,* 2005) (Fig 1.7). RP105 possess leucine rich repeats and it is expressed on B cells, dendritic cells and macrophages. Surface expression of RP105 is dependent on an accessory protein MD1. MD1 is a homologue of MD2 (Medzhitov *et al.,* 2001). RP105 does not possess a TIR domain, instead is has a small cytoplasmic region with a tyrosine-phosphorylation motif. It is of extreme importance that TLR signalling is kept under tight control otherwise it can lead to conditions such as septic shock. RP105 acts as a specific inhibitor of TLR4 and therefore regulates responses to LPS.

1.8 Polymorphisms in TLRs: Links with disease

Tightly controlled TLR signalling is required for the host to respond to microbial challenge. If TLR signalling is impaired it can lead to susceptibility to infection. If there is excessive signalling it can lead to septic shock and possibly to autoimmune diseases and allergies. TLRs have been implicated in numerous disease conditions. Several single nuclear polymorphisms (SNP) within individual TLRs have been identified and some are linked with disease progression.

Individuals possessing a co-segregating polymorphism in TLR4 (Asp299Gly and Thr399Ile) are hyporesponsive to LPS and they are more susceptible to gram negative bacterial infections (Arbour *et al.*, 2000). These TLR4 mutations Asp299Gly and Thr399Ile individually have been shown to predispose African children to severe malaria (Mockenhaupt *et al.*, 2006).

A polymorphism in the TLR2 gene (Arg753Gln) has been linked with an increased susceptibility to tuberculosis (Pandey and Agrawal, 2006) and reduced responses to gram positive bacteria especially streptococci (Lorenz *et al.*, 2000).There is another mutation in TLR2 (Arg677Trp) which has been associated with an increased risk of Lepromatous leprosy in a Korean population (Malhotra *et al.*, 2005).

A point mutation (392STOP) resulting in the introduction of a stop codon into TLR5 has been described. This stop codon severely impairs the signalling function of TLR5. Individuals with this mutation show increased susceptibility to Legionnaire's disease (Hawn *et al.*, 2003). The results from this study are quite controversial as this work was repeated on a much larger scale with Vietnamese typhoid patients and no disease association was observed (Dunstan *et al.*, 2005).

A number of polymorphisms have been identified within the adaptor molecules and downstream kinases used in the TLR signalling cascades. There is a reported disorder in which patients are deficient in IRAK4. These patients are highly susceptible to pyogenic gram positive bacteria, however they show resistance to viral infections (Khor *et al.*, 2007,Picard *et al.*, 2003). The results obtained from this IRAK4 study helps to confirm that TLRs are essential for host defence in humans although IRAK4 is also involved in IL-1 and IL-18 signalling.

A study carried out on individuals from Gambia, Kenya, United Kingdom and Vietnam found an SNP in the adaptor Mal (S180L). This polymorphism is associated with a protective effect against infectious diseases such as Malaria, bacteraemia; tuberculosis and pneumococcal bacteraemia (Khor *et al.*, 2007). Another Mal

polymorphism has been identified (C558T) and it is linked with an increased susceptibility to tuberculosis (Hawn *et al.*, 2006).

Hypomorphic mutations present in NFκB essential modulator (NEMO), for example C417R, cause the X-linked recessive disorder XR-EDA-ID. Patients suffering from this are highly susceptible to pyogenic bacterial such as *Streptococcus pneumonia* and *Staphylococcus aureas* and viral infections like *Herpes simplex virus* and *Cytomegalovirus*. These patients produce little cytokines in response to LPS or TNF-α. A missense mutation in the IKBA gene (S32I) which encodes IκBα can lead to the autosomal dominant condition AD-EDA-ID. Patients with this condition, much like the patients with XR-EDA-ID, show impaired responses to pyogenic infections. These patients show impaired TLR signalling as NFκB cannot be properly activated in response to TLR stimuli (Ku *et al.*, 2005).

Results obtained from genetic analysis of various polymorphisms must be interpreted with caution. Large scale case controlled studies are essential to obtain statistical significance to link a polymorphism with infectious disease susceptibility. It is believed by some that the observed polymorphisms in human TLRs indicate that this receptor family is completely redundant in protective immunity (Ku *et al.*, 2005). However it has also been suggested that certain polymorphisms may be maintained within a population as they are protective against specific pathogens and infectious diseases (Khor *et al.*, 2007). Overall the evidence provided by polymorphisms highlight the role that TLRs play in various infections and diseases in humans.

1.9 TLRs and the brain

This project concerns a novel LRR containing protein which shows high levels of expression in the brain. I will therefore now describe the role that TLRs play in the brain. TLRs have been found to play a role in central nervous system (CNS) inflammation and more recently in neuropathology. The brain is considered an immune privileged organ and therefore is mostly devoid of MHC expression and there are no antibodies present. Therefore the innate immune system must be of extreme importance to protect the CNS from infection. Although it would appear contradictory that inducing inflammation in the CNS could be beneficial, many studies are now revealing that TLRs have many roles within the brain some of which are neuroprotective. Following a brain insult such as stroke one would imagine that the presence of proinflammatory cytokines such as IL-6 and TNF-α would lead to increased brain injury. However studies on mice models deficient in these cytokines indicate that in fact they play a protective role during brain ischemia and the knockout mice show less long term recovery than the wild type mice (Bruce *et al.*, 1996, Scherbel *et al.*, 1999, Clark *et al.*, 2000). Interestingly the cytokine IL-1 has been shown to have a detrimental role in neural injury in inflammation during stroke. Knocking out the IL-1 gene leads to a reduction in brain damage (Boutin *et al.*, 2001). Therefore it appears that inflammation following brain injury can have a protective or destructive role depending on which cytokines are produced. It is believed that TLRs may be the key coordinators in the inflammatory response following stroke.

Astrocytes and Microglial cells appear to be the main immune cells capable of responding to pathogen invasion within the brain. Microglia are known as the macrophage cells of the brain and therefore are considered the principle immune

effector cells within the CNS. mRNA expression of all TLRs have been identified on microglia (Crack *et al.*, 2007). This expression has also been shown to be induced following bacterial infection.

1.9.1 TLR3 expression and function

TLR3 has been shown to be expressed throughout the CNS on microglia, neurons, oligodendrocytes and is most highly expressed on astrocytes (Bsibsi *et* al., 2002, Lafon *et* al., 2006, Farina *et al.,* 2004). The adaptor components of the TLR signalling pathway were also found to be constitutively expressed in astrocytes. TLR3 responds to dsRNA. It is believed that within the CNS TLR3 is upregulated when cells of the CNS become infected with viral components. This response would be rapid to prevent any deleterious inflammatory responses within the brain. All cells within the CNS expressing TLR3 have been shown to respond to the TLR3 ligand polyinosinic-polycytidylic acid (polyI:C) (Lafon *et* al., 2006, Olson *et al.*, 2004, Krasowska-Zoladek *et al.*, 2007). Activation of TLR3 within the brain has been shown to induce expression of neuroprotective cytokines such as IFN-β, neurotrophin 4, TGFb2 and ephrin type B receptor1 (Marsh and Stenzel-Poore *et al.*, 2008) (Fig 1.8). IFN-β has been shown to have more that just anti-viral functions within the CNS. It has been linked with a reduction in infarct volume in rats and rabbits following a stroke (Veldhuis *et al.*, 2003, Liu *et al*, 2002) and is currently in clinical trails as a potential treatment following acute stroke. Another study has shown that TLR3 can aid the entry of West Nile virus into the brain which can lead to lethal encephalitis (Wang *et al.,* 2004). West Nile virus is a ssRNA virus which is normally asymptomatic in humans but it can lead to encephalitis in some patients. They have shown that it is the

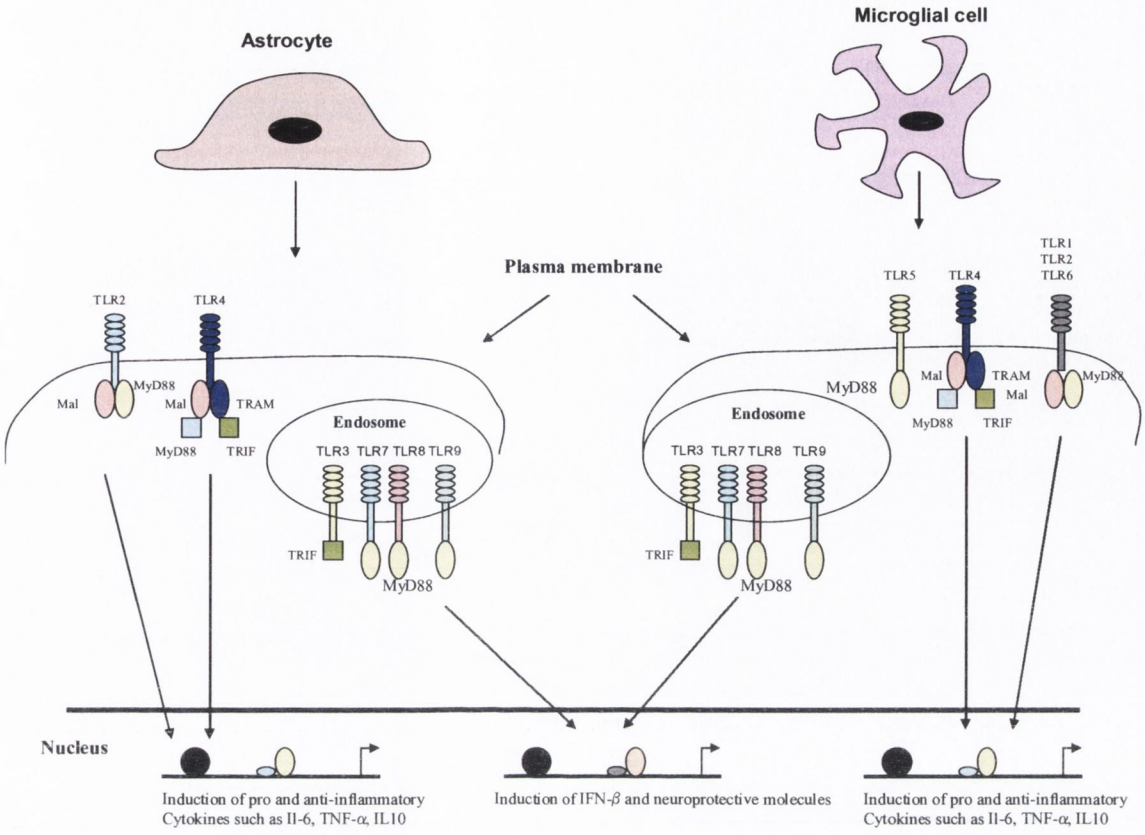


Figure 1.8 TLR expression and function within the brain. mRNA for TLRs 1-9 have been identified in astrocyte and microglial cells. TLR3 is the most highly expressed TLR in astrocytes and has been shown to repond to PolyI:C leading to the induction of IFN-β, neuroprotective proteins such as TGFb2 and also anti-inflammatory cytokines IL-10 and IL-9 (Marsh and Stenzel-Poore *et al*, 2008). TLR2 has also been shown to be functional in astrocytes and responds to *Staphylococcus aureus,* PGN from bacteria and the whole bacterium (Kelian *et al.*, 2005). TLR4 is expressed on microglial cells and studies carried out on TLR4 knockout microglia indicate that they are unable to respond to LPS (Esen and Kielian 2006). TLR5 has been shown to respond to flagellin from *Salmonella typhimurium* on microglial cells (Bowman *et al.*, 2005).

inflammatory response triggered by TLR3 that leads to a breakdown in the blood brain barrier allowing entry of the virus into the brain.

1.9.2 TLR2 and TLR4 expression and function

mRNA for TLR2 and TLR4 have been reported to be expressed in astrocytes and microglial cells (Carpentier *et al*, 2005, Bsibsi *et al.*, 2002) (Figure 1.8). TLR2 has been shown to be required to allow astrocytes to respond to peptidoglycan from *Staphylococcus aureus* (Kelian *et al.*, 2005). TLR2 and TLR4 have been reported to be involved in modulating adult hippocampal neurogenesis. Neurogenesis is the formation of new neurons in the brain which aids the brain in maintaining life long plasticity. This study showed that both TLR4 and TLR2 are expressed on adult neural stem/progenitor cells (NPCs). Studies carried out on TLR4 and TLR2 knockout mice indicated that TLR4 possesses an inhibitory role in adult neurogenesis whereas TLR2 has the opposite affect and enhances adult neurogenesis (Rolls *et al.*, 2007). TLR4 has also been shown to be involved in autoregulatory apoptosis of microglial cells. Although both TLR4 and TLR2 are expressed on microglia only activation of TLR4 is involved in apoptosis. This has been shown to be due to the fact that activation of TLR4 can lead to increased IFN-β expression which is believed to be the determinant for apoptosis (Jung *et al.*, 2005).

Recent reports have suggested that TLR4 plays an important role in inflammation within the brain following stroke. Stroke is one of the leading causes of death and it results from a blockage of blood or from a haemorrhage within the brain. TLR4 deficient mice have been examined following experimentally induced stroke and the mice have displayed lower infarct volumes and lower expression of proteins

associated with brain damage following stroke such as IRF-1, nitric oxide synthase, cyclooxygenase-2 and matrix metalloproteinase 9 (Caso *et al.*, 2007; Caso *et al.*, 2008). These results suggest that TLR4 is responsible for inflammatory mediated brain damage following stroke. Recent data has suggested that other TLRs such as TLR2 and TLR3 may have a role in stroke pathogenesis (Tiwari *et al.*, 2008). They show increases in TLR2, TLR4 and TLR3 mRNA expression following experimentally induced stroke in vivo.

Alzheimer's disease is a progressive neurodegenerative disease which is characterised post mortem by the presence of senile plaques and neurofibriller tangles within the brain. In recent years more and more research is being carried out in the field of TLRs and the brain and a number of links between TLRs and Alzheimer's disease have been made. It has been suggested that activation of TLR2 on microglial cells could play an important role in the disease by allowing the uptake of amyloid β peptide (Chen *et al.*, 2006). Amyloid β peptide is the main component of the senile plaques found in the brains of Alzheimer's patients. The LPS co-receptor CD14 has recently been shown to be over-expressed in the brains of Alzheimer's patients and is shown to be capable of phagocytosing amyloid β peptide (Yang *et al.*, 2005). Also the previously described polymorphism in TLR 4 Asp299Gly polymorphism has been linked with a protective affect against the development of late onset Alzheimer's disease (Minoretti *et al.*, 2005).

1.9.3 TLR7/8 expression and function

TLR7 and TLR8 have been shown to be expressed at the mRNA level in both astrocytes and microglial cells (Carpentier *et al*, 2005, Bsibsi *et al.*, 2002) (Fig 1.8).

Both TLRs have been shown to be functional and respond to their ligand R848 in microglial cells (Zhang *et* al., 2005). Stimulation of TLR8 with R848 has recently been shown to negatively regulate neurite outgrowth and is involved in the induction of neuronal apoptosis (Ma *et al.*, 2006). R848 activates both TLR7 and TLR8, however since TLR7 is not expressed on neurons the effects observed with this ligand are therefore specific to TLR8. For many years it has been believed that TLR8 is non functional in mice, however this paper indicates that TLR8 does in fact have a role in the brain which is distinct from the role in immunity. TLR8 is shown to be expressed on neurons and axons and once activated can lead to neurite outgrowth and apoptosis in an NFκB independent manner. They also suggest that activation of TLR3 with polyI:C can inhibit neurite outgrowth. These studies all suggest that TLRs play an important role in brain neuropathology. Some of these roles are distinct from the classical roles played by TLRs in immunity. These findings are not very surprising considering Toll was originally discovered in *Drosophila* and was linked to development prior to the link with immunity. This is an exciting field of research and many more questions remain to be answered involving the specific roles of TLRs within the CNS. It is still unclear whether the primary role of TLRs within the brain is for survival or damage. Many TLRs are being linked to certain diseases such as TLR3 with West Nile virus. Therefore TLRs are being studied more intensely to determine what role they may play in other CNS diseases and therefore are making attractive future therapeutic targets.

1.9.4 TLR9 expression and function

TLR9 mRNA has been identified in microglial and astrocytes and these cells have been shown to respond to CpG motifs (Dalpke *et al.*, 2002, Bowman *et al.*, 2003,

Carpentier *et al.*, 2005, Zhang *et al.*, 2005) (Fig 1.8). However most studies identifying TLR expression in the brain has been carried out in mice and rat brain samples and it has been reported that human fetal astrocytes do not express TLR9 (Farina *et al.*, 2005). Studies have not been carried out on age dependent expression of TLRs in the human brain and there are many contradicting papers showing different expression levels of TLRs in the various brain cell types. Therefore there is a lot more data required to fully understand the expression pattern and function of TLRs throughout the CNS.

1.10 Families of LRR containing proteins involved in neuro-immunity

There are many other leucine rich repeat containing proteins apart from the TLRs which play diverse and distinct roles within the CNS. This thesis investigates a novel LRR protein KIAA0644 which has been shown to be highly expressed in the brain. Bioinformatic studies have indicated that KIAA0644 is homologous to members of the TLR family and also to other LRR containing proteins which are shown to be highly expressed in the brain. I will therefore describe some of the other brain enriched families and their roles in neuroimmunology. These include the Amphoterin-induced gene and ORF-1 (AMIGO-1), the Nogo receptor (NgR1), LRR and Ig domain containing Nogo receptor interacting protein (LINGO-1), the netrin-G1 ligand NGL-1, the neuronal leucine rich repeat family (NLRR) and the fibronectin-leucine-rich transmembrane family (FLRT). Many of these proteins have role in neurite outgrowth and cell differentiation. However it is thought, that these proteins possess many other role in the CNS which are still to be determined.

1.10.1 NgR family

NgR1 is the best characterised member of the NgR family but it does possess two homologues, NgR2 and NgR3. NgR1 is the receptor for the protein Nogo A which is made up of a Nogo A domain and a Nogo 66 domain. This protein acts as an axon growth inhibitor. Once the NgR1 complex, which is made up of three proteins, p75 ^{NTR}, LINGO-1 and TROY/TAJ, becomes activated it leads to an inhibition in axon growth and nerve fibre regeneration (Schwab *et al.*, 2006). NgR1 receptor has also been linked with Phosphorylation of the EGF receptor. The use of EGF receptor kinase inhibitors led to an increase in axon outgrowth (Koprivica *et al.*, 2005). However it has been shown that the EGF receptor is not part of the NgR1 complex and it is cross talk between the two receptors which leads to different signalling cascades. Recently novel roles for NgR1 receptors in the inflammatory process have emerged. New data suggests that NgR1 may be involved in the removal of macrophages from neural tissue following injury to ensure there is no lasting inflammatory response (Fry *et al.*, 2007). This data shows that there is a lot more to the NgR1 receptor than was originally predicted.

1.10.2 FLRT family

The FLRT family consist of three proteins FLRT 1-3. These proteins contain 10 LRR and a fibronectin type III domain and are all expressed in the brain, although FLRT is also more globally expressed. They have been shown to have roles in neurite outgrowth, early embryonic development, in fibroblast growth factor signalling and cell adhesion (Karaulanov *et al.*, 2006).

1.10.3 AMIGO family

Three AMIGOs make up the AMIGO family. They are type 1 transmembrane proteins, with 6 LRR and an Ig domain and are only expressed within the nervous system. They have been implicated in cell adhesion and neuron outgrowth (Chen *et al.*, 2005).

All the families of LRR proteins described above are mainly brain enriched or localised throughout the CNS. Although some of them are more globally expressed, their functions to date are mostly linked with the neuronal system. Although most of these proteins appear to be involved in neuronal outgrowth either by promoting or inhibiting it, there is more recent data suggesting that these proteins may have other role within the CNS. The novel role for the NgR1 receptor in inflammation enhances the possibility that these other LRR containing proteins may have other roles to play that are distinct from their neuronal outgrowth functions.

1.11 Aims and Objectives

KIAA0644 is a novel leucine rich repeat containing protein that was initially identified in a microarray analysis carried out on embryonic stem cells of wild type and Mal knock out mice. The analysis identified a number of genes that exhibit differential expression levels in Mal knockout cells stimulated with LPS. Most of the genes identified had already been characterised to some extent while the remaining gene, KIAA0644, has no known function. The sequence of this gene is available on the Human Unidentified Gene-Encoded Large Proteins (HUGE) protein database as part of the Human cDNA project at the Kazusa DNA Research Institute (www.Kazusa.or.jp).

The specific goals are as follows:

1. To use bioinformatics analyses to identify all motifs present within the sequence and identify proteins homologous to the sequence.

2. Determine the mRNA and protein expression levels of KIAA0644 in cells both non-stimulated and stimulated with LPS and other TLR ligands. To investigate in which tissues the protein is most highly expressed.

3. To determine localisation of the protein at a basal level and following stimulation.

4. To address the possible function of KIAA0644 within the LPS signalling pathway.

Chapter Two

Materials and Methods

2.1 Materials

Rough (Serotype J5 TLR grade ™) and smooth Lipopolysaccharide (Serotype O55:B5 S form TLR grade ™) from *E.coli* was purchased from Alexis (Poole, Dorset, UK), Pam3Cys4 was obtained from Calbiochem (San Diego, California), PolyI:C was purchased from Amersham (GE healthcare UK LTD, Calfont St Giles, Bucks), Malp2 was purchased from Alexis (Poole, Dorset, UK), R848 was a purchased from 3M Pharmaceuticals (St. Paul, MN), TNFα and IL-1β were purchased from RnD systems (Abingdon, UK). Anti- β-actin antibody was purchased from Sigma (Poole, Dorset, UK). The anti-KIAA0644 antibodies were purchased from Eurogentic (Seraing, Belgium) and 21st Century biochemicals (Malboro, Massachusetts). The IκB antibody was a kind gift from Prof.R. Hay (University of Dundee, UK.). The polyclonal phospho-p38 antibody was obtained from Cell Signalling Technology, Inc (Danvers, Massachusetts). The anti-TLR4 antibody was purchased from Imgenex (San Diego, California). The anti-mouse IgG (whole molecule) peroxidase conjugate and anti-rabbit IgG (whole molecule) peroxidase conjugate antibodies were all purchased from Jackson Immunoresearch laboratories, Inc (Westgrove, Pennsylvania). The anti-mouse IgG (whole molecule) and anti-rabbit IgG (whole molecule) were obtained from Sigma (Poole, Dorset, UK). The anti-rabbit Alexflour 488 and anti-rabbit Alexaflour 647 antibodies were purchased from Invitrogen (Biosciences are distributors for Invitrogen ,Dun Laoghaire, Ireland).

The human embryonic kidney cell lines (HEK 293 stably expressing TLR2, HEK 293 stably expressing TLR3, HEK 293 stably expressing TLR4), U373 parental and U373 cells stably expressing CD14 cells were a gift from Dr Kate Fitzgerald (UMASS, Boston). The human macrophage THP1 cell line was obtained from the European

collection of Animal cell cultures (ECACC) (Salisbury, UK). The Wild type and Mal knockout mice were a gift from Prof Akira (Osaka Univeristy, Japan)..I made the mouse embryonic cell lines (both wild type and Mal knockout), the protocol is outlined in section 2.4.3). The L929 cell line was a gift from Dr Kate Fitzgerald. All cell culture materials, RPMI 1640, DMEM, IMDM, penicillin, streptomycin, trypsin and L-glutamine were purchased from Gibco, (Biosciences, Dun laoghaire Dublin). The antibiotic Ciprofloxacin was purchased from Mediatech Inc (Manassas, Virginia). Foetal calf serum was obtained from Biosera (East Sussex, UK). Genejuice was purchased from Novagen (Merck, Darmstadt, Germany). Cell culture flasks are all purchased from Starsted (Numbrecht, Germany). General laboratory chemicals were purchased from Sigma (Poole, Dorset, UK), with the exception of Glutathione-Sepharose 4B beads from Amersham (Buckinghamshire, United Kingdom), coelentrazine from Argus Fine Chemicals (Vernio, France), passive lysis buffer from Promega (Madison, Wisconsin, USA), broad band prestained protein and DNA markers from New England Biolabs Ltd. (Ipswich, Massachusetts), ECL reagent from Amersham and Supersignal West Pico chemiluminescent substrate from Pierce Biotechnology, Inc (Rockford, Illinois). Ponceau S stain was obtained from Sigma and collodiol blue stain was purchased from Perbio (Medical Supply Company Mulhuddart Dublin. TNF-α, IL-6, IL1-β and Rantes duosets were purchased from RnD systems (Abingdon, UK). Maxi prep, endotoxin free plasmid purification kits and Improm pt II RT-PCR kits were all purchased from Promega (Madison, Wisconsin, USA). Trizol reagent and chloroform were obtained from Sigma. RNA extraction kit was purchased from Qiagen (West Sussex, United Kingdom).

2.2 Expression Vectors

The sequencing vector PGEMT, the PGL3 basic and PGL3 enhancer vectors were purchased from Promega. The expression vector pCDNA-KIAA0644 was cloned within the lab by Dr Aisling Dunne. The dGL3-5κB-luc plasmid was a gift from Dr. R Hofmeister (Universitat Regensburg, Germany). The plasmid encoding CD14 was a kind gift from Dr Claire Bryant (University of Cambridge, UK). Flag tagged TLR4 was cloned in the lab by Dr Mikael Edjeback.

2.3 Cell culture

2.3.1 Growth and maintenance of cell lines

All cell lines used were stored in liquid nitrogen. All cells were stored at a concentration of 1×10^7 cells/ml in 95 % Fetal Calf Serum (FCS) and 5 % Dimethyl Sulphate (DMSO) in plastic cryo vials. Cells were thawed at 37 °C, 1 ml of FCS was added to the cells and then they were placed in the media specific to each cell line. U373, HEK293, HEK 293T and HEK 293TLR4 cells were placed in 10 ml of DMEM (10 % FCS), THP1 cells were placed in 10 ml of RPMI 1640 (10 % FCS). Cells were centrifuged at 1000 × g for 3 min. The pellet of cells were then resuspended in 10 ml of complete medium specific to the cell line, which was then placed in a T25 cell culture flask. Cells were maintained at 37 °C with 5 % CO_2. In order to use the cells in experimental assays they were grown in T175 cell culture flasks. For use in transfection assays, HEK 293, HEK 293TLR4, MEF and U373 cells were typically seeded at 1×10^5 cells/ml in 96 well, 6 well or 10 cm dishes 24 h prior to transfection, whereas THP1 cells were seeded at 2×10^5 cells/ml 24 h prior to

transfection. For continuing cell culture, all cell lines were seeded at 1×10^5 cells/ml and sub-cultured two or three times a week. HEK 293, U373 and MEF cells were removed from the surface of the cell culture flask by initially washing cells with 2 ml of TRYPSIN- EDTA followed by incubation with 5 ml of TRYPSIN- EDTA for 5 min. 10 ml of complete media was added to the cells and they were centrifuged at 1000 × g for 5 min. The contents of the flask were then transferred into a 30 ml sterilin and centrifuged at 1000 × g for 5 min. THP1 cells are a suspension cell line and therefore the cells can be poured directly into a sterile falcon tube and centrifuged at 1000 × g for 5 min. In all cases, the supernatant was removed from the cells and the pellet of cells was resuspended in 1 ml of complete media. Cells were counted using a haemocytometer and light microscope. Cell viability was determined using the dye Trypan blue, which is excluded from healthy cells but taken up by non-viable cells.

2.3.1 Cryo-preservation of cells

Cells were grown to a state of sub-confluency and were harvested and counted as previously described (section 2.3). Cells were centrifuged 1000 × g for 5 min and the pellet was resuspended in the correct volume of FCS containing 10 % DMSO, such that aliquots of 2×10^6 cells in 1 ml FCS/DMSO are placed in 1.5 ml cryotubes. These aliquots are placed in a container filled with isopropanol and placed at -80 ^{O}C overnight prior to being placed in liquid nitrogen.

2.4 Animals

Mice were obtained from the Bioresources unit at Trinity College Dublin. Animals were maintained in a controlled environment with a 12 h light/dark cycle at a temperature of 22-23 °C. When the animals were born they were removed from their litter box and placed into a ventilated box with bedding for transportation to the culture room suite where dissection occurred.

2.4.1 Preparation of mixed glial cells

All instruments used for dissection were sterilised by thorough cleaning with disinfectant followed by overnight baking at 200 °C (Sanyo-Gallenkamp Hotbox Overn, Model #OHG050, Loughborough, UK). Within the laminar airflow unit, neonatal mice were decapitated and the brains were removed in the following manner: The skin along the midline of the skull was cut and the skull was exposed. A small sharp scissors was inserted at the brain stem and incisions were made along both sides of the head ensuring that the point of the scissors was touching the skull at all times. The skull was then peeled back using a sterile forceps.

Once the skull was removed, the brain was scooped out and placed in a sterile Petri dish in normal orientation. A few drops of DMEM were added to the dish. The brain was bi-directionally chopped using a sterile disposable scalpel (Schwann-Mann, UK). They were then placed in a labelled sterile 15 ml falcon tube containing 2 ml of DMEM. The tubes were placed in the incubator for approximately 20 min. Each sample was triturated using a sterile Pasteur pipette and filtered though a 50 μl sterile nylon mesh filter (BD Biosciences, USA) into a fresh sterile 50 ml Falcon tube. Samples were

centrifuged at 1,700 × *g* for 3 min and the resulting supernatant was removed. Cells were resuspended in 2 ml of DMEM and gently triturated to produce a homogenous cellular suspension. Cells were counted as described in section 2.3 and were seeded at 1×10^6 cells/ml. For confocal analysis, 200 μl of cell suspension was pipetted onto the centre of a poly-L-lysine coated coverslip in the bottom of a 6 well plate. Once the cells adhered (2 h), each well was flooded with 1 ml of DMEM. For western blot analysis 200 μl of cell suspension was pipetted into 6 well plate. After 2 h the cells adhere and then 1 ml of DMEM was added to each well. The media was changed every 2-3 days and the cells were left to grow for 10 days prior to treatment.

2.4.2 Generation of bone marrow derived macrophages

Mice were euthenized in CO_2 chamber and confirmed dead by cervical dislocation.
The abdomen was cut and the skin was pulled back. Sterile utensils were used to remove as much skin as possible. Bone was removed by cutting as high up as possible to the hip joint and at the ankle. The knee was removed to expose the femur and tibia. Bone marrow was removed by flushing out the bone with 10 ml of complete DMEM in a 10 ml syringe with a 25 gauge needle. The needle was inserted into the bone caivity of the femur and the tibia, the cavity was flushed until the bone appeared white. Bone marrow cells were transferred and resuspended in a 50 ml tube. Cells were centrifuged at 1500 × *g* for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in Red Blood Cell Lysis buffer. This was then centrifuged again for 5 min at 1500 × *g*. This step was repeated two to thee times to ensure that all the red blood cells were lysed. Cells were resuspended in 5 ml of DMEM and were counted as previously described.

Cells were resuspended in DMEM with 10 % heat inactivated FCS, 0.1 % Ciprofloxacin (10 mg/ml) and 15 % MCSF containing media taken from L929 cells. Cells were seeded at 1×10^6 cells/ml into 30 cm dishes. Cells that failed to adhere after 24 h were placed into a fresh plate with medium and containing 15 % MCSF. Bone marrow derived macrophages were counted and set up for experiments on days 10-14 post isolation. For a 10 cm dish cells were seeded at 5×10^6 cells/ml in 10 ml of complete DMEM. For a 6 well plate cells were seeded at 2×10^6 cells/ml in 3 ml and for a 96 well plate cells were seeded at 2×10^6 cells/ml in 200 μl per well.

2.4.2.1 Testing the purity of the BMDM cells by FACs analysis

In order to ensure that all bone marrow is differentiated into BMDM, the cells were stained for the macrophage marker CD11b. 2×10^5 cells/ml BMDMs were used for extracellular staining of CD11b. Cells were seeded in 96 well conical bottom plate. The plate was centrifuged at 1200 × *g* for 5 min and resuspended in 50-100 μl of PBS containing 1 % FCS. CD11b antibody was added to each well at a final concentration of 2 μg/ml. The same concentration of isotypic control as the CD11b was added. All samples were mixed by pipetting. The plate was placed at 4 °C for 15-30 min, then centrifuged at 1200 × *g* for 5 min. Samples were washed with 100-200 μl of PBS-FCS. This step was repeated and finally the cells were resuspended in 100 μl of PBS-FCS. 100 μl of sample was transferred to a micronic tube, the plate was washed with a further 100 μl of PBS-FCS and this too was transferred to the micronic tube. Samples were analysed by flow cytometry using FACs Calibur machine on the same day.

2.4.3 Preparation of mouse embryonic fibroblast cells (MEFs)

Wild type and Mal knockout mice were sacrificed on day 13.5 of gestation and were removed to the cell culture room suite for dissection. All instruments used for dissection were sterilised by thorough cleaning with disinfectant followed by overnight baking at 200 °C (Sanyo-Gallenkamp Hotbox Overn, Model #OHG050, Loughborough, UK). Within the laminar airflow unit, the abdomen was cut and the skin was pulled back. Sterile utensils were used to remove as much skin as possible. The uterus was dissected out and the fetuses were removed. The head and liver of the fetuses were removed. All clots from the embryos were removed. Embryos were washed in 1-2ml of PBS and they were placed into a 10cm dish. Tissue was minced and 2mls of trypsin was added. Tissue was incubated at 37°C for 10 min. After incubation fetal tissue was vigorously pipetted until it was a single cell suspension. 1 ml was placed into 25cm^2 flask (Make two flasks). This was passage 1.

2.5 Generation of human peripheral blood mononuclear cells

Blood was obtained either directly from a healthy donor or taken from the blood bank at St. James hospital. Blood was transferred into a 50 ml falcon tube and diluted 1:2 with phosphate buffered saline (PBS). Ficoll plague plus was used to separate the blood into red blood cells, white blood cell ring and serum. The blood was slowly added to 20 ml of ficoll pague plus. The tubes were centrifuged at 1700 × *g* for 30 min. The white blood cell ring was transferred into a new 50 ml tube using a Pasteur pipette. The volume was adjusted to 50 ml and the samples were centrifuged again at 1700 × *g* for 10 min. The supernatant was removed. This step was repeated again, the pellet was then resuspended in 10 ml of complete IMDM media (10 % FCS, 0.1 % Ciprofloxacin (10 mg/ml). Cells

were counted and seeded at a concentration of 2×10^6 cells/ml. For 24 well plates 500 μl of media was added to each well, and for 6 well plates 3 ml of media was added to each well.

2.6 RNA Isolation

RNA isolations were carried out on the U373/CD14, THP1 and MEF cell lines and also on the human peripheral blood mononuclear cells (PBMC). 1×10^7 cells were obtained for the RNA isolation procedure. The cells were seeded in 10 cm dishes. The media was then removed; the cells were washed gently with 2 ml of sterile PBS. RNA was extracted using the Rneasy minikit from Qiagen. Cells were harvested in two different ways depending if the cells grew in suspension (THP1 cells) or in a monolayer (U373/CD14 etc):

2.6.1 Cells that grow in suspension

Cells were counted as described in section 2.3. 1×10^7 cells/ml were centrifuged at 300 $\times$ *g* in a centrifuge tube for 5 min. All supernatant was aspirated.

2.6.2 Cells that grow in a monolayer

All media on the cells were aspirated off and cells were lysed directly in the 10 cm dish as will be described in the next step.

2.6.3 Disruption of cells using buffer RLT

For pelleted cells, the pellet was loosened thoroughly by flicking the tube. 600 μl of buffer RLT was added to the cells. In order to lyse cells directly in the 10 cm dish, 600 μl of buffer RLT was added to the dish. The monolayer of cells was disrupted using a cell scraper. The lysate was collected into a microcentrifuge tube and vortexed for 1 min.

2.6.4 Homogenisation of the lysate

The lysate was passed up to 5 times though a blunt 20 gauge needle (0.9 mm in diameter) fitted to an RNase-free syringe. 1 volume of 70 % ethanol was added to the homogenised lysate and mixed well by pipetting. 700 μl of the sample was transferred to an RNeasy spin column in a 2 ml collection tube. The samples were centrifuged at 8000 × *g* for 15 sec. The flow though was discarded. 700 μl of buffer RW1 was added to the sample and again it was centrifuged at 8000 × *g* for 15 sec and the flow though was discarded. 500 μl of buffer RPE was added to the column and it was centrifuged at 8000 × *g* for 15 sec and the flow though was discarded. 500 μl of buffer RPE was added to the column and it was centrifuged at 8000 × *g* for 2 min and the flow though was discarded. The column was centrifuged for an additional 2 min and the flow though was discarded. The RNeasy spin column was placed in a sterile eppendorf and 30 μl of RNase free water was added. The column was centrifuged at 8000 × *g* for 1 min in order to elute the RNA off the column. RNA was quantified using a spectrophometer and reading OD 260 (DNA), 280 (proteins). Ratios of 260/280 are expected to be approximately 1.8/2 for pure RNA.

2.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Production of cDNA from the RNA template

Following RNA quantification the concentration of each sample was normalised by diluting more concentrated samples with Rnase free water. Once the concentration is equal in each sample for each template 4 μl of RNA and 1 μl of random primers were added to a minifuge tube. The sample was heated to 70 °C for 5 min and 4 °C for 5 min. This process produced denatured RNA. The next step was reverse transcription and the following table lists the components needed, they were added to a minifuge tube prior to the addition of the template and primers.

	Experimental reaction (μl)	**Water control (μl)**
Nuclease free water	5.5	5.5
Improm-II 5× Reaction buffer	4.0	4.0
$MgCl_2$, 25mM	3.0	3.0
dNTPs, 10mM	1.0	1.0
RRNasin	0.5	0.5
Improm-II reverse transcription (RT)	1.0	1.0
Total volume	15.0	15.0

Each component was added into minifuge tubes in the order they are depicted on the table above. All samples were kept on ice thoughout the experiment. 5 μl of denatured RNA was added to each minifuge tube. The tubes were placed into the PCR machine and the following program was used:

25 °C for 5 min (annealing occurs)

42 °C for 60 min (initial cDNA strand synthesis)

70 °C for 15 min (activation of reverse transcription)

Once the cDNA was synthesized it could be stored at this stage at 4°C, or used directly in a PCR reaction.

2.7.1 Polymerase Chain Reaction (PCR)

Each component required is outlined in the table below. In order to save time master mixes for each PCR reaction were made up in sterile eppendorfs and kept on ice. (Note: filter tips were used for all mixes made up and used in the PCR reactions)

A total of 20 μl of the master mix was needed for each PCR reaction. A total of 8 reactions were carried out for each experiment (in order to allow for pipetting error mix was made up for 9 samples). The following table shows the components needed for the master mixes:

	Experimental reaction	**Master Mix μl (9 samples)**	**Water control μl (9 samples)**
10× Reaction Buffer	2.5	22.5	22.5
dNTP mix, 10mM	0.5	4.5	4.5
$MgCl_2$, 2.25mM	0.75	10.125	10.125
Template	1	-----	5
Taq DNA polymerase	0.125	1.125	1.125
Nuclease free water	14.125	141.75	136.75
Total volume	20.0	180.0	180.0

RT-PCR primers
Forward KIAA0644 Primer
GCCTTGCGCCTCCTGCTCGTGGTG

Reverse KIAA0644 Primer
CCACCGCGAGAGCTTCTCGAAGGT (310bp)

Forward GapDH primer
GAACGGGAAGCTTGTCATCAA

Reverse GapDH primer
CTAAGCAGTTGGTGGTGCAG (350bp)

Primers were designed and obtained from MWG. Once the primers were received they were made up at a concentration of 100 pM. They were then made up to working stocks of 10pM. Each primer was made up into a mix consisting of the forward and reverse primers (10 μl of the forward primer and 10 μl of the reverse primer and 180 μl water (molecular biology grade water). 5 μl of each of the primer mixes were used per reaction bringing the total volume in each PCR tube to 25 μl. When all the samples were prepared in sterile PCR tubes they were placed into a preheated PCR machine (preheated to 95 °C).

The following PCR program was used.

95 °C for 5 min (Denaturation)
97 °C for 20 sec
64 °C for 1 min (Annealing)
72 °C for 45 sec (Extension)
5 Cycles

96 °C for 20seconds
62 °C for 45 seconds
72 °C for 1minute
35 Cycles

72 °C for 7 minutes

4 °C hold.

The annealing temperature had to be varied in different experiments as it is based on the melting temperature of the primers. Therefore each PCR reaction had to be optimized for each primer pair.

2.7.2 Agarose gel electrophoresis

In order to visualise the PCR products a 1 % agarose gel was made up using the following:

150 ml of TAE

1.5 g of agarose

5 μl of Ethidium bromide (ETBR)

When the gel set the combs were removed and the gel was placed into a gel box filled with TAE running buffer. 2 μl of DNA loading buffer (50 % (v/v) sterile glycerol in sterile H_20, and 10 mg of Bromophenol blue) was added to 20 μl of the PCR product. 15 μl of this was loaded onto the gel. A molecular weight marker was made up by adding 5 μl of a 1 KB marker and 1 μl of loading buffer. The gel was connected to a power supply and it was maintained at 100V for approximately 2 h. The gel was then visualized using a UV gel docking system.

2.8 Promoter Analysis

2.8.1 Promoter identification

Promoter analysis was carried out using the Genomatix website specifically using the Promoter inspector and Mat inspector tools. One promoter was identified for the gene KIAA0644. The sequence of the promoter was 564 bp long.

2.8.2 PCR

PCR Primers to the 564 bp fragment and two longer fragments 1 kb and 2 kb were designed. Restriction enzyme recognition sites were introduced into the 5' and 3' ends of DNA fragments during the amplification process in order to facilitate insertion into the PGEMT and PGL3 basic and enhancer vectors.

Primers

564bp forward primer

5' GCG GTA CCG TTT CCT CCT CCC CCT GC 3'
564bp reverse primer

5' GCA GAT CTT CGG ACT TCG CAG CGC CGT C 3'

1kb forward primer

5' GCG GTA CCG CCC ACA GCA GGA TGG AGT 3'
1kb reverse primer

5' GCA GAT CTT CGG ACT TCG CAG CGC CGT C 3'

2kb forward primer

5' GCG GTA CCC CAC GAA GTC ACC ACT GAC 3'
2kb reverse primer

5' GCA GAT CTT CGG ACT TCG CAG CGC CGT C 3'

Restriction enzymes: Forward = Kpn1 site

Reverse = Bgl2 site

A human genomic DNA library was used as 'target' and thermal cycling was performed

in an Eppendorf Mastercycler gradient. The reaction mixture in each PCR tube consisted of the following; 10X reaction buffer, 2.0mM final concentration of magnesium chloride, dNTPs at a final concentration of 0.2mM, 0.4μM of forward and reverse primers and 2.5 units of Taq polymerase made up to a final volume of 50μl using PCR grade water. The thermal cycles used are listed in the following table:

Hot start	94 °C for 3mins	
Denaturation	94 °C for 45sec	repeat steps
Annealing	55 °C for 45sec	×35 times
Extension	68 °C for 1min	
Final extension	68 °C for 5mins	

The PCR fragments were observed using agarose gel electrophoreses as previously Decribed in section 2.6.2. The 3 fragments were successfully amplified and they were extracted from the gel and purified using a Qiaquick gel extraction kit from Qiagen.

2.8.3 Gel extraction

DNA was excised from the gel using a sterile sharp scalpel. The gel was weighed in a colourless tube and 3 volumes of buffer QG was added to 1 volume of gel. The tube was incubated at 50 °C for 10 min. I volume of isopropanol was added to the tube and mixed. A QIAquick spin column was added to a 2 ml collection tube. In order to bind the DNA, the sample was added to the spin column and centrifuged at 17,900 × *g* for 1 min. 0.75 ml of buffer PE was added to the column and centrifuged again for 1 min. The flow though was discarded and the column was centrifuged at 17,900 × *g* for 1 min. The QIAquick column was added to a clean microcentrifuge tube and 50 μl of buffer EB was added to elute the DNA. The tube was centrifuged at 17,900 × *g* for 1 min.

2.8.4 Ligation reactions

10 μl of each amplified DNA fragment was added to 1 μl of PGemt vector, 11 μl of Buffer 2 and 1 μl of ligase. The samples incubated at 37 °C for 3 h.

2.8.5 Transformation

12 μl of each ligation product was transformed into 50 μl of DH5α competent cells using the following conditions; Cells were gently thawed in ice and once thawed were placed at 4 °C for 30 min, 42 °C for 2 min and back to 4 °C for 2 min. 1 ml of LB broth was added to each tube and they were then placed at 37 °C for 1 h. Tubes were centrifgued at 17,900 × *g* for 1 min. Each pellet was resuspended in 50 μl of LB broth. Each suspension was plated onto agar plates containing ampicillion antibiotic. Plates were placed at 37 °C overnight. Any colonies that grew were removed and placed in separate tubes containing 3 ml of LB broth with ampicillion antibiotic and incubated at 37 °C overnight.

2.8.6 Digestion of Plasmids

Approximately 1 μg of DNA was incubated with 1 unit of the appropriate restriction enzyme and 1 X buffer as recommended by the manufacturers in a final volume of 20 μl. The reaction was then incubated at 37 °C for 1 h. In order to ensure that the correct products were digested from the plasmid, the samples were electrophoreised on a 1% agarose gel.

2.8.7 Mini-preparation of plasmid DNA

DNA from bacterial colonies were isolated using Wizard Plus SV Miniprep DNA Purification System (Promega, Madison WI, USA). Bacterial cultures were grown overnight at 37 °C in 5 ml of LB medium (containing ampicillan antibiotic). Bacteria was pelleted and resuspended in 250 μl Cell Resuspension Solution. Cells were incubated for 5 min with the addition of 250 μl Cell Lysis Solution before adding 350 μl neutralization solution. The mixture was centrifuged at 17,900 × g for 10 min at room temperature. The cleared lysate was transferred to a spin column inserted into a 2 ml collection tube. The supernatant was centrifuged for 1 min at room. Then washed with 750 μl of Column wash solution and centrifuged for 1 min. This step was repeated with 250 μl Column wash solution. The DNA was eluted by addition of 50 μl of Nuclease Free H_2O. The insert was then sequenced to verify that the selected colonies contained the correct promoter sequences.

2.8.8 Subcloning

When the promoter fragments were successfully cloned into the PGemt sequencing vector they were digested out of this vector using the appropriate restriction enzymes (Kpn1 and Bgl2). Agarose gel electrophoesis was used to confirm that the fragments were removed from the vector. Amplified DNA fragments and expression plasmids, PGL3 basic and PGL3 enhancer were digested at 37 °C for 2 h using the appropriate restriction enzymes and purified as described previously in section 2.7.3.

2.8.9 Ligation

The ligation reactions were set up as follows; 1 μl vector, 15 μl insert, 2 μl of 2 X buffer, and 2μl of T4 DNA ligase. Ligation reactions were carried out at room temperature overnight.

2.8.10 Transformation

Transformation of the ligation products was carried out as described above in section 2.7.5. Following transformation and mini-preparation of the promoter products, they were again sent for sequencing to ensure the correct promoters were cloned.

2.8.11 Maxi-preparation of promoter plasmids

Once it was confirmed that the promoter sequences were correct a large volume of each of the promoters was obtained by maxi preparations (using the Qiagen endofree plasmid Maxi kit) of the promoter plasmids. 100 μl of the mini preparation of each plasmid was added to 100 ml of LB broth containing ampicillan. The cultures were grown overnight at 37 °C with gentle agitation. The cultures were transferred to 50 ml tubes and centrifuged at 6000 × *g* for 15 min at 4 °C. The bacterial pellets were resuspended in 10 ml of buffer P1. 10 ml of buffer P2 was added and the tubes were mixed vigorously. They were incubated at room temperature for 5 min. 10 ml of chilled Buffer P3 was added to the lysate, again this was mixed thoroughly. The lysate was poured into the barrel of a QIAfilter Cartridge and incubated for 10 min. The cap was removed from the Cartridge, the plunger was inserted and the cell lysate was placed into a clean 50 ml tube. 2.5 ml of buffer ER was added to the lysate and mixed and inverted 10 times, this was then

incubated in ice for 30 min. A QIAGEN tip was equilibrated by applying 10 ml of Buffer QBT, the column was left to empty by gravity flow. The filtered lysate was added to the QIAGEN tip and left to empty by gravity flow. The tip was washed with 2 × 30 ml of Buffer QC. The DNA was eluted with 15 ml of Buffer QN. The DNA was precipitated by the addition of 10.5 ml of isopropanol. This was centrifuged at 6,000 × *g* for 60 min. The DNA pellet was washed with 5 ml of endotoxin free 70 % ethanol and centrifuged at 6,000 × *g* for 30 min. The supernatant was removed and the pellet was left to air dry for 10 min and the redissolved in 500 μl of endotoxin free Buffer TE.

2.9 Transient transfection using GeneJuice®

Genejuice®, the liposomal based transfection reagent from Novagen was used to transfect of HEK 293 cells, MEF cells and A172 cells (2×10^5 cells/ml) in 96 well plates. Cells were transfected with different concentrations (10 ng, 20 ng, 50 ng, 100 ng) of promoters in PGL3 basic and also in the PGL3 enhancer vectors. In all cases the amount of DNA used per transfection was normalised using the appropriate amount of relevant empty vector control. Control plasmids used were the κB reporter plasmid and the normalization control plasmid, TK renilla. The κB reporter plasmid is used to test to ensure the cells are responding to certain TLR ligands such as LPS and PamCys. 0.8 μl of Genejuice® was mixed with 9.2 μl of serum free DMEM per transfection and incubated at room temperature for 5 min. 30 μl of this was then added to DNA and incubated for 15 min at room temperature. Each DNA transfection was carried out in triplicate. 10 μl of the DNA and Genejuice mix was added to the cells, which were incubated at 37°C for 16 h prior to stimulation. For 6 well plate transfections, the total DNA used was 1-2 μg, 8 μl

Genejuice® and 92 μl serum-free DMEM. To transfect 10 cm dish, 5-10 μg of DNA was used in combination with 15 μl Genejuice® and 235 μl serum-free DMEM.

2.10 Luciferase gene reporter assay

2.10.1 Cellular lysate preparation

Cells were transfected in 96 well plates as described in 2.6. 6 h post-stimulation or 24 h post transfection (if no stimulation), media was removed from cells. They were washed with PBS, they were then lysed for 15 min at room temperature with 50 μl of 1X Passive Lysis Buffer (promega). 20 μl of the lysed cell mix was taken and tested for firefly luciferase activity and 20 μl of the lysed cell mix was tested for Renilla luciferase activity.

2.10.2 Measurement of Luciferase Activity

Firefly luciferase activity was assayed by the addition of 40 μl of luciferase assay mix (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to the sample and Renilla luciferase was read by the addition of 40 μl of a 1:1000 dilution of Coelentrazine in PBS (Final concentration 1μg/ml). Luminesence was read using a Mediators PHL luminometer. Firefly luminescence readings were corrected for Renilla activity and expressed as fold stimulation over unstimulated empty vector (EV) control.

2.11 Western Blot analysis

2.11.1 Cell Stimulation and extract preparation

Cells were seeded at 2×10^5 cells/ml in 6 cm dishes and stimulated with a number of TLR ligands (LPS, PolyI:C, Malp 2 and Pam3Cys4) for a number of different time points. The reactions were terminated by removal of media from the cells followed by the addition of PBS to the dishes. The cells were washed for a total of 3 times in PBS. Cells were either lysed directly using sample buffer or they were lysed in high stringency buffer or RIPA buffer.

2.11.2 Cell lysis with sample buffer

100μl of sample buffer (5X sample buffer: 250mM TrisHCL ph6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) was added to each well of a 6 well plate. Cells were removed using a rubber cell scraper and transferred into eppendorfs. The samples are then sonicated for 10 sec at 80 % strength, boiled at 100 ^{o}C for 5 min and then centrifuged at 17,900 $\times$ *g* for 5 min.

2.11.3 Cell lysis with high stringency buffer or RIPA buffer

Between 150μl of high stringency buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 % glycerol (v/v), 1 % NP-40 (v/v) containing 10 μg/ml PMSF, 30 μg/ml aprotinin and 1 μg/ml sodium orthovanadate) or RIPA buffer (PBS buffer containing 1 % Nonidet P40, 0.5 % (w/v) sodium deoxycholate and 0.1 % SDS containing 10 μg/ml PMSF, 30 μg/ml aprotinin, and 1 μg/ml sodium orthovanadate) was added to each dish.

Cells were scraped and transferred into eppendorfs. These were mixed with gentle rotation at 4°C for 1 h. Following the incubation period samples were centrifuged at 17,900 × *g* for 10 min. Supernatants were removed and placed in clean eppendorfs.

The lysates were assayed for protein concentration.

2.11.4 Protein concentration determination

Protein concentration determination was carried out by use of a Bradford assay. A 96 well flat bottomed plate was used for this. 15 μl of sterile distilled water was added to each well using a multi-channel pipette. 5 μl of each sample was added to the wells, this was carried out in duplicate. Finally 200 μl of Bradford reagent (0.01 % (w/v) Coomassie Brillinat Blue G-250, 4.7 % (v/v) ethanol, 8.5 % (v/v) orthophosphoric acid) at room temperature, was added to each well. The plate was placed into a Dynatech MR5000 plate reader which read at 595nm. The results were then used to calculate the protein concentration using an equation taken from a Bradford standard curve. The standard curve was constructed using BSA concentrations in the range 0-20 μg/μl. Each sample was then equalised against the lowest protein concentration using high stringency buffer. 25μl of sample buffer was added to each eppendorf and the samples were boiled at 100 °C for 3 min.

2.11.5 Sodium Dodecyl Sulfate- polyacrylamide gel electrophoresis (SDS- PAGE)

Samples were resolved on Sodium Dodecylsulphate (SDS) polyacrylamide gel using a constant current of 25 mA per gel. Samples were first electrophoresed though a stacking gel (1ml 30% bisacrylamide mix, 0.75 ml 1M Tris pH 6.8, 60μl 10% ammonium

persulphate and 6μl TEMED made up to 6ml with H_2O) to condense protein and then resolved according to size using 8-12% polyacrylamide gels (30% bisacrylamide mix, 3.75ml 1.5M tris pH 8.8, 150μl 10% (w/v) ammonium persulphate, 6μl TEMED made up to 15ml with H_2O). Pre-stained protein markers (New England Biolabs) were also placed on the gel as molecular weight standards.

2.11.6 Transfer of proteins to membrane

The resolved proteins were then transferred to polyvinyidene diflouride (PVDF) using a wet transfer system with all components soaked first in transfer buffer (25mM Tris-HCL pH 8.0, 0.2 M glycine, 20% methanol). The gel was placed on a layer of filter paper and sponge overlaid with the membrane. A second piece of filter paper was placed on top followed by a second sponge. The entire assembly was placed in a cassette. An ice pack was placed in the chamber, which was then filled with transfer buffer and a constant current of 150 mA was applied for 2 h.

2.11.7 Blocking the membrane

Membranes were blocked for non specific binding by incubation in 50 ml of 5 % (w/v) non fat dried milkin 1 % (v/v) Tris Buffered Saline (TBS)-Tween for 1 h at room temperature. The membrane was washed three times for 5 min in 1 % (v/v) TBS-Tween.

2.11.8 Antibody incubation

The membrane was incubated for 1 h at room temperature or overnight at 4 ^{o}C with the primary antibody of interest at 1:100 to 1:1000 dilution depending on the particular

antibody. Following incubation the membrane was washed for 5 min three times in 1 % (v/v) TBS-Tween, and incubated with the appropriate secondary horseradish peroxidase linked enzyme for 1 h at room temperature. Again the membranes were washed for 5 min three times in 1 % (v/v) TBS-Tween. Membranes were developed by enhanced chemiluminesence (ECL) according to manufacturers instructions (Amersham).

2.11.9 Stripping and reprobing

The same membrane can be probed for more than one protein if the membrane is stripped of the initial antibodies and reprobed with the new antibody of interest. The membrane was washed once in 1 % (v/v) TBS-Tween. The membrane was striped by incubating it in 100 mM glycine at pH 3 for 30 min. The membrane was blocked again in 5% (w/v) non fat dried milk for 1 h and probed with primary and secondary antibodies and developed as described in section 2.10.6.

2.11.10 Colloidal Blue staining

Resolved gels were soaked in Colloidal Blue Stain overnight and then washed several times with filtered H_2O until bands become visible.

2.12 Membrane Fractionation Assay

Cells were seeded at 1×10^5 cells/ml in 10 cm dishes and incubated at 37 °C overnight. After 24 h media was removed and the cells were washed three times with sterile PBS. Cells were resuspended in 500μl membrane resuspension buffer and subjected to 30 strokes of a tight fitting Dounce homogenizer. Samples were centrifuged at 97,000 × *g*

for 1 h at 4 °C. Supernatant (cytosolic fraction) was removed and 100 μl of sample buffer with 10 % mercaptoethanol was added. 100 μl of sample buffer was added to the pellets (membrane fraction). The samples were then examined by western blotting as described in section 2.10.

2.13 siRNA assays

2.13.1 Transient transfection of siRNA oligos using oligofectamine

siRNAs were designed and obtained from Dharmacon and Qiagen. U373/CD14 cells were seeded at 5×10^4 cells/ml in 6 well plates. Oligos were made up to a final concentration of 20 μM. Oligos were then diluted 1/10 in serum free medium. A 1/5 dilution of oligofectamine was made up using serum free media (SFM. Oligofectamine was added to siRNA and incubated for 20 min. Cells were washed once with 1 ml of SFM. 880 μl of SFM was then added to the cells. siRNA and oligofectamine mix was added to the cells. After 6 h, 1 ml of media containing 20 % FCS and 2 × L glutamine was added to the cells. Cells were harvested after 48 h. Samples were examined by western blot according to the protocol outlined in section 2.10.

2.13.2 Transfection of siRNA oligos using the amaxa system

Human PBMC cells are semi adherent and THP1 cells are a suspension cell line, therefore they are difficult to transfect by conventional lipid based transfection methods. Therefore the amaxa system was used as it is capable of transfecting suspension cells. The Cell Line Nucleofector® Kit V was used for all siRNA transfections. 1×10^6 cells/ml PBMC or THP1 cells were used per point. Between 0.5-3 μg of siRNA was added to the

cells, then combined with 100 μl of Nucleofector® solution V and transferred to an amaxa certified cuvette. Each sample was processed separately to avoid storing cells in Nucleofector® solution V for more than 15 min. The S-019 program was set on the Nucleofector®, the sample was inserted and electroporated. An amaxa pipette was used to transfer the sample into 500μl of pre-warmed RPMI containing 10 % FCS and 0.1 % Penicillin, streptomycin for THP1 cells and IMDM containing 10 % FCS and 0.1 % Ciprofloxacin for PBMC. Cells were incubated at 37 °C for 72 h. Cells were lysed directly in sample buffer as described in section 2.10.2 and analysed by western blot as described in section 2.10.

2.14 Immunoprecipitation

2.14.1 Antibody precoupling

The relevant antibodies were precoupled with protein G sepharose beads, by incubating 15-30μl of antibody with 40μl of beads overnight with gentle rotation at 4°C.

2.14.2 Immunoprecipitation

Cells were seeded at 2×10^5cells/ml in 10cm dishes. The cells were grown for 24 h in media containing 10% FCS. Transfections were carried out using Genejuice® Transfection Reagent (Novagen) as previously described in section 2.8. 10 μg of DNA plasmid was transfected into the cells, which were harvested 24 h post-transfection. The cells were washed 3 times in sterile PBS. 800 μl of high stringency lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 % glycerol (v/v), 1 % NP-40 (v/v) containing 10 μg/ml PMSF, 30 μg/ml aprotinin and 1 μg/ml sodium orthovanadate) was

added to the cells. The cells were removed from the 10 cm dish using a rubber cell scraper and transferred into a clean eppendorf. Tubes were placed at 4 °C for 30 min with continuous gentle rotation. Samples were then centrifuged at 17,900 × *g* for 10 min. Supernatants were removed and added to the relevant pre-coupled antibody. Samples were either incubated overnight at 4 °C or for 2 h at room temperature. 50 μl of each lysate was retained to confirm expression of the protein of interest, this was added to sample buffer and boiled at 100 °C for 5 min. Following incubation the immune complexes were washed twice with 1 ml lysis buffer and once with ice-cold PBS. All supernatant was removed and beads were resuspended in 30 μl 5 X sample buffer. The samples were boiled for 5 min and SDS-PAGE analysis was performed on the precipitated complexes as described in section 2.10.

2.15 Dimensional SDS-PAGE analysis

2.15.1 Sample preparation

100% of foetal calf serum (FCS) was used as the detection sample for this procedure. Immunoprecipitation was carried out as described in section 2.13 using the anti-KIAA0644 antibody precoupled to protein A/G beads. After the immune complex was washed, samples were resuspended in 40μl of sample solubilization solution (8M Urea, 50mM DTT, 4% CHAPS, 0.2% carrier ampholytes, 0.0002% bromophenol blue.

2.15.2 Isoelectric Focusing

The sample was transferred to a rehydration tray. The 8cm IPG strip (ph 6-11) obtained from Amersham was placed over the sample, and left to rehydrate overnight at room

temperature. Rehydrated strips were then moved to the focusing tray and were overlaid with mineral oil. The focusing conditions were as follows:

Step 1: Linear Slope to 300 V for 30 min

Step 2: Constant 1000 V for 30 min

Step 3: Linear slope to 5000 V for 90 min

Step 4: Constant 5000 V for 24 min

Step 5: Hold at 50 V

2.15.3 Rehydration of IPG strips

IPG strips were incubated in DTT equilibration buffer (6 M Urea, 375 mM Tris, pH 8.8, 2 % SDS, 20 % glycerol and 2 % DTT) for 10 min, followed by Iodoacetamide equilibration buffer (6 M Urea, 375 mM tris, pH 8.8, 2 % SDS, 20 % glycerol and 2.5 % iodoacetamide) for 10 min.

2.15.4 Second Dimension

IPG strips were positioned on top of the second dimension gel (8 %) (4 ml of 30 % Bisacrylamide, 6.9 ml H_2O, 3.8 ml 1.5 M Tris-HCL pH 8.8, 150 μl 10 % SDS, 150 μl 10 % APS and 6 μl of TEMED. The IPG strip was secured in place by overlaying it with 0.5 % molten agarose prepared in SDS-PAGE running buffer with a small amount of bromopheonol blue, used to track the ion front during electrophoresis. Samples were resolved on a SDS polyacrylamide gel using a constant current on 20 mA per gel. Samples were transferred and immunoblotted as described in sections 2.10.

2.16 Mass Spectrophotometry

KIAA0644 was immunoprecipitated from 100 % and 10 % Foetal Calf serum as described in section 2.13.2 and the samples were electorphorised on 8 % SDS-PAGE gels (section 2.10.5). One of the gels was then transferred onto PVDF membrane and probed for KIAA0644 as explained in section 2.10. The second gel was placed in a Pyrex dish and washed thee times for 10 min with millipure H_2O. Colloidal Blue stain was added to the gel and it was left gently shaking overnight at room temperature. The gel was destained with millipure H_2O for 3 h (or until bands were clearly visible).

2.16.1 In Gel protein digestion and protein identification by MALDI-TOF Mass Spectrometry

Protein spots were excised manually from the 1D gel. Gel pieces were washed thee times with millipure H_2O (500µl per spot) for 5 min each with gentle agitation. Gel pieces were then washed for 5 min with 50 nM NH_4HCO_3 containing 30 % acetonitrile (ACN). The spots were then dehydrated with 100 % ACN for 5 min. The spots were then placed in a speed-vacuum (ThermoSavant) for 5 min on low heat. For trypsin digestion, a 100 µg/ml aliquot of sequencing grade tryspin (Roche Applied Science, Mannheim, Germany) dissolved in 1 mM HCL was diluted 1:10 in digestion buffer (25 mM NH_4HCO_3, 0.1 n-octyl β-D-glucopyranoside). 2 µl of trypsin solution (20 ng) was pipetted directly onto the desiccated gel pieces. After allowing the gel piece to rehydrate for 5 min, a further 10 µl of digestion buffer was added, and the samples were incubated at 37°C overnight.

After trypsin digestion, peptides were extracted twice into 40 µl of 66 % acetonitrile and 0.1 % trifluoroacetic acid in a sonicating water bath, followed by lyophilization in a Speed-Vac at room temperature. For mass spectrometric analysis, peptides were solubilized by sonication in 5 µl of 5 % formic acid. Digested samples (0.5-1 µl) were applied to a Teflon-coated 96 well MALDI target plate (Applied Biosciences) followed by the addition of 0.5-1 µl of a 10 mg/ml matrix solution of α-cyano-4-hydroxy-cinnamic acid in 60 % acetonitrile and 0.1 % trifluoroacetic acid. Samples were allowed to air dry at room temperature before analysis in positive reflection mode in a Voyager DE Pro MALDI mass spectrometer (Applied Biosciences, Foster City, USA).

2.17 Confocal Microscopy

2.17.1 Preparation of coverslips

Sterile coverslips were coated with 1mg/ml poly-L-lysine. Excess poly-L-lysine was aspirated off after 1min. The coverslips were left to dry for 1 h in a laminar flow hood.

2.17.2 Preparation of slides

Coverslips were placed in 6 well plates and cells were seeded at a concentration of 2×10^5 cells/ml. After 24 h cells were transfected with KIAA0644 and cells were stimulated with 1 µg/ml Pam Cys for 24 h. Cells were then rinsed gently in PBS twice. Cells were fixed by incubating them in 2 % formaldehyde (2 g paraformaldehyde was dissolved in 10 ml 10 X PBS, NaOH was added dropwise to aid dissolving, once dissolved sterile deionised H_2O was added to a final concentration of 1 X PBS, pH 7.4) for 15 min at room temperature. The formaldehyde was removed and slides were washed three times in 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 min. Triton was then removed and the cells were rinsed thee times in PBS. At this point the coverslips were removed and placed in new 6 well plates. Cells were rinsed once in 2 % (w/v) Bovine serum albumin (BSA) in sterile PBS. Primary antibody was diluted 1:200 in 2 % (w/v) BSA-PBS and placed on the slides for 1 h. The antibody was removed and cells were washed in 2 % BSA-PBS three times. The secondary antibody was diluted 1:250 in 2 % BSA-PBS and 100 μl was added to each coverslip in the dark and left to incubate for 45 min. Slides were removed and rinsed in distilled water. Approximately 10 μl of mounting solution (50 % (v/v) glycerol, 0.5 X PBS, 2 mg/ml para-p-phenylendiamine, 1 μl/ml DAPI stain) was dropped onto a slide. The coverslip was placed cell-side down onto the mounting solution and sealed with nail polish to prevent drying and movement under the confocal microscope. At no stage during this procedure were the coverslips allowed to dry out.

2.17.3 Cryosectioning and staining protocol

Whole mice brains were dissected and placed in 4 % PFA pH 7.4 overnight at 4 ^{o}C and subsequently washed 4 X 15 min with PBS. The brain was cryoprotected using a 10 %, 20 % and 30 % sucrose gradient, and 12 µm frozen sections were subsequently cut on a cryostat at - 20 ^{o}C and placed on poly-lysine coated slides. Primary antibodies were incubated on sections overnight at 4 ^{o}C. Following incubation, sections were washed 3 times in PBS and subsequently blocked again with 5 % NGS for 20 min at room temperature. Secondary antibodies were incubated with the sections at 37 ^{o}C for 2 h followed by 3 washes with PBS. All sections were counterstained with 4', 6-diamidine-2-phenylindole-dihydrochloride (DAPI; Sigma Aldrich, Ireland) for 30 sec at a dilution of

1:5000 of a stock 1 mg/ml solution, and sections were visualized using an Olympus FluoView TM FV1000 Confocal microscope.

2.17.4 Perfusion protocol

The biotinylated reagent EZ-Link TM Sulfo-NHS-Biotin (Pierce) (1 ml/g body weight of 2 mg/ml EZ-Link TM Sulfo-NHS-Biotin, 443 Da) was perfused @ 37 °C for 5 min through the left ventricle of the beating heart of anaesthetized mice. Following perfusion with the tracer molecule, the whole brain was dissected and placed in 4 % PFA pH 7.4 overnight at 4 °C and subsequently washed 4 X 15 mins with PBS. Following cryoprotection using a 10 %, 20 % and 30 % sucrose gradient, 12 µm frozen sections were cut on a cryostat at - 20 °C and incubated with streptavidin conjugated to the fluorescent probe FITC. This allowed for the visualization of microvessels in the brain.

2.18 Enzyme linked immunosorbant assays (ELISA)

2.18.1 Sample preparation

BMDM were seeded at 1×10^5 cells/ml in a 96 well plate. U373/CD14 cells were seeded at 2×10^5 cells/ml and PBMC were seeded at 1×10^6 cells/ml in 24 well plates. 24 h later the cells were either left untreated or stimulated with the appropriate ligand for 24 h. The supernatants were then removed to a new sterile 96 well plate or into sterile eppendorfs and were assayed on that day or stored at -80°C indefinitely.

2.18.2 Plate preparation

Capture antibody was diluted to the working concentration in PBS pH 7.4 (0.2 μm filtered).

ie:	Human TNFα	working concentration of 4.0 μg/ml
	Human IL-6	working concentration of 2.0 μg/ml
	Human IL-1β	working concentration of 4.0 μg/ml
	Human Rantes	working concentration of 1.0 μg/ml
	Murine Rantes	working concentration of 2.0 μg/ml
	Murine IL-6	working concentration of 2.0 μg/ml
	Murine TNFα	working concentration of 0.8 μg/ml

A 96 well plate was immediately coated with 100 μl per well of the diluted capture antibody, the plate was sealed and incubated overnight at 4 ^{o}C. The capture antibody was aspirated from each well, and the plate was washed with Wash buffer (0.05 % Tween 20 in PBS, pH 7.2-7.4), for a total of three washes. Complete removal of liquid at each step was essential for good performance. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Te plate was then blocked by adding 300 μl of reagent diluent (1 % BSA in PBS, pH 7.2-7.4) to each well. Plates were incubated at room temperature for a minimum of 1 h. The reagent diluent was aspirated from each well, and each well was then washed with wash buffer for a total of three washes. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels.

2.18.3 Assay procedure

The standards were prepared by diluting the recombinant protein in reagent diluent with a high standard concentration of 2000 pg/ml. A seven point standard curve using 2-fold

serial dilutions in Reagent Diluent was then prepared. 100 μl of sample or standard in Reagent Diluent was added to each well. The plates were covered with an adhesive strip and incubated for 2 h at room temperature. The samples and standards were then aspirated from each well, and each well was washed with wash buffer for a total of three washes. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. 100 μl of the working concentration of detection antibody, diluted in reagent diluent, was then added to each well.

ie.	Human TNFα	working concentration of 4.0 μg/ml
	Human IL-6	working concentration of 200 ng/ml
	Human IL-1β	working concentration of 300 ng/ml
	Human Rantes	working concentration of 10 ng/ml
	Murine Rantes	working concentration of 400 ng/ml
	Murine IL-6	working concentration of 200 ng/ml
	Murine TNFα	working concentration of 150 ng/ml

Once the detection antibody was added the plate was covered with a new adhesive strip and incubated for 2 h at room temperature. The detection antibody was then aspirated from each well, and each well was washed with wash buffer for a total of three washes. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. 100 μl of the working dilution of streptavidin-HRP (1:200 dilution of streptavidin-HRP in 1 % BSA-PBS) was added to each well. The plate was covered and incubated for 20 min at room temperature away from direct light. The streptavidin-HRP was then aspirated from each well, and each well was washed three times with wash buffer. After the last wash, any remaining wash buffer was removed by

inverting the plate and blotting it against clean paper towels. 100 μl of substrate solution (A 1:1 mixture of Colour Reagent A (H_20_2) and Colour Reagent B (tetramethybenzidine)) was then added to each well. Once the substrate solution was added the plates were incubated for 20 min at room temperature away from direct light. 50 μl of stop solution (2N H_2SO_4) was then 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 450 nm.

Chapter Three

Bioinformatic analysis of KIAA0644

Chapter 3

3.1 Introduction

As mentioned in chapter 1, TLR4 is the receptor for LPS. In order for TLR4 to respond to LPS it requires a number of co-receptors such as MD2 and CD14. Once the TLR4 complex is assembled it recruits various intracellular molecules to initiate a signal response which culminates in production of proinflammatory cytokines. Although the TLR4 signalling pathway has been studied intensely for many years, a number of questions still remain unanswered. In particular, how LPS binds and signals via the co-receptors.

CD14 is a co-receptor vital for TLR4 responses to LPS. It is believed that LPS binds to LPS binding protein which transfers LPS monomers to CD14. The role of CD14 is to concentrate the LPS and therefore increase the sensitivity of the TLR4 complex to the ligand. CD14 has been reported to be only required for responses to smooth LPS and not to rough LPS (Jiang *et al.*, 2005). There is still much speculation over the importance of CD14 in the LPS signalling pathway and the hypothesis put forward by Jaing *et al* has raised many questions. It is unclear why rough LPS is capable of binding to the TLR4/MD2 complex without CD14 and how it can lead to differential downstream responses. In our experiments it is clear that cells that do not express CD14 are capable of responding to rough or smooth LPS in some way, indicating that there may be another receptor in the complex which has not yet been identified.

TLRs are expressed on many cell types throughout the body. mRNA for most TLRs have been identified in the brain, however very little work has been carried out on protein expression levels of TLRs within the brain. TLR2 and TLR3 have been shown to be expressed at a high level, however TLR4 is not. Since the brain lacks

MHC expression it is difficult to understand why it would not possess all the innate immune receptors, which are designed to respond rapidly to infectious agents. There are a number of possible explanations for this apparent anomaly. Either TLR4 is expressed at a very low basal level in the brain and can be rapidly induced upon stimulation, or there are other receptors present in the brain capable of substituting for TLR4.

The aim of this investigation was to identify novel LPS inducible genes in wild type and MAL knockout cells. Five genes were identified in a microarray carried out on embryonic stem cells from wild type and MAL knockout mice. Only one of these genes was uncharacterised and it was annotated at KIAA0644. Due to it's novelty we decided to focus on this gene and try to characterise it and determine its role in the LPS signalling pathway. The initial goal of this study was to use bioinformatic analysis tools to characterise the gene and determine if it was worth pursuing through to experimental studies and ultimately determine what function this novel gene may have within the LPS signalling pathway.

3.2 Results

3.2.1 KIAA0644 was identified in a microarray analysis

A Preliminary microarray analysis identified a number of genes that exhibit differential expression levels in Mal knockout embryonic stem cells stimulated with LPS. Several of the genes identified have been characterised to some extent while one of them, KIAA0644, has no known function. The sequence of this gene is available on the HUGE (Human Unidentified Gene-Encoded Large Proteins) protein database as part of the Human cDNA project at the Kazusa DNA Research Institute (www.Kazusa.or.jp). The ultimate aim of this project is to characterise and determine a function for this novel gene.

3.2.2 KIAA0644 is a leucine rich repeat containing protein located on human chromosome 7

KIAA0644 was mapped to chromosome 7 at 7p15 using the map viewer tool available from the NCBI website (www.ncbi.nlm.nih.gov). It is 4.7kb in length and is transcribed in the anti parallel direction. It is flanked on either side by the genes CREB5 and CPVL carboxypeptidase (Fig 3.1).

3.2.3 KIAA0644 has 13 LRR, a fibronectin domain and a transmembrane domain

I inserted the protein sequence of KIAA0644 into a number of proteomic tools available from the expasy website (www.expasy.org) in order to further characterise the predicted protein. The signal P program revealed that there is a signal sequence at the N terminus of the protein (Fig 3.2). Combinations of programs were used to identify the domains within this protein. The Simple Modular Architecture Research Tool (SMART) program locates motifs present in a protein sequence. This program

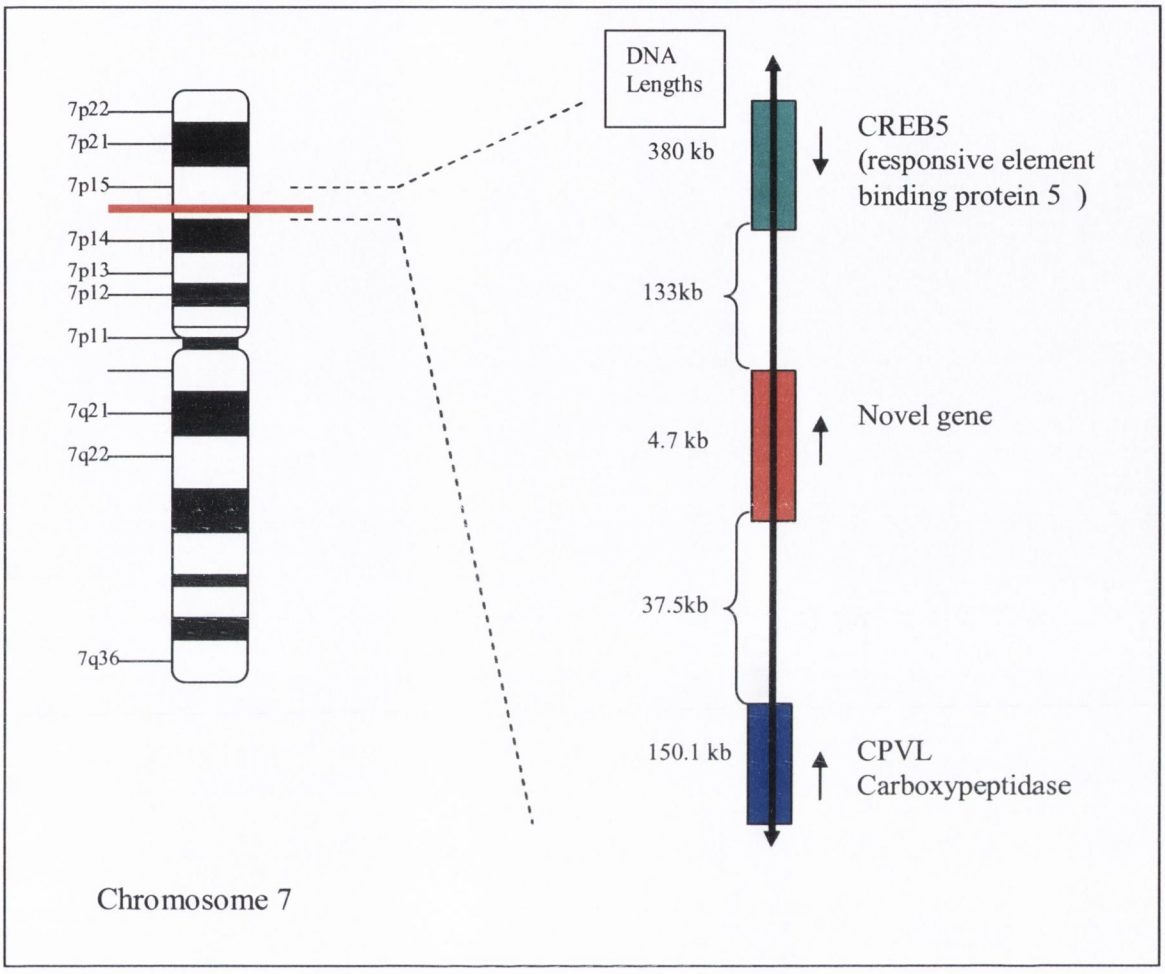


Figure 3.1 Chromosomal Location of Human KIAA0644. The human genome map viewer tool available from the NCBI website was used to determine that KIAA0644 is located on chromosome 7 at 7p15, which is indicated by the red line. It is 4.7 kb in length and is flanked by the genes CREB5 and CPVL. The direction of transcription is indicated by the arrows, KIAA0644 is transcribed in the anti-parallel direction.

revealed that there are 12 leucine rich repeats present in the protein sequence of KIAA0644. TMHMM program showed that there is a predicted transmembrane region close to the C terminus of the protein

Once it was revealed that this protein may contain LRR domain we decided to use more sophisticated and technical computer programs to identify the specific motifs in more detail. The sequence of KIAA0644 was therefore submitted to the Meta Server 3D-Jury (1) for domain identification and protein structure and function analysis. Domain identification was viewed as being reliable when the '3djury' score was >50. Searches for structural domains using the 3D-Jury Metaserver (1) identified both an LRR (residues 25-374) and a type III Fibronectin domain (residues 588-686) in KIAA0644. This program however identified 13 leucine rich repeat motifs whereas SMART only identified 12. The top ranked significant structural homologues for the LRR region in KIAA0644 were the Lingo-1 ectodomain (pdb 2id5; Jscores 195.88 – 210.0), followed by the TLR3 ectodomain (pdb 2a0z; Jscore 166.0). For the type III fibronectin domain the top three structural homologues were the type III fibronectin domain from human contactin (pdb 2ee2; Jscore 68.5) and the first and fifth domains from human neogenin (pdbs 1x5f and 1x5j; Jscores 65.5 and 65.25). Interestingly the type III fibronectin domain from KIAA0644 was identified even in the absence of a significant psiblast result, suggesting that the underlying sequence differs from those previously identified in these domains (Fig 3.2).

Homology models for both the KIAA0644 LRR and type III fibronectin domains were generated using Modeller 9v3 (Fig 3.3 and Fig 3.4). For the type III fibronectin domain separate models were generated with either 2ee2 (Fn_III_A), or both 1x5f and 1x5j (Fn_III_B) as templates. Model analysis showed good stereochemistry with the Verify3D scores being 96.58 (LRR), 97.22 (Fn_III_A), and

Signal sequence N terminal cap

MEAARALRLLLVVCGCLALPPLAEPVCPERCDCQHPQHLLCTNRGLRVVPK

TSSLPSPHDVLTYSLGGNFITNITAFDFHRLGQLRRLDLQYNQIRSLHPKTFEK

LSRLEELYLGNNLLQALAPGTLAPLRKLRILYANGNEISRLSRGSFEGLESLVK

LRLDGNALGALPDAVFAPLGNLLYLHLESNRIRFLGKNAFAQLGKLRFLNLS

ANELQPSLRHAATFAPLRSLSSLILSANSLQHLGPRIFQHLPRLGLLSLRGNQL

THLAPEAFWGLEALRELRLEGNRLSQLPTALLEPLHSLEALDLSGNELSALHP

ATFGHLGRLRELSLRNNALSALSGDIFAASPALYRLDLDGNGWTCDCRLRGL

C terminal cap

KRWMGDWHSQGRLLTVFVQCRHPPALRGKYLDYLDDQQLQNGSCADPSPS

ASLTADRRRQPLPTAAGEEMTPPAGLAEELPPQPQLQQQGRFLAGVAWDGA

ARELVGNRSALRLSRRGPGLQQPSPSVAAAAGPAPQSLDLHKKPQRGRPTRA

DPALAEPTPTASPGSAPSPAGDPWQRATKHRLGTEHQERAAQSDGGAGLPPL

Fibronectin domain

VSDPCDFNKFILCNLTVEAVGADSASVRWAVREHRSPRPLGGARFRLLFDRF

GQQPKFHRFVYLPESSDSATLREIRGDTPYLVCVFGVLGGRVCPVAPRDHCA

Transmembrane domain

GLVTLPEAGSRGGVDYQLLTLALLTVNALLVLLALAAWASRWLRRKLRARR

KGGAPVHVRHMYSTRRPLRSMGTGVSADFSGFQSHRPRTTVCALSEADLIEF

PCDRFMDSAGGGAGGSLRREDRLLQRFAD

Figure 3.2 Protein sequence of human KIAA0644. The predicted protein is 811 amino acids in length and contains an N-terminal signal sequence as predicted using the Signal P prediction program from Swissprot, which is underlined and displayed in red. The N-terminus of the putative protein also contains 13 leucine rich repeats highlighted in blue. TMHMM program was used to determine if a potential transmembrane region is present in the protein. This predicted transmembrane region is depicted in orange. A predicted fibronectin domain is highlighted in purple.

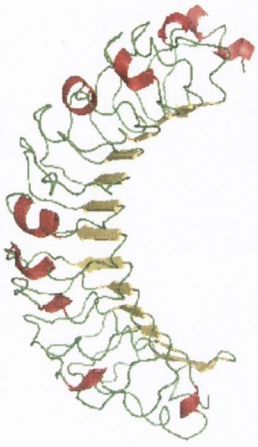


Figure 3.3 The predicted structure of the ectodomain of KIAA0644 shows a horseshoe shapped solenoid. The homology model for KIAA0644 was generated using Modeller 9v3. This final model was prepared using PyMol (http://www.pymol.org). The KIAA0644 LRR domain consists of 13 LRR motifs with both an N- and C-terminal cap. These caps are not contained in the model structure. The asparagine ladder is conserved throughout and these contribute hydrogen bonding networks which allow for stabilization of the solenoid structure.



Figure 3.4 Predicted Structure of the fibronectin domain of KIAA0644. Modeller 9v3 was used to design the homology structure of the fibronectin domain of KIAA0644. Separate models were generated with either 2ee2 (Fn_III_A), or both 1x5f and 1x5j (Fn_III_B) as templates. Model analysis showed good stereochemistry with the Verify3D scores being 96.58 (LRR), 97.22 (Fn_III_A), and 95.19 (Fn_III_B). Similarly the proportions of outlying residues in the Ramachandran plots were 3.2 % (LRR), 0 % (Fn_III_A), and 2.0 % (Fn_III_B). As this figure shows the fibronectin domain contains seven beta sheets and folds with a beta sandwich, or greek-key, topology.

95.19 (Fn_III_B). Similarly the proportions of outlying residues in the Ramachandran plots were 3.2 % (LRR), 0 % (Fn_III_A), and 2.0 % (Fn_III_B).

As anticipated from the degree of structural homology the KIAA0644 fibronectin domain contains seven beta sheets and folds with a beta sandwich, or greek-key, topology. The KIAA0644 LRR domain consists of 13 LRR motifs with both an N- and C-terminal cap. These caps are not contained in the model structure. The consensus sequence of the KIAA0644 LRRs is xLxxLxLxxN with the exception of LRR1 in which the first two leucines are replaced with valine and tyrosine respectively, and LRR4 where the third leucine is replaced with an alanine. The asparagine ladder is conserved throughout. Including the variable region each LRR in KIAA0644 is 24 residues long, with the exception of LRR7 which contains an additional two residues in the variable region.

3.2.4 The protein sequence of KIAA0644 is highly conserved across species

The protein sequences of KIAA0644 in human, mouse and rats were obtained from NCBI website. The sequences were obtained in fasta format and an alignment was obtained using the clustalw alignment program (Fig 3.5). The result shows that the sequences are highly conserved between the three species.

The protein sequence was inserted into the blast 2 program available from the NCBI website to obtain a table of the most closely related proteins to KIAA0644. The results are shown in table 3.1. The mouse and rat sequences show highest homology. A number of other leucine rich repeat containing proteins show quite high similarity. Previous results showed that the ectodomain of TLR3 showed high structural homology to KIAA0644. This blast 2 method used the entire protein sequence to determine related proteins and interestingly TLR3 in the Japanese flounder displays a

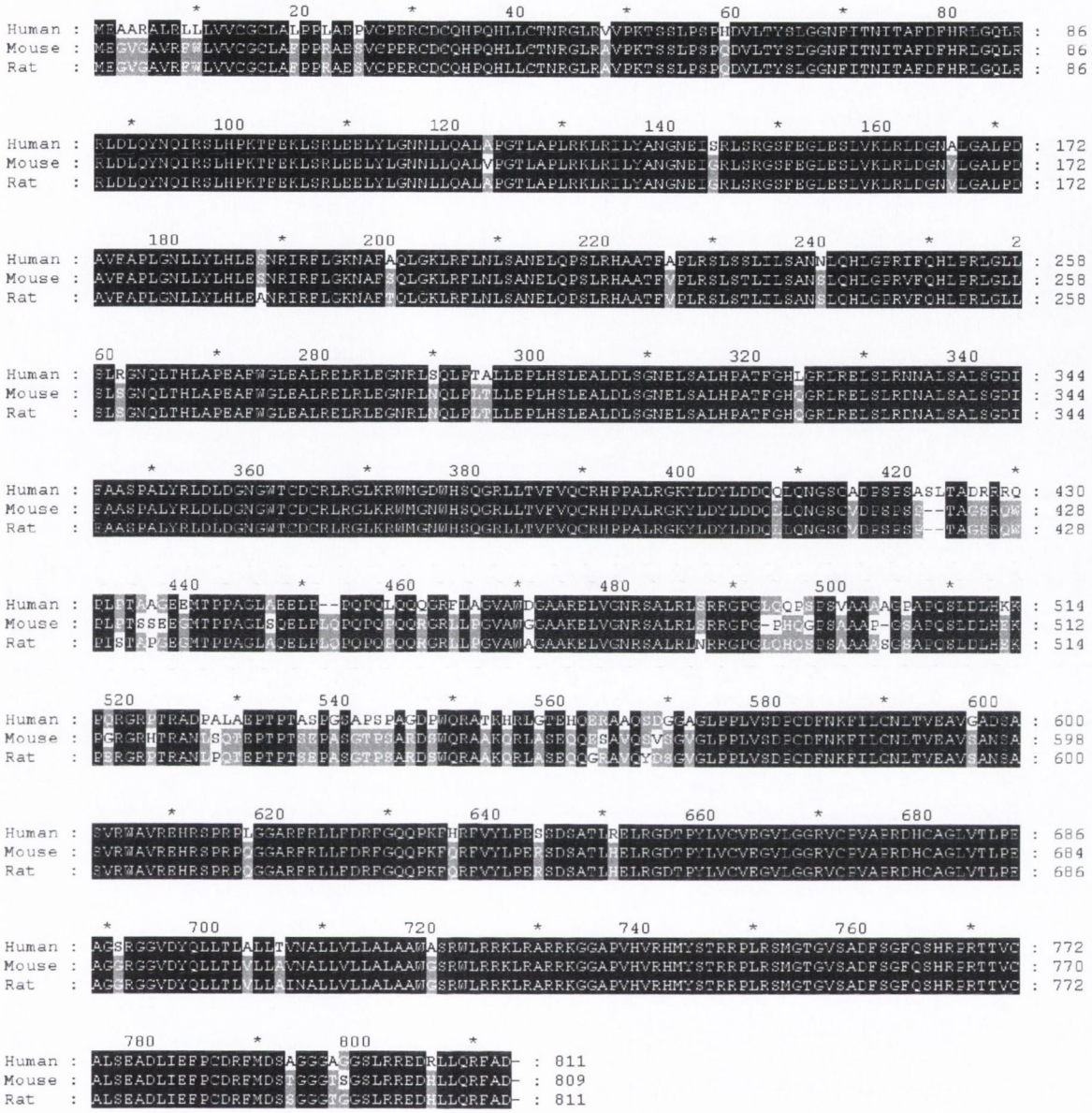


Figure 3.5 The protein sequence of KIAA0644 is highly conserved across species. Human, mouse and rat sequences were obtained from NCBI. The sequences in fasta format were inserted into Clustalw alignment program. Areas of highest homology are highlighted in black. Areas with close homology are highlighted in grey. White areas show least homology.

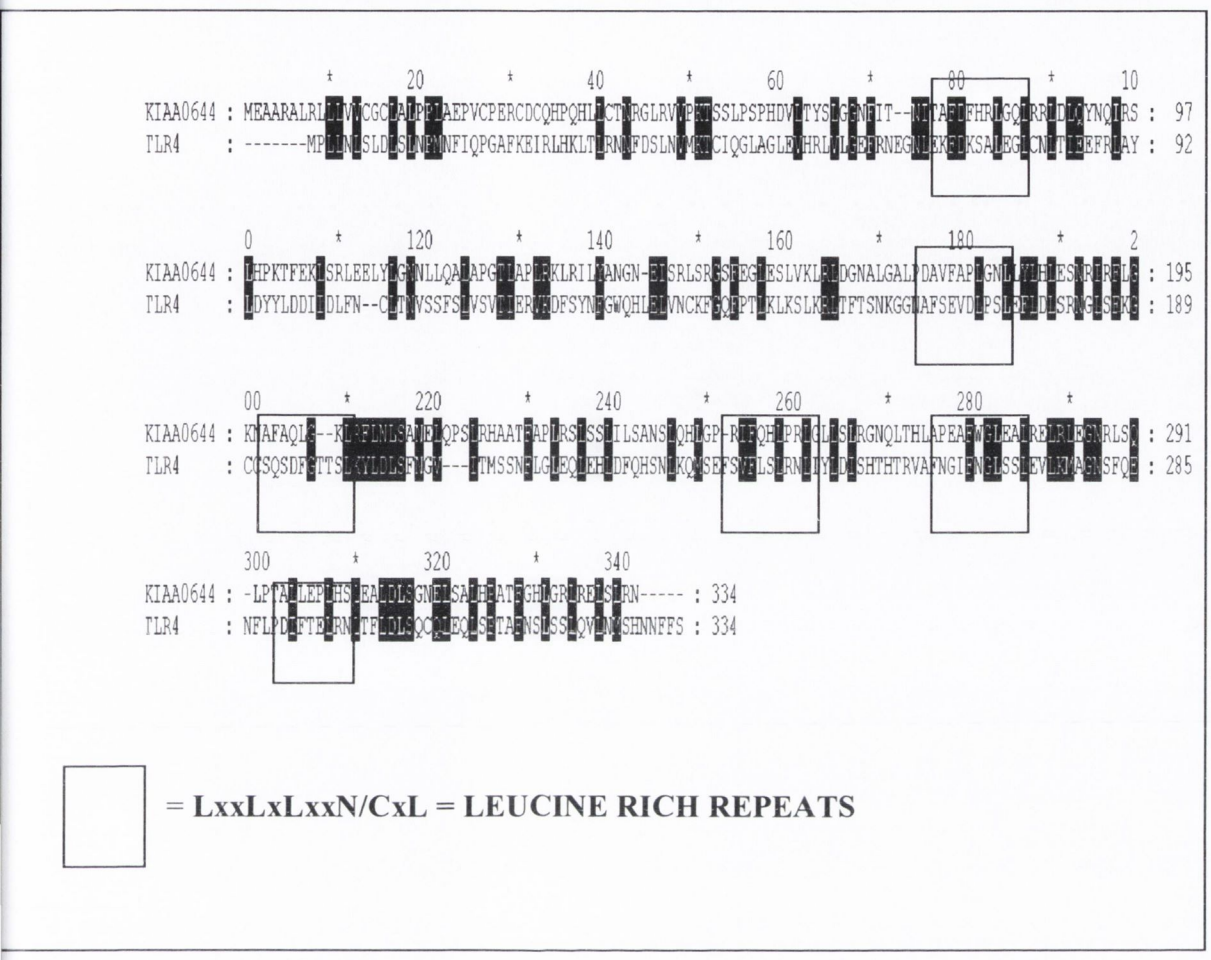
ID	Length	Identity%	%Positives
KIAA0644 human	**811**	**100**	**100**
KIAA0644 macaca mulatta	**811**	**96**	**96**
KIAA0644 rat	**811**	**88**	**90**
KIAA0644 mouse	**809**	**88**	**90**
Carboxypeptidase N subunit 2 precursor	**545**	**37**	**49**
Leucine rich repeat containing protein 15 precursor mouse	**579**	**35**	**45**
LRRC15 protein human	**581**	**33**	**45**
Insulin like growth factor binding protein acid labile chain	**603**	**34**	**46**
TLR4 Human	**839**	**32**	**47**
Synleurin	**622**	**31**	**47**
TLR3 Human	**961**	**28**	**44**
Leucine rich repeat and immunoglobulin-like domains protein 2 precursor	**1054**	**27**	**42**

Table 3.1 Table of Proteins most related to KIAA0644. This table was constructed from results obtained using the Blast 2 program from NCBI. The protein sequence of KIAA0644 was inserted into the program and the results above are the proteins with the closest homology to KIAA0644.

significantly high sequence homology to KIAA0644. TLR4 shows a sequence homology to KIAA0644. A sequence alignment of the ecto-domains and TIR domains of KIAA0644 and TLR4 are shown in Figs 3.6 and 3.7 respectively.

3.2.5 KIAA0644 appears to have a cytosolic TIR domain

As mentioned in the introduction, members of the TLR family all contain a cytosolic TIR domain. This domain spans about 200 amino acids, with varying degrees of sequence similarity among family members. Three particular boxes can be identified which are highly conserved among family members. Box 1 is considered the signature sequence of the family whereas boxes 2 and 3 contain amino acids critical for signalling. The crystal structure of the TIR domains of TLR1 and TLR2 has revealed a core structural element centered around box 2 (Xu *et al*., 2000). This region, termed the BB loop, forms an exposed surface patch and contains a critical proline or alanine residue. These amino acids are located at the tip of the loop and are thought to form a point of contact with downstream signalling components. Close inspection of KIAA0644 reveals that it also contains a highly conserved box 2 and an identifiable box 1 and 3 (Fig 3.7). TLR3 is the only TLR that has a critical alanine in BOX 2 of the TIR domain instead of a proline. KIAA0644 like TLR3 has an alanine in box 2 of its TIR domain (Fig 3.8). This finding raised the possibility that KIAA0644 is a member of the TLR family.



= **LxxLxLxxN/CxL = LEUCINE RICH REPEATS**

Figure 3.6 Alignment of TLR4 and KIAA0644 Ectodomains. Sequences of KIAA0644 and TLR4's ectodomains were obtained from NCBI. Alignment of the two proteins was carried out using clustalw. Regions showing highest homology between the two sequences are highlighted in black. Leucine rich repeats can be identified and are highlighted by boxes.

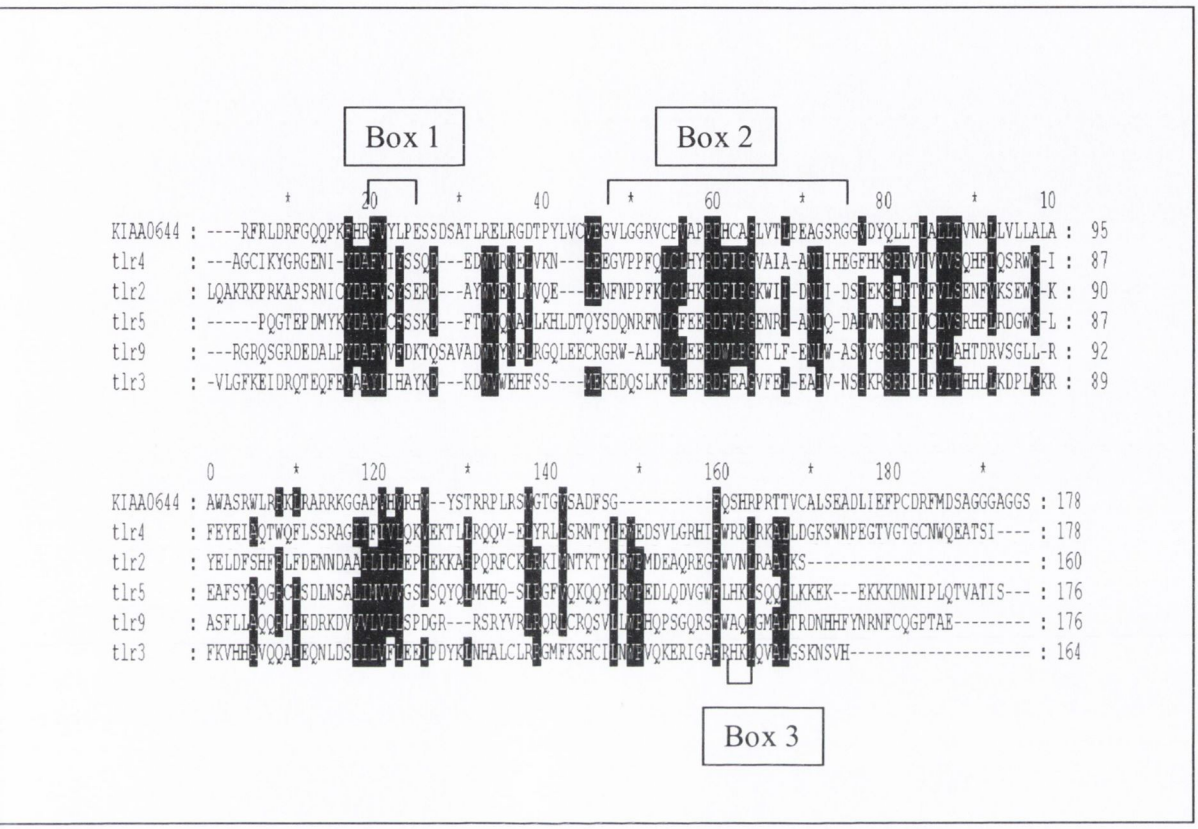


Figure 3.7 Alignment of the cytoplasmic region of KIAA0644 with other members of the TLR family. Sequences of the cytoplasmic regions of TLR 2, 3, 4, 5 and 9 and KIAA0644 were obtained from NCBI. Alignment of the cytoplasmic regions was carried out using clustalw. Exact sequence matches are highlighted in black.

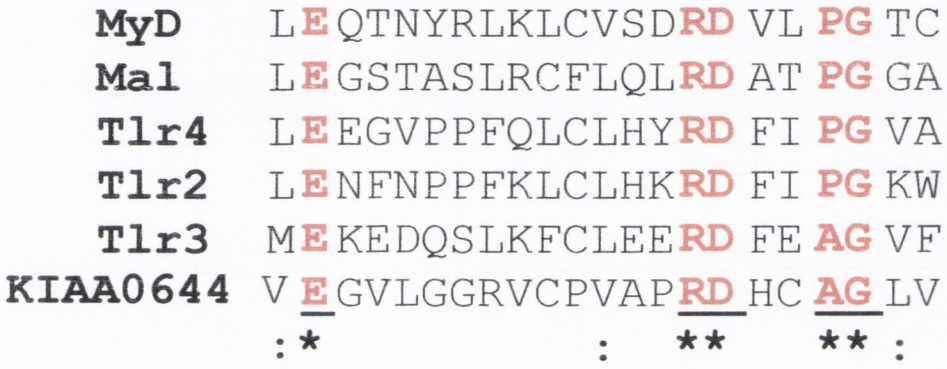


Figure 3.8 Box2 of the TIR domains of TLR3 and KIAA0644 are highly homologous. The sequences of the box 2 region of MyD88, MAL, TLR 2, 3, 4 and KIAA0644 were aligned using genedoc. The conserved amino acids within the TIR domain are highlighted in red.

3.2.6 KIAA0644 shows sequence similarity to the Nogo 66 receptor (NgR) and Fibronectin-leucine-rich transmembrane family (FLRTs)

Results obtained from Opsona Therapeutics identified a close sequence homology between KIAA0644 and members of the NgR family. This information led me to carry out a phylogentic analysis which confirmed this association. The full sequences of a number of leucine rich repeat containing proteins were inserted into the computer program PHYLIP. PHYLIP is a progam used for inferring phylogenies. This program is used to construct a phylogenetic tree grouping proteins with the highest sequence similarities. NgR is a protein which binds to Nogo 66. It is mainly present in the central nervous system (CNS) in neurons and it is involved in mediating the inhibition of axon growth. It is the NgR receptor that inhibits axon regrowth in injured adult CNS (McGee et al, 2003). The FLRT family is a group of leucine rich repeat containing proteins that are involved in early embryonic development, neurite regrowth and fibroblast growth factor signalling (Karaulanov et al., 2006). The AMIGOs are a family of proteins highly enriched in the CNS, which include Amphoterin-induced gene and ORF-1 (AMIGO-1). AMIGO-1 is believed to be involved in axon tract development (Kuja-Panula *et al.*, 2003). A LRR and Ig domain containing Nogo Receptor interacting protein I (LINGO-1) is also a member of this family and it is involved in oligodendrocyte differentiation and myelination (Chen *et al.*, 2006). As Fig 3.9 displays, KIAA0644 groups with the NgR and FLRT families, suggesting that KIAA0644 is more closely related to these families than with the AMIGO or TLR families.

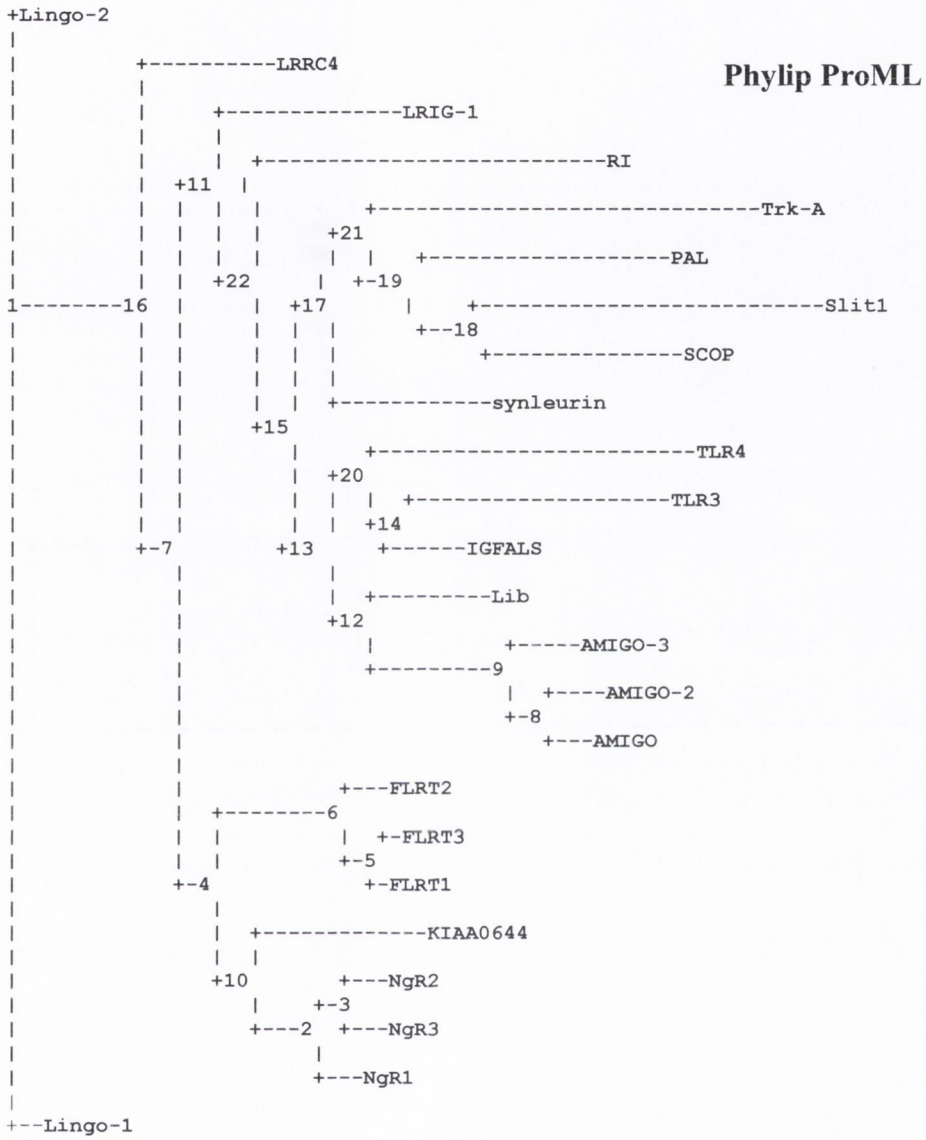


Figure 3.9 Phylogenetic tree analysis groups KIAA0644 with the Nogo receptor and FLRT families. The sequences of all the proteins displayed above were inserted into the phylip phylogenetic tree program PromL. The tree groups families or homologous proteins together into branches.

SNP ID	cSNP	Heterozygosity	Function
Rs13226391	Leu/Pro 702	N.D.	Missense
Rs13226590	Ala/Ser 679	N.D.	Missense
Rs3735562	Gly/Asp 369	0.498	Missense
Rs3735561	Ala/Thr 347	0.015	Missense
Rs740250	Asn/Ser 240	0.185	Missense
Rs34205455	Trp 274	N.D.	Frame-shift
Rs5883174	Leu 22	N.D.	Frame-shift
Rs 33979900	Arg 22	N.D.	Frame-shift

Table 3.2 Table of all annotated small nuclear polymorphisms (SNPs) in human KIAA0644. This table contains all SNPs that lead to changes in amino acid sequence or frame-shift changes. All SNPs were taken from the NCBI website (www.ncbi.nlm.nih.gov).

3.2.7 KIAA0644 contains a number of single nucleotide polymorphisms

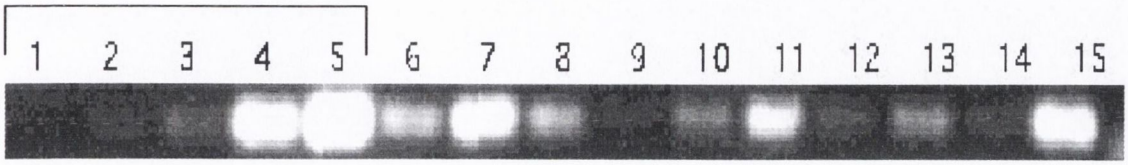
The single nucleotide polymorphism (SNP) database is available from www.ncbi.nlm.nih.gov. I searched this database for all annotated SNPs present in KIAA0644 which cause amino acid changes. Table 3.2 shows that completed list of SNPs present in KIAA0644. Many of these SNPs could potentially alter the protein structurally. The leu/Pro 702 SNP occurs in the transmembrane region, however both amino acids are neutral and non polar, therefore should not cause any structural changes. Ala/Ser 679 SNP does not occur within any domain in particular however it is a change from a non polar hydrophobic amino acid to a polar hydrophilic amino acid which could affect the protein structure. The Gly/Asp 369 SNP could be important as it occurs between LRR13 and the C terminal Cap. It is also a change from a hydrophobic to a hydrophilic amino acid. Again the Ala/Thr 347 SNP could have functional importance as it occurs just before LRR 13 and again is a change from a hydrophobic to a hydrophilic amino acid. Like the two previous SNPs the Asn/Ser 240 occurs in the leucine rich repeat region. It occurs at LRR 8, however it is not as significant a change in amino acid as both asparagines and serine are hydrophilic and therefore this would probably not affect the protein structurally.

3.2.8 KIAA0644 is highly expressed in the brain

Expression profiles for the human and murine form of KIAA0644 are available from the HUGE protein database, in which RT-PCR reactions were performed with primers targeting the 3'untranslated region of the mRNA encoding protein from this database. Expression was detected in all tissues tested with highest levels occurring in the spleen, brain and ovary (Fig 3.10 part A). RT-PCR ELISA results were also obtained from the HUGE datasabe (Fig 3.10 part B). This ELISA is a combination of RT-PCR

(A)

amplified from serial tenfold dilutions of plasmid DNA template (from 0.1 fg to 1 pg, respectively).



6 = Heart	11 =Kidney
7 = Brain	12 =Pancreas
8 = Lung	13 = Spleen
9 = liver	14 = Testis
10 =Skeletal muscle	15 = Ovary

(B)

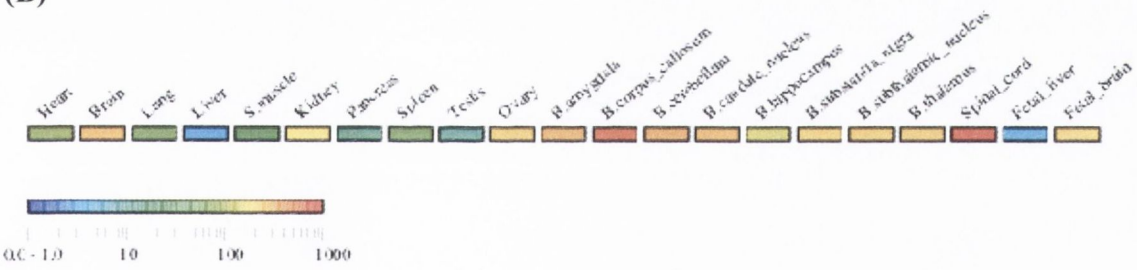


Figure 3.10 Human KIAA0644 is expressed in several tissues. RT-PCR results (part A) obtained from the Kazusa DNA Research Institute shows KIAA0644 is highly expressed in the brain (No.7), spleen (No. 13), kidney (No.11) and ovary (No.11). It is also expressed but at a much lower level in Heart (No.6), skeletal muscle (No. 10) and pancreas (No. 12). Numbers 1 to 5 are control DNA at varying concentrations, showing KIAA0644 is present in the higher concentrations of control DNA. Elisa results (part B) obtained from the Kazusa DNA research institute indicate that KIAA0644 is most highly expressed in the spinal cord and brain, specifically the Corpus Callosum region of the brain. Lowest expression is seen in the liver.

followed by the quantification of products by ELISA. The results show that KIAA0644 is highly expressed in the spinal cord and corpus callosum in the brain.

Data available from symatlas (http://symatlas.gnf.org/SymAtlas) shows expression levels of KIAA0644 in various tissue samples and in various cell lines. The results again show that KIAA0644 is highly expressed in the brain specific tissue samples such as the cerebellum and hypothalamus (Fig 3.11). KIAA0644 is also most highly expressed in brain specific cell lines such as the SHSYSY which are a brain neuroblastoma cell line (Fig 3.12).

I examined the expression profile of KIAA0644 in the mouse brain from data available from the brain atlas website (www.brain-map.org). It again shows that KIAA0644 is highly expressed in the brain, particularly in the cerebellum; cortex and hippocampus regions (Fig 3.13) and the olfactory areas and the Striatum-like amygdalar nuclei (Fig 3.14).

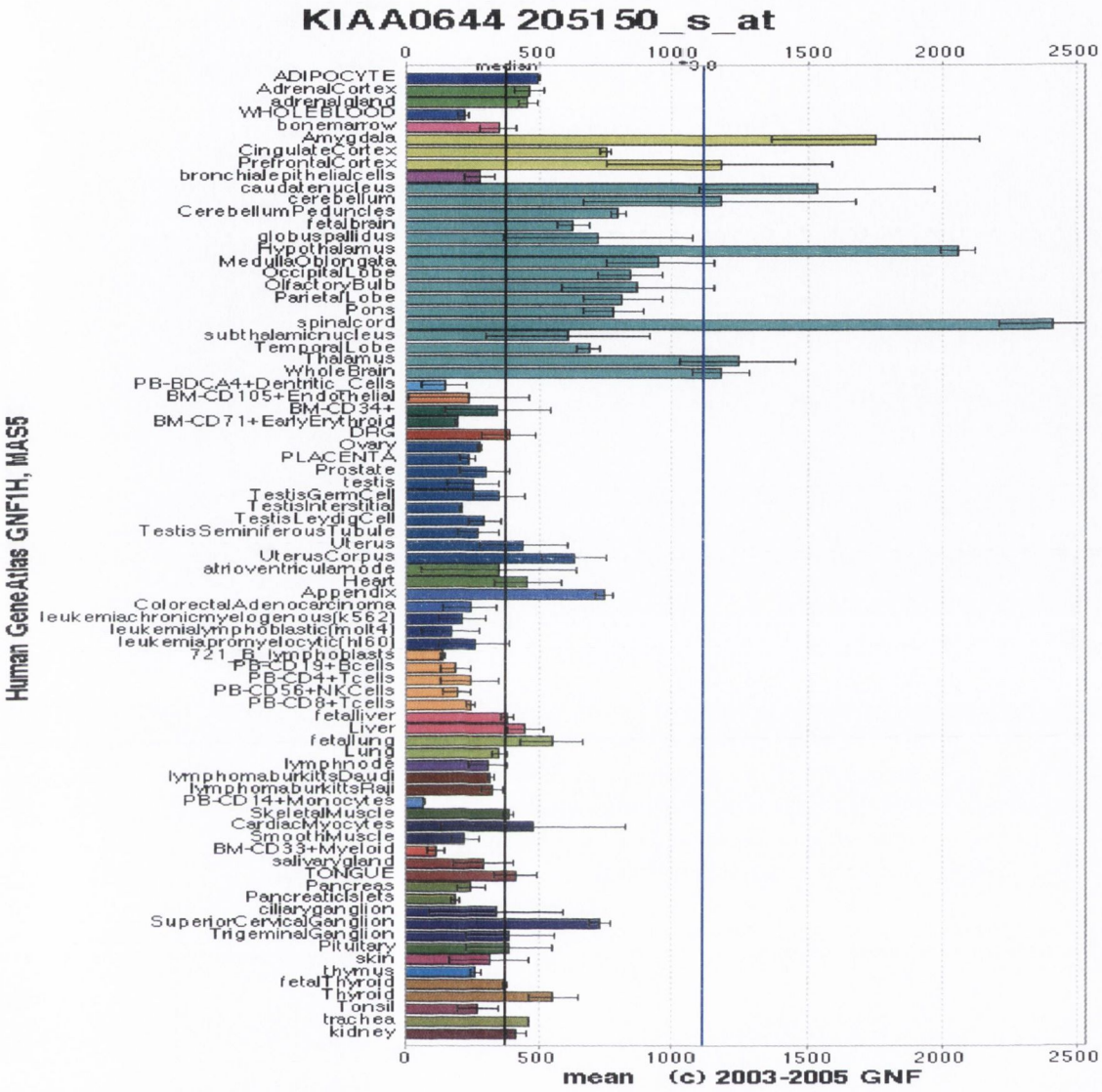


Figure 3.11 KIAA0644 is expressed in a number of tissues. Expression levels of KIAA0644 in a number of tissue samples were obtained from symathlas (http://symatlas.gnf.org/SymAtlas/). KIAA0644 is most highly expressed in brain specific tissue.

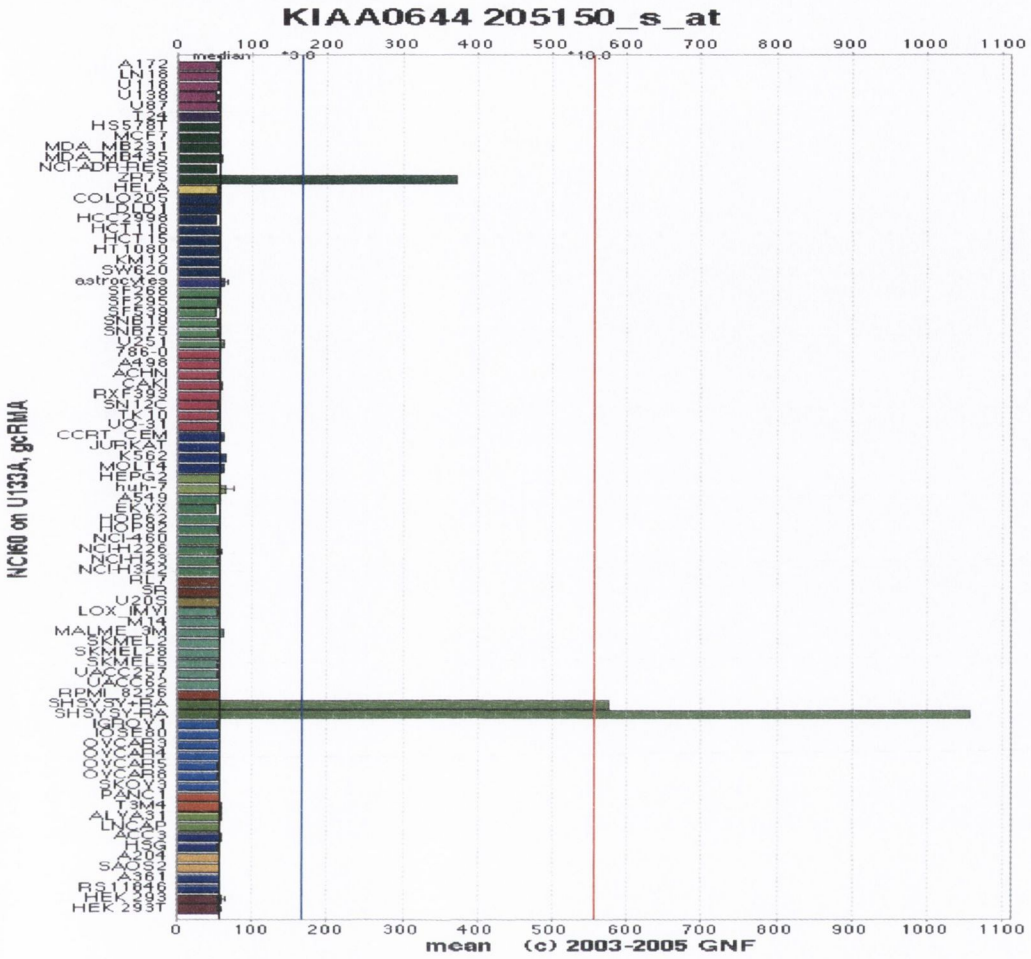


Figure 3.12 KIAA0644 is expressed in brain specific cell lines. Expression levels of KIAA0644 in various cell lines were obtained from symathlas (http://symatlas.gnf.org/SymAtlas/). Highest expression of KIAA0644 is in the neuroblastoma cell line SHSYSY.

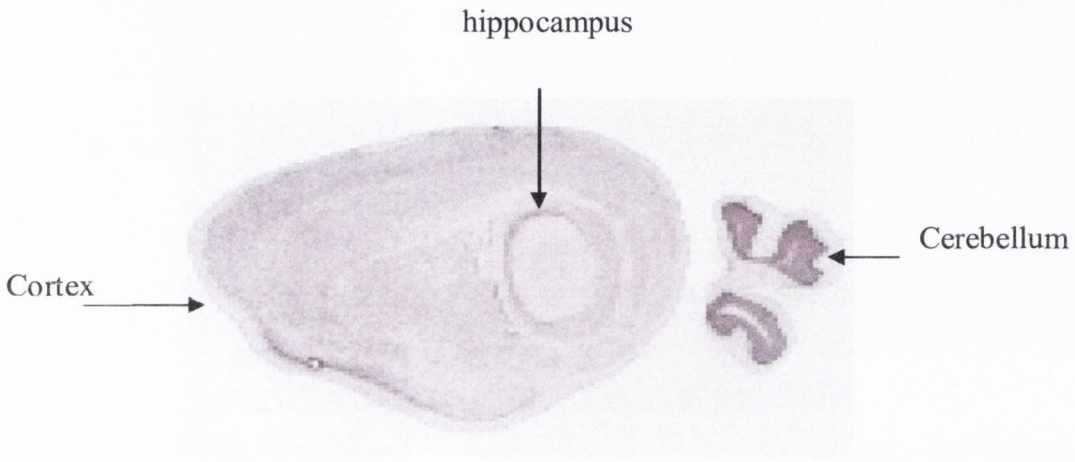
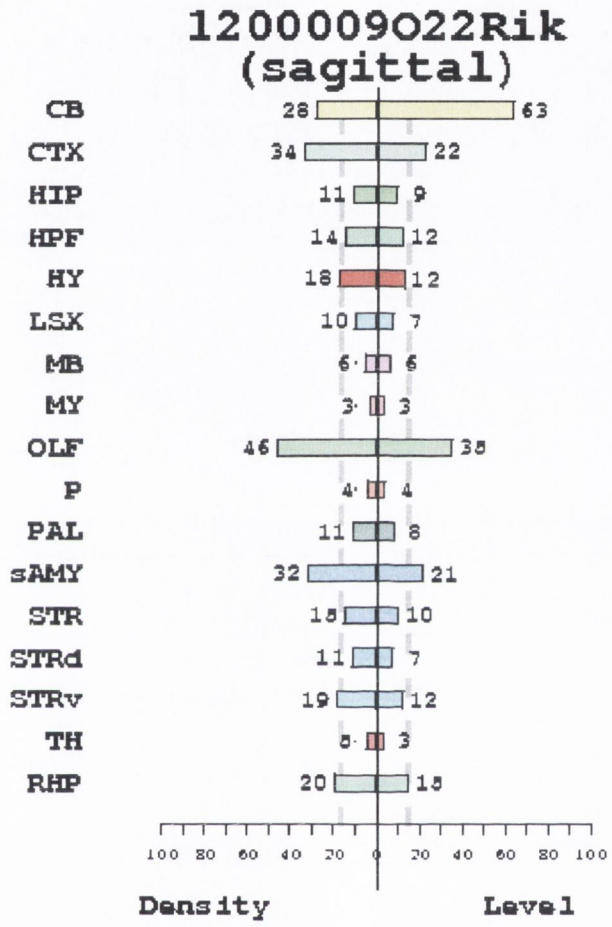


Figure 3.13 KIAA0644 is highly expressed in the brain. Data available from the brain atlas website (www.brain-map.org) shows that KIAA0644 in the mouse brain is highly expressed particularly in the cerebellum, cortex and hippocampus regions.



CB: Cerebellum
CTX: Cerebral Cortex
HIP: Hippocampal region
HPF: Hippocampal formation
HY: Hypothalamus
LSX: Lateral septal complex
MB: Midbrain
MY: Medulla
OLF: Olfactory areas
P: Pons
PAL: Pallidum
sAMY: Striatum-like amygdalar nuclei
STR: Striatum
STRd: Striatum dorsal region
STRv: Striatum ventral region
TH: Thalamus
RHP: Retrohippocampal region

Figure 3.14 KIAA0644 is highly expressed in specific regions of the brain. Data available from the brain atlas website (www.brain-map.org) shows that KIAA0644 in the mouse brain is highly expression in the cerebellum, olfactory areas and the Striatum-like amygdalar nuclei (Gene Expression Density and Level values normalized to a range of [0,100]).

3.3 Discussion

This project concerns an LPS inducible protein annotated as KIAA0644. This protein is predicted to possess 13 leucine rich repeat domains, a fibronectin domain, a transmembrane domain and a possible TIR domain. It is highly expressed in the brain and could be important for LPS action.

The LPS signalling pathway is one of the most intricate and important signalling cascades in our immune system. Understanding how the LPS signal gets transduced from the extracellular environment to within our cells is crucial for the maintenance of homeostasis. LPS is a potent immunostimulant and is the causative agent of endotoxic shock and it is estimated that over 1400 people die each day worldwide from sepsis. Therefore it is essential to fully comprehend this pathway in order to obtain treatments for such conditions as septic shock. A key breakthrough in the study of LPS signalling occurred in 1998 when it was first identified that TLR4 is the receptor for LPS (Poltorak *et al.,* 1998, Qureshi *et al.,* 1999). The initial evidence proving that TLR4 is the receptor for LPS came from a mutant mouse strain C3H/HeJ, which is hyporesponsive to LPS challenge. The mutation is a missense point mutation within the cytoplasmic region which results in the highly conserved proline within the BB loop region being substituted for a histidine (Poltorak *et al.*, 1998). Cells from mice deficient in TLR4 have also been shown to be hyporesponsive to LPS (Hoshino *et al.*, 1999).

Once TLR4 was identified as the receptor the next challenge was to understand how TLR4 functions. These studies are still ongoing and many questions still remain around this pathway. TLR4 alone cannot respond to LPS, as described in the

introduction it requires a number of accessory proteins and co-receptors such as MD2 and CD14 to allow signalling to occur in response to LPS stimulation. In addition for TLR4 to respond it recruits a number of intracellular proteins. TLR4 recruits the signalling adaptor molecules MAL, MyD88, TRIF and TRAM. MyD88 recruits the serine/threonine kinases IRAK1, IRAK2 and IRAK4, which then activate TRAF6, which in turn links the TLRs to the IKK complex which regulates NFκB activation. At the beginning of this project a preliminary microarray analysis was carried out on wild type and MAL knockout embryonic stem cells stimulated with LPS to try and identify new proteins which may have a role in LPS signalling. In total five genes were identified that showed differential expression following LPS stimulation. Four of these genes had been previously characterised to some extent therefore we decided to concentrate on a novel unidentified gene KIAA0644 which had not been previously characterised or linked to the LPS signalling pathway.

The sequence of KIAA0644 was available from the human unidentified gene database available from the Kazusa research institute. Once the sequence of the protein was identified I carried out bioinformatics analysis to try and determine as much information as possible about this gene prior to experimental work.

The first thing I identified was the chromosomal location of KIAA0644. Using the map viewer tool available from NCBI, I identified that KIAA0644 is present on human chromosome 7p15. It is flanked on either side by CREB5 or a carboxypeptidase CPLV. KIAA0644 is 4.7kb in length; it consists of a single exon and is transcribed in an anti-parallel direction. The next focus was to try and get as much information about the sequence of KIAA0644. A number of proteomic tool

searches were used from the expasy website. Signal P program identified a signal sequence at the N terminus of KIAA0644 (1-18 aa), a feature common to all membrane localized proteins. TMHMM program revealed a predicted transmembrane domain from amino acid 696-720. The motif identification program SMART indicated that there were 12 leucine rich repeats present in KIAA0644. However when the sequence was put into 3D-Jury Metaserver (1) it showed 13 leucine rich repeats as well as a fibronectin domain. When the sequence is checked by eye and the LRR domains are isolated it is clear that there are 13 leucine rich repeats. Structural homology searches using only the LRR region of KIAA0644 revealed that Lingo-1 and TLR3 showed the highest homology. The closest hits showing homology to the fibronectin domain of KIAA0644 were human contactin and neogenin. The fibronectin domain of KIAA0644 was found to be a unique sequence in that it was even identified without the significant psiblast result, suggesting that this sequence actually differs from other fibronectin domain sequences. The homology structural models of the ectodomain and the fibronectin domain of KIAA0644 were identified using the Modeller 9v3 (2) program. The fibronectin domain contains seven beta sheets and folds with a beta sandwich, or greek-key, topology and the LRR region shows a horse shoe shapped solenoid. Each LRR in KIAA0644 is 24 amino acids long except for LLR7 which contains an additional two residues in the variable region. This could be of importance to the function of the protein as LRRs which differ in length tend to be involved in such things as ligand binding.

The sequence of KIAA0644 is highly conserved across species. Sequence alignment shows how highly homologous KIAA0644 is across human, mouse and rat. The blast 2 program was used to show proteins with show highest sequence similarity to

KIAA0644. KIAA0644 in the *macaca mullata* show is 96% homology to the human protein. Other leucine rich repeat containing proteins show sequence similarity to KIAA0644 such as LLRC15, TLR4 and TLR3. Another recently identified protein Synleurin possess similar sequence to KIAA0644 using the blast 2 program. This protein is a leucine rich repeat containing protein (Wang *et al.,* 2003). It is also a single spanning transmembrane protein. Both Synleurin and KIAA0644 are quite homologous to insulin like growth factor binding proteins and TLRs. The only function so far elucidated for synleurin is that it renders cells sensitive to activation by cytokines and LPS.

KIAA0644 shows sequence homology to some of the TLR family members. Both the blast 2 program and the the 3D-Jury Metaserver (1) program show that TLR3 shares sequence and structural homology to KIAA0644. When the ectodomains of KIAA0644 are aligned with the ectodomains of the TLRs their leucine rich repeat regions align. Members of the TLR superfamily possess a cytosolic TIR domain. Within this domain three particular boxes can be identified which are highly conserved among family members. Box 1 is considered the signature sequence of the family whereas boxes 2 and 3 contain amino acids critical for signalling. Close inspection and alignment with other TLR family members reveals that KIAA0644 may contain a highly conserved box 2 and an identifiable box 1 and 3. Box 2 of KIAA0644 was also shown to be particularly similar to that of TLR3. These results provide evidence that KIAA0644 could be a member or is in some way related to the TLR superfamily. The main concern with suggesting that KIAA0644 possess a cytosolic TIR domain is that KIAA0644 contains a predicted transmembrane domain in the same region as the predicted TIR. It is possible that KIAA0644 does not contain

a TIR domain, or it could also be possible that KIAA0644 contains a transmembrane region in a different part of the protein. However the predicted explanation would be that the predicted transmembrane domain is correct and that KIAA0644 does not contain a TIR domain.

Phylogenetic analysis of a number of leucine rich repeat families indicates that KIAA0644 is more closely related to the Nogo 66 receptor family and the FLRT family compared to the AMGIO or TLR families. As mentioned previously the NgR, FLRT and AMIGO families are all highly expressed in the CNS. NgR is involved in the inhibition of axon growth. Therefore it has been studied intensely in the area of spinal injury. Many inhibitors to NgR have been investigated in the hope that this could lead to nerve regeneration in adults with CNS injury. FLRT proteins are involved in fibroblast growth factor signalling and nerve outgrowth. Therefore it is a very interesting observation that KIAA0644 associates closely with these two families. Data available from the Kazusa research institute indicates that KIAA0644 is highly expressed in the brain and spinal cord. Therefore it is possible that it has a role in signalling in neurons. A recent paper carried out an analysis of the evolutionary relationships and expression patterns of all leucine rich repeat containing family members (Dolan *et al.*, 2007). Within this paper they identify 139 LRR containing proteins in humans and group them into three families. The LRR_Ig_FN3 family, which is made up of proteins which contain LRRs and immunoglobulin domains or fibronectin domains. The FLRT and Amigo families would belong to this group. The LRR_Tollkin group is made up of the TLR family and other LRR containing proteins that do not possess TIR domains. Finally the LRR_Only group is made up of proteins that only possess TIR domains. The very interesting finding in this paper is that they

cluster KIAA0644 into the LRR_Tolkin group and not the LRR_Ig_FN3 group, even though KIAA0644 possess a fibronectin domain. Another interesting observation from this paper is that they group the protein Synleurin with the LRR_Only group. We identified Synleruin using bioinformatics as a homologous protein to KIAA0644, due to its sequence similarity across the leucine rich repeats and transmembrane domain. However this paper suggests that KIAA0644 is more closely related to the TLR family than Synleurin. Although our phylogenetic analysis groups KIAA0644 more with the NgR and FLRT families, this paper suggests that KIAA0644 is more linked to the TLR family. However it must be noted that all these findings are prediction based and therefore must be confirmed experimentally to fully understand where KIAA0644 belongs functionally. LRR proteins play a diverse functional role within the immune system. They are required for the detection of antigens (West *et al.*, 2006), they are involved in neurite outgrowth (Chen *et al.*, 2006) and cell adhesion (Karaulanov *et al.*, 2006). Recent studies have suggested that TLRs are expressed and involved in neuron functioning (Ma *et al.*, 2006). Perhaps KIAA0644 has evolved as a relative of the TLRs and NgR and FLRT families to facilitate functioning of various LRR proteins, or to carry out a role that these families are unable to do. However it must be noted that all these programs are purely prediction based and therefore need to be confirmed with more in depth experimental analysis. This information offers good background knowledge of the protein and provides hints to help with experimental design to try and confirm a function for this protein using in-vitro and in-vivo techniques.

Many genes contain single nucleotide polymorphisms (SNPs), which occur when a single nucleotide changes among members of a species. SNPs may occur within

coding regions or in non coding regions of a gene. They may also cause synonymous or non- synonymous changes to occur within the protein sequence. SNP genotyping is important as it can show risk factors within a population in association with certain diseases. The SNP within the TLR4 TIR domain helps to highlight the functional importance that SNPs can have on proteins. This single missense point mutation from a proline to a histidiene results in an inability to respond to LPS.

There are a number of SNPs present in KIAA0644. Only those which lead to amino acid changes were recorded. It is noted that a number of the SNPs lead to changes from hydrophobic to hydrophilic amino acids. Many of these SNPs could potentially lead to structural changes within the protein which may be crucial to ligand binding and signalling. These SNPs could also be important in later studies involving disease association and KIAA0644.

RT-PCR and ELISA results available from the Kazusa research institute indicates that KIAA0644 is highly expressed in the brain, spleen, kidney and spinal cord. I carried out searches on symathlas website which show expression levels of genes in tissues and cell lines. This data also confirmed that KIAA0644 is highly expressed in the brain and also in the brain neuroblastoma cell line SHSYSY. In comparison TLR4 is found mainly in whole blood, myeloid and macrophage cells. However TLR4 is found to be highly expressed in a glioblastoma cell line SNB19 and small cell lung cancer cell line Hop 92. It is interesting that the two areas where KIAA0644 is basally highly expressed are the brain and lung. TLR 4 under conditions of homeostasis is not expressed in the brain or lung or may be at a very low level. Perhaps KIAA0644 is present constitutively at a high level in brain and lung to compensate for TLR 4. Then

under conditions of extreme stress such as chronic inflammation or cancer TLR 4's expressions levels are greatly increased to combine with KIAA0644 to attempt to return the body to a state of homoeostasis. FLRT and NgR expression is mainly limited to the brain. Many of their known functions are brain specific. It is possible that KIAA0644 may posses functional abilities related to these families.

Data taken from the brain atlas website showed that KIAA0644 is highly expressed in the cerebellum, cortex and hippocampus of the brain. Other data from brain atlas showed expression was highest in the cerebellum, olfactory areas and the Striatum-like amygdalar nuclei.

The data from our preliminary microarray experiment suggested that KIAA0644 could be an LPS inducible gene. Bioinformatic analysis has shown that KIAA0644 is a leucine rich repeat containing protein which shares homology with the TLR superfamily, the nogo receptors and the FLRT receptors. KIAA0644 is highly expressed in a number of tissues around the body and specifically in the brain, lung, kidney ovaries and spleen, which is a very interesting observation. The sequence of KIAA0644 is highly conserved across species again highlighting the potential importance of this gene. All the computer based research suggests that this is a very important gene and therefore warrants more detailed experimental research to help determine a role for this novel protein within the immune system.

Chapter 4

4.1 Introduction

In the previous chapter bioinformatics analysis of KIAA0644 revealed that it is a leucine rich repeat containing protein with a fibronectin domain, a transmembrane domain and a possible TIR domain. Data available from the Kazusa research institute showed that KIAA0644 is highly expressed at the RNA level in brain, lung, heart kidney spleen and spinal cord. Other web based databases suggested that KIAA0644 is expressed in the cerebellum and olfactory areas of the brain. Overall the computer based research suggested that this is a highly novel gene which may be related to the TLR, NgR or FLRT families.

The initial identification of KIAA0644 came from a microarray analysis carried out on wild type and MAL knockout embryonic stem cells. It arose as a gene displaying differential gene expression in response to LPS. This data along with the bioinformatics analysis would suggest that KIAA0644 may be involved in the LPS and potentially the TLR signalling pathways.

TLRs are indispensable for innate recognition of microbial products (Takeda *et al.*, 2003). The subcellular distribution of TLRs is optimal to their recognition of different ligands. TLR 1, 2, 4, 5 and 6 are all expressed on the cell surface whereas TLR3, 7, 8 and 9 are expressed on endosomal membranes. The mechanisms which regulate the subcellular distribution of TLRs are poorly understood. In order for TLR4 to respond to LPS it requires MD2. One of the functions of MD2 is to increase cell surface expression of TLR4 (Shimazu *et al.*, 1999), MD2 associates with TLR4 allowing glycosylation of TLR4 to occur which then leads to increased cell surface expression

(Ohnishi *et al.*, 2003). Another newly identified protein PRAT4A has been reported to in involved in cell surface expression of TLR4 (Wakabayashi *et al.*, 2006). It is interesting to note that LPS is recognised by TLR4/MD2 complex at both the cell surface on macrophages (Akashi *et al.*, 2003) and also in the Golgi apparatus in intestinal epithelial cells (Latz *et al.*, 2002, Hornef *et al.*, 2003). The mechanism by which TLR4/MD2 resides in the Golgi or moves to the plasma membrane remain unknown. This data shows how complex the LPS signalling cascade is. Whether these distinct sites of LPS recognition lead to similar downstream responses is also unknown. In view of these findings it is clear that many mechanisms require elucidation to fully understand the LPS signalling pathways. A number of other proteins may be identified that provide a clearer role in regulating responses to LPS.

Data thus far indicates that KIAA0644 is an LPS inducible gene highly expressed in the brain. The aim of this investigation is to ultimately try and determine a function for this novel protein. Initial experiments concentrated on expression and induction of KIAA0644 in human and mouse cells. Localisation of this novel protein could also provide evidence for its role in the LPS signalling pathway.

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Chapter Four

Investigation into the expression and induction of KIAA0644

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4.2 Results

4.2.1 Expression of KIAA0644 is induced by TLR2 and TLR4 ligands

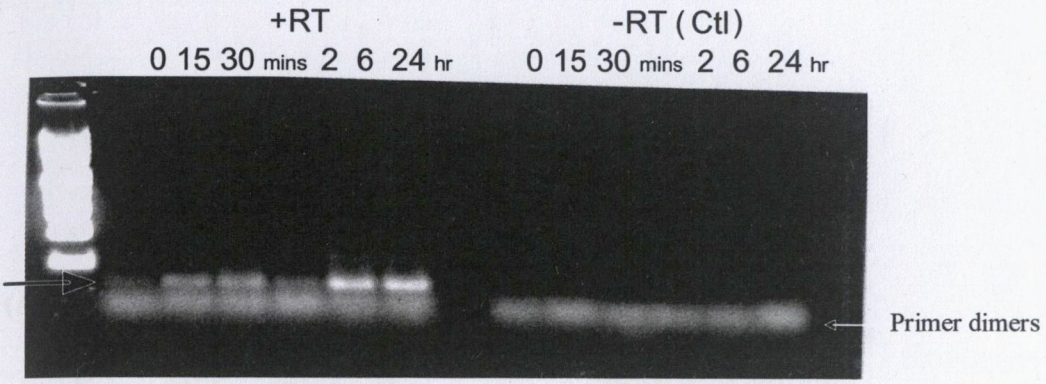
The preliminary microarray analysis carried out, which led to the identification of this protein, showed that the expression of KIAA0644 was abolished in MAL knock out cells stimulated with LPS. This would suggest that this gene could be regulated by LPS or perhaps other TLR ligands. The induction of KIAA0644 mRNA expression was analysed by RT-PCR. Bioinformatic analysis suggested that KIAA0644 is highly expressed in the brain, therefore a brain specific cell line was chosen to carry out the RT-PCR. U373/CD14 cells are a human astrocytoma cell line. Under normal conditions these cells do not express CD14. Therefore in order to make them responsive to LPS CD14 was stably transfected into them. Since these cells are highly responsive to LPS, they were chosen as an initial model system. I also carried out RT-PCR experiments on MEFs. PCR conditions were optimised and induction of KIAA0644 was observed in both cell types (Fig 4.1). In U373/CD14 cells stimulated with LPS the greatest induction of KIAA0644 was observed at 6 and 24 h. Induction of the gene in MEF cells stimulated with LPS and also with Malp2 was observed from 6 h.

4.2.2 KIAA0644 mRNA expression is induced in vivo in mouse brain treated with LPS

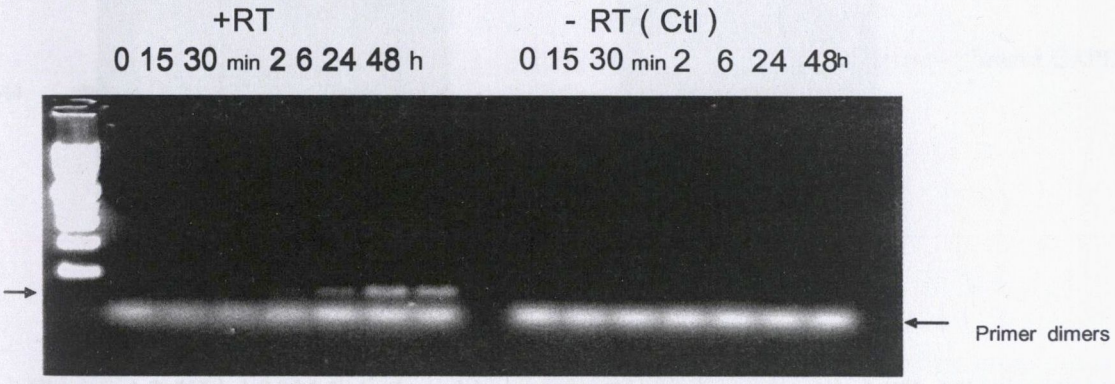
Mice received an intracerebroventricular (ICV) injection of LPS or water as a control. After 3 h the mice were sacrificed, brains were dissected and analysed for KIAA0644 mRNA by RT-PCR. As Fig 4.2 shows mRNA expression of KIAA0644 is induced upon LPS stimulation and not in the control sample.

Figure 4.1 KIAA0644 expression is induced by LPS and MALP2 in human astrocytoma cells U373/CD14 and primary mouse embryonic fibroblasts (MEF). U373/CD14 (A) and MEFs (B, C) were cultured in 10 cm dishes at a concentration of 1×10^5 cells/ml. After 24 h they were treated with 1 μg/ml LPS for the times indicated above. Expression of KIAA0644 was also measured in MEFs treated with 2nM Malp2 (C). RNA from the cell lines were used as target libraries for PCR reactions. (+RT and RT indicates with and without reverse transcriptase). These results are representative of three independent experiments.

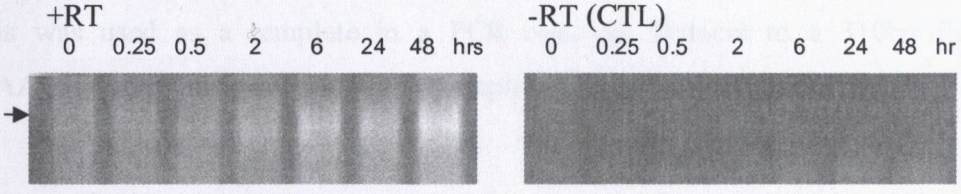
(A) U373/CD14 cells stimulated with 100 ng/ml LPS



(B) Wild type MEFs stimulated with 100 ng/ml LPS



(C) MEFs stimulated with 2nM Malp2



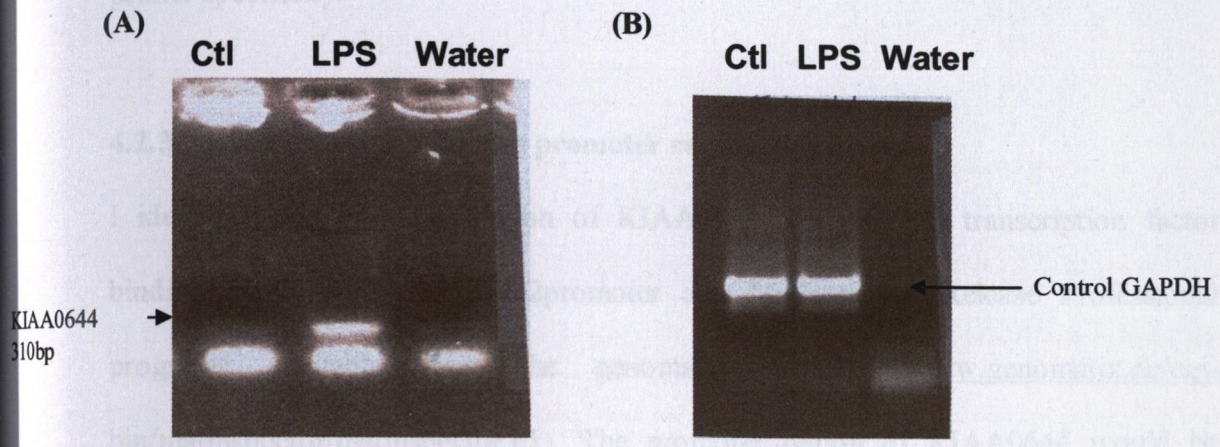


Figure 4.2 KIAA0644 is induced in mouse brain treated with LPS. Mice received an ICV injection of LPS (1μg/ml) for 3 hr. The brains were removed and homogenised in Tri reagent. RNA was extracted and converted to cDNA using an ImpromtII RT-PCR kit. This was used as a template in a PCR reaction. Primers to a 310bp fragment of KIAA0644 were designed and used to amplify KIAA0644 in a PCR reaction.

4.2.2.1 Confirmation that primers used in the RT-PCR experiments are amplifying the correct sequences

RT-PCR was carried out on MEF cells. The amplified sequence was excised and gel purified. The product was inserted into a sequencing vector (PGEMT). This was then minipreped, lyophilised and sent to MWG for sequencing. The sequence results show that the amplified region is the 310bp fragment of KIAA0644 (Fig 4.3), confirming primer specificity.

4.2.3 Characterisation of the promoter region of KIAA0644

I identified the promoter region of KIAA0644 and possible transcription factor binding sites using the Gene2promoter and MatInspector Release Professional programs available from the genomatix website (www.genomatix.de/cgi-bin/matinspector/matinspector.pl). The promoter region of KIAA0644 would be predicted to be contained within the 2 kb region proximal to exon 1. The MatInspector program revealed putative binding sites for several transcription factors within this region, such as AP2, IRF7 and Ets-1 (Fig 4.4).

Primers were designed to 3 different lengths of promoter (564bp, 1kb and 2kb) within the region 2kb next to exon 1 of the gene. These regions were amplified from genomic DNA, they were gel purified and inserted into the sequencing vector PGEMT. Once the sequences were confirmed to be correct they were subcloned into PGL3basic and PGL3enhancer vectors. The promoters were transfected into a variety of cell lines to determine their activity using the luciferase reporter assay. A number of TLR ligands were added to the cells to examine whether they could drive the activity of the promoters. HEK293TLR4 cells were transfected with a number of concentrations of the plasmids containing the promoters (10, 20, 50 and 100ng). Cells were stimulated for 24 h with LPS. Constitutive activity of each promoter was

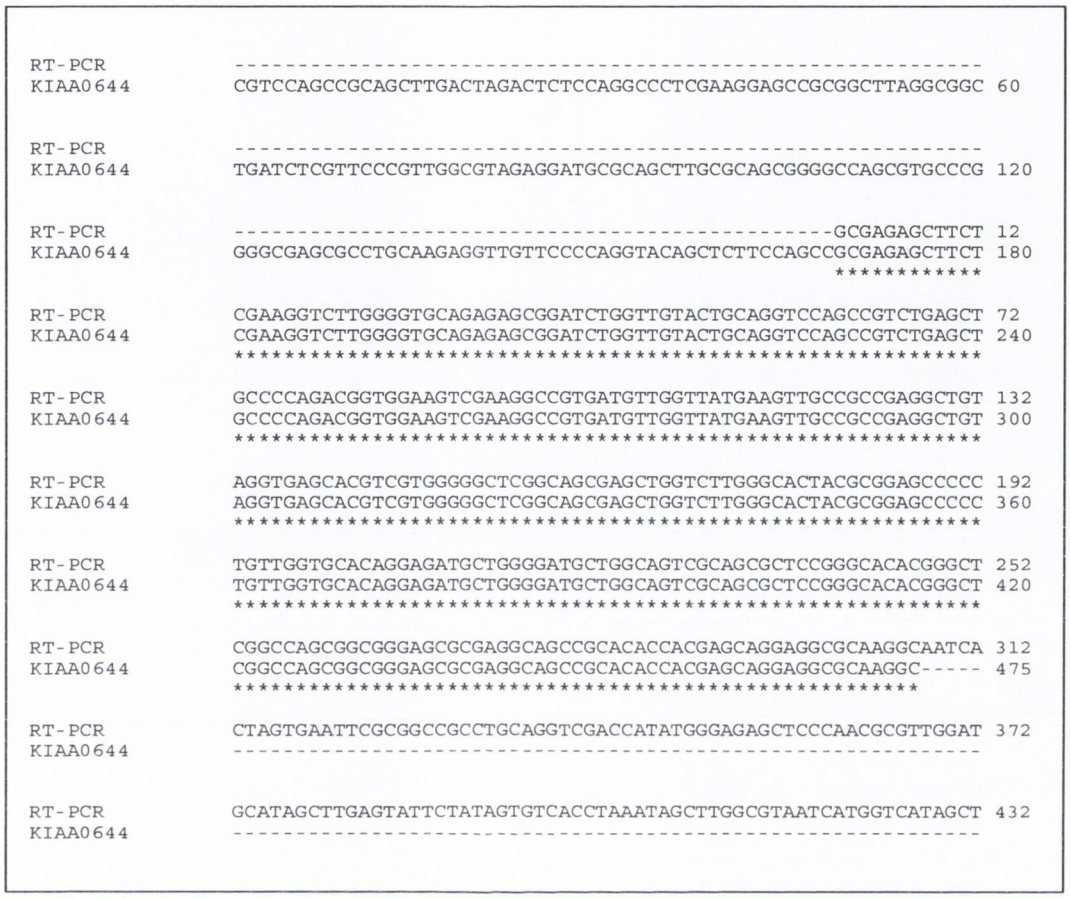


Figure 4.3 Primers used in RT-PCR experiments are specific for KIAA0644.

MEF cells were cultured in 10cm dishes at a density of 1×10^5cells/ml. They were treated with 1μg/ml LPS for 24 hr. mRNA was extracted from the cells, and used as a target library for PCR reactions. The primers designed to KIAA0644 amplified a 310bp fragment which was excised and gel purified using a Qiagen kit. 10μl of the product was inserted into a sequencing vector PGEMT. This was grown in DH5α competent cells, minipreped and lyophilised. This was sent to MWG for sequencing. The RT-PCR sample above was the sample sent to MWG for sequencing. KIAA0644 in the figure above represents the sequence for KIAA0644 available from NCBI. Both sequences are the same indicating that the primers used in this PCR are specific to KIAA0644.

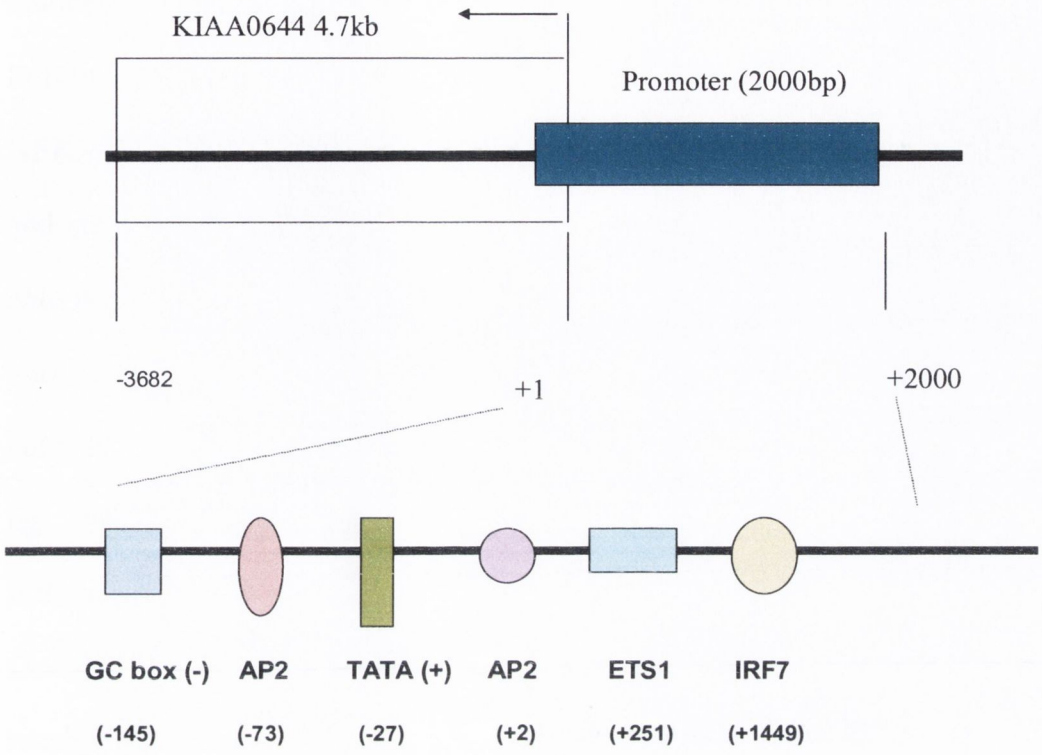


Figure 4.4 Putative Promoter Region of Human KIAA0644. The putative promoter region of KIAA0644 was identified using Promoter Inspector and Mat Inspector. All the transcription factors above have a matrix score of greater than 0.8. The matrix score measures how closely the sequences within the promoter correspond to the conserved nucleotides within the transcription factor matrix. A significant match is >0.8

observed prior to treatment with a ligand. No increase in promoter activity was observed when LPS was added to the cells. These experiments were repeated using a number of stimulation time points. A variety of TLR ligands were also investigated: PolyI:C was tested in HEK293TLR3 cells, MALP 2 and PamCys were tested in HEK293TLR2 cells. U373/CD14 cells were transfected with the promoter constructs and stimulated with a number of ligands. All results were similar to Fig 4.5 (other data is not shown). Only constitutive activity of the promoters was observed and there was no increase in activity when a ligand was added. It appears that there is less constitutive activity with the 50 ng and 100 ng quantities of plasmid compared to 20 ng. This could be due to the limitations of the assay. Perhaps these quantities are a little too high and are therefore affecting the luciferase activity of the NFκB reporter. Or this promoter may just be more active in smaller quantities. However the overall result observed in these experiments show that there is only constitutive activity of the promoters and there is no increase in activity of any quantity when a ligand is added. This was a disappointing finding however there are a number of possible explanations for the lack of stimulation over basal activity. This will be dealt with in the discussion.

4.2.4 Characterisation of the anti-KIAA0644 antibody

The predicted protein is 811aa (83kd) in length. A polyclonal antibody to a peptide sequence in the C-terminus of the protein was obtained from Eurogentec.

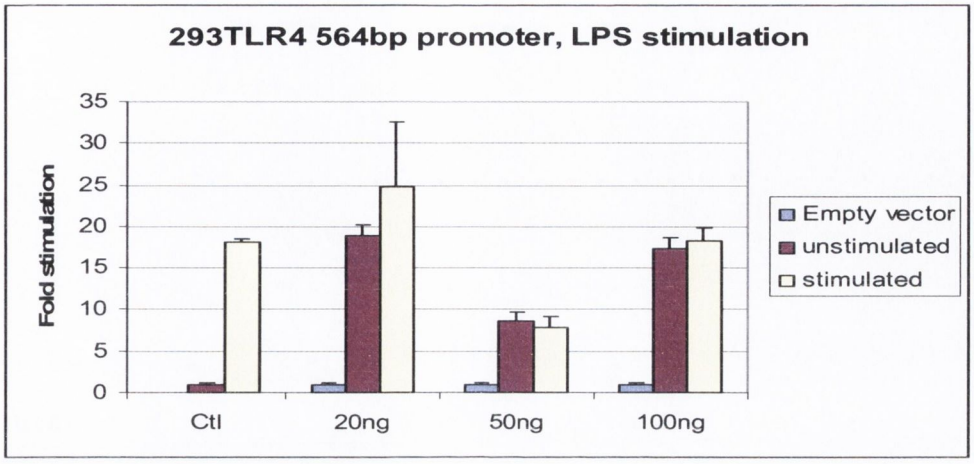
The peptide sequence to which the antibody were raised:

Amino acids 797-811: **SLRREDRLLQRFAD**

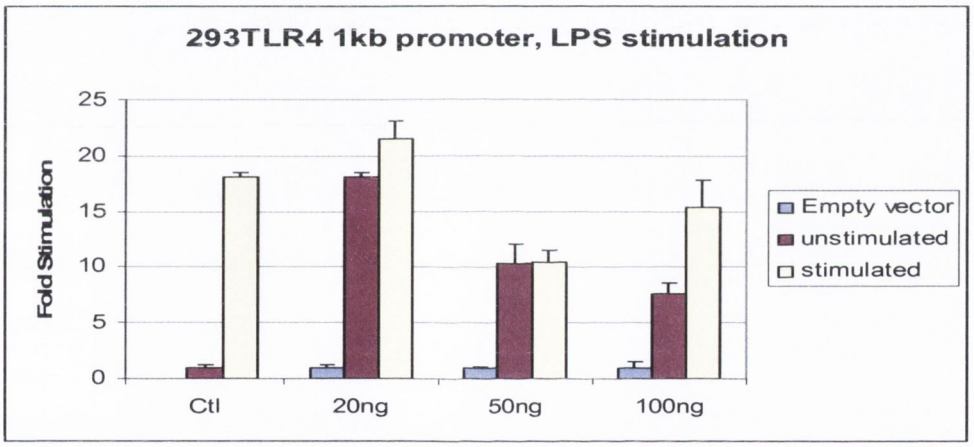
This peptide sequence is highly conserved across species (Fig 4.6) and therefore these antibodies should be capable of cross reacting and hence can be used to probe for

Figure 4.5 All 3 promoters of KIAA0644 (564bp, 1kb and 2kb) show constitutive activity in a κB luciferase assay. HEK293TLR4 cells were cultured at 1 $\times 10^5$ cells/ml in 96 well plates and cultured overnight. Cells were transfected with κB-luciferase (80 ng) and Renilla-luciferase (40 ng) together with empty vector control (Ctl) or with each promoter of KIAA0644 at the concentrations indicated above. After 24 h, cells were stimulated with LPS (1 μg/ml) for 24 h before being harvested and analysed for reporter gene activity. Results were normalised for *Renilla* luciferase activity and represented as fold stimulation over the non stimulated empty vector controls. Results are expressed as mean ± standard deviation for triplicate determinations and are representative of six separate experiments.

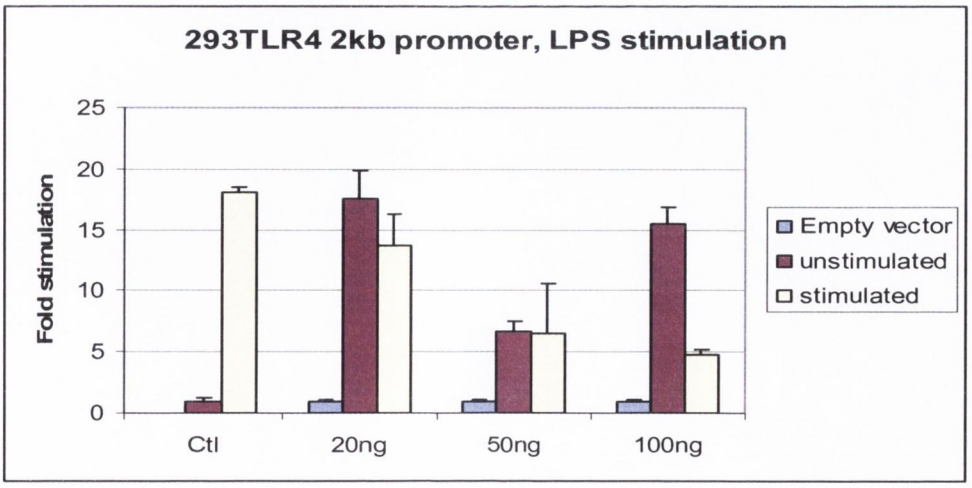
(A)


293TLR4 564bp promoter, LPS stimulation
Fold stimulation
35
30
25
20
15
10
5
0
Ctl
20ng
50ng
100ng
Empty vector
unstimulated
stimulated

(B)


293TLR4 1kb promoter, LPS stimulation
Fold Stimulation
25
20
15
10
5
0
Ctl
20ng
50ng
100ng
Empty vector
unstimulated
stimulated

(C)


293TLR4 2kb promoter, LPS stimulation
Fold stimulation
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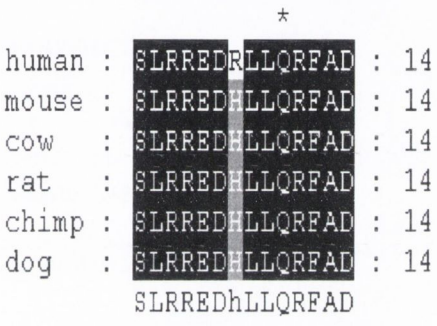


Figure 4.6 Anti-KIAA0644 antibody should cross react with a number of species. The peptide sequence to which the antibody was raised against is highly conserved across species as outlined in the Gene Doc alignment above. This antibody can therefore be used on a variety of species to detect KIAA0644.

KIAA0644 from different species. Later an affinity purified antibody generated to the same peptide sequence was obtained from 21st Century Biochemicals.

Initial experiments were carried out to determine optimum concentrations and specificity of the antibody obtained from Eurogentec. A test bleed of the anti-KIAA0644 was first obtained and characterised. Three concentrations (1:200, 1:500, 1:1000) of the antibody were tested in cell lysates of U373/CD14 cells which were non-stimulated. The antibody detected the protein at 83kd. There is quite a large nonspecific band appearing at the 63kd marker (Fig 4.7)

A booster bleed of the antibody was then obtained and characterised in the same way as the test bleed. KIAA0644 is detected at the 83kd marker. The non specific band is no longer visible at the 63kd marker (Fig 4.8). All further experiments carried out used either antibody at a dilution of 1:1000.

4.2.4.1 Mass spectrophotometry confirms presence of KIAA0644 in fetal calf serum

Mass spectrophotometry was used in order to confirm antibody specificity and cross reactivity with other species. I found that KIAA0644 is highly abundant in FCS (Fig 4.9). 1 ml of 100% FCS and 1ml of 10 % FCS was taken (diluted with sterile water) and KIAA0644 was immunoprecipitated from these samples. The immunoprecipitate was electrophorised on two 8% gels. One gel was used in a western blot and the other gel was stained with colloidal blue. The western blot result shows how highly expressed KIAA0644 is in FCS (Fig 4.9 part A). The same bands are visible on the colloidal stained gel (Fig 4.9 part B). Bands were taken from the colloidal stained gel, trypsin digested and analysed by Mass Spectrophotometry. The spots labeled KIAA0644 obtained a MOWSE score of 1.224e + 06, indicating that

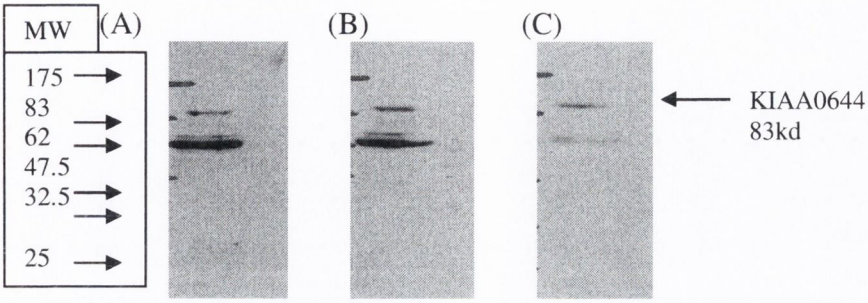


Figure 4.7 Characterisation of the test bleed of anti-KIAA0644 antibody. 1:200 dilution (A), 1:500 dilution (B), 1:1000 dilution (C) of the antibody was used to detect KIAA0644 in cell lysates of U373/CD14 cells. Samples were analysed by Sodium Dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted for KIAA0644.

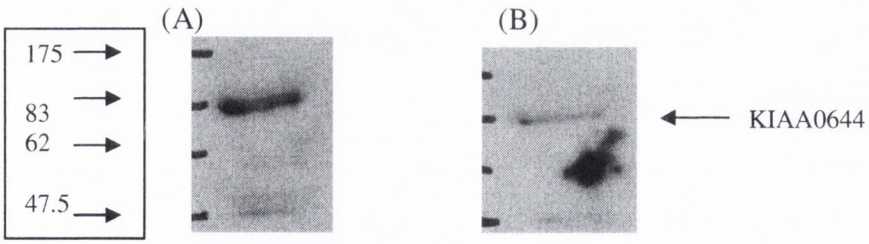
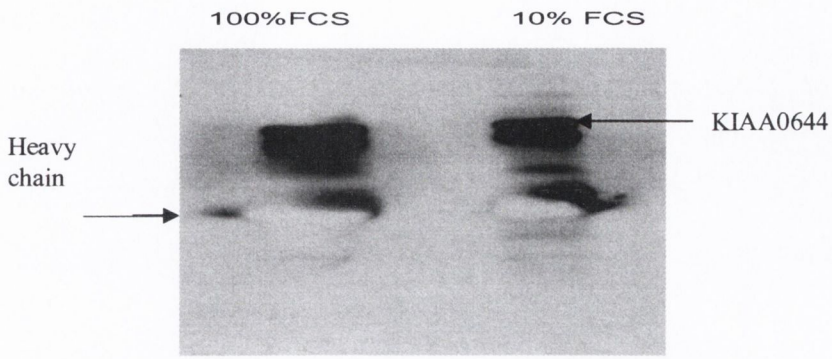


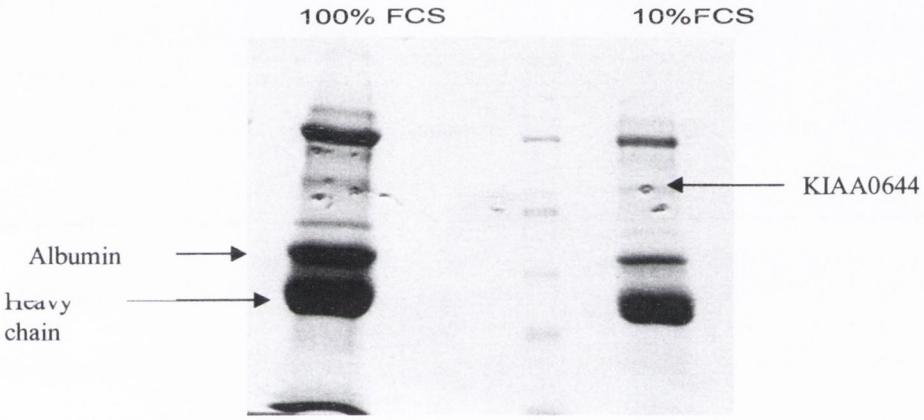
Figure 4.8 Characterisation of the booster bleed of anti-KIAA0644 antibody. 1:500 dilution (A) and 1:1000 dilution (B) of the booster bleed antibody was used to detect the protein in U373/CD14 cell lysates (non-stimulated). Samples were analysed by SDS-PAGE and immunoblotted for KIAA0644.

Figure 4.9 KIAA0644 is highly expressed in fetal calf serum and the anti-KIAA0644 antibody immunoprecipitates it. KIAA0644 was immunoprecipitated from 100% and 10% FCS. FCS samples were added to pre-coupled beads with anti-KIAA0644 antibody and incubated for 24 hr at 4°C with gentle rotation. Following incubation the immune complexes were washed three times with 1ml of high stringency lysis buffer. The beads were then resuspended in 40μl of sample buffer. Samples were resolved on two 8% SDS gels at 25mV for 1 hr. One gel was then transferred onto PVDF membrane, and immunoblotted using anti-KIAA0644 antibody (Part A). The second gel was stained with colloidal blue stain (Part B). Spots were taken from the colloidal blue stained gel tyrpsin digested and analysed by mass spectrophotometry. A graph showing trypsin digested peaks of KIAA0644 were obtained (part C). These peaks were transferred into a database Mascot and compared to the sequence database for the cow (Bos Taurus) available from NCBI. A highly significant Mowse score of 1.224e + 06 was obtained indicating that this is bovine KIAA0644.

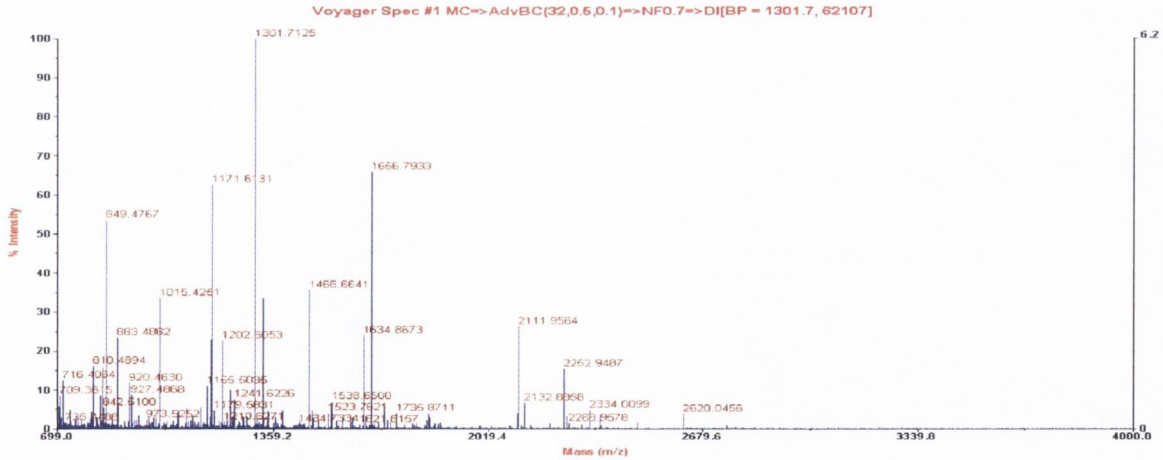
(A)



(B)



(C)



Mowse Score	% Coverage	Protein MW	Protein name
1.224e + 06	19.0	109217	Similar to Insulin like binding protein acid labile chain (Bovine KIAA0644)

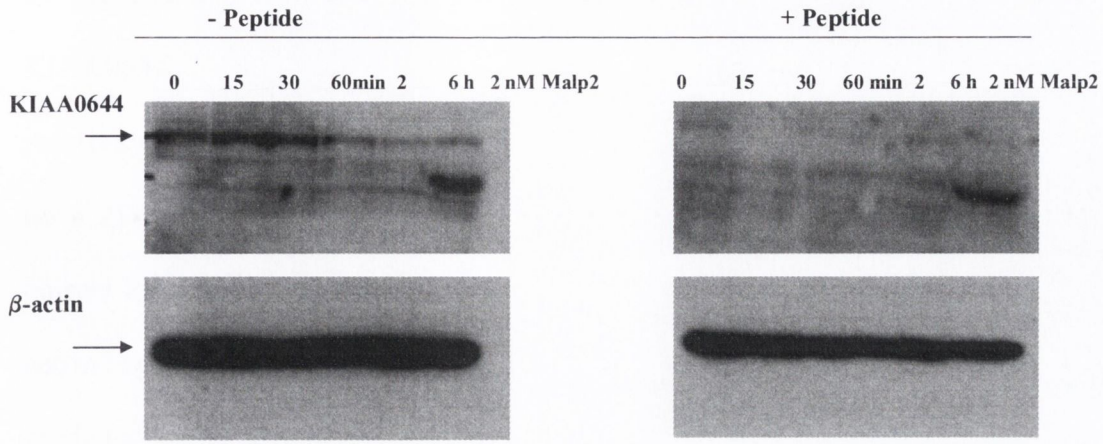
KIAA0644 is one of the most abundant proteins in the sample thus confirming antibody specificity.

4.2.4.2 Peptide competition assay confirms antibody specificity

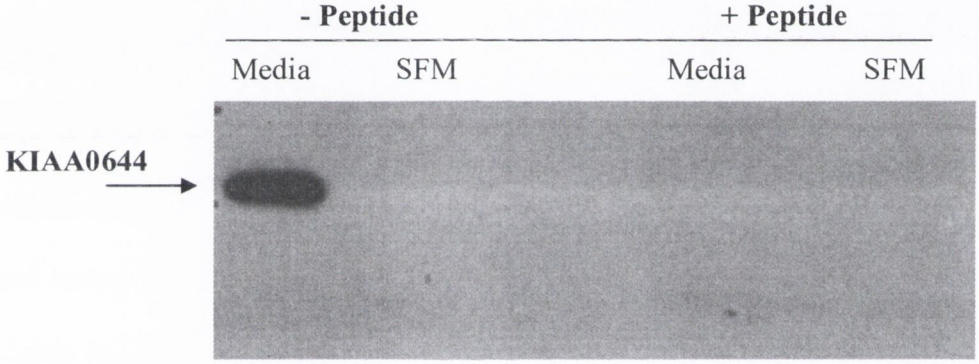
In order to further confirm antibody specificity I decided to carry out peptide competition assays. Peptides were obtained from Eurogentec and 21st Century biochemicals. The competition assay was carried out on multiple cell types and samples. In Fig 4.10 part A, the human glioma cell line A172 was tested for antibody specificity. Cells were seeded in 6 well plates and stimulated with Malp2. In Part B media containing 10 % FCS were tested for KIAA0644 expression. This was tested as I found KIAA0644 was highly expressed in FCS (Section 4.10). Serum free media was used as a control. The same protocol was used in Part A and B of Fig 4.10. Membranes were either probed directly using anti-KIAA0644 C terminal antibody (Eurogentec), or 10 μg/ml of competing peptide was first added to the primary anti-KIAA0644 C terminal antibody. In both A and B it is clear that the corresponding 83 kd band is competed out with the peptide. In part C the affinity purified antibody obtained from 21st Century Biochemicals was tested for specificity. HEK293 cells were seeded in 6 well plates and either transfected with 3 μg/ml KIAA0644 or were not transfected (NT). Samples were analysed by western blot. Membranes were probed directly with anti-KIAA0644 antibody (21st Century) or the antibody was first incubated for 30 min at 30 °C with 20 μg/ml of competing peptide. The results show that peptides can block both endogenous and over expressed KIAA0644.

Figure 4.10 Peptide competition confirms antibody specificity. In part (A) A172 glioma cells were seeded at 1×10^5 cells/ml into 6 well plates. Cells were treated with 2 nM Malp2 for the indicated time points. Cells were harvested directly into sample buffer, sonicated for 5 min and boiled at 100 °C for 5 min. Samples were electrophoresed on 10 % SDS gels, transferred to PVDF membrane and blocked in 5 % (w/v) dried milk in TBS-Tween buffer. One membrane was probed using primary KIAA0644 antibody (1/1000 dilution). The second membrane was probed using primary KIAA0644 antibody (1/1000 dilution) also containing 10 μg of competing peptide. In both cases β-actin was used as a loading control. In part B, media containing 10 % FCS and serum free media were electrophoresed on two 10 % SDS gels and again probed using primary antibody and primary antibody containing 10 μg of competing peptide. In part the antibody obtained from 21st Century antibodies was tested for specificity. HEK293 cells were seeded at 1×10^5 cells/ml into 6 well plates. Cells were either not transfected (NT) or transfected with 3 μg of KIAA0644 (TF). Samples were harvested and electrophoresed on 10 % SDS gels, transferred to PVDF membrane and blocked in 5 % (w/v) dried milk in TBS-Tween buffer. Samples were either probed directly using anti-KIAA0644 antibody, or the primary antibody was incubated at 30 °C for 30 min with 20 μg/ml of its corresponding competing peptide.

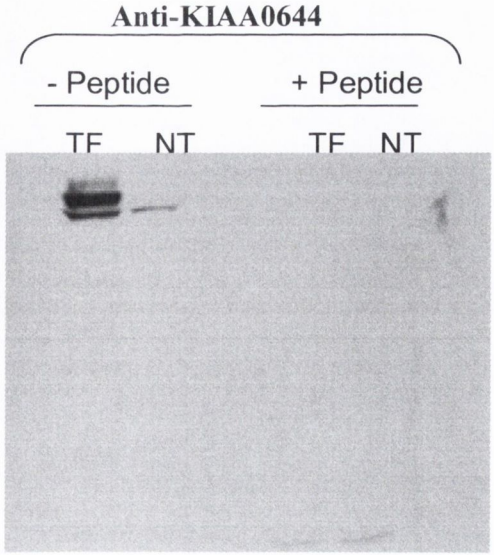
(A)


- Peptide
+ Peptide
0 15 30 60min 2 6 h 2 nM Malp2
0 15 30 60 min 2 6 h 2 nM Malp2
KIAA0644
β-actin

(B)


- Peptide
+ Peptide
Media SFM
Media SFM
KIAA0644

(C)


Anti-KIAA0644
- Peptide
+ Peptide
TF NT
TF NT

4.2.4.3 KIAA0644 has a PI of 9.7

PI prediction programs available from www.expasy.org suggest that the PI of KIAA0644 is approximately 9.7. To further confirm the antibody specificity and cross reactivity, KIAA0644 was immunoprecipitated from 100 % FCS and electrophoresed on a 2D gel to determine the PI of KIAA0644. A distinct spot is detected at the correct PI and molecular weight predicted by the PI prediction program (Fig 4.11). A second spot can be seen at the same molecular weight but at a much lower PI which is predicted to be KIAA0644 being held in the gel by a more abundant protein such albumin.

4.2.5 KIAA0644 is highly expressed in the brain

I next examined the protein expression of KIAA0644 in various tissue samples. Whole tissue protein samples were obtained, electrophoresed on a 10 % SDS page gel and tested for expression of KIAA0644 (Fig 4.12). Results show that KIAA0644 is highly expressed in the brain and lung confirming the RT-PCR results obtained from the HUGE database (Fig 3.10). It appears from the Fig 4.12 that the band expressed in the brain sample possesses a higher molecular weight than the lung sample, however this is due to the position with which the gel electrophoresed and transferred. From these tissue samples it appears that KIAA0644 is not expressed in the spleen. The RT-PCR results from the HUGE database indicated that KIAA0644 was present at the RNA level in the spleen, kidney and heart. It is possible that KIAA0644 does not get translated into protein in these tissues or it may not have been detectable due to low levels of expression.

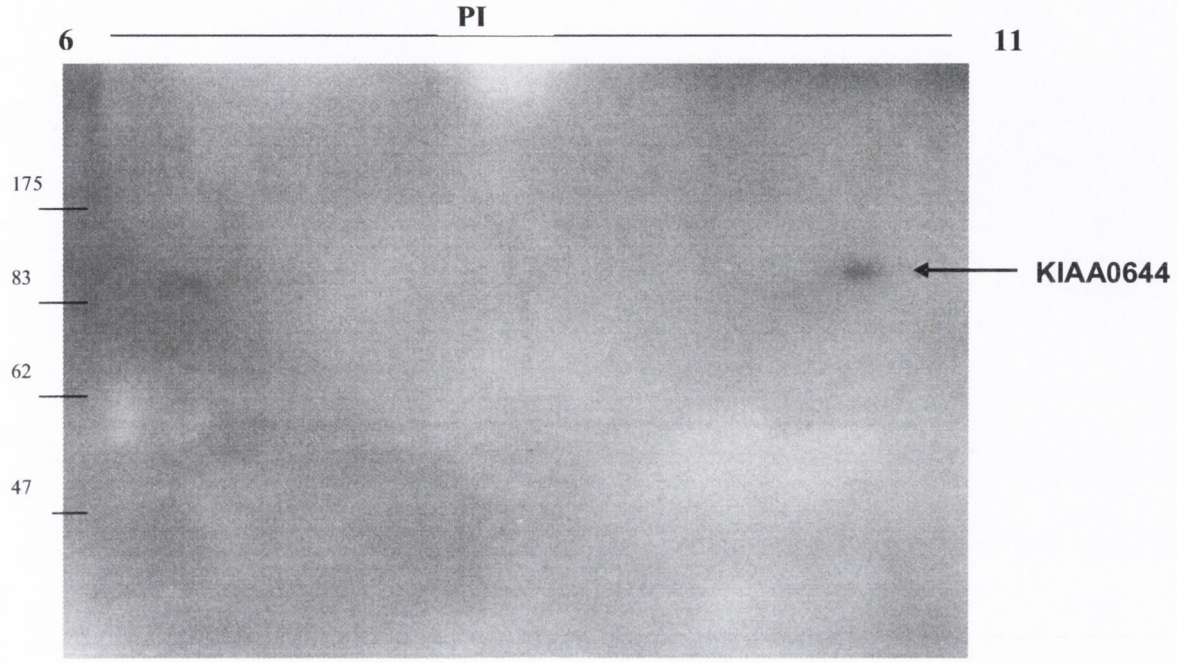


Figure 4.11 KIAA0644 present in FCS has a PI of about 9.7. KIAA0644 was immunoprecipitated from 100% FCS. FCS was added to the pre-coupled beads with anti-KIAA0644 antibody and incubated for 24 hr at 4°C with gentle rotation. Following incubation of the samples, the immune complexes were washed three times with 1 ml of high stringency lysis buffer. Beads were then resuspended in 40μl of sample buffer and focused on IPG strips with a PI from 6-11. The strip was then placed on top of an 8% SDS gel and resolved at 25mV for 1 hour. The gel was then transferred for 1.5 hr at 100mV onto PVDF membrane. The membrane was probed for KIAA0644 using the C terminal antibody. KIAA0644 appears from the figure to have a PI of 9.7.

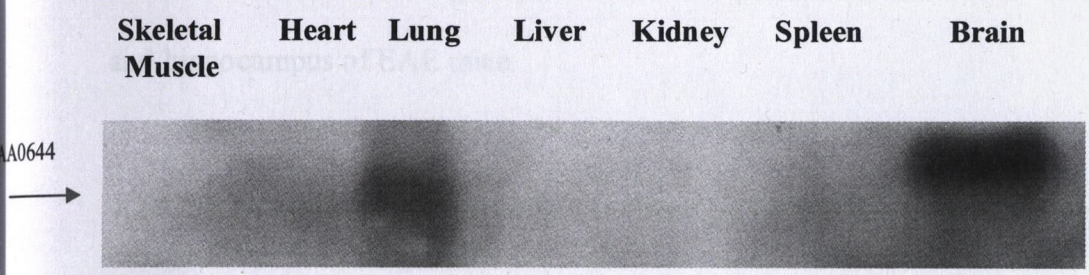


Figure 4.12 KIAA0644 is highly expressed in the brain and lung. Human tissue protein samples were obtained and analysed by SDS-PAGE. They were immunoblotted for KIAA0644. KIAA0644 is highly expressed in the brain and lung tissue samples.

4.2.5.1 KIAA0644 is expressed in the cortex, hippocampus and cerebellum of both wild type and experimental autoimmune encephalomyelitis (EAE) mice

EAE mice are an animal model of the autoimmune condition Multiple Sclerosis. All mice were sacrificed and brains were dissected out. Brains were divided into the cortex, hippocampus and cerebellum. Samples were analysed on 10 % SDS gels. Fig 4.13 shows that KIAA0644 is highly expressed in the cortex, hippocampus and cerebellum. There is a slight increase in the expression of KIAA0644 in the cortex and hippocampus of EAE mice.

4.2.5.2 KIAA0644 is expressed and induced by LPS in the human astrocytoma cell line U373/CD14

Fig 4.12 shows that KIAA0644 is highly expressed in the brain. Therefore I wanted to study a number of brain specific cell lines to determine the expression and induction of KIAA0644 in response to TLR ligands. U373/CD14 cells were used as the model to examine KIAA0644 expression and induction at the RNA level (Fig 4.1), therefore they were the first cells examined at the protein level also. Cells were seeded in 6 well plates and stimulated for a number of time points with 100 ng/ml LPS. Cells were harvested and examined for KIAA0644 expression. Fig 4.14(A) shows that KIAA0644 is most highly induced following 2 and 4 h stimulation with LPS. Previous RT-PCR results indicated that KIAA0644 gets induced after 15 min and is highest after 6 h stimulation. Perhaps it's the early induction of KIAA0644 at the RNA level that accounts for the increase at the protein level after 2 h. It appears from the protein expression that KIAA0644 returns to basal level after 24 h stimulation. Fig 4.14 (B) shows a more rapid induction of KIAA0644 in U373/CD14 cells after only 30 and 60 min stimulation with LPS. In these cells there is a much lower basal level of KIAA06444 expressed at time 0, and perhaps this allows for the induction to be

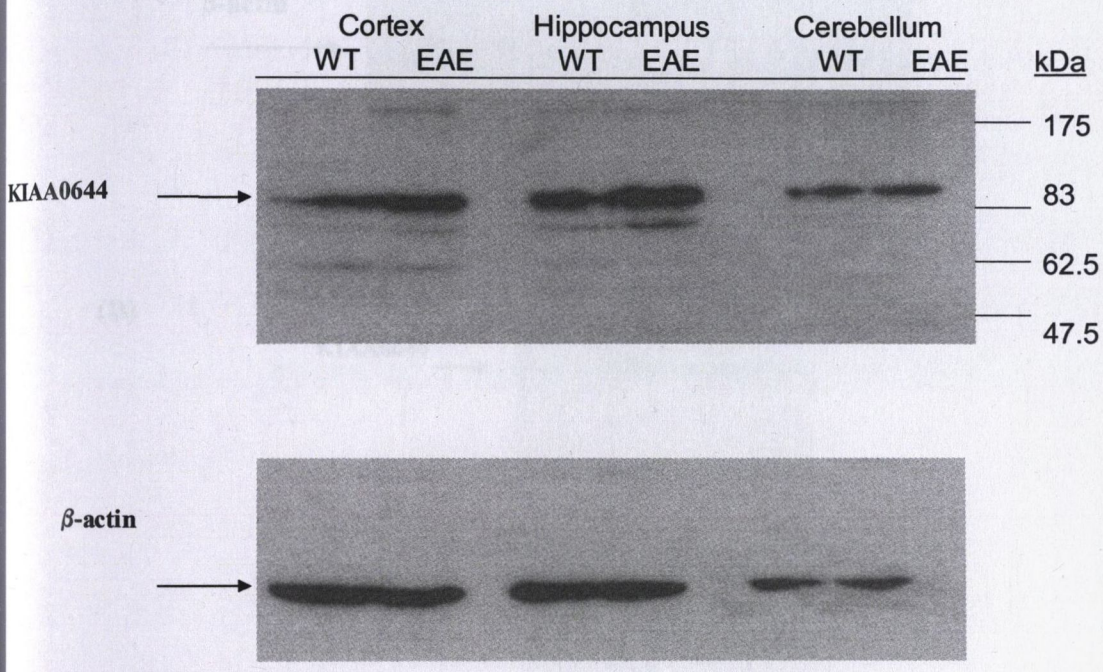


Figure 4.13 KIAA0644 is expressed in the cortex, hippocampus and cerebellum of normal and EAE mice. Brains were removed from wild type and EAE mice. They were dissected into the cortex, hippocampus and cerebellum. Samples were homogenized in Krebs buffer, analysed by SDS-PAGE and immunoblotted for KIAA0644. As a control to ensure equal protein loading expression the samples were probed for β-actin.

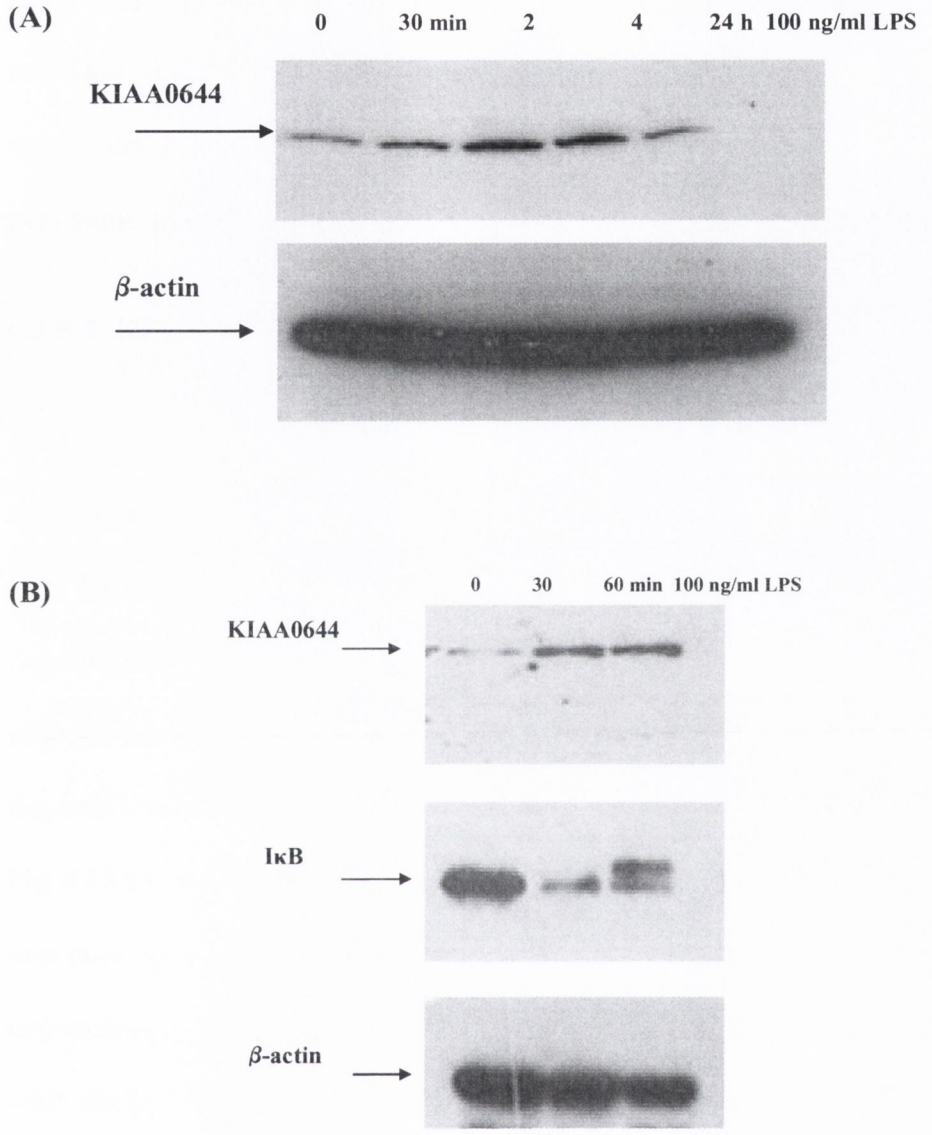


Figure 4.14 KIAA0644 is expressed and induced by LPS in the human astrocytoma cell line U373/CD14. U373/CD14 cells were seeded at a density of 2×10^5 cells/ml in 6 well plates. After 24 h they were stimulated with 100ng/ml LPS for various time points in part A and B. Samples were harvested, resolved on 10% SDS gel, transferred to PVDF membrane and probed for expression levels of KIAA0644 and β-actin in part (A), and for KIAA0644, IκB and β-actin in part (B).

observed more clearly. The IκB results in Fig 4.14 (B) shows that these cells are responding to LPS stimulation. From all results obtained from U373/CD14 cells I conclude that KIAA0644 gets induced by LPS stimulation. Depending on the experiment this can be observed as early as 30 min post stimulation or as late as 4 h post stimulation.

4.2.5.3 KIAA0644 is expressed and induced by LPS in the astrocytoma cell line 1321-N1

1321-NI cells are another astrocytoma cell line. The cells were seeded in 6 well plates and stimulated with 100 ng/ml LPS for a number of time points (Fig 4.15). Cells were then harvested and tested for KIAA0644 expression and induction and also IκB degradation. KIAA0644 is expressed at all time points. The protein appears to be induced with LPS stimulation at 15 min, 1 and 2 h (Fig 4.15). In order to confirm that this cell line is capable of responding to LPS stimulation I examined IκB-degradation. Fig 4.15 (B), shows that the cells are responding as IκB-α was degraded at 15 and 30 min post stimulation. β-actin is used as a control as it is a house keeping gene whose expression should be equal in all samples, Fig 4.15 (C). These results are consistent with those observed in the other astrocytoma cell line U373/CD14. However the induction appears to be more rapid in this cell line with KIAA0644 being induced after only 15 min. I did not test these cells at the RNA level. Perhaps they respond more rapidly in general to LPS compared to the U373/CD14 and this may account for the induction observed at 15 min post LPS stimulation.

4.2.5.4 KIAA0644 is induced in the glial cell line A172 in response to Pam3Cys4 stimulation

A172 are a brain glial cell line. Cells were cultured and stimulated with PamCys for the indicated time points. Cells were then harvested and tested for KIAA0644

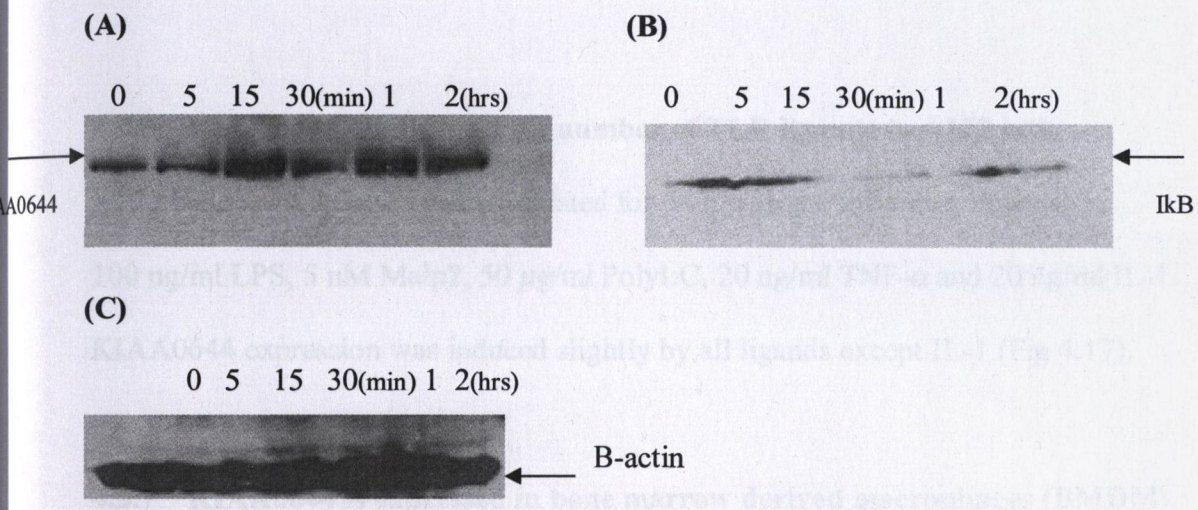


Figure 4.15 KIAA0644 is induced by LPS in 1321-N1 cells. 1321-N1 cells were seeded at a density of 2×10^5 cells/ml in 10cm dishes. After 24 hr they were stimulated with 100ng/ml LPS for various time points. Samples were harvested, resolved on 10% SDS gel, transferred to PVDF membrane and probed for KIAA0644 (A), IκB (B) and β-actin (C) expression levels.

expression and induction. The protein is present at a very low basal level at time point 0. Its expression is greatly induced at times 2, 6 and 24 h following stimulation (Fig 4.16 (A)). β-actin control shows equal protein loading (B). Interestingly KIAA0644 is appearing as a doublet in these samples. This could be due to post translational modification such as glycosylation. Since KIAA0644 only possesses one exon it is not possible for these bands to be splice variants of the protein.

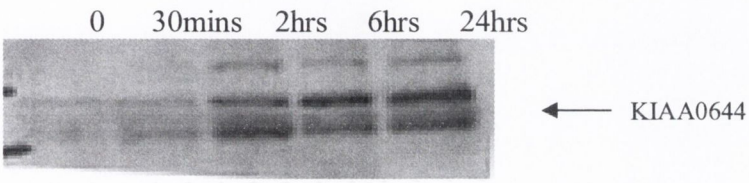
4.2.6 KIAA0644 is induced by a number of TLR ligands in A172 cells

A172 cells were cultured and stimulated for 24 h with the following ligands:
100 ng/ml LPS, 5 nM Malp2, 50 μg/ml PolyI:C, 20 ng/ml TNF-α and 20 ng/ml IL-1. KIAA0644 expression was induced slightly by all ligands except IL-1 (Fig 4.17).

4.2.7 KIAA0644 is expressed in bone marrow derived macrophages (BMDM) obtained from mice

BMDM cells respond to a number of TLR ligands, therefore they were a good model to test for KIAA0644 induction. BMDM were removed from wild type (WT) mice. Cells were cultured for ten days in DMEM supplemented with 10 % FCS and 10 % L929 supernatant in order to differentiate the cells into macrophages. Cells were then treated with 100 ng/ml LPS to determine if KIAA0644 could be induced. Fig 4.18 part (A) clearly shows that KIAA0644 is induced by LPS 3 h and 24 h post stimulation. β-actin is used as a loading control. This experiment was repeated and the cells were also stimulated with 50 μg/ml PolyI:C and 300 hau Sendai virus for 24 h. Fig 4.18 (B) shows that KIAA0644 is only induced following LPS stimulation. The loading control used for this experiment is Ponceau S stain as at this time there was no β-actin antibody available. This stain is red in colour and is used to show the most

(A)



(B)

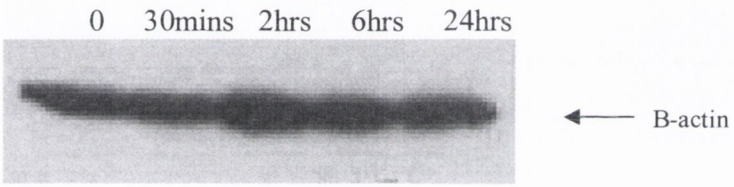


Figure 4.16 KIAA0644 is induced by PamCys in A172 cells. A172 cells were seeded at 1×10^5 cells/ml in 10 cm dishes and were stimulated with 1 μg/ml PamCys for the time points indicated. Cells were washed with PBS and 20 μl of sample buffer containing 10% mercaptoethanol was added. Samples were resolved on a 10 % SDS gel, transferred onto PVDF membrane, then incubated with anti KIAA0644, and anti β-actin respectively. This figure is representative of three separate experiments.

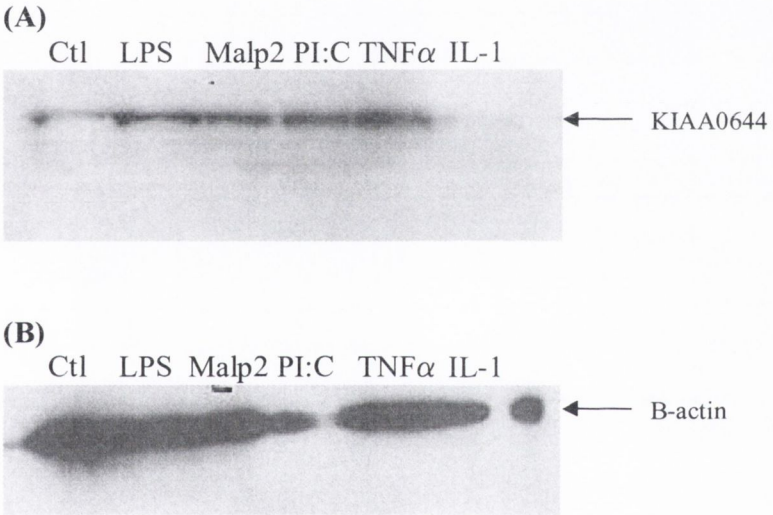


Figure 4.17 KIAA0644 is induced by a number of TLR ligands. A172 cells were seeded at 1×10^5 cells/ml and cultured overnight at 37 °C in 10 cm dishes. They were stimulated with the following ligands 100 ng/ml LPS, 5 nM Malp2, 50 µg/ml PolyI:C, 1 µg/ml TNF-α and 1 µg/ml IL-1 for 24 h. Cells were washed with PBS. 20 µl of sample buffer containing 10 % mercaptoethanol was added. Samples were sonicated, boiled for 5 min and then resolved on a 10 % SDS gel. The gel was transferred onto PVDF membrane, and incubated with anti KIAA0644 (A) and anti β-actin (B) antibodies respectively.

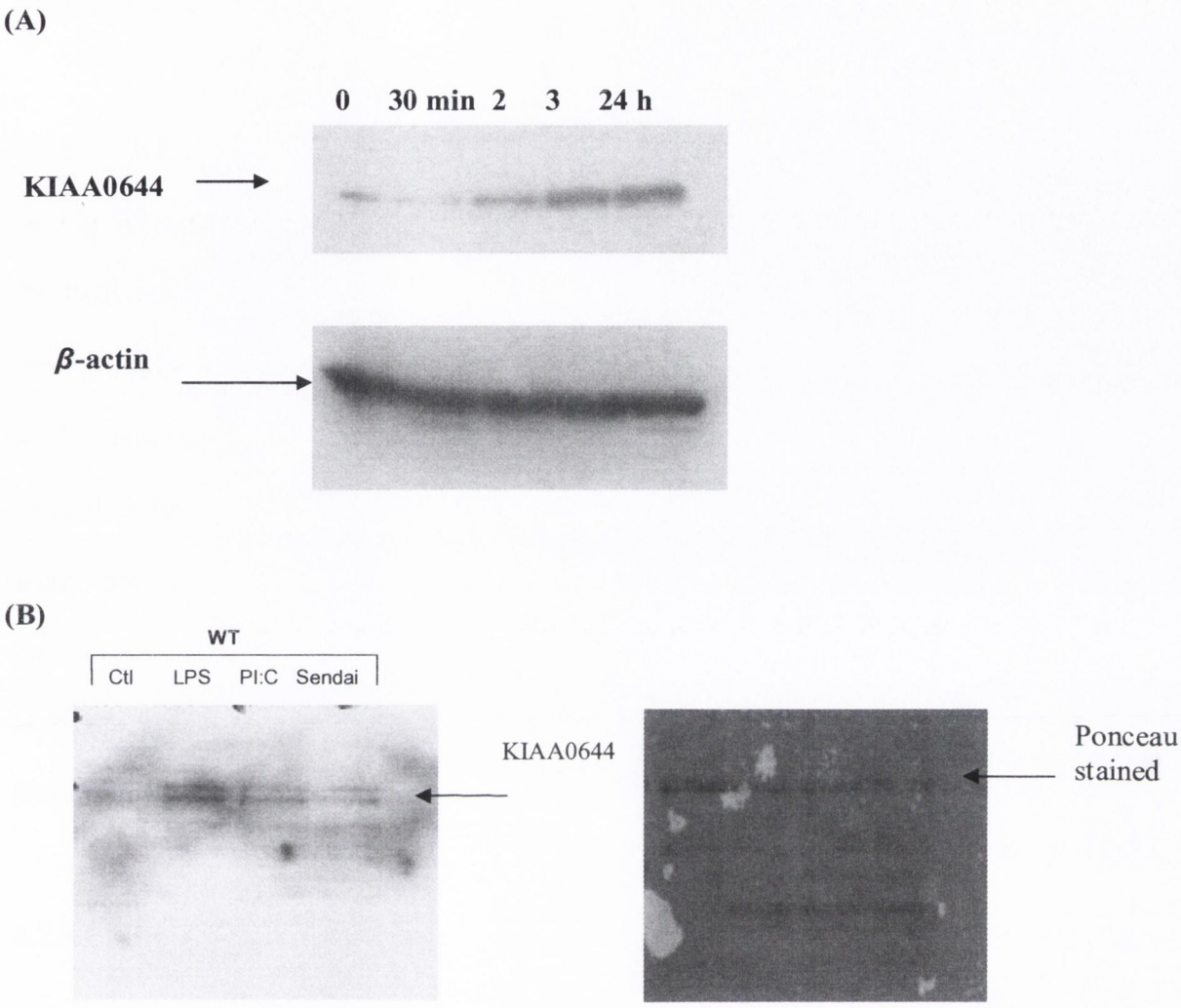


Figure 4.18 KIAA0644 expression is induced by LPS in BMDMs. BMDM cells were extracted from wild type mice. Cells were cultured in DMEM containing 10 % FCS and 10 % L929 supernatant for 10 days. Cells were seeded at a density of 2×10^5 cells/ml and cultured overnight at 37 °C in 10 cm dishes. They were then stimulated with 100 ng/ml LPS, 100 μg/ml PolyI:C, and 300 hau Sendai Virus for 24 h. Samples were resolved on a 10 % SDS gel, then transferred onto PVDF membrane and probed for KIAA0644. Ponceau S stain was added to the PVDF membranes after they were developed to confirm equal protein loading.

highly abundant proteins present on the membrane. These proteins should be present in equal amounts in each lane and can therefore act as a loading control.

4.2.8 KIAA0644 is induced by LPS in total mice brain lysates

As Fig 3.9 and 4.12 previously showed, KIAA0644 is highly expressed in the brain. RT-PCR results show that there is high mRNA expression of KIAA0644 when mice were stimulated with LPS for 3 h (Fig 4.2). I wanted to examine if this result would be the same at the protein level. Mice received an ICV injection of LPS or water as a control. After 3 h mice were sacrificed, brains were dissected and total protein lysates were obtained. KIAA0644 is induced by LPS in the protein lysates (Fig 4.19). As mentioned previously KIAA0644 is appearing as a double band in these samples. Only the upper band is being induced in these samples. Part (B) of Fig 4.19 shows there is equal protein loading in each lane.

4.2.8.1 KIAA0644 is induced by LPS in human primary peripheral mononuclear cells (PBMC)

Human primary PBMC were obtained from a healthy donor and isolated using a ficoll gradient. They were cultured in IMDM media and after 2 h they were stimulated for 6 and 24 h with the following TLR ligands, 100 ng/ml LPS, 1 μg/ml Pam Cys and 50 μg/ml PolyI:C. KIAA0644 is induced by LPS following 6 h stimulation. There are low levels of KIAA0644 present in the cells after stimulation with the other ligands at both time points (Fig 4.20).

4.2.8.2 KIAA0644 is induced after 30 min stimulation with LPS in PBMC

Human PBMC were cultured in IMDM media and after 24 h the cells were stimulated with LPS for 30 min and 60 min. As Fig 4.21 shows KIAA0644 is induced by LPS

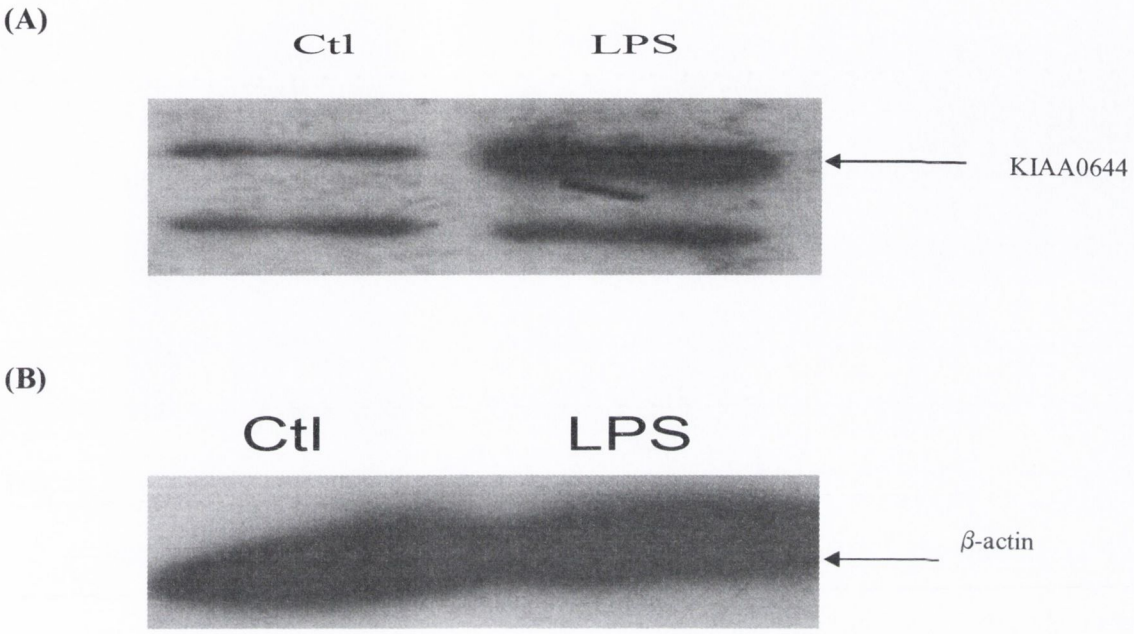


Figure 4.19 KIAA0644 is induced by LPS in vivo in mice brains. Mice were injected by ICV with 1 μg/ml LPS or water as a control. After 3 h brains were dissected and placed in krebs calcium, homogenized and sample buffer was added. Samples were resolved on 10% SDS gels, and transferred onto PVDF membrane and immunoblotted with anti- KIAA0644 and anti β-actin antibody respectively. Data shown is representative of two separate experiments.

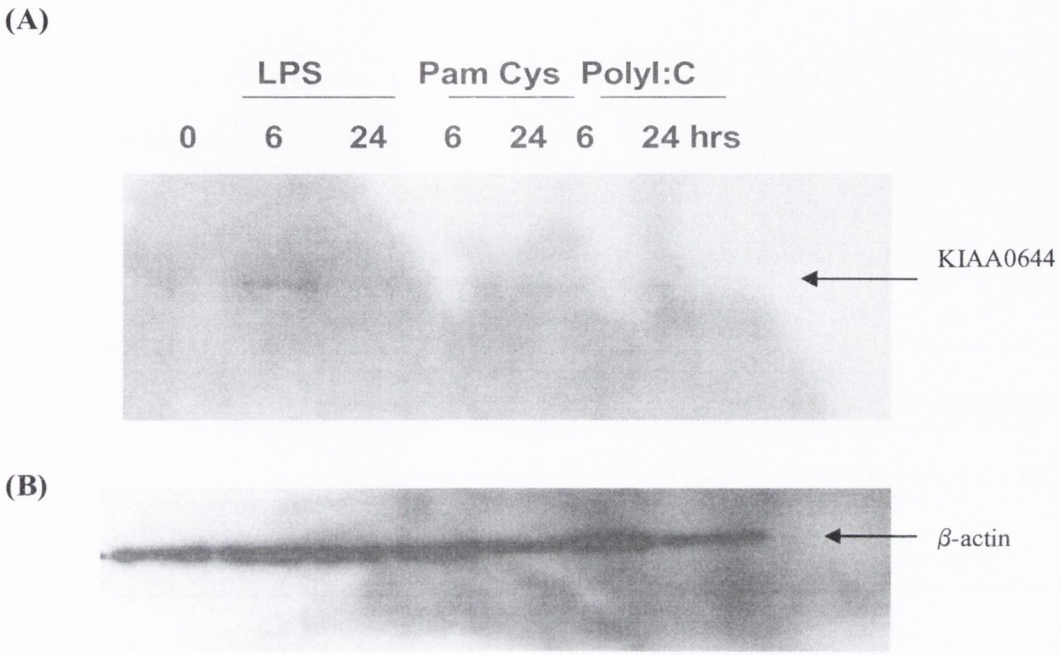


Figure 4.20 KIAA0644 is induced in PBMC. 20 ml of blood was taken from a healthy donor. PBMC were obtained from the blood using a ficoll gradient. Cells were seeded in 12 well dishes at a concentration of 2×10^6 cells/ml and cultured overnight in IMDM media containing 10 % FCS at 37 °C. The media was then changed to serum free and the cells were stimulated for 6 and 24 h with 100 ng/LPS, 1 μg/ml Pam Cys and 50 μg/ml PolyI:C. Cells were lysed in 100 μl of total cell lysis buffer for 30 min. Supernatant was transferred into a fresh tube and 25 μl of sample buffer containing mercaptoethanol was added. Samples were resolved on 10 % SDS gels, and transferred onto PVDF membrane and immunoblotted with anti KIAA0644 and anti β-actin antibodies respectively. Data shown is representative of two separate experiments

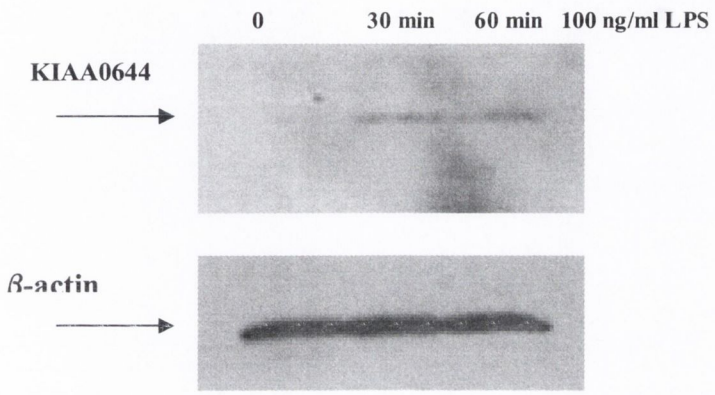


Figure 4.21 KIAA0644 is induced by LPS in human PBMC. 20 ml of blood was taken from a healthy donor. PBMCs were obtained from the blood using a ficoll gradient. Cells were seeded in 12 well dishes at a concentration of 2×10^6 cells/ml. After 24 h the cells were stimulated for 0, 30 and 60 min with 100 ng/ml LPS. Cells were lysed in 100 μl of total cell lysis buffer for 30 min. Samples were resolved on 10% SDS gels, and transferred onto PVDF membrane and immunoblotted with anti KIAA0644 and anti β-actin antibodies respectively. Data shown is representative of three separate experiments

after 30 and 60 min LPS stimulation. This is a very rapid induction of the protein. These cells may be responding better to LPS stimulation and hence allowing more rapid induction of KIAA0644. Also there appears to be very little KIAA0644 expressed basally making the induction easier to observe.

4.2.8.3 KIAA0644 is induced by LPS but not by TNF-α in PBMC

Human PBMC were cultured in IMDM media. After 24 h the cells were stimulated with LPS or TNF-α. As Fig 4.22 shows KIAA0644 is induced following LPS but not TNF-α stimulation. In these samples KIAA0644 is appearing as a doublet, where as it was only a single band in Figs 4.20 and 4.21. This has also been observed in other cells such as U373/CD14 cells. In Fig 4.22 the upper band is weaker than the lower band, however both bands get induced following LPS stimulation. These bands could be the result of post translational modification such as glycosylation.

4.2.8.4 KIAA0644 is induced by LPS in THP1 cells

THP1 cells are a human monocytic cell line. They were cultured in RPMI and after 24 h were stimulated with LPS for a number of time points. Fig 4.23 shows KIAA0644 is highly induced after 30 min stimulation with LPS. This induction is consistent with that observed in PBMC and U373/CD14 cells. The only difference is that it appears that in THP1 cells the levels of KIAA0644 return to normal following 60 min stimulations with LPS. However it must be noted that when this experiment was carried out on other occasions the levels of KIAA0644 can stay increased over time, but the consistent finding is that KIAA0644 gets induced by LPS approximately 30 min post stimulation.

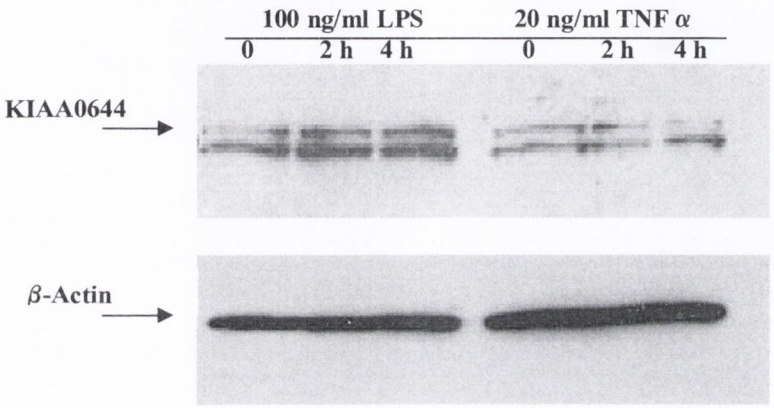


Figure 4.22 KIAA0644 is induced by LPS but not by TNF-α in human PBMCs. 20 ml of blood was taken from a healthy donor. PBMCs were obtained from the blood using a ficoll gradient. Cells were seeded in 12 well dishes at a concentration of 2×10^6 cells/ml and cultured overnight in IMDM media containing 10 % FCS at 37 °C. Cells were stimulated for 2 and 4 h with 100 ng/ml LPS or 20 ng/ml TNF-α. Cells were lysed in 100 μl of total cell lysis buffer for 30 min. Samples were resolved on 10 % SDS gels, and transferred onto PVDF membrane and immunoblotted with anti KIAA0644 and anti β-actin antibodies respectively. Data shown is representative of three independent experiments.

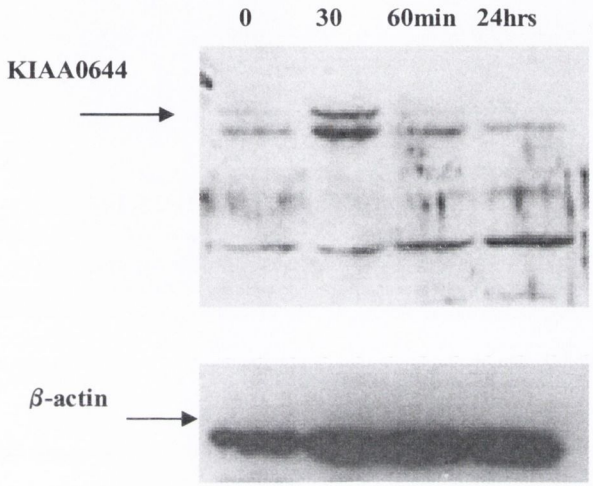


Figure 4.23 KIAA0644 is induced by LPS in THP1 cells. THP1 cells were set up at 1×10^6 cells/ml in RPMI supplemented with 10 % FCS. After 24 h the cells were stimulated with 100 ng/ml LPS for the indicated times. Cells were lysed in RIPA buffer, normalized for protein expression and resolved on 10 % SDS gel. Samples were transferred to PVDF membrane and probed for expression of KIAA0644 and β-actin respectively.

4.2.8.5 KIAA0644 is induced by LPS in primary mice mixed glial cells

Mixed glial cells (70% astrocytes, 30% microglial cells) were obtained from 1 day old neonatal mice. Cells were cultured in 6 well plates for 10 days. They were then stimulated with LPS for a number of time points and analysed by western blot. Fig 4.24 clearly indicates that KIAA0644 can be induced by LPS after 30 min. The strongest induction of KIAA0644 occurs after 6 h LPS stimulation.

4.2.9 KIAA0644 is a cytosolic protein

Membrane fractionation experiments were carried out in order to determine the localisation of KIAA0644 within the cell. Initial localisation experiments were carried out on U373/CD14 cells. Membrane fractionation of the cells was carried out and this separated the sample into membrane (pellet) and cytosol (supernatant). KIAA0644 is clearly present in the cytosolic fraction. It is also induced following 2 h stimulations with LPS (Fig 4.25 (A)). The same blot was reprobed with an anti β actin antibody to show equal protein loading (Fig 4.25 (B)).

A control experiment was then set up to ensure that the protocol for membrane fractionation studies was correct. The expression of a known membrane bound protein epidermal growth factor receptor (EGF- receptor) was investigated to ensure that it is appearing only in the membrane fraction. Fig 4.26(A) shows that the protein is only present in the membrane. However an unexpected and interesting finding from this experiment was that the EGF receptor is clearly being induced following LPS stimulation.

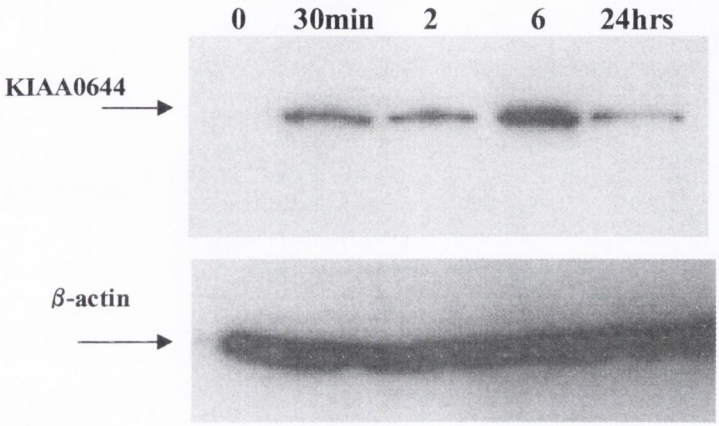
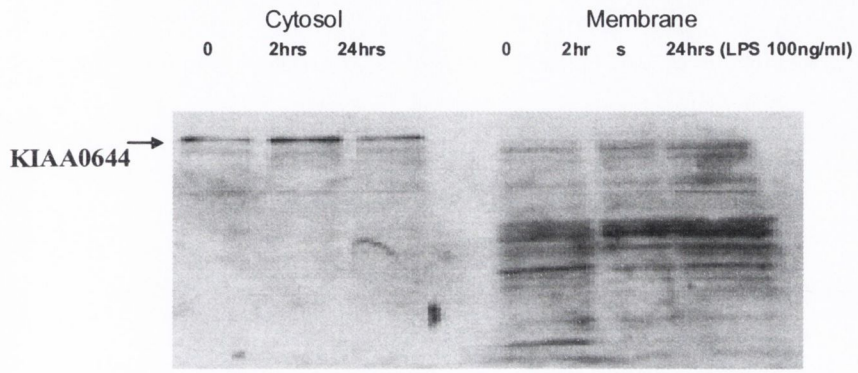


Figure 4.24 KIAA0644 is induced by LPS in primary mouse mixed glial cells.
Primary mixed glial cells were obtained from the brains of 1 day old neonatal mice. Each time point is representative of one mice brain. Cells were set up in 6 well plates and cultured for 10 days, then stimulated with 100 ng/ml LPS for the indicated time points. Cells were lysed in sample buffer and resolved on 10 % SDS gels. They were transferred onto PVDF membrane and immunblotted for KIAA0644 and β-actin.

(A)



(B)

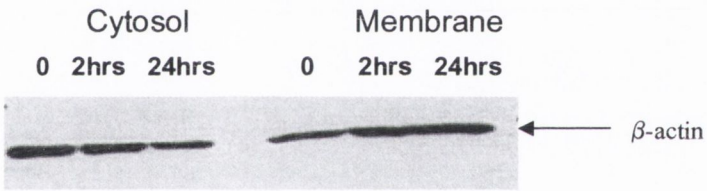
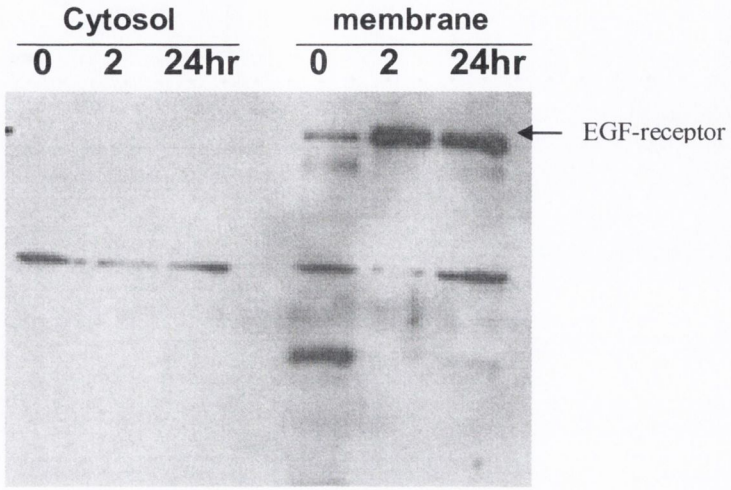


Figure 4.25 KIAA0644 is present in the cytosol and is induced by LPS in U373/CD14 cells. U373/CD14 cells were seeded in 10 cm dishes at 2×10^5cells/ml and cultured overnight in DMEM media containing 10 % FCS. Cells were stimulated with 100 ng/ml LPS for 2 and 24 h. Samples were lysed in 500 μl of membrane lysis buffer. Samples were centrifuged at 97,000 × *g* for 1 h. This separates the sample into a cytosolic fraction (supernatant) and the membrane fraction (pellet). Samples were resolved on a 10 % gel, and transferred onto PVDF membrane and immunoblotted with an anti-KIAA0644 antibody and β-actin antibody. This result is representative of three independent experiments.

(A)



(B)

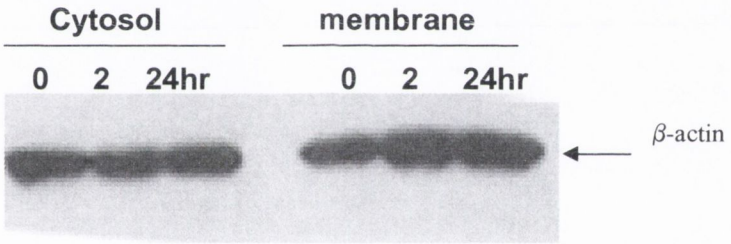


Figure 4.26 EGF- receptor is a membrane bound protein and it only appears in the membrane fraction. U373/CD14 cells were seeded in 10 cm dishes at 2×10^5cells/ml and cultured overnight in DMEM media containing 10 % FCS. Cells were stimulated with 100 ng/ml LPS for 2 and 24 h. The media was aspirated off and the cells were washed (3 times) with PBS and then lysed in 500 μl of membrane lysis buffer. Samples were centrifuged at 97,000 × *g* for 1 h. The sample was separated into a cytosolic fraction (supernatant) and the membrane fraction (pellet), 125 μl of sample buffer was added to the cytosolic fraction and 80 μl was added to the membrane fraction. The membrane fraction was sonicated and both fractions for centrifuged for 2 min at 1000 × *g* and boiled for 5 min. Samples were resolved on a 10 % gel, and transferred onto PVDF membrane and immunoblotted with anti-KIAA0644 and anti β-actin antibody respectively.

4.2.9.1 Transiently over-expressed KIAA0644 localises to the membrane

U373/CD14 cells were transfected with 4.5 μg of plasmid encoding KIAA0644. After 24 h the cells were stimulated with 100 ng/ml LPS for 2 h. Membrane fractionation was carried out on the samples and they were analysed by western blot. Fig 4.27 shows that although there is KIAA0644 present basally in the cytosolic fractions, all the transfected KIAA0644 is localising to the membrane fraction. This could be an artifact of over expression, or perhaps endogenous KIAA0644 can localize to small undefined membranes, therefore when a large amount of KIAA0644 is expressed it all moves into these membranes and is centrifuged down with the membrane fraction.

4.2.10 The C terminus of KIAA0644 is present inside the cell

The KIAA0644 antibody was raised to the C terminus of the protein (Fig 4.6). It is predicted that KIAA0644 has a transmembrane region (Fig 3.2) therefore we would expect that the C terminus of the protein will be present inside the cell. To confirm this I carried out confocal microscopy on non-permeabilised cells to determine if KIAA0644 could still be detected. U373/CD14 cells were seeded in 6 well plates containing poly-L-lysine coated coverslips. After 24 h the samples were probed with anti-KIAA0644 antibody and an alexflour-488 conjugated secondary antibody. The coverslips were mounted on slides and observed under the confocal microscope. As Fig 4.28 shows there is no staining of KIAA0644 confirming that the C terminus of KIAA0644 is inside the cell and can only be observed when the cells are permeablised. Therefore for all future confocal experiments the cells had to be permeablised.

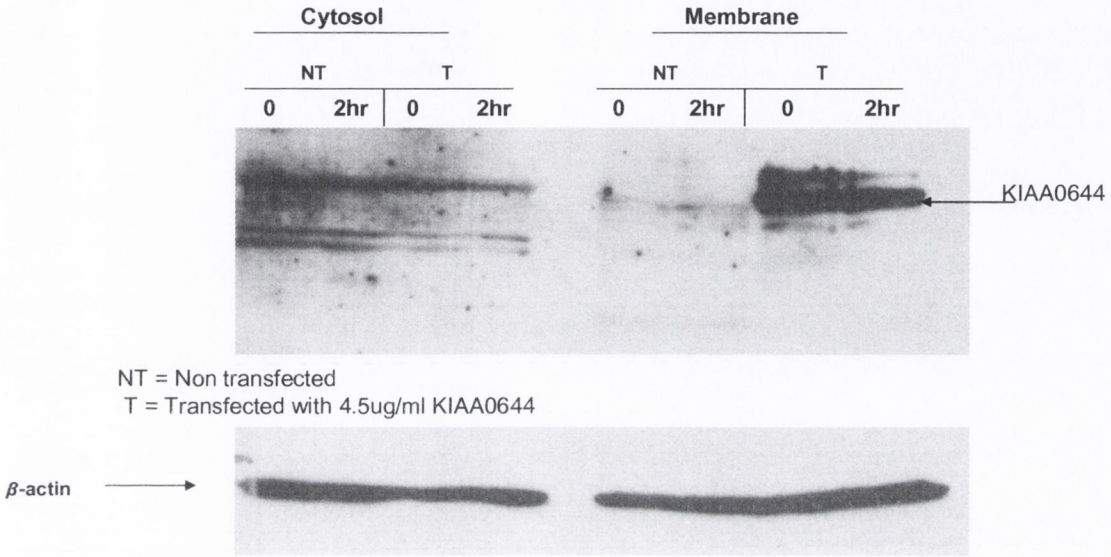
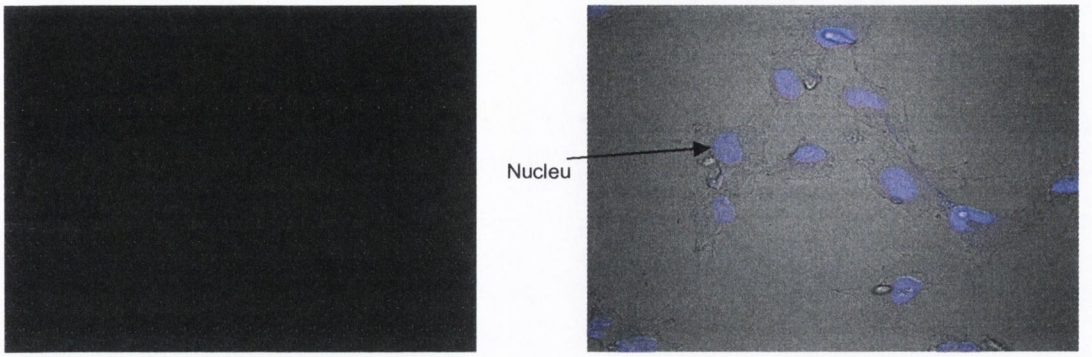


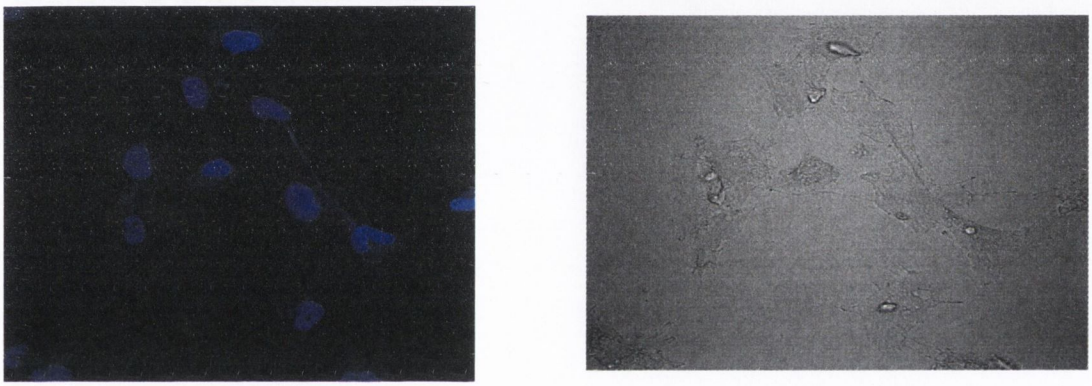
Figure 4.27 Transfected KIAA0644 is localized to the membrane. U373/CD14 cells were seeded in 6cm dishes at 2×10^5 cells/ml and cultured overnight in DMEM media containing 10 % FCS. Cells were transfected with 4.5 μg of plasmid encoding KIAA0644 using genejuice. After 24 h they were stimulated with 100 ng/ml LPS for 2 h. Samples were lysed in 500 μl of membrane lysis buffer and centrifuged at 97,000 $\times$ g for 1 h. Samples were resolved on a 10 % gel, and transferred onto PVDF membrane and immunoblotted with an anti-KIAA0644 antibody and β-actin antibody. This blot is representative of three individual experiments.

Figure 4.28 C-terminal antibody to KIAA0644 is undetectable in non permeabilised U373/CD14 cells. U373/CD14 cells were cultured at 2×10^5 cells/ml in 6 well plates containing sterile coverslips coated with 1 mg/ml poly-L-lysine. After 24 h the cells were fixed by incubating them in 2% formaldehyde for 15 min at room temperature. Cells were rinsed once in PBS. In part (A) the cells were incubated win anti-KIAA0644 antibody for 1 h, and then incubated in a alexa-flour 488 conjugated anti-rabbit secondary antibody for 30 min. In part (B) the cells were only incubated with an alexa-flour 488 conjugated anti-rabbit secondary antibody for 30 min. Dap 1 stain was added to the cells to highlight the nucleus. Coverslips were mounted onto slides and observed with a confocal microscope. Arrows indicate the nuclei of the cells stained in blue.

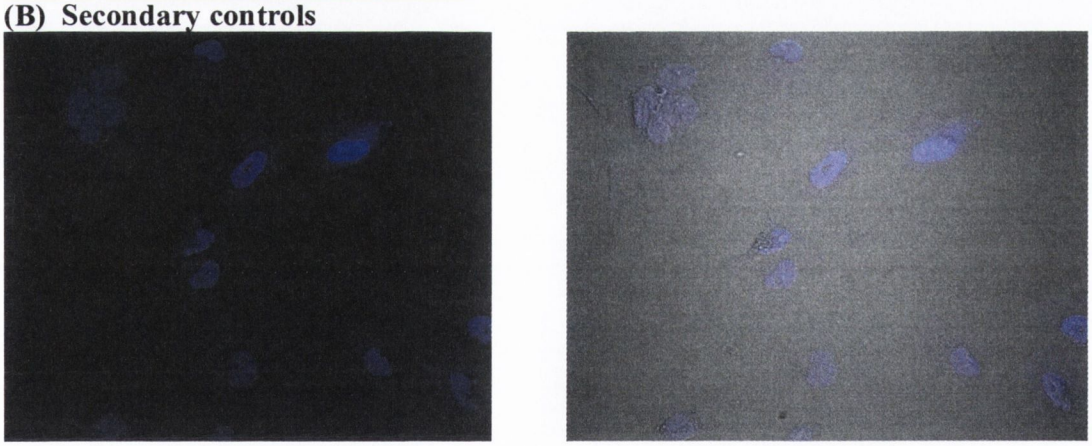
C terminal anti KIAA0644

Phase, dapi staining and anti





(B) Secondary controls



4.2.11 KIAA0644 can exist in a secreted form

4.2.11.1 KIAA0644 is highly abundant in fetal calf serum

Due to the initial observations that KIAA0644 appears to be cytosolic (Fig 4.26) and possesses a signal sequence I decided to examine the possibility that it may get secreted from cells. I took supernatant directly off U373/CD14 cells and analyzed them on a 10 % SDS gel. There were large amounts of the protein present (Fig 4.29). I decided to examine the expression of KIAA0644 in fetal calf serum to see if this would account for the large volume of protein present in the supernatant. I found KIAA0644 to be highly abundant in fetal calf serum (Fig 4.30). There was no KIAA0644 detected in serum free media. The region to which the antibody was originally designed is highly conserved across species (Fig 4.6) and hence accounts for this ability to detect KIAA0644 in fetal calf serum. Therefore all future experiments examining the secretion of KIAA0644 had to be done on cells cultured in serum free media. KIAA0644 may not have initially been detected in the serum free medium taken from cells as it is very dilute, therefore in all further experiments investigating the expression of KIAA0644 in media, KIAA0644 was immunoprecipitated.

In the previous experiments to determine that the C terminal antibody to KIAA0644 was specific I used FCS to immunoprecipitate KIAA0644 (Fig 4.9. Mass spectrophotometry results also revealed that KIAA0644 is expressed in FCS and therefore confirms the existence of a secreted form of the protein.

4.2.11.2 KIAA0644 is secreted from U373/CD14 cells following LPS stimulation

Following on from the observation that KIAA0644 is highly abundant in fetal calf serum, I wanted to determine if it can get secreted from U373/CD14 cells at a basal

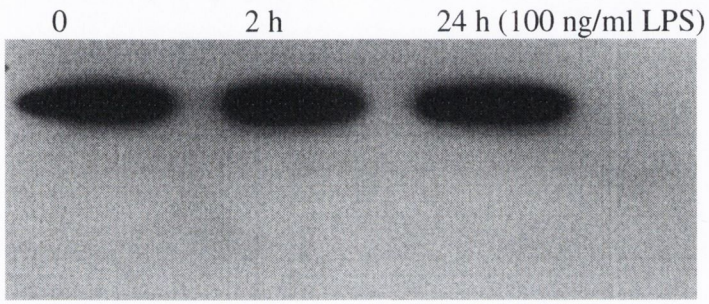


Figure 4.29 KIAA0644 is highly abundant in the supernatants of U373/CD14 cells. U373/CD14 cells were seeded at 2×10^5 cells/ml in 10 cm dishes. After 24 h they were stimulated with 100 ng/ml LPS for 2 and 24 h. 20 µl of supernatant was taken off the cells and 5 µl of sample buffer containing mercaptoethanol was added. Samples were resolved on a 10% SDS-PAGE gel and then transferred onto PVDF membrane and immunoblotted with anti KIAA0644. Data shown is representative of two separate experiments.

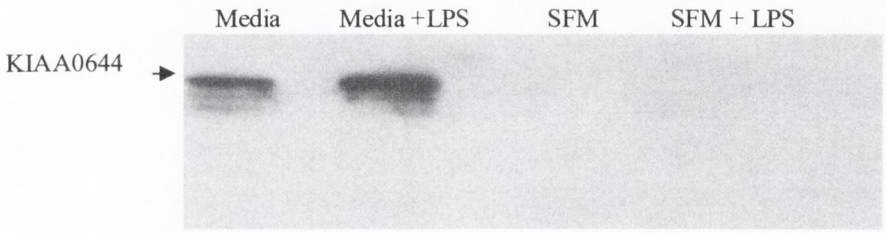


Figure 4.30 KIAA0644 is an abundant protein in fetal calf serum. U373/CD14 cells were seeded at 2×10^5 cells/ml in 10 cm dishes, half the cells were cultured in media containing 10 % FCS and the other cells were cultured in serum free media. After 24 h the cells were stimulated with 100 ng/ml for a further 24 h. 20 µl of supernatant was removed and 5 µl of sample buffer containing mercaptoethanol was added. Samples were resolved on an SDS gel, transferred to PVDF membrane and immunoblotted with anti KIAA0644.

level and with stimulation. U373/CD14 cells were cultured in serum-free media. They were stimulated with LPS for 30 min, 2 h 6 h and 24 h. 1 ml of each supernatant was removed and added to protein G beads which had been pre-coupled to KIAA0644 antibody overnight. In order to immunoprecipitate KIAA0644 the beads were incubated with the supernatant overnight. The control used was a rabbit IgG control. In the control KIAA0644 should not be immunoprecipitated as there is no anti-KIAA0644 present. Only non specific proteins should be immunoprecipitated using the IGG control antibody.

Samples were analysed by western blot and probed for KIAA0644. Fig 4.31 shows, that KIAA0644 can be immunoprecipitated from the supernatants of U373/CD14 cells. There is basal secretion of KIAA0644 at time 0 with the optimum secretion occurring after 6 h LPS stimulation.

4.2.11.3 KIAA0644 is secreted from THP1 cells following LPS stimulation

Once I confirmed that KIAA0644 is getting secreted from U373/CD14 cells, I wanted to see it occurring in another cell type to ensure that it was not cell type specific response.

THP1 cells are a monocytic suspension cell line. I investigated the secretion of KIAA0644 in THP1 cells that were cultured in serum-free media. KIAA0644 was immunoprecipitated from the supernatants following stimulation with LPS. The results (Fig 4.32 (A)) show that KIAA0644 is secreted from the cells when stimulated with LPS for 30 min and 2 h. The control used is a rabbit IgG control, KIAA0644 should not be immunoprecipitated as there is no anti-KIAA0644 present. Only non specific proteins should be immunoprecipitated using the IGG control antibody. Fig

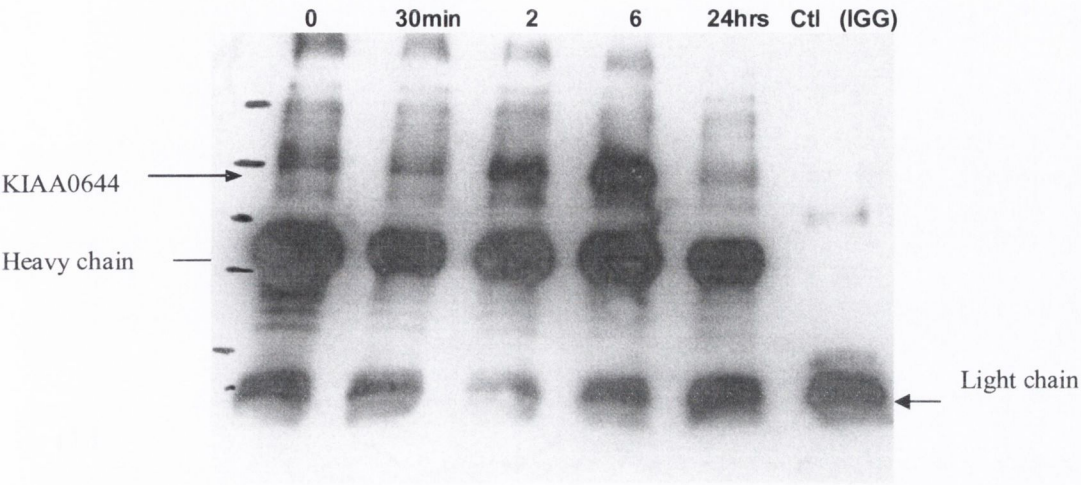
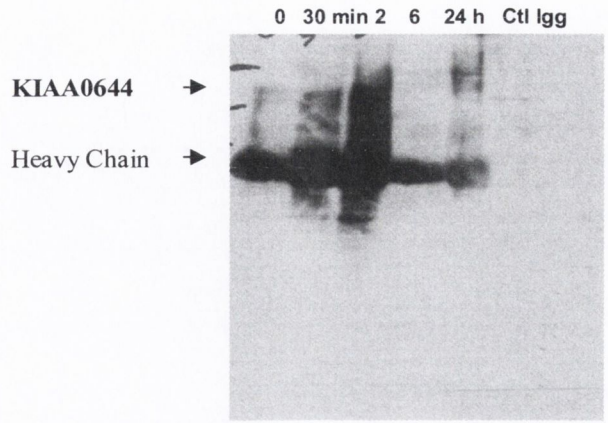


Figure 4.31 KIAA0644 is secreted from U373 cell following LPS stimulation. U373/CD14 cells were seeded at 1×10^5cells/ml in 10 cm dishes and cultured overnight in media containing 10 % FCS. After 24 h the cells were washed and serum free media was added. They were then stimulated with 100 ng/ml LPS for the time points indicated above. 1 ml of supernatant was removed from each 10 cm dish. 15 μl of anti-KIAA0644 was coupled to 40 μl of protein G beads overnight at 4 °C. For the control 30 µl of rabbit secondary antibody was coupled to 40 μl of protein G beads overnight at 4 °C. Supernatants were then added to the beads and were placed at 4 °C and gently rotated overnight. Beads were washed three times with high stringency buffer. 25 μl of sample buffer was added to each sample then resolved on a 10 % SDS gel and transferred onto PVDF membrane and immunoblotted with anti-KIAA0644 antibody. This result represents two independent experiments.

(A)



(B)

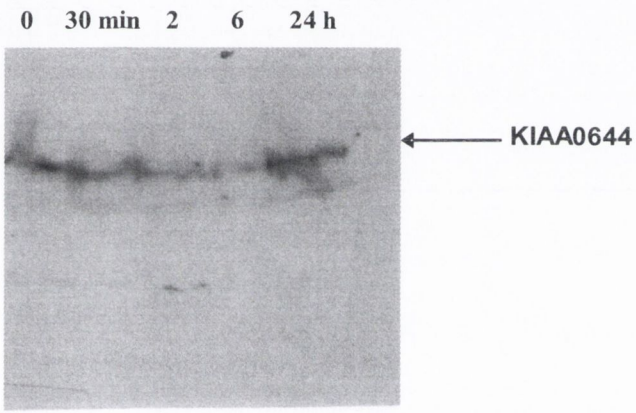


Figure 4.32 KIAA0644 gets secreted from THP1 cells after LPS stimulation. THP1 cells were seeded at 1×10^6cells/ml in 10 cm dishes and cultured overnight in media containing 10 % FCS. Cells were washed and serum free media was added. They were stimulated with 100 ng/ml LPS for the time points indicated above. 1 ml of supernatant was removed from each 10 cm dish. 15 μl of anti-KIAA0644 was coupled to 40 μl of protein G beads overnight at 4 °C, for the control 30 µl of rabbit secondary antibody was coupled to 40 μl of protein G beads overnight at 4 °C. Supernatants were then added to the beads and left rolling at 4 °C overnight. 25 μl of sample buffer was added to each sample; they were resolved on a 10 % SDS gel, transferred onto PVDF membrane and immunoblotted with anti-KIAA0644 antibody. This result is representative of three independent experiments.

4.32 (B) shows that KIAA0644 is present at low levels in the whole cell lysates of THP1.

4.2.11.4 Confocal images show KIAA0644 to be cytosolic and present in undefined pockets

A172 cells were cultured on poly-L-lysine coated coverslips. After 24 h the slides were removed and treated with anti-KIAA0644 primary antibody. This antibody is not labeled; therefore a secondary anti-rabbit antibody conjugated to alexaflour-488 was used to aid in the detection of KIAA0644. The coverslips were mounted onto slides and observed under the confocal microscope. The nuclei of the cells were stained with DAP1 (blue) and Fig 4.33 shows that the nuclei are round and therefore in a healthy and viable state. The results show that in untreated cells, endogenous KIAA0644 is present thoughout the cytosol with the protein concentrating in undefined pockets throughout the cell (Fig 4.33 part A and B). Similar localisation of KIAA0644 can be seen in U373/CD14 cells in Fig 4.34.

Transiently over-expressing KIAA0644 in U373/CD14 cells leads to a gathering of the protein into undefined pockets throughout the cell. As expected, there is a lot more of the protein present throughout the cell when it is over-expressed (Fig 4.35). These results are consistent with those observed during membrane fractionation. Fig 4.33 and 4.34 show that endogenous KIAA0644 is mainly present in the cytosolic fraction. However when KIAA0644 is over expressed in Fig 4.35 is all appears to localise to the membrane. These images show that KIAA0644 is not localising to the plasma membrane, perhaps they are localising to endosomal membranes.

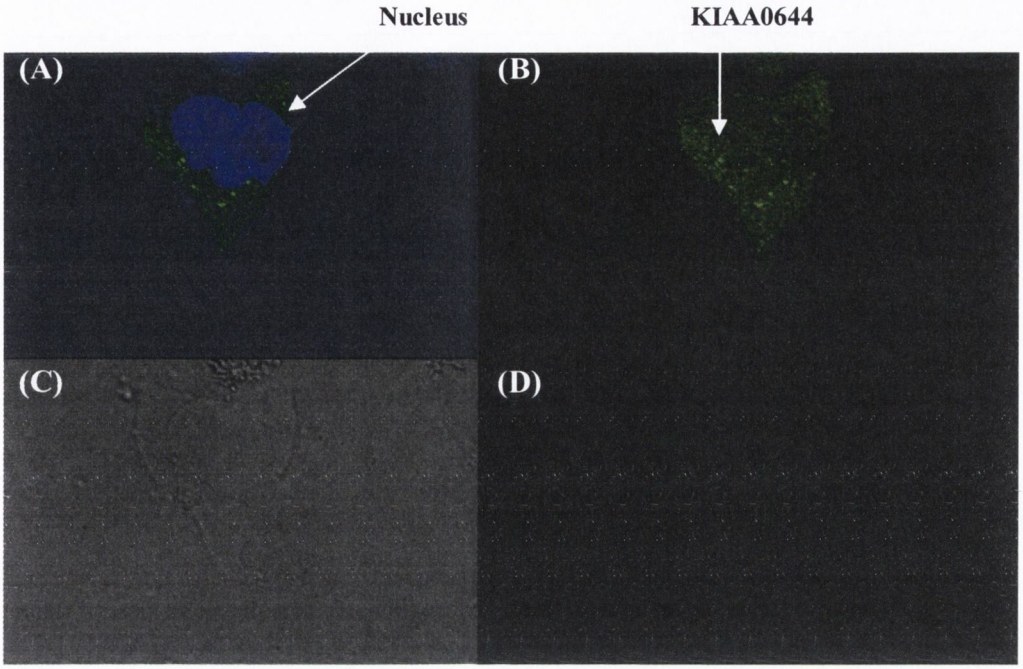


Figure 4.33 KIAA0644 is present in the cytosol and in undefined bodies thoughout the cytosol of A172 cells. A172 cells were cultured at 2×10^5 cells/ml in 6 well plates containing sterile coverslips, coated with 1 mg/ml poly-L-lysine. Cells were fixed by incubating them in 2 % formaldehyde for 15 min at room temperature. Cells were permeabilised and left on ice for 10 min. Cells were rinsed once in PBS and blocked in 2.5 % BSA for 30 min. Anti-KIAA0644 antibody was then placed on the slides for 1 h. An alexflour-488 conjugated anti-rabbit secondary antibody was added in the dark and incubated for 30 min. 4', 6-diamidine-2-phenylindole-dihydrochloride (DAPI) stain was added to the cells to highlight the nucleus (A). Coverslips were mounted onto slides and observed with a confocal microscope. Arrows indicate the nuclei of the cells stained in blue and the KIAA0644 which is stained green (A and B). (C) shows the phase display of the cell. Part D is the secondary control panel.

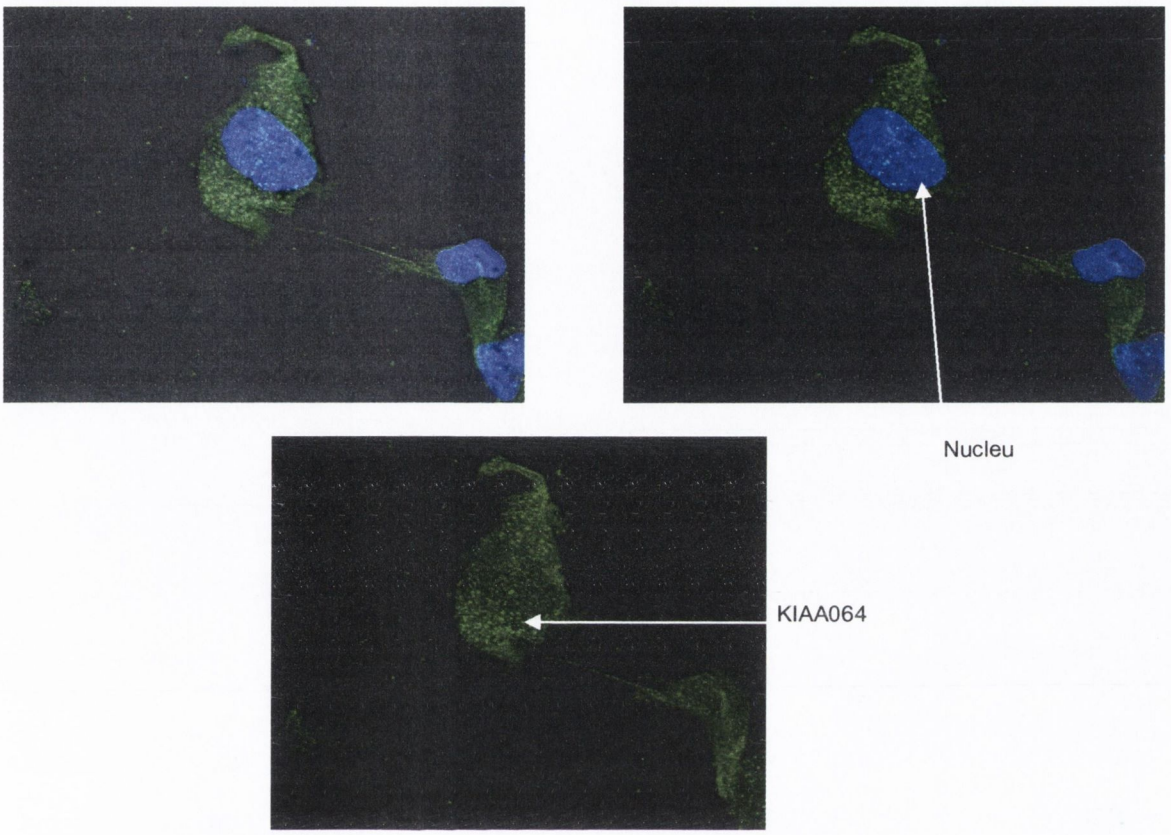


Figure 4.34 KIAA0644 is present in the cytosol and in undefined bodies thoughout the cytosol of U373/CD14 cells. U373/CD14 cells were cultured at 2×10^5cells/ml in 6 well plates containing sterile coverslips coated with 1 mg/ml poly-L-lysine. Cells were fixed by incubating them in 2 % formaldehyde for 15 min at room temperature. Cells were permeabilised and left on ice for 10 min. Cells were rinsed once in PBS and blocked in 2.5 % BSA for 30 min. Anti-KIAA0644 antibody was then placed on the slides for 1 h. An alexaflour-488 conjugated anti-rabbit secondary antibody was added in the dark and incubated for 30 min. DAPI stain was added to the cells to highlight the nucleus. Coverslips were mounted onto slides and observed with a confocal microscope. Arrows indicate the nuclei of the cells stained in blue and the KIAA0644 which is stained green.

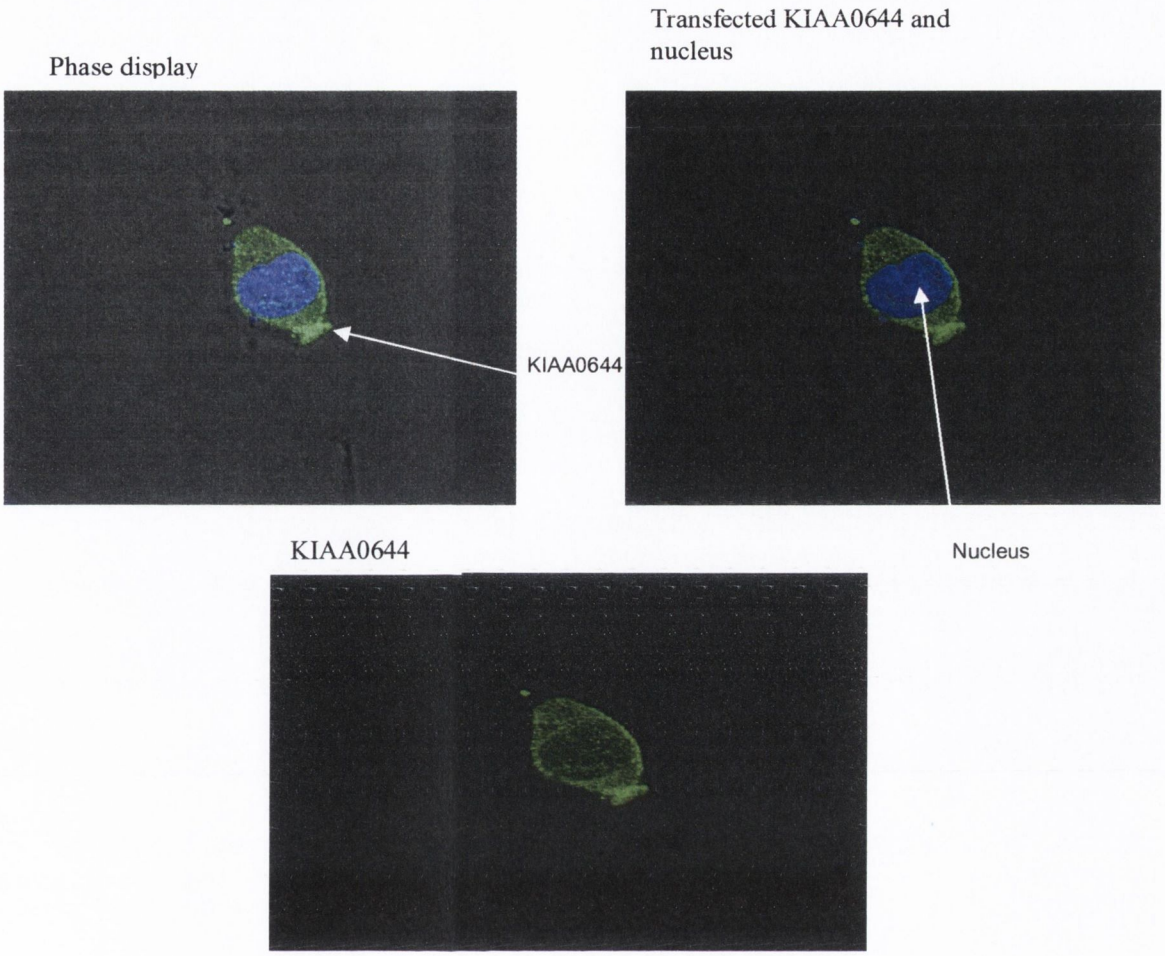


Figure 4.35 Transfected KIAA0644 localises to undefined bodies in U373/CD14 cells. U373/CD14 cells were cultured at 2×10^5cells/ml in 6 well plates containing sterile coverslips coated with 1 mg/ml poly-L-lysine. After 24 h 3 μg of plasmid encoding KIAA0644 was transfected into the cells using genejuice. After 24 h the cells were fixed by incubating them in 2 % formaldehyde for 15 min at room temperature. Cells were permeabilised and left on ice for 10 min. Cells were rinsed once in PBS and blocked in 2.5 % BSA for 30 min. Anti-KIAA0644 antibody was then placed on the slides for 1 h. An alexaflour-488 conjugated anti-rabbit secondary antibody was added in the dark and incubated for 30 min. DAPI stain was added to the cells to highlight the nucleus. Coverslips were mounted onto slides and observed with a confocal microscope. Arrows indicate the nuclei of the cells stained in blue and the KIAA0644 which is stained green. This figure represents two independent experiments.

Fig 4.36 shows that there is no background staining occurring with the secondary Alexa-flour 488 antibody and therefore all staining using KIAA0644 antibody is specific.

4.2.12 KIAA0644 is expressed on astrocytes and microglial cells in primary mixed glial cells

4.2.12.1 GFAP is a marker for astrocyte cells

Mixed glial cells were obtained from 1 day old neonatal mice. Cells were incubated for 10 days on coverslips coated with Poly-L-lysine in 6 well plates. Cells were treated with GFAP and mounted on slides and observed under the confocal microscope. A mixed glial cell population is made up of approximately 70% astrocytes and 30 % microglial cells. Fig 4.37 clearly shows the astrocytes staining red within the cell population. The secondary controls confirm that there is no background staining.

4.2.12.2 KIAA0644 is expressed on astrocytes and microglial cells

From previous results it was clear that KIAA0644 is highly expressed in the brain, specifically in the hippocampus, cerebellum and cortex. KIAA0644 is highly expressed in the astrocytoma cell line U373/CD14 and the Glial cell line A172. I wanted to determine what cells KIAA0644 is expressed in healthy mice primary cells. The two main cell types in the brain are astrocytes and microglial cells. Therefore I choose to make a mixed glial population to determine if KIAA0644 is expressed on these cells. Fig 4.24 shows that KIAA0644 is expressed at the protein level in the mixed glial cell population. Therefore confocal imaging was chosen as a technique to determine if KIAA0644 is expressed on astrocytes, microglial cells or both. GFAP was used to stain for the astrocytes in the population.

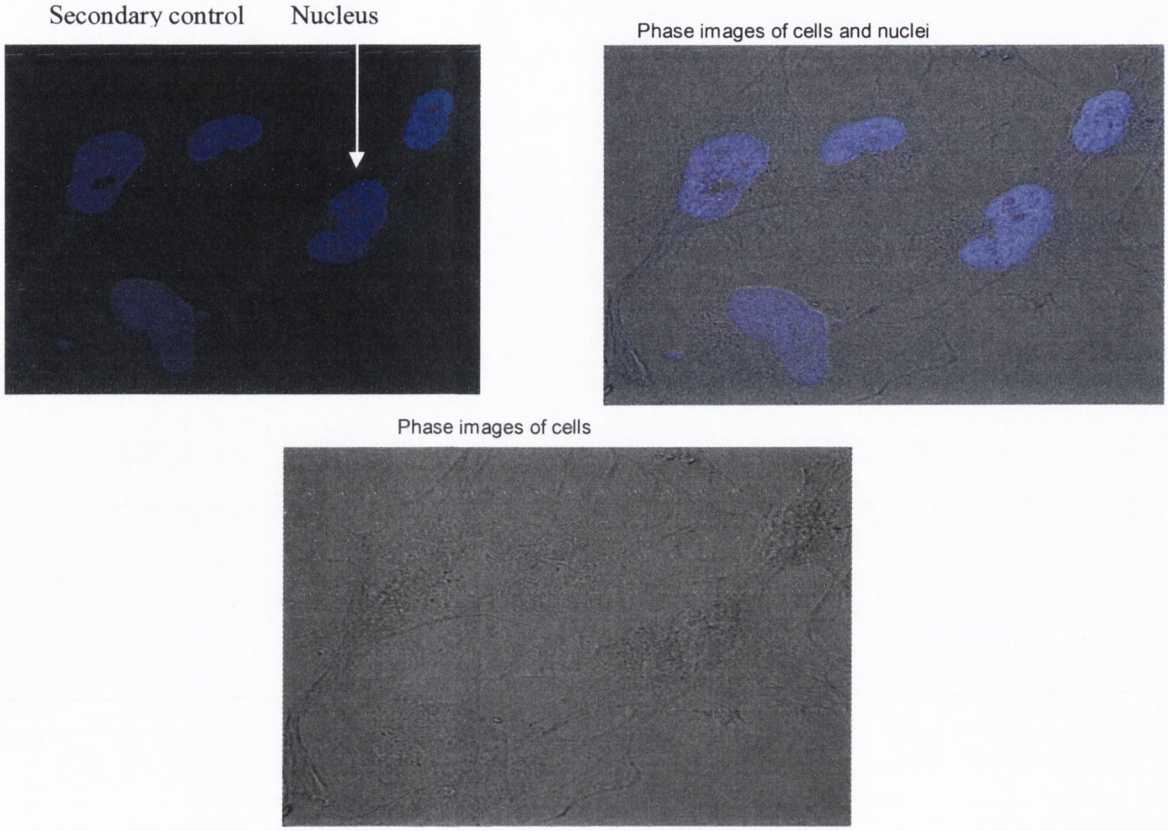


Figure 4.36 Secondary controls confirm that KIAA0644 antibody staining is specific. U373/CD14 cells were cultured at 2×10^5 cells/ml in 6 well plates containing sterile coverslips coated with 1 mg/ml poly-L-lysine. After 24 h 3μg of plasmid encoding KIAA0644 was transfected into the cells using genejuice. After 24 h the cells were fixed by incubating them in 2 % formaldehyde for 15 min at room temperature. Cells were permeabilised and left on ice for 10 min. Cells were permeabilised and left on ice for 10 min. Cells were rinsed once in PBS and blocked in 2.5 % BSA for 30 min. An alexaflour-488 conjugated anti-rabbit secondary antibody was added in the dark and incubated for 30 min. DAPI stain was added to the cells to highlight the nucleus. Coverslips were mounted onto slides and observed with a confocal microscope. Arrows indicate the nuclei of the cells stained in blue.

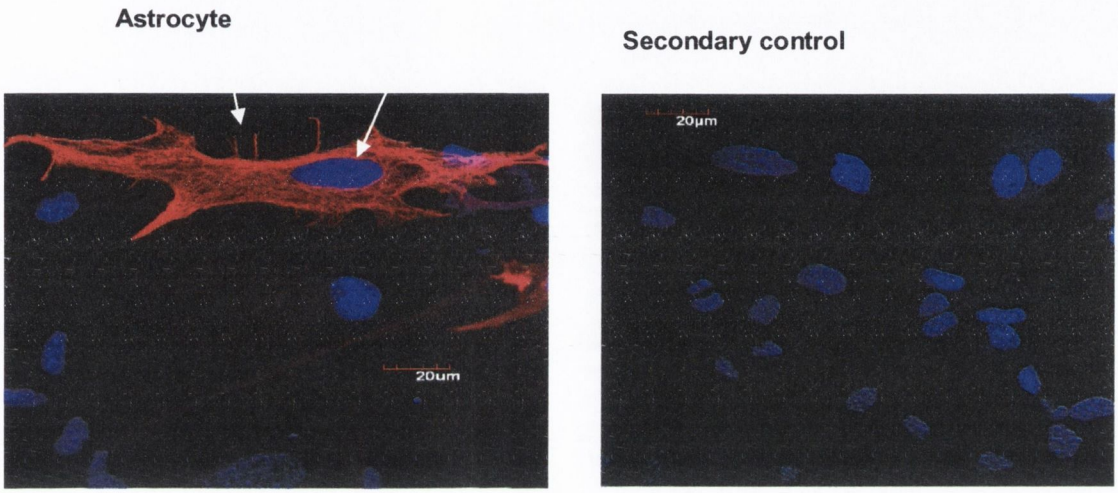


Figure 4.37 GFAP stains Astrocytes red in primary mixed glial cells. Primary mixed glial cells (70 % astrocytes, 30 % glial cells) were obtained from the brains of 1 day old neonatal mice. Cells were set up in 6 well plates containing sterile coverslips were coated with 1 mg/ml poly-L-lysine. After 10 days the cells were fixed by incubating them in 2 % formaldehyde for 15 min at room temperature. Cells were permeabilised and left on ice for 10 min. Cells were rinsed in PBS and blocked with 2.5 % BSA. GFAP antibody was added in the dark and incubated for 60 min. DAPI stain was added to the cells to highlight the nucleus. Coverslips were mounted onto slides and observed with a confocal microscope. Arrows indicate the nuclei of the cells stained in blue, astrocytes are stained red.

Mixed glial cells were obtained from 1 day old neonatal mice. Cells were incubated at 37 °C for 10 days on poly-L-lysine coated coverslips in 6 well plates. Cells were probed with anti-KIAA0644 antibody and detected using a secondary alexaflour-488 conjugated antibody. The samples were also probed with GFAP in order to stain the astrocytes in the population. Samples were mounted on slides and observed under the confocal microscope. Fig 4.38 shows astrocytes staining red within the cell population. KIAA0644 is stained green and it is present on all cells in the population, indicating that KIAA0644 is expressed on astrocytes and microglial cells.

4.2.12.3 KIAA0644 is expressed in mouse brain samples and specifically localises around blood vessels.

Previous results have shown that KIAA0644 is highly expressed in the brains of mice (Fig 4.13). Once it was confirmed by western blot that KIAA0644 is highly expressed I wanted to see by microscopy where KIAA0644 is localised to within the brain.

The blood vessels within the brain can be stained with a tracer molecule by first perfusing the animal for 5 min with biotinylated reagent EZ-Link TM Sulfo-NHS-Biotin. The whole brain was then removed cyro-protected using a sucrose gradient, cut on a cryostat and mounted onto slides. Each slide was probed with anti-KIAA0644 primary antibody. Since the blood vessels were staining green a red secondary alexaflour-647 conjugated antibody was used to aid detection of KIAA0644. DAPI was added to the mounting solution in order to stain the nuclei. Slides were observed under the confocal microscope.

Fig 4.39 clearly shows the blood vessels staining in green and the red KIAA0644 appearing to localise around these blood vessels. Fig 4.39 B is a higher magnification image showing the opening of the blood vessel. A distinct staining of

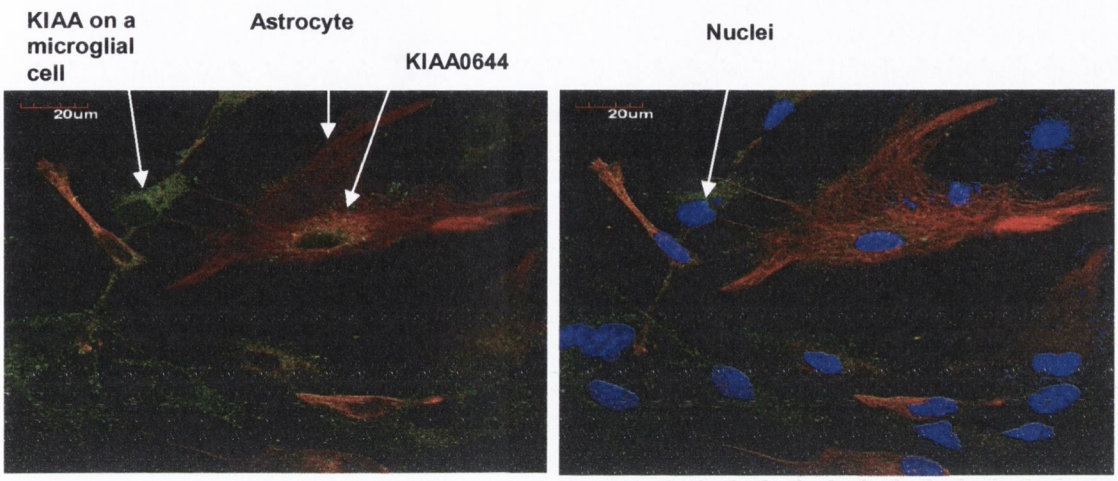
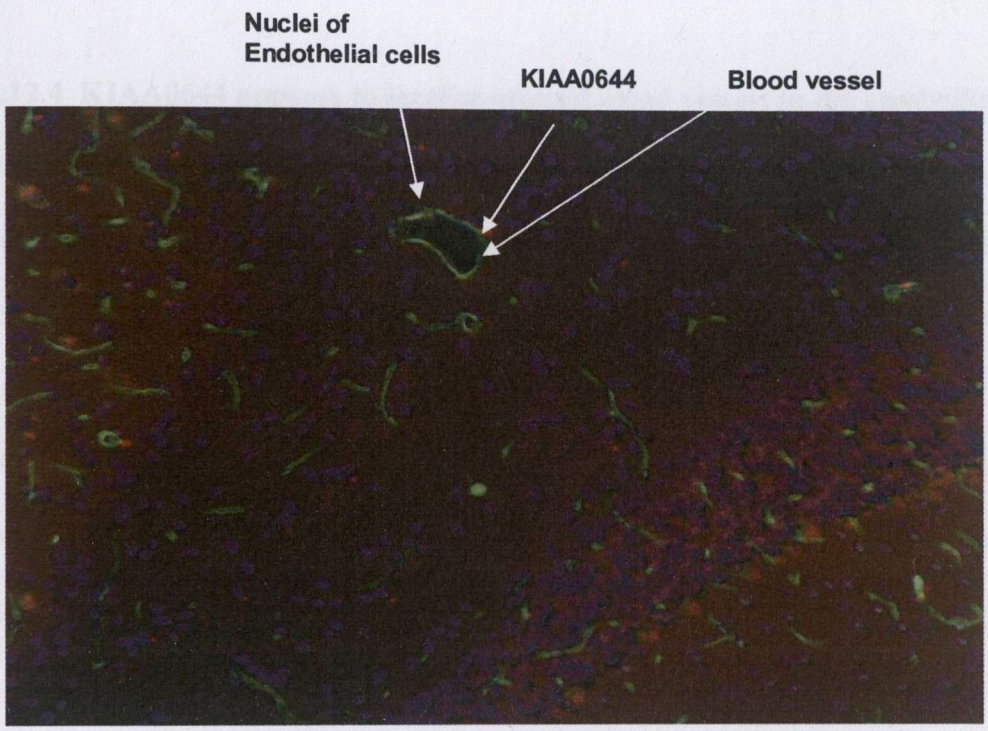


Figure 4.38 KIAA0644 is expressed on both astrocytes and microglial cells. Primary mixed glial cells (70 % astrocytes, 30 % glial cells) were obtained from the brains of 1 day old neonatal mice. Cells were set up in 6 well plates containing sterile coverslips were coated with 1 mg/ml poly-L-lysine. After 10 days the cells were fixed by incubating them in 2 % formaldehyde for 15 min at room temperature. Cells were permeabilised and left on ice for 10 min. Cells were rinsed in PBS and blocked with 2.5 % BSA. Astrocytes were stained with GFAP antibody, all cells were probed for KIAA0644, using anti KIAA0644 antibody and an alexaflour-488 conjugated secondary antibody. DAPI stain was added to the cells to highlight the nucleus. Coverslips were mounted onto slides and observed with a confocal microscope. Arrows indicate the nuclei of the cells stained in blue, astrocytes are stained red and KIAA0644 is stained green.

Figure 4.39 KIAA0644 localises around blood vessels in the brain

The whole brain was dissected and placed in 4 % PFA pH 7.4 overnight at 4 °C and subsequently washed 4 X 15 mins with PBS. The brain was cryoprotected using a 10 %, 20 % and 30 % sucrose gradient, and 12 µm frozen sections were subsequently cut on a cryostat at - 20 °C and placed on poly-lysine coated slides. Primary anti-KIAA0644 antibody was incubated on sections overnight at 4 °C. Following incubation, sections were washed 3 times in PBS and subsequently blocked again with 5 % NGS for 20 min at room temperature. Secondary alexaflour-647 antibody was incubated with the sections at 37 °C for 2 h followed by 3 washes with PBS. All sections were counterstained with 4', 6-diamidine-2-phenylindole-dihydrochloride (DAPI; Sigma Aldrich, Ireland) for 30 sec at a dilution of 1:5000 of a stock 1 mg/ml solution, and sections were visualized using an Olympus FluoView TM FV1000 Confocal microscope. Arrows indicate the nuclei of the cells stained in blue, blood vessels are stained green and KIAA0644 appears as red.

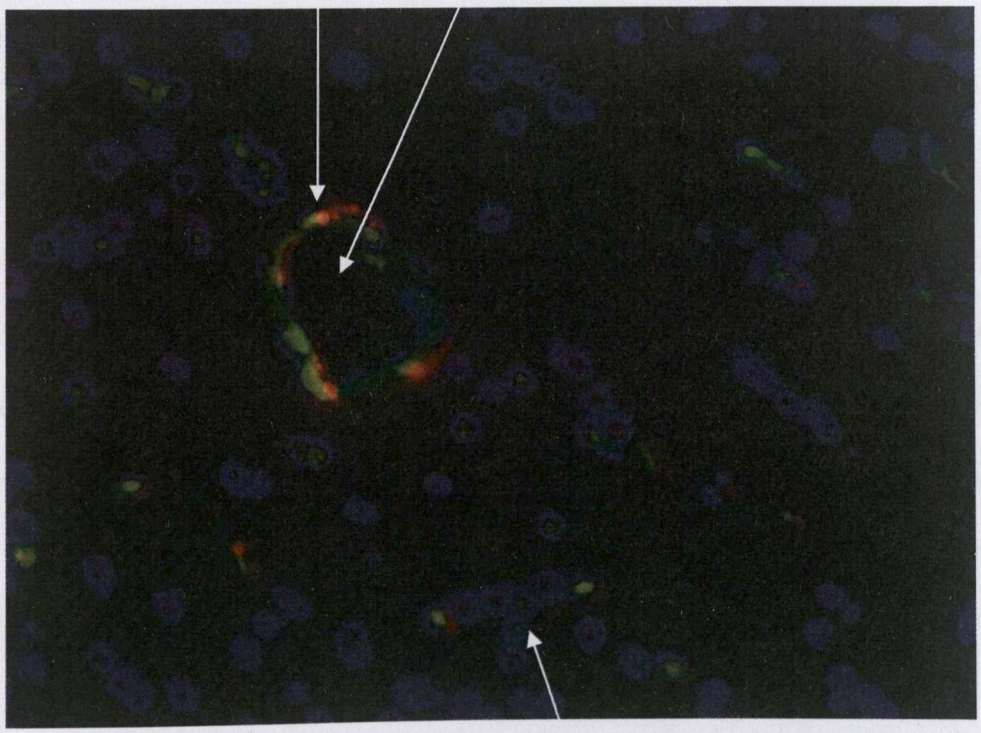
(A)



Red: KIAA0644 Blood vessel

(B)

The blood vessel is stained green



nuclei

KIAA0644 is observed around this blood vessel. Since KIAA0644 is highly expressed on astrocytes and microglial cells (Fig 4.38) it is probably these cells that are present along the blood vessels.

4.2.12.4 KIAA0644 appears to localise around blood vessels in the cerebellum

It was previously confirmed by western blot that KIAA0644 is highly expressed in the cerebellum. Again I wanted to confirm this and its localisation using confocal microscopy. The whole brain was removed cyro-protected using a sucrose gradient, cut on a cryostat and mounted onto slides. Each slide was probed with anti-KIAA0644 primary antibody. A red secondary alexaflour-647 conjugated antibody was used to aid detection of KIAA0644. DAPI was added to the mounting solution in order to stain the nuclei. Slides were observed under the confocal microscope.

Fig 4.40 shows that KIAA0644 is highly expressed in the cerebellum and it displays a distinct staining pattern. We would predict that again KIAA0644 is localising around blood vessels within the cerebellum.

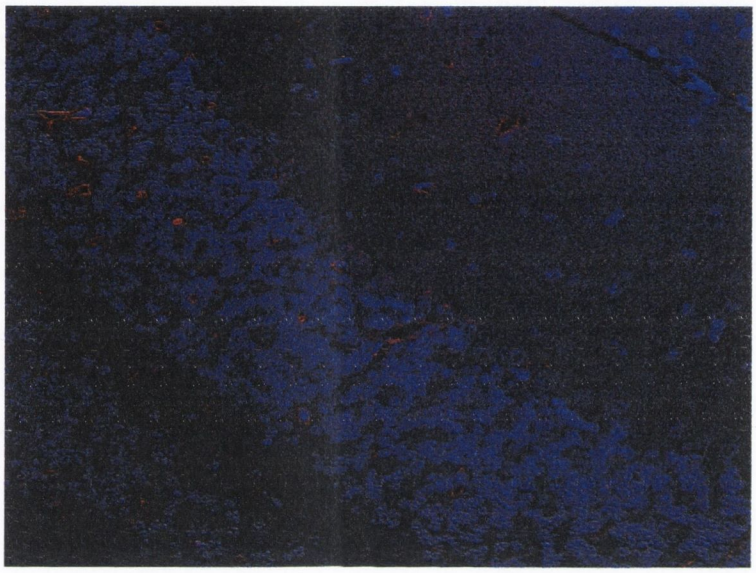
4.2.12.5 KIAA0644 is highly expressed throughout the hippocampus

I showed in Fig 4.13 that KIAA0644 is highly expressed in the hippocampus at the protein level. I wanted to confirm this expression study using confocal microscopy.
The whole brain was then removed cyro-protected using a sucrose gradient, cut on a cryostat and mounted onto slides. Each slide was probed with anti-KIAA0644 primary antibody. A red secondary alexaflour-647 conjugated antibody was used to aid detection of KIAA0644. DAPI was added to the mounting solution in order to stain the nuclei. Slides were observed under the confocal microscope.

Figure 4.40 KIAA0644 is expressed and appears to be localised around vessels in the cerebellum. The whole brain was dissected and placed in 4 % PFA pH 7.4 overnight at 4 °C and subsequently washed 4 X 15 min with PBS. The cerebellum was removed and cryoprotected using a 10 %, 20 % and 30 % sucrose gradient, and 12 µm frozen sections were subsequently cut on a cryostat at - 20 °C and placed on poly-lysine coated slides. Anti-KIAA0644 primary antibody was incubated on sections overnight at 4 °C. Following incubation, sections were washed 3 times in PBS and subsequently blocked again with 5 % NGS for 20 min at room temperature. Secondary Alexaflour-488 conjugated antibody was incubated with the sections at 37 °C for 2 h followed by 3 washes with PBS. All sections were counterstained with 4', 6-diamidine-2-phenylindole-dihydrochloride (DAPI; Sigma Aldrich, Ireland) for 30 sec at a dilution of 1:5000 of a stock 1 mg/ml solution, and sections were visualized using fluorescent microscope. Arrows indicate the nuclei of the cells stained in blue, KIAA0644 is stained green. Part (B) is a higher magnification image of part (A). Arrows indicate the nuclei of the cells stained in blue, KIAA0644 is stained red.

(A)

KIAA0644



(B)

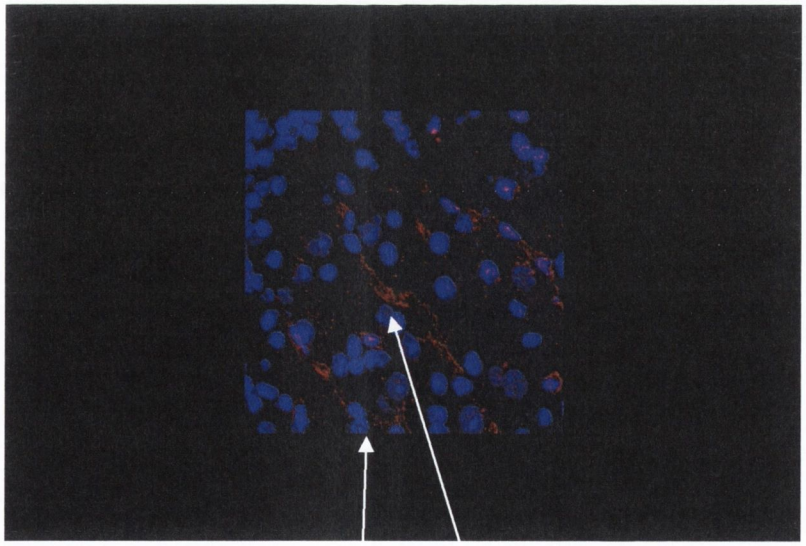


Fig 4.41 shows that KIAA0644 is highly expressed throughout the hippocampus in a pattern which would suggest it is closely linked with the blood vessels.

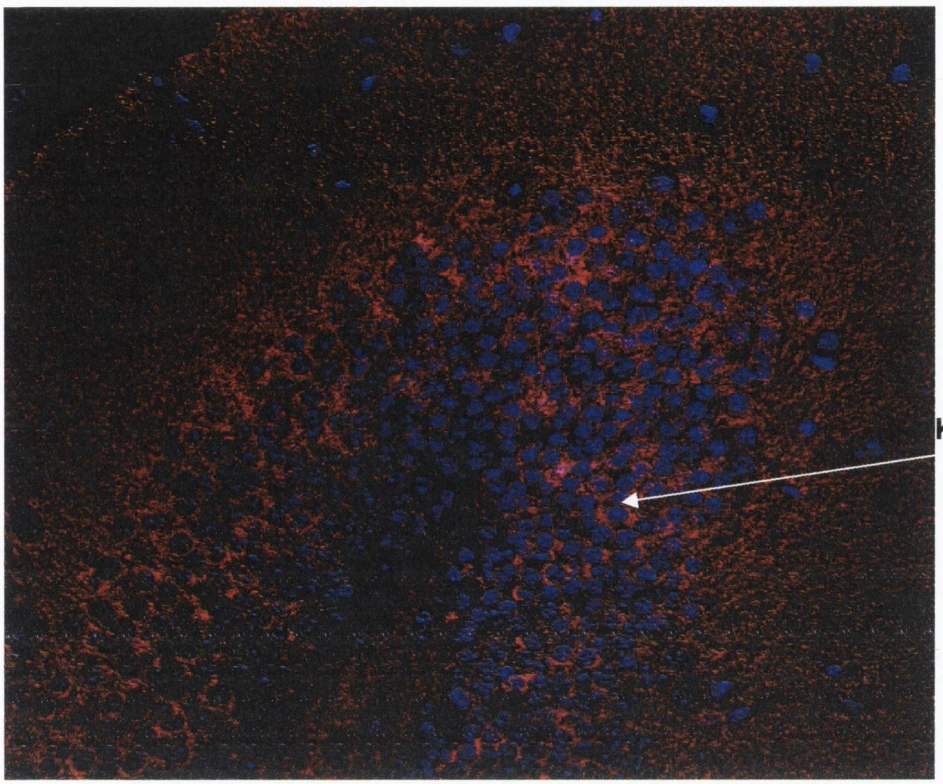


Figure 4.41 KIAA0644 is widely expressed in the hippocampus. The whole brain was dissected and placed in 4 % PFA pH 7.4 overnight at 4 °C and subsequently washed 4 X 15 min with PBS. The hippocampus was removed and cryoprotected using a 10 %, 20 % and 30 % sucrose gradient, and 12 µm frozen sections were subsequently cut on a cryostat at - 20 °C and placed on poly-lysine coated slides. Anti-KIAA0644 primary antibody was incubated on sections overnight at 4 °C. Following incubation, sections were washed 3 times in PBS and subsequently blocked again with 5 % NGS for 20 min at room temperature. Secondary Alexaflour -488 conjugated antibody was incubated with the sections at 37 °C for 2 h followed by 3 washes with PBS. All sections were counterstained with 4', 6-diamidine-2-phenylindole-dihydrochloride (DAPI; Sigma Aldrich, Ireland) for 30 sec at a dilution of 1:5000 of a stock 1 mg/ml solution, and sections were visualized using fluorescent microscope. Arrows indicate the nuclei of the cells stained in blue, KIAA0644 is stained green. Arrows indicate the nuclei of the cells stained in blue, KIAA0644 is stained red.

4.3 Discussion

The preliminary microarray carried out identified genes displaying differential expression levels in wild type and MAL knockout embryonic stem cells in response to LPS. KIAA0644 was the only uncharacterised gene in the list and therefore held the most novelty as a potentially new gene on the LPS signalling pathway. Bioinformatic analysis predicted KIAA0644 to possess 13 leucine rich repeat domains, a fibronectin domain, a transmembrane domain and a possible TIR domain. Data available from the Kazusa research institute suggested that KIAA0644 is highly expressed in the brain, lung, ovaries, spleen and spinal cord. Other online databases provided similar results suggesting that its expression levels are high in the brain and also in brain specific cell lines. Combining the bioinformatics data with and our initial microarray data the first experiment I decided to carry out was to determine if KIAA0644 is expressed at the RNA level in a human brain cell line U373/CD14 and to determine if it can get induced by TLR ligands. Since the sequence of KIAA0644 is highly conserved across species I also examined a mouse specific cell line (MEF) for expression and induction. Results show that KIAA0644 is induced quite rapidly at the RNA level in U373/CD14 cells stimulated with LPS. It is more delayed in the MEFs in response to LPS and Malp2. In vivo studies in mice showed that KIAA0644 is induced in response to LPS stimulation.

All data thus far confirms that KIAA0644 is expressed in the brain and can be induced by LPS. In order to try and examine the regulation of the gene I decided to try and study the possible promoter of KIAA0644. Again bioinformatics tools were used to try and identify the region expressing the promoter. In order to ensure that I did not miss the promoter region or any regulatory factors needed for the promoter to

function I cloned 3 promoters 564bp, 1 kb and 2 kb into pGL3 basic and also into the pGL3 enhancer vector. The enhancer vector contains the SV40 enhancer element and it was tested as it can work better to verify functional promoter elements. The results obtained from the promoter analysis were not what had been anticipated. I transfected the promoters into a number of cell types, at various concentrations and using many TLR ligands. The results were consistent across all experiments; all promoters displayed quite high constitutive activity. Unfortunately there was no activity above this when the promoters were stimulated with different TLR ligands. There is a number of reasons why this may be the case. The promoter could be missing an important enhancer element which may be present upstream from the start of the promoter sequence. Secondly the promoter may be constitutively active and therefore regulated post-transcriptionally either by mRNA stabilisation or perhaps by the expression of KIAA0644 microRNAs. Under normal circumstances mRNA that contains adenylate uridylate-rich elements (AREs) in the 3'UTR region has a rapid turnover rate. KIAA0644 does contain a number of AREs in its 3'UTR, therefore the promoter may just be constitutively active and the mRNA sequence is stabilised when the gene is stimulated with a ligand. MicroRNAs (miRNA) are small molecules only 21-23 nucleotides in length and their main function is to regulate gene expression normally by downregulation (Lee *et al.*, 1993). miRNA are normally complementary or partially complementary to sequences in the 3'UTR. The computer program TargetScan 4.2 suggests a number of miRNAs that would be involved in the regulation of KIAA0644, the main one is miRNA 23 and this is conserved across human, mouse, rat, dog, and chicken. Another possible explanation for the lack of promoter activity in response to stimulation is that the gene is under the control of a long range enhancer element. A paper was published (Lehoczky *et al.*, 2004), which

suggested that KIAA0644 is expressed in the forebrain and the posterior limb and is located upstream of the *Hoxa13* gene. In this paper they hypothesise that many of the *Hox* genes expressed in the distal limb are all under the control of a long range enhancer element instead of individual promoter elements. Perhaps this could also be the case for KIAA0644 and the regions I cloned may not be the promoter region for the gene, instead it may be controlled by an upstream long range global enhancer.

The next aim in the project was to raise an antibody to KIAA0644 to try and confirm the expression and induction observed at the RNA level. Two antibodies were made to a C terminal sequence, one was obtained from Eurogentec and the other from 21st Century Biochemicals. The antibodies were optimised and examined for specificity. The peptide to which the antibody was raised is highly conserved across species allowing for use in human and mouse cells. I demonstrated that KIAA0644 is highly expressed at the protein level in human lung and brain. This was consistent with the RT-PCR results; however I did predict that there would be expression also in the spleen, heart and kidney tissue. I have observed expression of KIAA0644 in human embryonic kidney (HEK293) cells. Therefore I would conclude that it is probable that KIAA0644 is expressed more globally in tissues and due to low expression levels in the particular samples used in this experiment the protein was not detected.

KIAA0644 is expressed in the hippocampus, cortex and cerebellum of wild type mice. Interestingly there appears to be a slight increase in expression of KIAA0644 in the Multiple Sclerosis model EAE mice. Since KIAA0644 is highly expressed in the brain it is possible that it could be implicated in the pathogenesis of the disease. Its expression could be induced due to increased inflammatory responses. KIAA0644 is

expressed and induced by LPS in two human astrocytoma cell lines, human monocytes, primary human PBMCs, mouse macrophages and primary mixed glial cells. KIAA0644 also appears to be induced by Malp2 in the glioma cell line A172, however its induction appears to be a little more delayed compared to LPS stimulation. At the RNA level KIAA0644 was induced by LPS at 15 min with the greatest induction at 6 h. At the protein level induction can vary from cell type to cell type, and even within the one cell type depending on how responsive the cells are to stimulation at the time. However there are consistent findings and that KIAA0644 is induced 30 min post LPS stimulation. In human primary PBMC it was observed that KIAA0644 was inducible by LPS and not TNF-α, lending specificity towards the LPS signalling pathway. In vivo studies in mice brains showed basal KIAA0644 expression which was greatly enhanced following LPS stimulation. KIAA0644 can appear as a doublet. In the case of the in vivo samples only the upper band is getting induced. This could be because the lower band is a non specific band or the doublet could also be the result of post translational modification. Other cell types such as the A172 cells indicate that both bands get equally induced by LPS stimulation therefore the most probable explanation is that the extra band is due to post translational modification. This study into the post translational modification of KIAA0644 was carried out in the Opsona facility. There they found that KIAA0644 does not undergo phosphorylation but the protein does get glycosylated. In the doublet it is possible that one of the bands is the result of glycosylation. The gene is only made up of a single exon therefore the bands cannot be splice variants.

The central nervous system (CNS) is an immune privileged site which displays highly complex immunoreactivity. It is nearly devoid of MHC expression and designed to

respond rapidly to infection to ensure there is no lasting inflammation. Therefore the innate immune system is key to CNS homeostasis. Many TLRs are currently studied for their expression levels and functions within the brain. TLR3 has been shown to be expressed and function on astrocytes (Farina *et al.*, 2005) suggesting that astrocytes are important in fighting viral infections within the CNS. However expression of TLR3 can be exploited by West nile virus and cause lethal encephalitis (Wang *et al.*, 2004). Microglial cells have also been shown to express TLRs at the RNA level and they have been shown to be capable of responding to LPS, CpG motifs and peptidoglycans (Town *et al.*, 2005). Activation of TLR2 on microglial cells has been shown to allow uptake of amyloid β peptide which is associated with Alzheimer's disease (Chen *et al.*, 2006). Interestingly a recent paper has shown that TLR8 function as a negative regulator of neurite outgrowth and this role for TLR8 is distinct its role in immunity (Ma *et al.*, 2006). TLR2 and TLR4 have also been shown recently to have opposing roles in hippocampal neurogenesis, TLR2 appears to promote neurogensis while TLR4 inhibits it (Rolls *et al.*, 2007). In view of the recent findings that TLRs have distinct role in the CNS some of which are separate to their immune function I decided to use fluorescent and confocal imaging to determine exactly which cells in the brain KIAA0644 is expressed. I demonstrated that KIAA0644 is highly expressed on both astrocytes and microglial cells. Studies carried out on whole mice brain sections revealed high expression of KIAA0644 in the hippocampus and cerebellum. Expression patterns of KIAA0644 in the whole tissue sections suggest that KIAA0644 localises around blood vessels. Astrocytes perform many functions within the brain such as support for endothelial cells which form the blood brain barrier (BBB). The BBB is essential in the protection of the brain and is impermeable to vascular molecules. Astrocytes are also involved in repair and they provide

nutrients to neurons. KIAA0644 has been previously found to be highly expressed on astrocytes, therefore it would be predicted to localise around blood vessel as astrocytes are vital in supporting endothelial cells at the BBB.

Having established expression and induction I demonstrated the localisation of KIAA0644 using two methods; membrane fractionation and Confocal microscopy. The results from the membrane fractionation studies clearly show that endogenously KIAA0644 is primarily expressed in the cytosolic fraction even after LPS stimulation. Transient over-expression of KIAA0644 localises solely to the membrane fraction. The assay used for membrane fractionation is quite crude, involving one fast centrifugation. It is possible that at a basal level KIAA0644 is expressed on small membranes, perhaps endosomal membranes that are not concentrated to the membrane fraction during the procedure. When over-expressed KIAA0644 may concentrate onto these membranes and hence descend into the membrane fraction. Confocal images obtained support this hypothesis. The C terminus of KIAA0644 was confirmed to be intracellular using confocal imaging. The antibody was only observed when the cells were permeablised. This result also confirmed that there is transmembrane domain within KIAA0644. Localisation studies using confocal imaging showed that KIAA0644 is expressed throughout the cytosol, in some cases it appeared to be present in undefined pockets or membranes. When it was over-expressed these regions become more visible and appear to look like endosomal membranes. KIAA0644 never localised to the plasma membrane. These localisation studies were difficult to understand in terms of function in relation to LPS signalling. Most TLRs are expressed on the plasma membrane, however TLR3, TLR7/8 and TLR9 localise to endosomal compartments. Recent published confocal images of

other proteins involved in the TLR signalling pathways show similar staining patterns to KIAA0644. Confocal images of the distribution of MyD88 are characterised as condensed regions throughout the cytosol of the cell. The functional consequences of this are not understood (Honda *et al.*, 2004, Nishiya *et al.*, 2007). It is suggested that the non TIR region within MyD88 is critical for its subcellular localisation (Nishiya *et al.*, 2007). It is possible that the localisation of KIAA0644 to the membrane during membrane fractionation and its condensed forms in the confocal images are just an artefact of over-expression. Many proteins when over-expressed form aggregates and misfold and lead to false subcellular localisation. However it is also possible that since KIAA0644 possess a transmembrane domain that it is expressed on endosomal membranes or on some undefined vesicle. MyD88 condenses in the cytoplasm and markers for the golgi, endosomes and lysosomes were examined and none of them co-localised with MyD88 (Nishiya *et al.*, 2007) suggesting that there is an uncharacterised organelle present within the cytoplasm that is critical to TLR signalling. Also TLR4/MD2 have been shown to be expressed on either the cell surface of macrophages and also in the golgi in intestinal epithelial cells (Latz *et al.*, 2002, Hornef *et al.*, 2003). This data along with the MyD88 data suggests that TLR functional responses occur throughout the cell, maybe depending on the cell type. All data obtained on KIAA0644 indicates that this is an LPS inducible protein therefore it is possible that like MyD88, KIAA0644 is localising to an undefined organelle in the cell, or perhaps KIAA0644 is localising to intracellular pools of TLR4.

A number of components of the TLR signalling pathways are present as both membrane bound proteins and also in soluble forms. CD14 acts as a co-receptor for TLR4 signalling. It can be present as a GPI-anchored protein but also in a soluble

form. Soluble CD14 plays many roles within the immune system, primarily it acts as a shuttle for LPS but it can also affect LPS priming of neurtophils (Troelstra *et al.*, 1997), lead to the influx and efflux of phospholipids (Sugiyama *et al.*, 2001) and induce B cell growth (Filipp *et al.*, 2001). A soluble and functional form of TLR2 has also been identified in human plasma and breast milk (LeBouder *et al.*, 2003). Since there is a large documented number of soluble proteins involved in the TLR signalling pathway I examined the possibility of KIAA0644 existing in a secreted form. Fetal Calf Serum (FCS) consists of a large number of growth factors and proteins such as albumin. KIAA0644 was found to be highly abundant in FCS. The bovine sequence of KIAA0644 is highly homologous to the human protein therefore the same antibody was used to identify bovine KIAA0644. Bovine KIAA0644 is slightly bigger than human KIAA0644, it is 109kda compared to 83kda. Identifying KIAA0644 as a highly abundant protein in FCS confirmed that a secreted form does exist. I next demonstrated that KIAA0644 can get secreted from U373/CD14 cells and THP1 cells following LPS stimulation. However it appears that the full length protein (83kda) gets secreted and that is does not get cut or processed. The transmembrane domain is close to the C terminus of the protein, if the protein was being cut at this point a change in molecular weight of approximately 9-10kda would be predicted, however this did not occur. Perhaps the entire protein is capable of moving outside the cell in response to stimulation. Having a soluble form of KIAA0644 may possess duel roles in relation to LPS signalling. Perhaps like sCD14, sKIAA0644 is needed for cells that do not express KIAA0644 endogenously, or it could function to act as a decoy receptor to inhibit excessive activation of membrane bound KIAA0644 in response to LPS.

Data presented in this chapter confirms that KIAA0644 is an LPS inducible protein, highly expressed throughout the brain and on macrophages. KIAA0644 appears to localise to undefined organelles throughout the cytoplasm of the cell and secreted forms of the protein also exist.

Chapter Five

Functional analysis of KIAA0644 and its role in the LPS signalling pathway

Chapter 5

5.1 Introduction

Results from the previous chapter confirm that KIAA0644 is an LPS inducible protein. KIAA0644 is induced following LPS stimulation in a number of mouse and human cell lines and in primary human PBMC and mouse BMDM cells. KIAA0644 expression levels in the brain were also confirmed at both the RNA and protein level and in vivo studies showed that it is highly inducible by LPS within the brain. KIAA0644 appears to be mainly localised to the cytosol, however over-expression studies show localisation to the membrane. Therefore it is possible that KIAA0644 is expressed on undefined vesicles throughout the membrane. Another interesting observation was the identification of a secreted form of KIAA0644. LPS also appears to lead to an increase in the secretion of KIAA0644 from the cell. All this data suggests that KIAA0644 is a functional protein that may have a role in the LPS signalling pathway.

As mentioned previously TLR4 requires additional co-receptors to form a complex and allow for the recognition of LPS. This is a highly regulated and complex pathway. Many questions still remain unanswered as to exactly how this complex functions and what the specific roles are for each receptor within the complex. LBP is required for transferring LPS to CD14 (Yu *et al.*, 1996), and it has also been shown to greatly enhance the sensitivity to LPS (Wurfel *et al.*, 1997). As discussed previously CD14 is an integral part of the LPS signalling pathway. Importantly $\beta 2$ integrans have been reported to be capable of substituting in some way for CD14 in response to LPS (Ingalls *et al.*, 1995). MD2 is another co-receptor that is critical for TLR4 recognition of LPS. MD2 has been reported to have a number of functions within the LPS

signalling pathway, it is need to bind LPS and it also is required for cell surface expression of TLR4. CD14, TLR4 and MD2 display differential expression patterns throughout the body. There have been cell lines reported to lack CD14 or MD2 and still possess the ability to respond to LPS. This could be explained by the presence of soluble forms of both MD2 and CD14 which could compensate for the lack of either membrane bound protein. However it also remains possible that there are other proteins present in the immune system that can compensate for these receptors.

This study has identified KIAA0644 as a novel LRR containing protein, expressed in brain, lung and ovaries and highly inducible by LPS. This chapter explores the biological functions of KIAA0644 using RNAi techniques, over-expression studies and co-immunoprecipitation assays. Gene silencing of KIAA0644 greatly affects the LPS signalling pathway, but shows no affect on the TNF-α signalling pathway. Over-expression of KIAA0644 enhances LPS signalling. Physical association between CD14 and KIAA0644 and also between TLR4 and KIAA0644 is shown. All data presented confirms that KIAA0644 is a novel protein involved in the LPS signalling pathway.

5.2 Results

5.2.1 Transient over-expression of KIAA0644 enhances LPS signalling

Work in chapter four confirmed that KIAA0644 is widely expressed at the protein level throughout the body and is LPS inducible in a number of cell lines and primary cells. The next step in this study is to further understand the biochemical and biological function of KIAA0644 role in this pathway. In order to achieve this I have over-expressed the protein and determined what affect this has on LPS signalling.

A luciferase assay was the first approach I investigated to determine the effects of over-expressing KIAA0644 on the LPS pathway. U373/CD14 cells were transfected with a plasmid expressing KIAA0644 or an equivalent empty vector control plasmid along with an NFκB reporter plasmid. Fig 5.1 shows that over-expressing KIAA0644 alone can drive the NFκB reporter gene 3 fold. Once stimulated with LPS, KIAA0644 drives the NFκB reporter over 7 fold.

5.2.1.1 Transient over-expression of KIAA0644 in U373 parental cells leads to an increase in IL-6 and Rantes production following LPS stimulation

As mentioned earlier TLR4 requires co-receptors for LPS recognition. Therefore I wanted to investigate if KIAA0644 could compensate in the absence of some of these co-receptors. U373 parental cells do not express CD14. Therefore they were chosen as a suitable cell line to determine if KIAA0644 can compensate for a loss of CD14.

U373 parental cells were transfected with KIAA0644 or CD14 and after 24 h half the cells were cultured in full media (containing 10 % FCS) and the remaining cells were cultured in media without FCS. The cells were cultured differently as it is known that CD14 is highly expressed in FCS and this could therefore compensate for the lack of CD14 in the cells. The cells were then stimulated for an additional 24 h

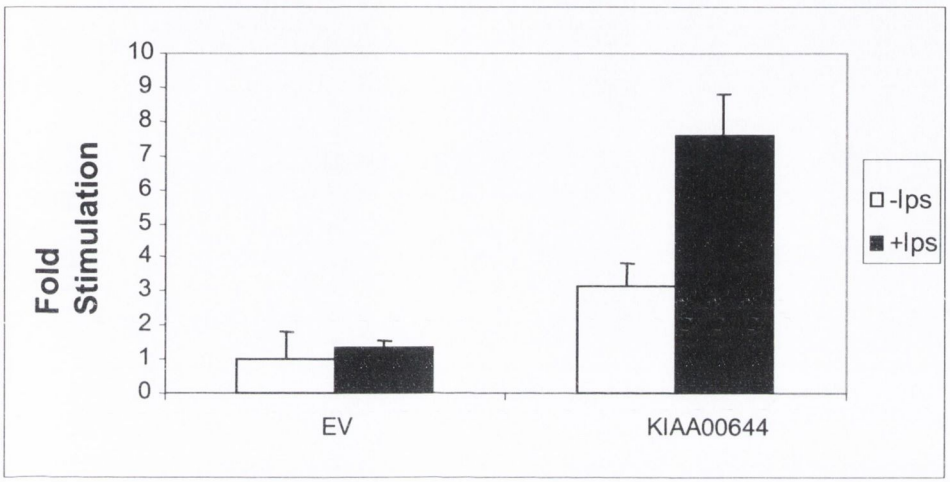


Figure 5.1 KIAA0644 enhances LPS signalling. U373/CD14 cells (1×10^5 cells/ml) were transfected with κB-luciferase (80 ng) and Renilla-luciferase (40 ng) together with 50 ng of empty vector control or KIAA0644 expression plasmid. After 24 h, cells were stimulated with LPS (100 ng/ml) for 6 h before being harvested and analysed for reporter gene activity. Results are normalised for *Renilla* luciferase activity and represented as fold stimulation over the non stimulated empty vector controls. Results are expressed as mean ± standard deviation for triplicate determinations and are representative of three separate experiments.

with LPS. Supernatants were removed and cytokine ELISA were carried out. Fig 5.2 A, shows that even when the cells are cultured in full media containing FCS, when KIAA0644 is over-expressed it leads to an increase in IL-6 production from the cells following LPS stimulation. In part B and C the cells were cultured in serum free media and over-expression of KIAA0644 greatly increases the LPS induced secretion of Il-6 and Rantes.

5.2.1.2 Over-expression of KIAA0644 in MEF cells enhances LPS signalling

It was been reported that TLR4/MD2 is capable of responding to rough LPS in the absence of CD14. However there have also been some contradictory reports and therefore the results are not conclusive. I wanted to investigate if over-expression of KIAA0644 had different effects on rough or smooth LPS stimulation. All over-expression experiments so far were carried out in U373 cells, hence I choose to use MEF cells to see if the results were consistent with those observed in the U373 cells.

Like U373 parental cells MEF cells do not express CD14. The cells were therefore transfected with KIAA0644, or with CD14 or with KIAA0644 and CD14 together. The cells were then stimulated with rough or smooth LPS. Supernatants were removed and Rantes elisas were carried out. Fig 5.3 shows that over-expression of KIAA0644 can enhance Rantes production following LPS stimulation to the same extent as over-expressing CD14. Fig 5.4 shows that when the plasmids are transfected in alone without stimulation only a basal level of IL-6 is recorded. In this experiment over-expression of CD14 causes a slightly higher increase in IL-6 production compared to KIAA0644 over-expression following LPS stimulation. When CD14 and KIAA0644 are transfected together there is no further increase in IL-6 or Rantes

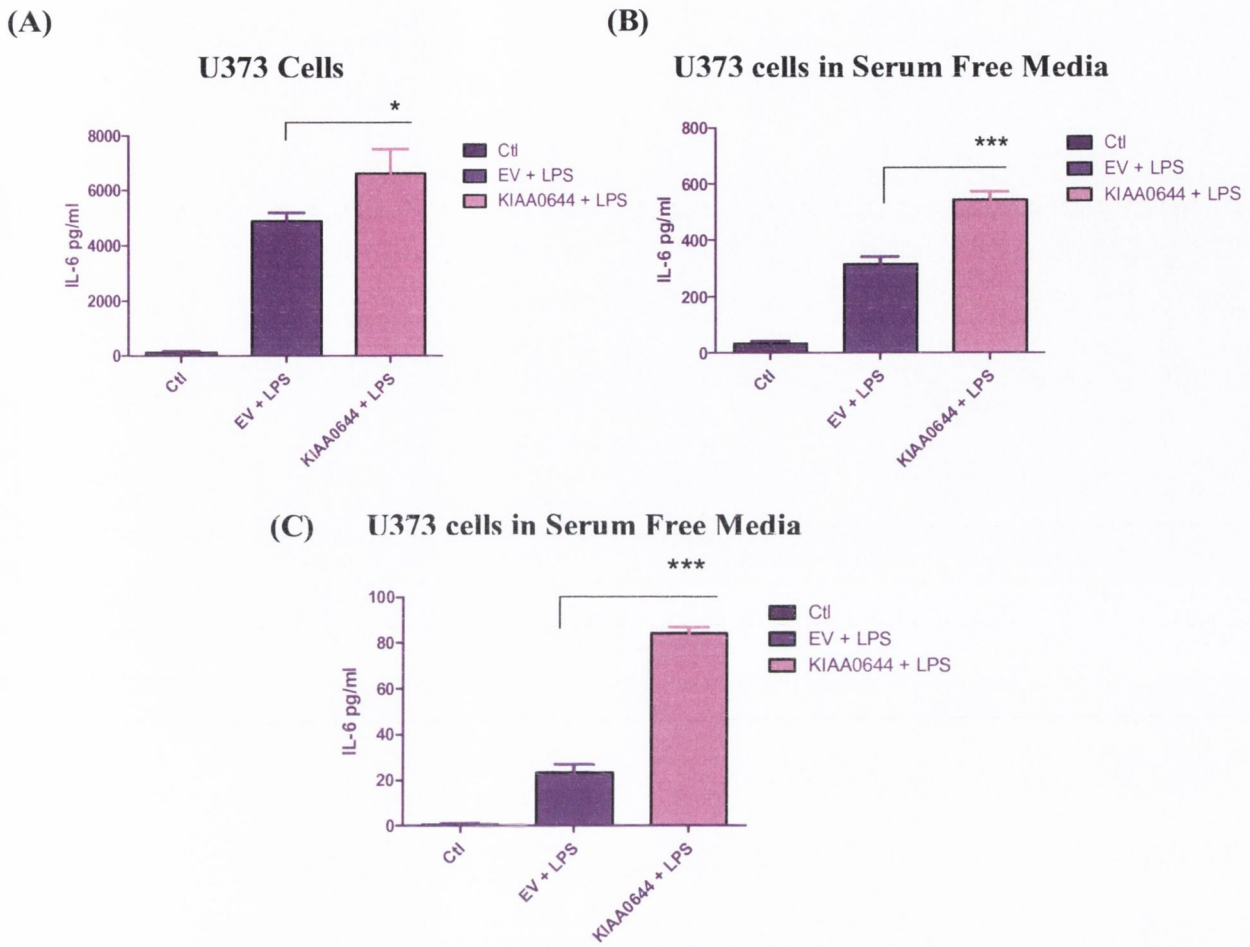


Figure 5.2 Transient over-expression of KIAA0644 enhances IL-6 and Rantes production in U373 parental cells. U373 cells were set up at a concentration of 2×10^5 cells/ml in 6 well plates. After 24 h 3 μg of empty vector (pcDNA3.1) or 3 μg of pcDNA-KIAA0644 was transfected into cells using genejuice. After 24 h the media was changed in part (B) and (C) to serum free media. Cell used in part (A) were maintained in complete media. Cells were all stimulated with 100 ng/ml LPS for 24 h. Supernatants were then removed from the cells and IL-6 and Rantes elisas were carried out. Tukey-Kraner multiple comparisons test was performed on all data. These figures represent three independent experiments.

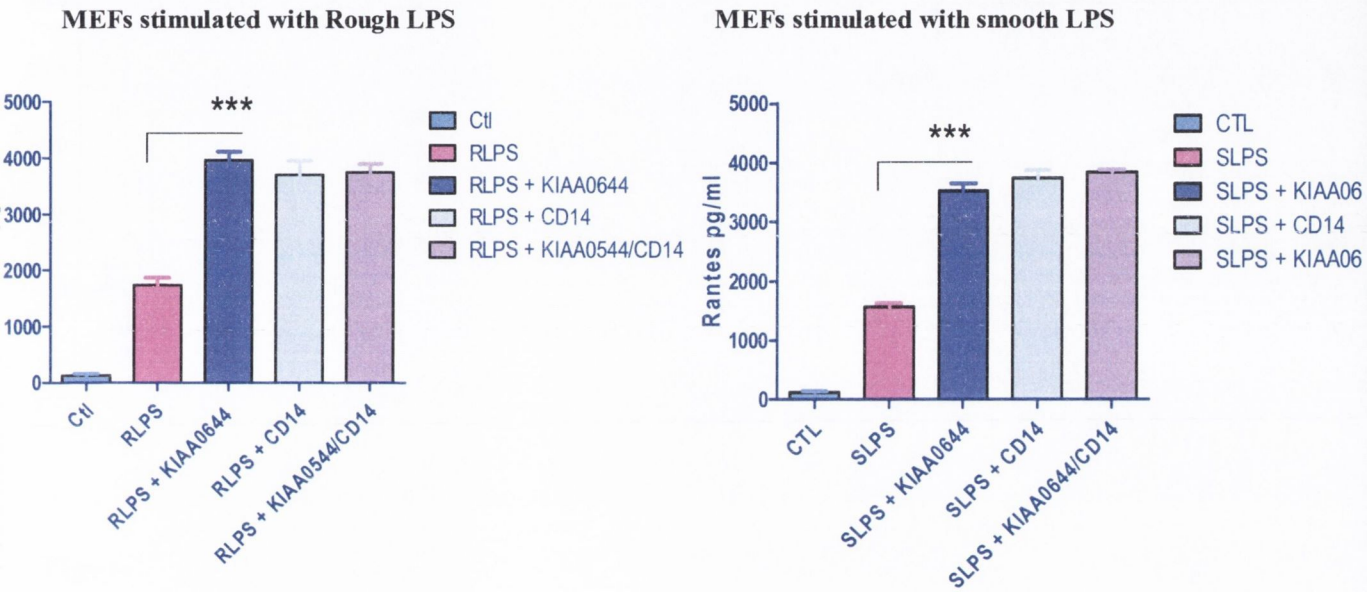


Figure 5.3 Transient over-expression of KIAA0644 in MEF cells causes an increase in Rantes production in response to both rough and smooth LPS stimulation. MEF cells were set up at 1×10^5 cells/ml in 6 well plates. After 24 h the cells were transfected with 3 μg of pcDNA-KIAA0644, 3 μg of pcDNA-CD14 or 1.5 1.5 μg of pcDNA-KIAA0644 together with 1.5 μg of pcDNA-CD14. After 48 h cells were stimulated with 100 ng/ml of rough or smooth LPS for an additional 24 h. Supernatants were removed and rantes elisas were carried out. Tukey-Kraner multiple comparisons test was performed on all data.

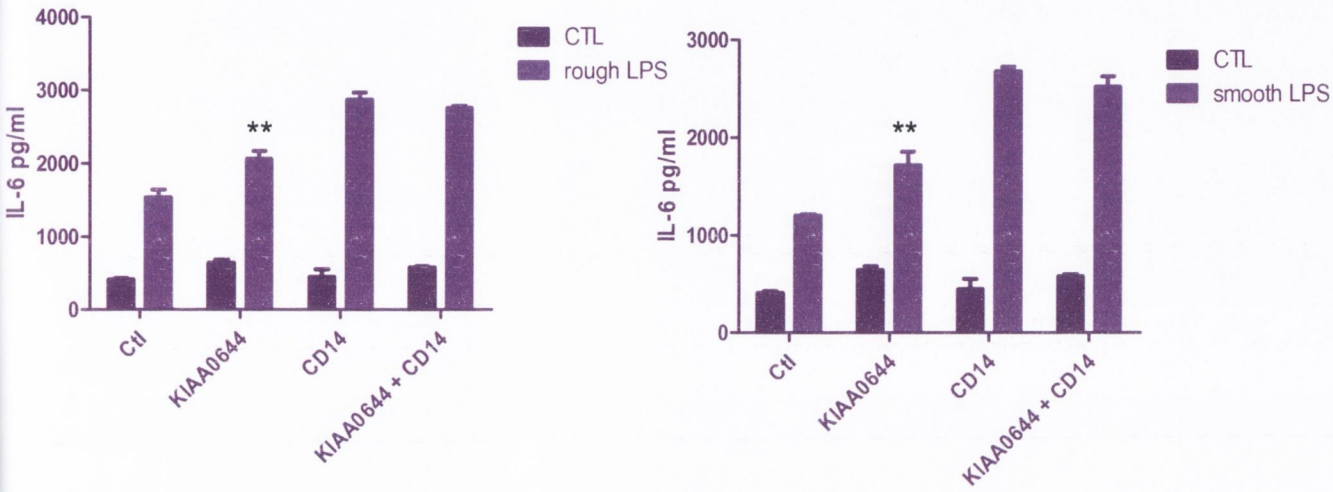


Figure 5.4 Transient over-expression of KIAA0644 in MEF cells causes an increase in IL-6 production in response to both rough and smooth LPS. MEF cells were set up at 1×10^5 cells/ml in 6 well plates. After 24 h 3 μg of plasmid expressing KIAA0644 or 3 μg of plasmid expressing CD14 or both together were transfected into cells using genejuice. The control has 3 μg of empty vector transfected in. After 48 h the cells were stimulated with 100 ng/ml of rough or smooth LPS for an additional 24 h. Supernatants were removed and rantes elisas were carried out. Tukey-Kraner multiple comparisons test was performed on all data.

production over the single transfections. Also it is important to note that the increase in Rantes and IL-6 release occurs to the same extent with both rough and smooth LPS.

5.2.1.3 Over-expressing KIAA0644 enhances LPS but not TNF-α signalling

Over-expression of KIAA0644 can enhance IL-6 and rantes production from U373 and MEF cells following LPS simulation. In order to determine if this effect is LPS specific I wanted to study another stimulant. I choose to look at TNF-α as a control ligand for the over-expression studies.

U373 parental cells were transfected with KIAA0644 or CD14 and after 24 h the media was changed to serum free media. The cells were then stimulated for an additional 24 h with LPS or TNF-α. Supernatants were removed and an IL-6 elisa was carried out. Fig 5.5 shows, that over-expression of CD14 or KIAA0644 alone without stimulation does not induce IL-6 production, however over-expression of KIAA0644 and CD14 lead to an increase in IL-6 production in response to LPS but not TNF-α stimulation. Co-transfecting CD14 and KIAA0644 together gives the same result as transfecting them on their own.

5.2.2 KIAA0644 binds LPS

5.2.2.1 KIAA0644 can bind LPS. This interaction can be competed with unlabeled LPS

Results obtained thus far clearly indicate that KIAA0644 is an LPS inducible protein. In order to establish the role for KIAA0644 within the TLR4 signalling complex the ability of KIAA0644 to bind LPS was investigated.

HEK293 cells were transfected with KIAA0644. Cells were harvested and incubated with biotinylated LPS and unlabelled LPS were added to the samples in

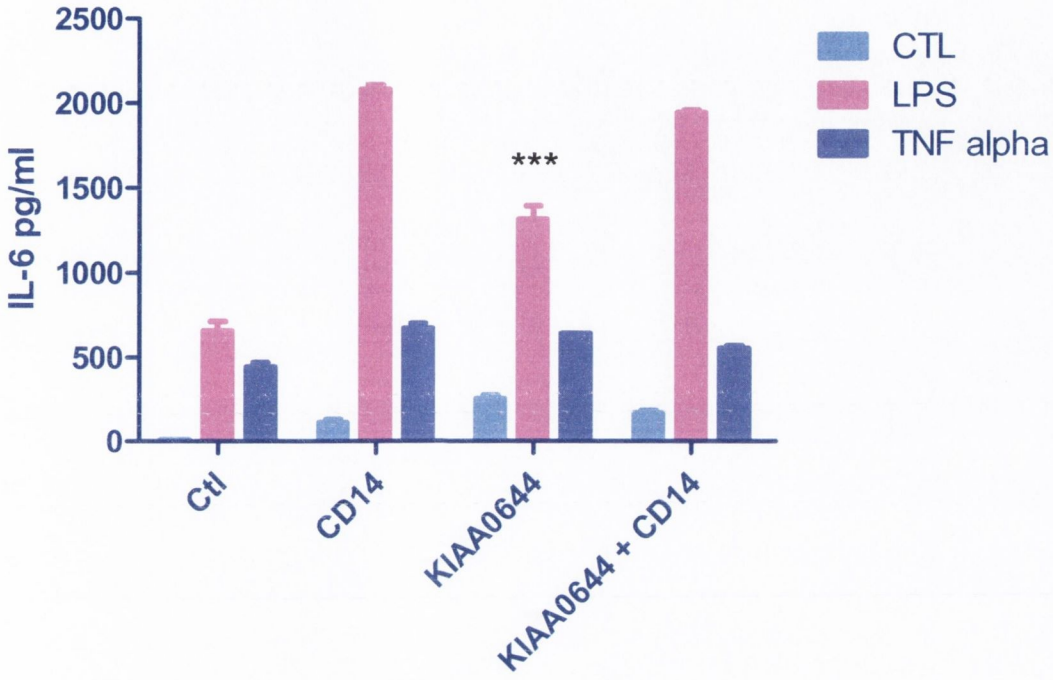


Figure 5.5 Reconstitution of U373 parental cells with KIAA0644 boosts the LPS signalling pathway but not the TNF-α signalling pathway. U373 cells were set up at a concentration of 1×10^5 cells/ml in 6 well plates. After 24 h 3 μg of empty vector (pcDNA3.1) or 3 μg of pcDNA-KIAA0644 or both together were transfected into cells using genejuice. After 48 h the media was changed to serum free media. Cells were stimulated with 100 ng/ml LPS or 20 ng/ml TNF-α for 24 h. Supernatants were then removed from the cells and an IL-6 elisa was carried out. Tukey-Kraner multiple comparisons test was performed on all data. This figure represents two independent experiments.

increasing amounts. Samples were analysed by western blot. Fig 5.6 shows that KIAA0644 interacts with LPS and this interaction can be competed with unlabelled LPS.

5.2.3 KIAA0644 interacts with TLR4 and CD14

5.2.3.1 KIAA0644 can bind to TLR4. KIAA0644 binds weakly to the TLR4 P/H (proline to histidene) mutant

All the evidence to date indicates that KIAA0644 is part of the LPS signalling complex. KIAA0644 gets induced by LPS and over-expression of the protein can enhance signalling. Fig 5.6 indicates that KIAA0644 can bind to LPS. The next focus of study is to determine KIAA0644's role within the complex. I wanted to determine if KIAA0644 is acting in some way as an accessory protein or a co-receptor to TLR4. To investigate this I wanted to establish if KIAA0644 can bind to TLR4.

Human embryonic kidney cells (HEK293) are easily transfected and hence were chosen as the cell line to study interactions. TLR4 and KIAA0644 or TLR4 P/H and KIAA0644 were transfected into HEK293 cells. TLR4 plasmid contains a flag tag and this was used to immunoprecipitate out the complex. Cell lysates were added to flag beads, these samples were then probed for KIAA0644 expression. Fig 5.7 shows that KIAA0644 is strongly interacting with TLR4. It is still capable of binding to the mutant TLR4 P/H however this is a much weaker interaction than observed with the wild type TLR4. To ensure that KIAA0644 and TLR4 were transfected into the cells their expression levels were also checked.

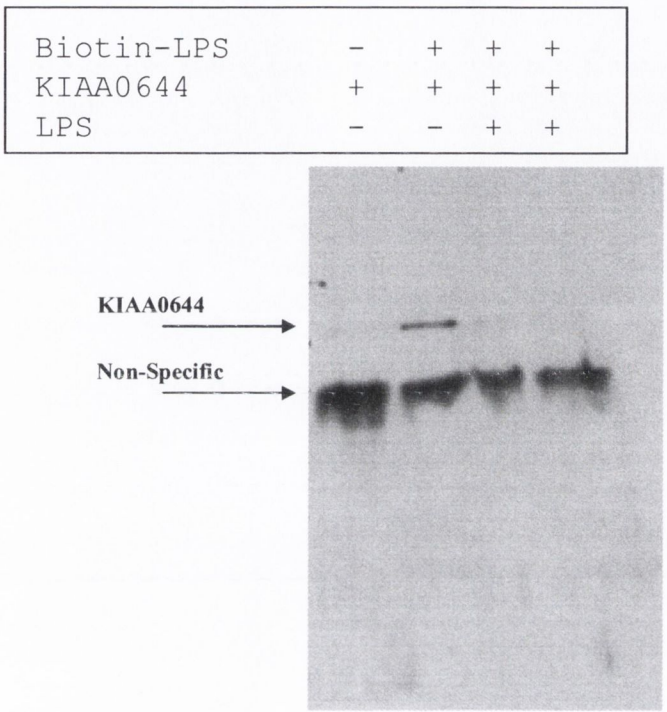


Figure 5.6 KIAA0644 can bind LPS. This can be competed with unlabeled LPS

HEK 293 cells were set up at 1×10^5 cells/ml in 10 cm dishes. After 24 h they were transfected with 10 ug of KIAA0644. After 24 h the cells were harvested in high stringency buffer. Lysates were incubated with biotinylated LPS for 2 h at room temperature. Increasing amounts of unlabeled LPS was added again for 2 h. Samples were then washed; sample buffer containing β-mercaptoethanol was added. Samples were resolved on a 10% SDS gel, transferred into PVDF membrane and immunoblotted with anti-KIAA0644. This figure represents three independent experiments.

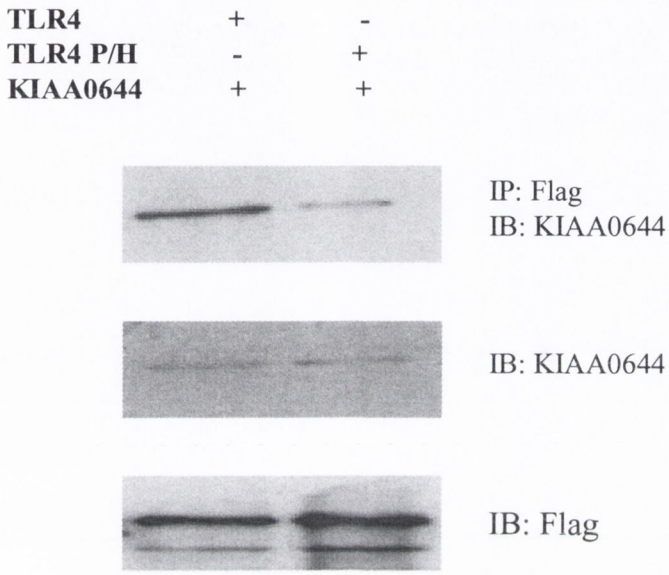


Figure 5.7 KIAA0644 can interact with TLR4. KIAA0644 is unable to interact as strongly with the TLR4 P/H (proline to histidine) mutant. Human embryonic kidney cells (HEK 293) cells were set up at 1×10^5 cells/ml in 10cm dishes. After 24 h they were transfected with 10 ug of KIAA0644 and 10 ug of Flag TLR4. After a further 24 h the cells were harvested in high stringency buffer. A sample of each lysate was removed and blotted for KIAA0644 and Flag TLR4 respectively. The remaining lysate was added to flag tagged beads for 2 h. Immunocomplexes were assayed for KIAA0644 (top panel). This result represents three independent experiments.

5.2.3.2 Endogenous KIAA0644 can bind TLR4

Fig 5.7 shows that KIAA0644 interacts with TLR4, however both proteins are over-expressed and this can lead to false positive interactions. Therefore I wanted to confirm that transfected TLR4 can interact with endogenous KIAA0644.

U373 cells were transfected with Flag tagged TLR4. Cells were stimulated with LPS then harvested. Flag beads were used to immunoprecipitate the TLR4 complex, then the samples were probed for KIAA0644 expression. The control sample was added to sucrose beads with mouse non-conjugated IgG antibody added. There should be no interaction observed in the control sample as there is no flag antibody present. Fig 5.8 shows that endogenous KIAA0644 can interact with Flag tagged TLR4 and this interaction increases upon LPS stimulation.

5.2.3.3 LPS stimulation increases the interaction between KIAA0644 and TLR4

Fig 5.8 indicated that LPS stimulation can increase the interaction between KIAA0644 and TLR4. I wanted to further investigate this interaction by extending the LPS time course.

U373 cells were transfected with Flag tagged TLR4. Cells were stimulated with LPS then harvested. Flag beads were used to immunoprecipitate the TLR4 complex, then the samples were probed for KIAA0644 expression. The control sample was added to sucrose beads with mouse non-conjugated IgG antibody added. Fig 5.9 shows that endogenous KIAA0644 can interact with Flag tagged TLR4 and this interaction increases upon LPS stimulation. The strongest interaction occurs after 2 and 24 h stimulation with LPS.

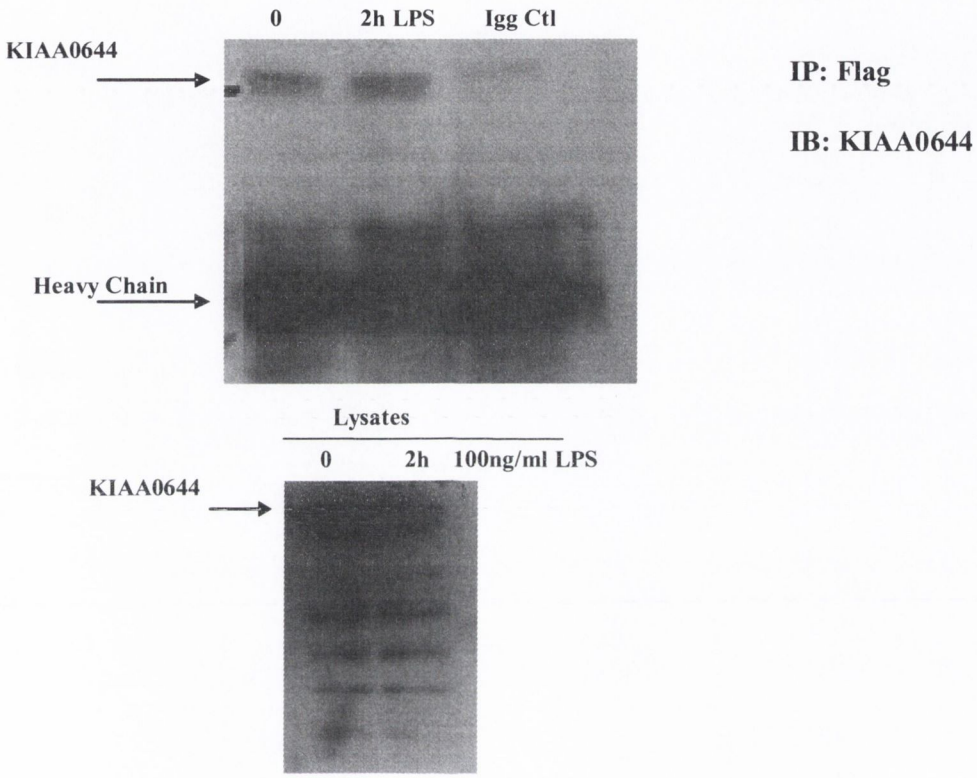


Figure 5.8 TLR4 interacts with endogenous KIAA0644. Human embryonic kidney cells (HEK 293) were set up at 1×10^5 cells/ml in 10 cm dishes. After 24 h they were transfected with 10 ug of Flag TLR4. After a further 24 h the cells were stimulated with 100 ng/ml LPS for 2 h, after which the cells were harvested in high stringency buffer. 50 μl of each sample was removed and assayed for KIAA0644 expression. The remaining lysate was added to flag tagged beads, or to a mouse non-conjugated Igg control for 24 h at 4°C. Immunocomplexes were assayed for KIAA0644.

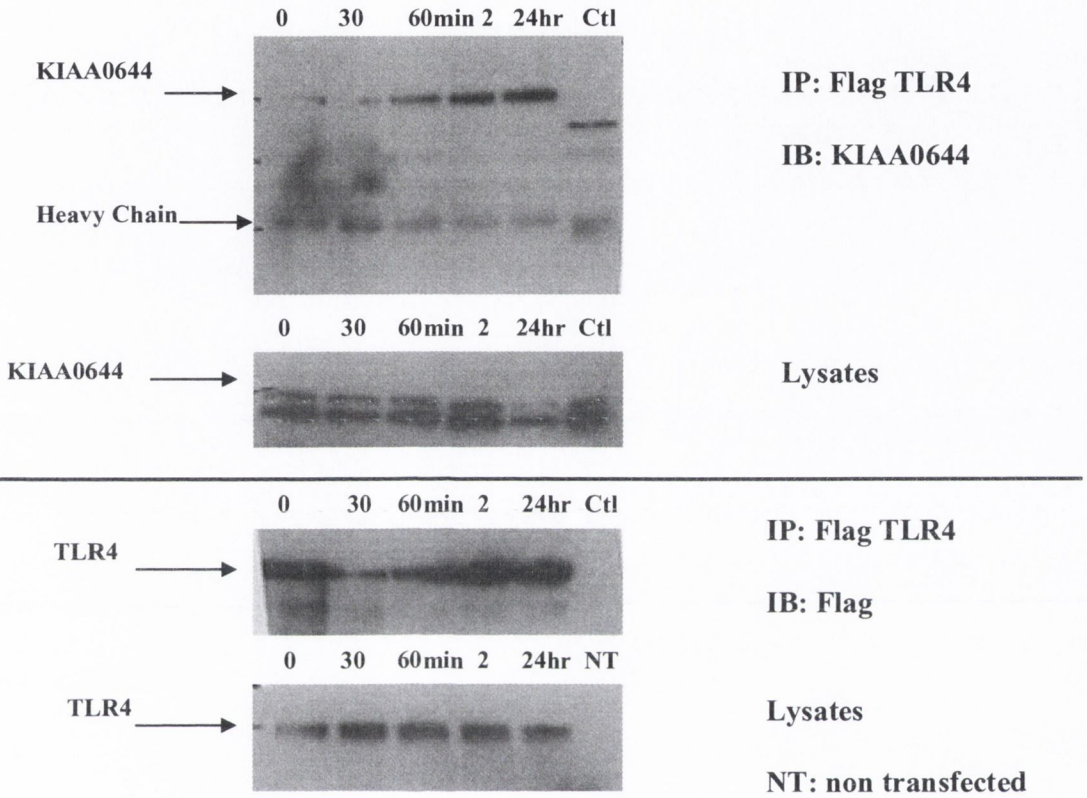


Figure 5.9 TLR4 interacts with endogenous KIAA0644. This interaction increases when stimulated with LPS. HEK 293 cells were set up at 1×10^5 cells/ml in 10 cm dishes. After 24 h they were transfected with 10 ug of Flag TLR4. After a further 24 h the cells were stimulated with 100 ng/ml LPS for 2 h, after which the cells were harvested in high stringency buffer. 50μl of each sample was removed and blotted for KIAA0644 and Flag TLR4. The rest of the lysate was added to flag tagged beads or to mouse agarose control beads (Ctl) for 24 h at 4°C. Immunocomplexes were assayed for KIAA0644 and Flag TLR4 respectively.

5.2.3.4 KIAA0644 can interact with CD14

It is clear that KIAA0644 interacts with TLR4 and this interaction is enhanced upon LPS stimulation. These results again confirm that KIAA0644 is part of the LPS signalling complex. CD14 is another crucial protein involved in the LPS signalling pathway. It acts as a co-receptor responsible for binding to LPS monomers. However there is still a lot unknown about the exact role of CD14 within the signalling complex. It has been reported that CD14 is only needed for responding to smooth LPS. However I have found that in cells reconstituted with CD14, they respond to both rough and smooth LPS (Fig 5.3 and Fig 5.4). Once I confirmed that KIAA0644 interacts with TLR4, I wanted to determine if it could bind to CD14.

U373 cells were transfected with CD14 expressing plasmid. Cells were stimulated with LPS and then harvested. The immunoprecipitation was carried out in two ways. The samples were immunoprecipitated with protein A/G beads cross-linked with CD14 antibody and then probed for KIAA0644. The other samples were immunoprecipitated with protein A/G beads cross-linked with KIAA0644 antibody and probed for CD14 expression. The control sample was added to protein A/G beads cross-linked with rabbit non-conjugated IgG antibody. Fig 5.10 shows that endogenous KIAA0644 can interact with CD14 and this interaction decreases after 24 h stimulation with LPS.

In summary the interaction between KIAA0644 and CD14 decreases with LPS stimulation and the interaction between KIAA0644 and TLR4 increases. Therefore it is possible that KIAA0644 is needed to transfer LPS from CD14 onto TLR4.

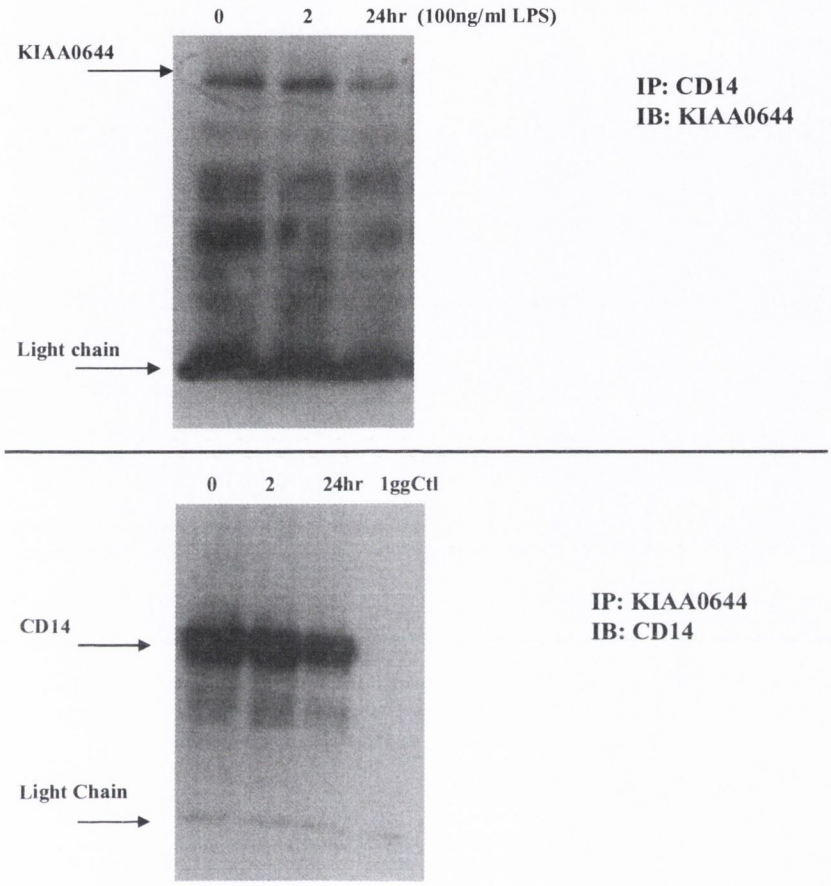


Figure 5.10 Endogenous KIAA0644 can interact with CD14. This interaction decreases upon stimulation with LPS. HEK 293 cells were set up at 1×10^5 cells/ml in 10 cm dishes. After 24 h they were transfected with 10 ug of CD14. After a further 24 h the cells were stimulated with 100 ng/ml LPS for 2 h and 24 h, after which the cells were harvested in high stringency buffer. 20ul of KIAA0644 and CD14 antibodies were pre-coupled overnight to protein A/G beads. Beads were washed and the lysates were added for 24 h at 4°C. Immunocomplexes were assayed for KIAA0644 and CD14 respectively.

5.2.4 Knockdown of KIAA0644 affects that LPS signalling pathway in U373/CD14 cells

5.2.4.1 siRNA specifically knocks down KIAA0644 expression in U373/CD14 cells

The data I have complied so far indicates that KIAA0644 is an LPS-inducible protein. Over-expression of KIAA0644 clearly enhances cells responses to LPS and it is also capable of binding to LPS. The results indicating that KIAA0644 binds to CD14 at a basal level and moves away following LPS stimulation could also be an important indicator of the function of the protein. KIAA0644 has also been shown to interact with TLR4 and this interaction increases following LPS stimulation. The main way to show that a protein has a vital function is to generate knockout mice. However in the absence of knockout cells another very useful method to determine function is RNAi. Therefore the next step in this study was to examine what effect knocking down KIAA0644 would have on LPS signalling. siRNA constructs to the gene were designed and obtained from the company Dharmacon. Previous experiments show that KIAA0644 is present and inducible in U373/CD14 cells and therefore this cell line was chosen to optimise the initial siRNA experiments.

One siRNA construct specific to KIAA0644 was transfected into U373/CD14 cells using oligofectamine. After 48 h the cells were harvested and KIAA0644 expression levels were determined by western blot. This siRNA construct successfully knocked down KIAA0644 (Fig 5.11, lane 2 and 3 siRNA). A control non targeting construct was also added into U373/CD14 cells to act as a negative control to ensure that the siRNA to KIAA0644 is specific. No decrease in KIAA0644 expression levels should be observed when the non targeting control is transfected into cells.

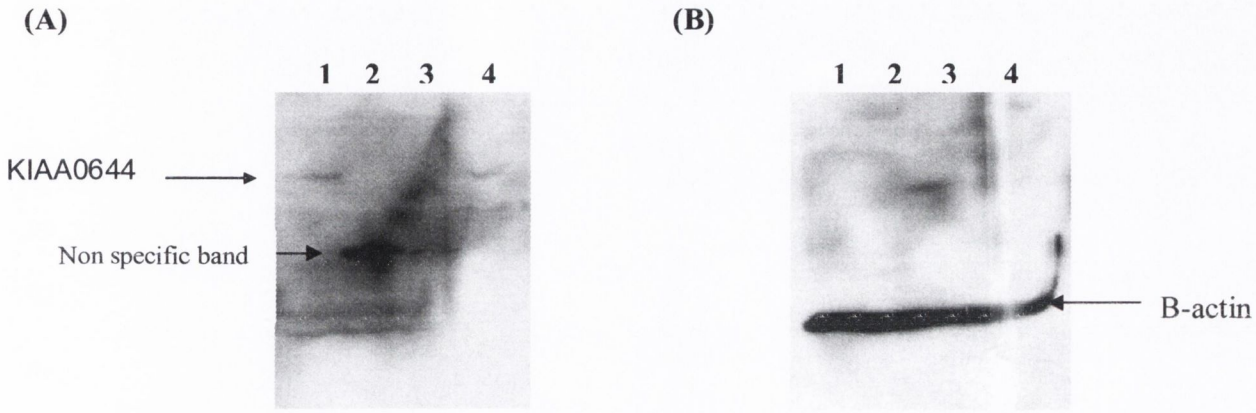


Figure 5.11 KIAA0644 is specifically knocked down in U373/CD14 cells using siRNA. siRNA oligos specific to KIAA0644 were designed and obtained from the company Dharmacon. U373/CD14 cells were set up at 5×10^4 cells/ml in 6 well plates. After 24 h the media was changed to serum free and siRNA oligos were transfected in using oligofectamine. After 6 hs media containing 20 % FCS and 2×L glutamine was added to the non transfected cells (lane1), the cells transfected with siRNA (200 nM) to KIAA0644 (lane2) and to the cells transfected with the non targeting siRNA control (200 nM) (lane 4). Lane 3 contains cells transfected with siRNA to KIAA0644 and these cells were cultured only in serum free media. Cells were then harvested after 48 h. 60 μl of sample buffer was added directly to the cells. Samples were sonicated, resolved on a 10% SDS gels, transferred onto PVDF membrane, and immunoblotted with anti-KIAA0644 (figureA) and anti-β-actin (figureB) respectively.

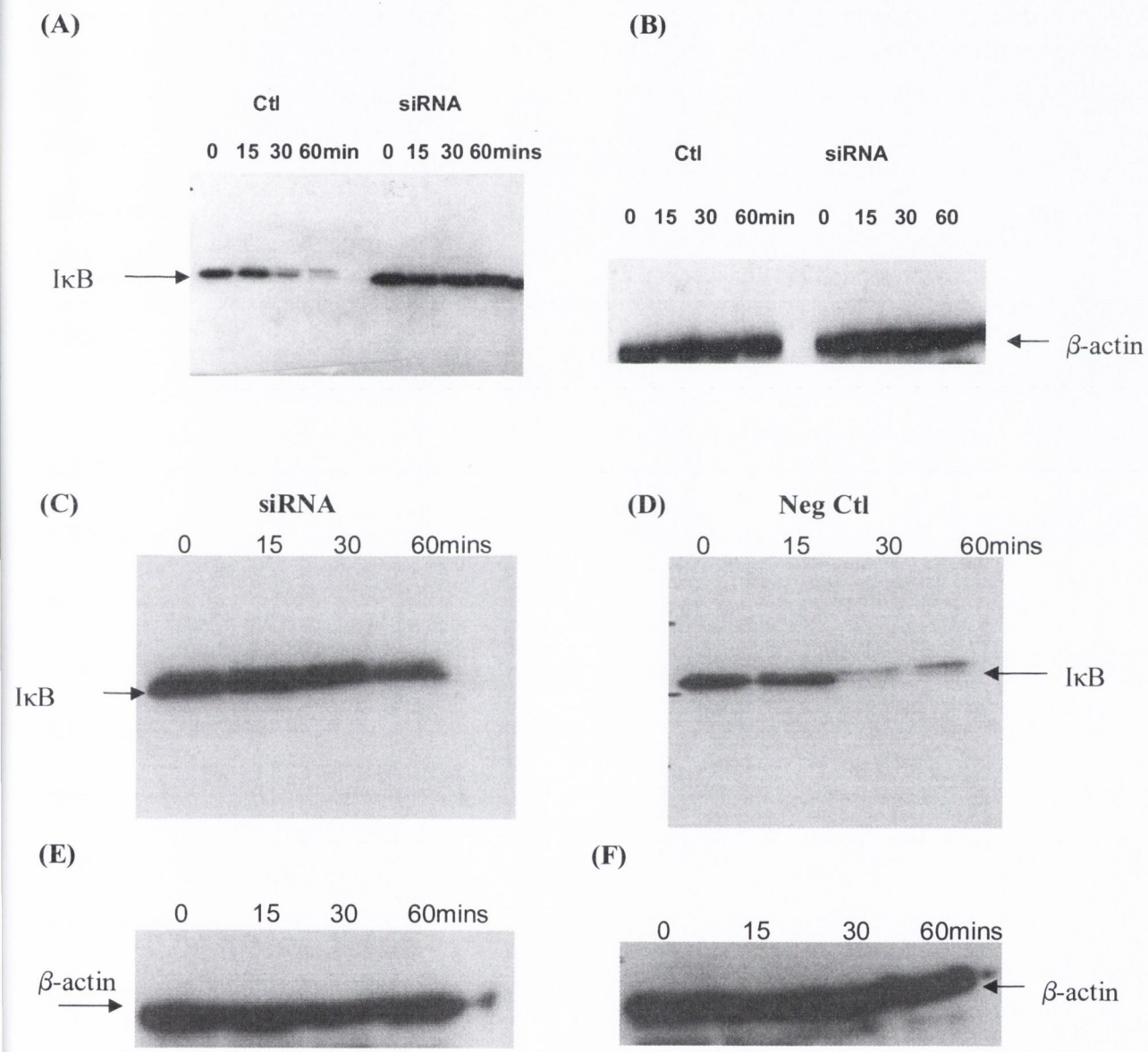
5.2.4.2 Knocking down KIAA0644 in U373/CD14 cells causes an inhibition of IκB degradation following LPS stimulation

I have shown in previous results that KIAA0644 is induced by LPS in U373/CD14 cells (Fig 4.1). Once it was confirmed that KIAA0644 was being consistently knocked down, I went on to examine any effects that knocking down this protein would have on LPS signalling. The first read out for LPS stimulation I investigated was IκB degradation. U373/CD14 cells respond to LPS which leads to IκB degradation normally following 30 and 60 min stimulations with 100 ng/ml LPS (Fig 5.12 part A). When siRNA to KIAA0644 is added to these cells there is no longer any IκB degradation observed (Fig 5.12 part (A). This result was repeated in part C. This suggests that KIAA0644 is somehow involved in LPS signalling leading to IκB degradation. A non targeting siRNA was used as a negative control to ensure that the effects observed with the siRNA to KIAA0644 were specific. When this construct was transfected into the cells IκB degradation was observed as normal, again confirming that siRNA to KIAA0644 and its effects on the cell are exact (Fig 5.12 part D). Each blot in Fig 5.12 was reprobed with anti β-actin antibody to show equal loading of protein in each lane (Fig 5.12 part B, E and F).

5.2.4.3 siRNA from Qiagen specifically knocks down KIAA0644 in U373/CD14 cells

The siRNA from dharmacon was capable of knocking down KIAA0644 in U373/CD14 cells (Fig 5.11). However in order to confirm that the results observed are specific and correct I wanted to confirm the knockdown and the effects on the LPS signalling pathway using a different siRNA oligo. A number of oligos were purchased from Qiagen, however only siRNA no.4 worked consistently.

Figure 5.12 Knockdown of KIAA0644 in U373/CD14 cells inhibits IκB degradation following LPS stimulation. U373/CD14 cells were seeded at 5×10^4 cells/ml in 6 well dishes. In figure A, cells were either transfected with siRNA specific to KIAA0644 using oligofectamine or not transfected (Ctl). In figure C, cells were transfected with siRNA (200 nM) to KIAA0644 using oligofectamine and in figure D the cells were transfected with a non targeting siRNA (200 nM) (negative control). All samples were treated with 100 ng/ml LPS for the time points indicated above. They were then harvested after 48 h. 60 μl of sample buffer was added directly to the cells. Samples were sonicated, resolved on a 10 % SDS gels, then transferred onto PVDF membrane, and immunoblotted with anti IκB-α (figure A, figure C and D) and anti β-actin (figure B, E and F) antibodies respectively. This data represents thee separate experiments.


(A)
Ctl
siRNA
0 15 30 60min 0 15 30 60mins
IκB
(B)
Ctl
siRNA
0 15 30 60min 0 15 30 60
β-actin
(C)
siRNA
0 15 30 60mins
IκB
(D)
Neg Ctl
0 15 30 60mins
IκB
(E)
0 15 30 60mins
β-actin
(F)
0 15 30 60mins
β-actin

The same protocol was used as described previously and siRNA No.4 was transfected into U373/CD14 cells using oligofectamine. After a total of 72 h the cells were stimulated with 100 ng/ml LPS for the indicated time points. Expression levels of KIAA0644 were analysed by western blot. In the previous experiments IκB degradation was used as the LPS read out (Fig 5.12), this time phosphorylation of p38 was examined to determine if knockdown of KIAA0644 can affect this as well as IκB degradation in response to LPS. Fig 5.13 (A) shows that siRNA causes a decrease in KIAA0644 expression levels in U373/CD14 cells. Fig 5.13 (B) shows that when KIAA0644 expression is decreased there is no phosphorylation of p38 in response to LPS. β-actin is measured to ensure equal protein loading in each lane.

5.2.4.4 Partial knockdown of KIAA0644 still affects the LPS signalling pathway

siRNA as a technique is quite technically difficult and is therefore very hard to obtain complete knockdown of a protein. However even 50 % knockdown can be sufficient to see effects. While carrying out siRNA on U373/CD14 cells I often observed that KIAA0644 was only partially knocked down. Importantly however this partial decrease in KIAA0644 expression at 0 and 30 min stimulations is enough to affect the LPS signalling pathway. Fig 5.14 shows partial knockdown of KIAA0644 at the protein level. IκB degradation was recorded in response to LPS. When KIAA0644 is knocked down there is impaired IκB degradation in response to LPS compared to the controls. Since KIAA0644 ordinarily gets induced 30 min post LPS stimulation it is not surprising that when it is knocked down using siRNA it could begin to get induced at a later time point, which is what is observed in Fig 5.14.

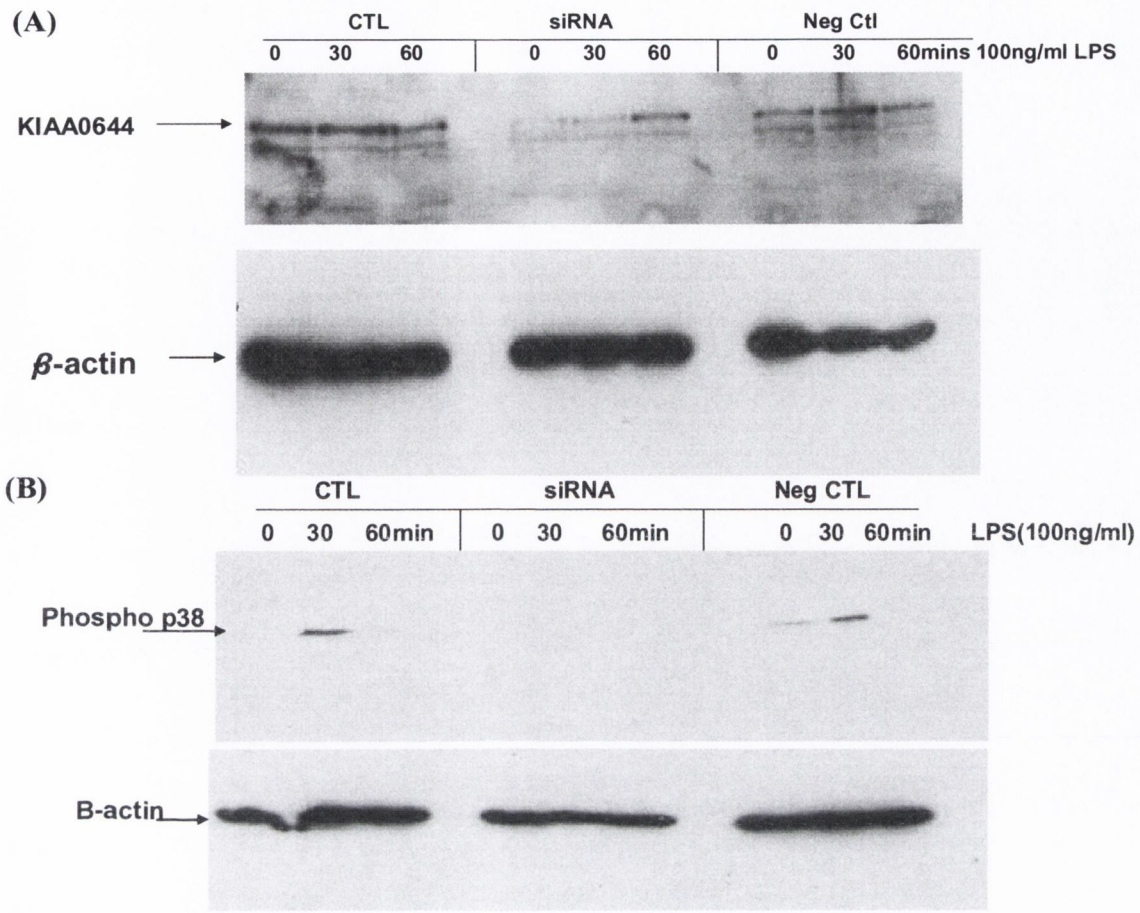


Figure 5.13 Knockdown of KIAA0644 in U373/CD14 cells affects the LPS signalling pathway. U373/CD14 cells were set up at 5×10^4 cells/ml in 6 well plates. After 24 h the media was changed to serum free media. The control cells were un-transfected. siRNA from qiagen was transfected at a concentration of 50 nM using oligofectamine. The negative control cells were transfected with a non targeting siRNA at a concentration of 50 nM. Cells were incubated for 48 h before being stimulated with 100 ng/ml LPS for the time points indicated above. 60 μl of sample buffer was added directly to the cells. Samples were sonicated, resolved on a 10 % SDS gels, transferred onto PVDF membrane, and immunoblotted for phosphorylated p38 and β-actin respectively. This result represents three independent experiments.

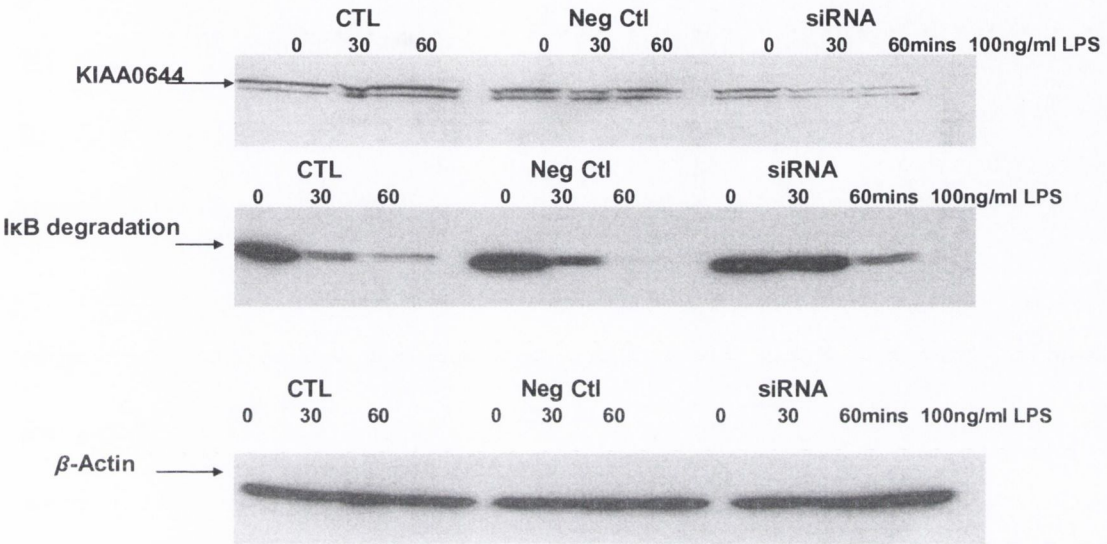


Figure 5.14 Partial knockdown of KIAA0644 in U373CD14 cells affects the LPS signalling pathway. U373/CD14 cells were set up at 5×10^4 cells/ml in 6 well plates. After 24 h the media was changed to serum free media. The Control cells were untransfected. siRNA from qiagen was transfected at a concentration of 50 nM using oligofectamine. The negative control cells were transfected with a scrambled version of siRNA at a concentration of 50 nM. Cells were incubated for 72 h before being stimulated with 100 ng/ml LPS for the time points indicated above. 60 μl of sample buffer was added directly to the cells. Samples were sonicated, resolved on a 10% SDS gels, transferred onto PVDF membrane, and immunoblotted for IκB and β-actin respectively. This result represents two independent experiments.

5.2.4.5 Knockdown of KIAA0644 does not affect the TNF-α signalling pathway in U373/CD14 cells

In order to determine if KIAA0644 is exclusively part of the LPS signalling pathway I had to examine if knockdown of it's expression would affect another signalling pathway such as the TNF-α signalling pathway. I had previously shown that KIAA0644 is not inducible by TNF-α (Fig 4.22) therefore we would predict that knocking KIAA0644 down should not have an affect on the TNF-α signalling pathway.

U373/CD14 cells were transfected with siRNA No.4 from Qiagen using oligofectamine. After a total of 72 h the cells were stimulated with TNF-α (20 ng/ml) for the indicated time points. Expression levels of KIAA0644 and IκB degradation were analysed by western blot. Fig 5.15 shows that KIAA0644 expression is decreased with the siRNA and this does not have any affect on the TNF-α signalling pathway.

5.2.5 siRNA no.4 from Qiagen does not display off target effects

5.2.5.1 siRNA to KIAA0644 does not affect TLR4 expression levels

Clearly knocking down KIAA0644 expression affects the LPS signalling pathway in a number of cells lines. However it was feared that siRNA may be non-specifically knocking down other proteins involved in the LPS signalling pathway. Clearly TLR4 is the crucial receptor required for the recognition of LPS within the immune system. Therefore I chose to examine its expression levels to ensure that the siRNA to KIAA0644 was not having off target effects and non-specifically knocking down TLR4.

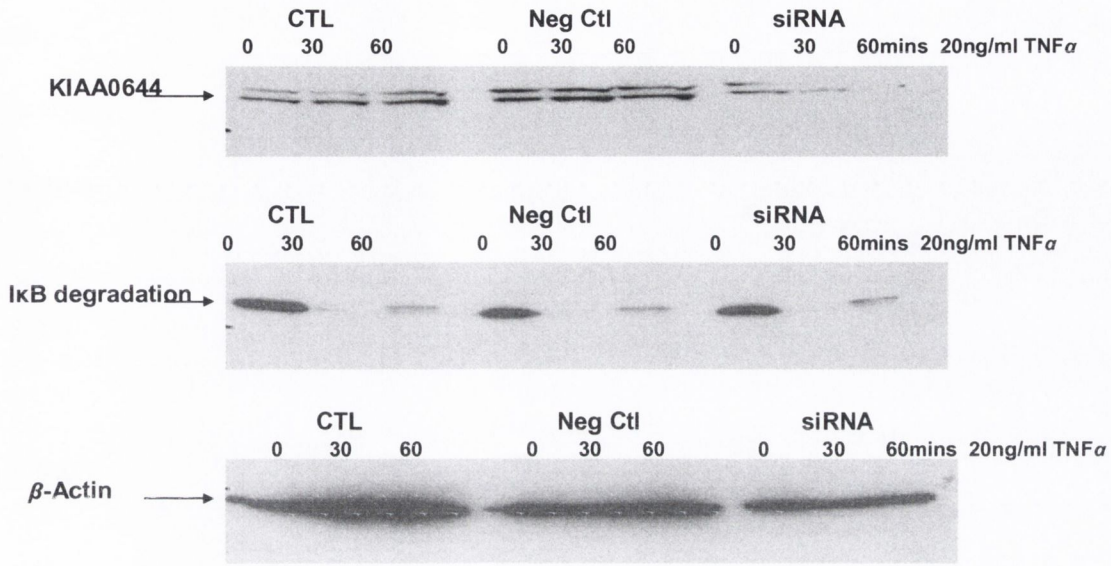


Figure 5.15 Knockdown of KIAA0644 in U373/CD14 cells does not affect the TNF-α signalling pathway. U373/CD14 cells were set up at 5×10^4 cells/ml in 6 well plates. After 24 h the media was changed to serum free media. The control cells were untransfected. siRNA from qiagen was transfected at a concentration of 50 nM using oligofectamine. The negative control cells were transfected with a scrambled version of siRNA at a concentration of 50 nM. Cells were incubated for 72 h before being stimulated with 20 ng/ml TNF-α for the time points indicated above. 60 μl of sample buffer was added directly to the cells. Samples were sonicated, resolved on a 10% SDS gels, transferred onto PVDF membrane, and immunoblotted for IκB and β-actin respectively. This result represents two independent experiments.

TLR4 is expressed in U373/CD14 cells as they are capable of responding to LPS stimulation. siRNA to KIAA0644 was transfected into these cells using oligofectamine. After 72 h the cells were harvested and TLR4 expression levels were analysed by western blot. Fig 5.16 shows that siRNA to KIAA0644 does not alter the expression levels of TLR4 in these cells.

5.2.6 Knockdown of KIAA0644 in THP 1 cells affects the LPS signalling Pathway

5.2.6.1 siRNA (qiagen) specifically knocks down KIAA0644 expression in THP1 cells

All previous siRNA results were carried out in the astrocytoma cell line U373/CD14. In order to ensure that the effects observed are not cell type specific I decided to carry out siRNA studies in the monocytic cell line THP1. THP1 cells are a suspension cell line and therefore are unable to be transfected using oligofectamine. Therefore the siRNA oligos transfection was optimized using the amaxa based transfection system.

THP1 cells were electroporated in the amaxa machine using the S-019 program. Samples were then added to 12 well plates containing RPMI. After 72 h the cells were stimulated with 100 ng/ml LPS for a number time points, the negative control used in these experiments is a scrambled version of siRNA no.4 from qiagen. KIAA0644 expression levels and IκB degradation were recorded by western blot. Fig 5.17 shows that KIAA0644 gets induced by LPS after 30min stimulation in the negative control sample. When siRNA is transfected in KIAA0644 expression is abolished. This knockdown of KIAA0644 has only a marginal effect on LPS induced IκB degradation. β-actin levels are recorded to ensure equal protein loading. The IκB degradation is not inhibited as much in the THP1 cells when KIAA0644 is knocked down compared to the U373/CD14 cells. Perhaps in these particular cells KIAA0644

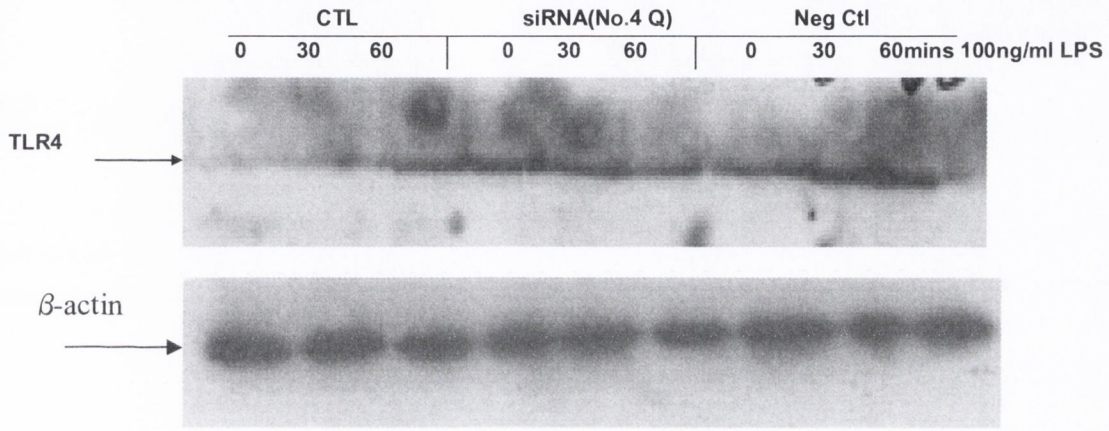


Figure 5.16 siRNA to KIAA0644 does not affect TLR4 expression levels. U373/CD14 cells were set up at 5×10^4 cells/ml in 6 well plates. After 24 h the media was changed to serum free media. The control cells were untransfected. siRNA from qiagen was transfected at a concentration of 50 nM using oligofectamine. The negative control cells were transfected with a scrambled version of siRNA at a concentration of 50 nM. Cells were incubated for 72 h before being stimulated with 100 ng/ml LPS for the time points indicated above. 60 μl of sample buffer was added directly to the cells. Samples were sonicated, resolved on a 10% SDS gels, transferred onto PVDF membrane, and immunoblotted for TLR4 and β-actin respectively.

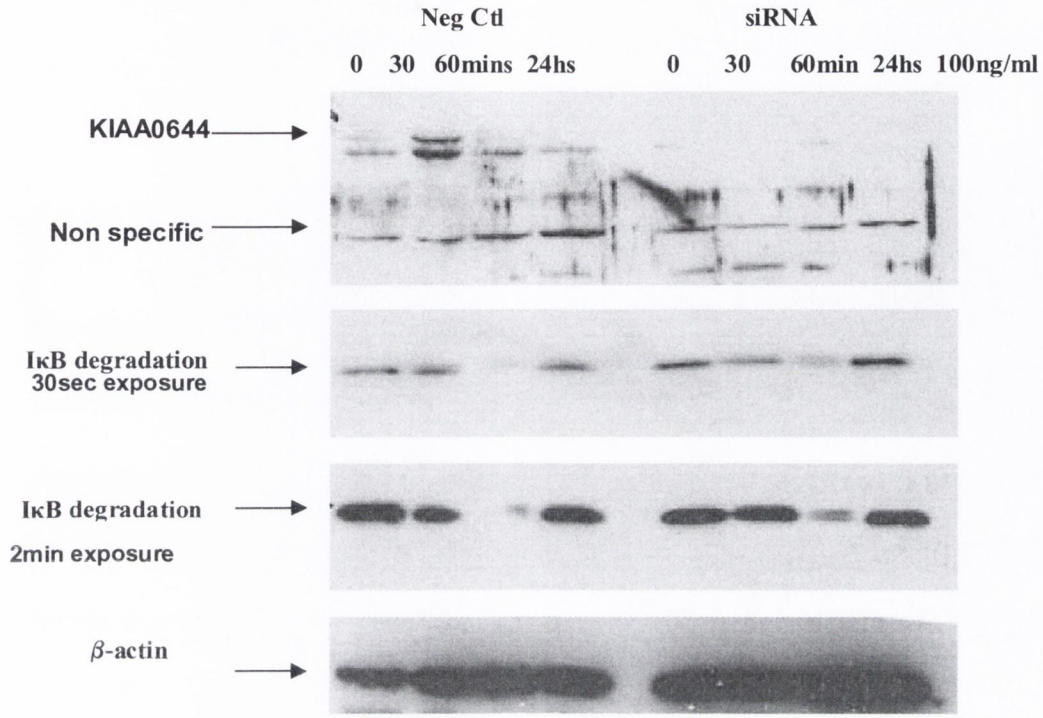


Figure 5.17 Knockdown of KIAA0644 in THP1 cells using the amaxa system affects the LPS signalling pathway. THP1 cells were set up at a concentration of 1×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was transfected into THP1 cells using the amaxa program S-019. Following electorporation of the samples, they were added to complete RMPI medium and incubated at 37°C for 72 h. Samples were stimulated with 100 ng/ml LPS for the time points indicated. Samples were harvested, lysed and sonicated in sample buffer, resolved on a 10% SDS gels, transferred onto PVDF membrane, and immunoblotted with anti-KIAA0644, anti- IκB and β-actin respectively. This result represents two independent experiments.

is not as crucial for LPS induced IκB degradation as it is in the U373/CD14 cells. There may also be a small residual pool of KIAA0644 present which is enough to allow the pathway to be triggered.

5.2.6.2 Decreased KIAA0644 expression leads to less TNF-α, IL-6 and IL1-β cytokine release in response to LPS

Knockdown of KIAA0644 expression has been shown to affect the LPS signalling pathway by causing a decrease in IκB degradation and phosphorylation of p38 in response to LPS stimulation. I decided to study the cytokine release from THP1 cells in response to LPS stimulation to determine if knockdown of KIAA0644 expression levels would have any effect on this process.

THP1 cells were electroporated using program S-019 on the amaxa system. Cells were grown in RPMI for 72 h. Cells were stimulated with 100 ng/ml LPS for 24 h. Supernatants were removed from the cells and TNF-α and IL-6 elisas were carried out. The remaining cells were lysed in buffer RLT and RNA was extracted in order to carry out RT-PCR to confirm knockdown of KIAA0644 expression at the RNA level. Fig 5.18 shows, that there is a decrease in both TNF-α and IL-6 cytokine production in response to LPS when KIAA0644 is knocked down compared to the scrambled negative control samples. Fig 5.19 shows the RT-PCR results which indicate that there is approximately a 50% decrease in KIAA0644 RNA expression when siRNA is transfected into the cells.

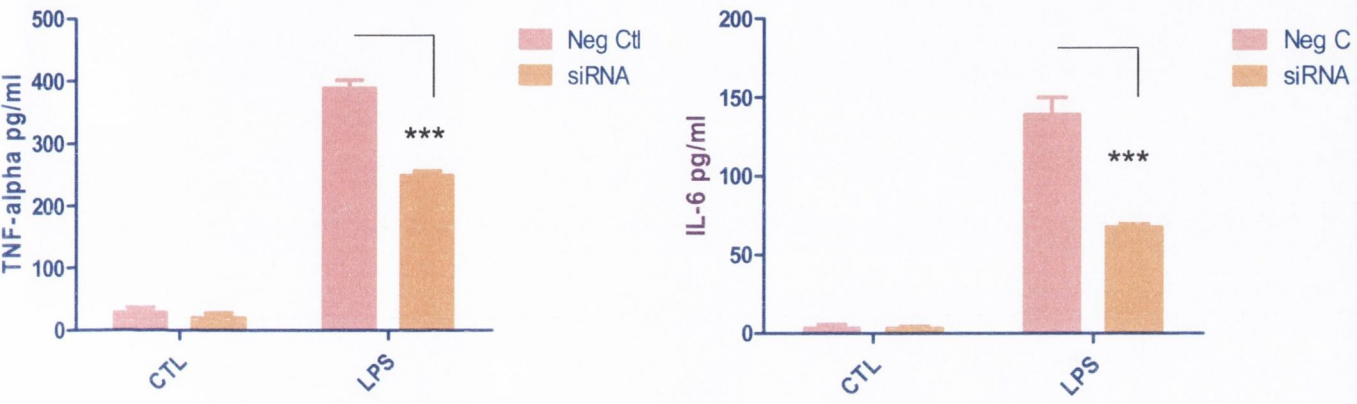


Figure 5.18 Knockdown of KIAA0644 in THP1 cells affects the levels of cytokines released following LPS stimulation. THP1 cells were set up at a concentration of 1×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was tranfected into THP1 cells using the amaxa program S-019. Following electroporation, cells were added to complete RMPI media in 24 well plates and incubated at 37°C for 72 h. Samples were stimulated with 100 ng/ml LPS for 24 h. They were removed and centrifuged at 1000 *g* for 5 min. Supernatants were removed, TNF-α and IL-6 elisas were carried out. A Tukey-Kramer multiple comparisons test was performed on all data. This represents three separate experiments.

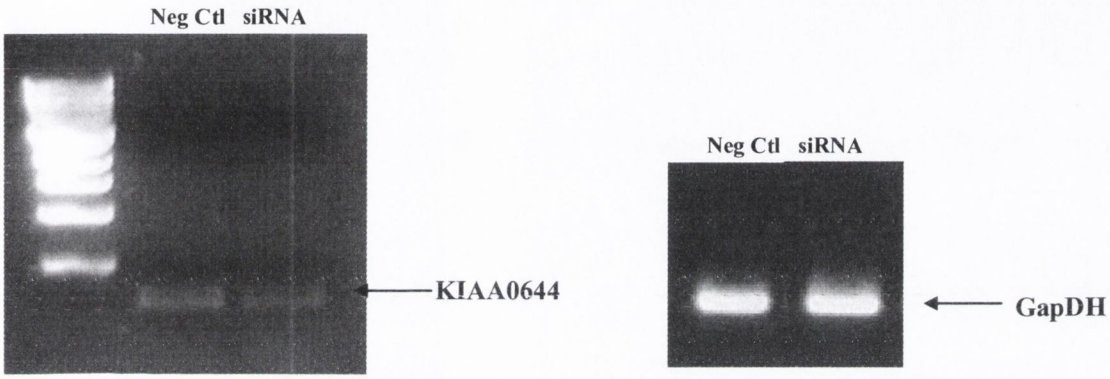


Figure 5.19 Confirmation of knockdown of KIAA0644 in THP1 cells by RT-PCR. THP1 cells were set up at a concentration of 1×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was transfected into THP1 cells using the amaxa program S-019. Following electroporation the cells were added to complete RMPI medium in 24 well plates and incubated at 37 ^{o}C for 72 h. Cells were harvested and lysed in buffer R1. Primers specific to KIAA0644 were designed and RT-PCR was performed.

5.2.7 Knockdown of KIAA0644 in human PBMC affects the LPS signalling pathway

5.2.7.1 siRNA (qiagen) specifically knocks down KIAA0644 protein expression in human PBMC

All siRNA results so far have been carried out in cell lines which are known to respond to LPS stimulation. I wanted to confirm the siRNA results in a primary cell type, therefore we choose to look at human PBMC. Human PBMC respond to LPS stimulation and previous results had indicated that KIAA0644 is inducible by LPS in these cells (Fig 4.21). PBMC are a semi-adherent cell line and are difficult to transfect, therefore the amaxa system was used to carry out transfections.

Human blood was obtained from the blood bank. PBMC were isolated using a ficoll gradient. Cells were electroporated with siRNA or a scrambled negative control siRNA using program S-019 on the amaxa machine. After 72 h cells were stimulated with 100 ng/ml LPS for 60 and 30 min. Samples were harvested and KIAA0644 expression levels were observed. Fig 5.20 shows that KIAA0644 is knocked down using siRNA oligos and not with the scrambled negative control oligos. In the negative control samples it is seen that KIAA0644 get induced by LPS stimulation after 30 and 60 min.

5.2.7.2 siRNA knocks down KIAA0644 at the RNA level in human PBMC

Fig 5.20 confirms that KIAA0644 is knocked down at the protein level. I wanted to ensure that it was also being affected at the RNA level.

PBMC were isolated from human blood using a ficoll gradient. Cells were electroporated with siRNA or a scrambled negative control siRNA using the amaxa program S-019. After 72 h the samples were lysed in RLT buffer. RNA was extracted from the cells and quantified using a spectrophometer. Each sample was normalized

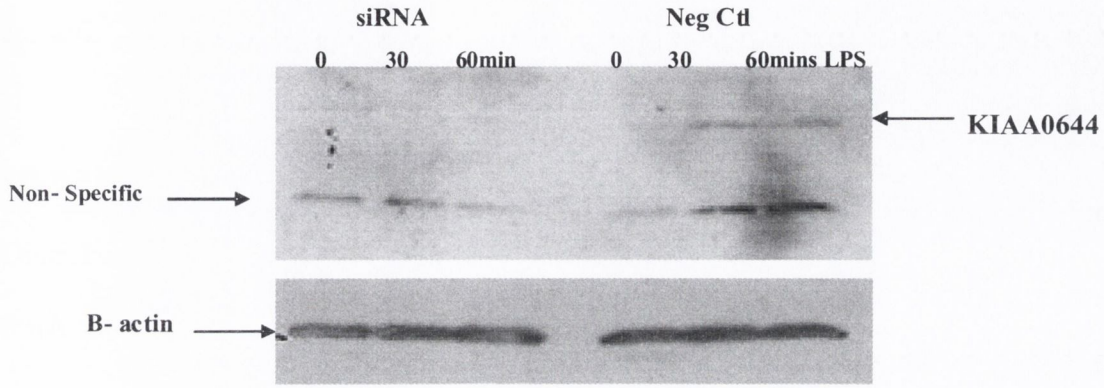


Figure 5.20 KIAA0644 can be knocked down using siRNA in human peripheral blood mononuclear cells. PBMC were isolated from whole blood using a ficoll gradient. Cells were set up at a concentration of 2×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was tranfected into the cells using the amaxa program S-019. Following electroporation the cells were added to complete IMDM media in 24 well plates and incubated at 37 ^{o}C for 72 h. Samples were stimulated with 100 ng/ml LPS for 24 h. Samples were harvested, lysed and sonicated in sample buffer, resolved on a 10 % SDS gels, transferred onto PVDF membrane, and immunoblotted with anti-KIAA0644 and β-actin respectively. This result represents 4 independent experiments.

to ensure equal RNA concentrations are in each sample. Primers to KIAA0644 were designed and obtained from MWG. PCR was carried out to determine the RNA levels of KIAA0644 in the cells. Fig 5.21 clearly shows decreased RNA levels of KIAA0644 when siRNA is transfected into the cells. GapDH is used as a loading control.

5.2.7.3 Knockdown of KIAA0644 affects the LPS signalling pathway in PBMC

Once I was able to confirm knockdown of KIAA0644 at both the protein and the RNA level in PBMC I wanted to determine what affect this decrease in KIAA0644 expression would have on the LPS signalling pathway.

PBMCs were electroporated with siRNA or a scrambled negative control siRNA using program S-019 on the amaxa machine. After 72 h cells were stimulated with 100 ng/ml LPS for 60 and 30 min. Samples were harvested and IκB degradation and phosphorylation of p38 were used as LPS signalling read outs. Fig 5.22 show that when KIAA0644 is knocked down using siRNA is there a delay in the degradation of IκB after 30 min stimulation with LPS when compared to the negative control sample. Phosphorylation of p38 is seen in the negative control sample following 60 min stimulation with LPS. This is abolished when KIAA0644 is knocked down (Fig 5.23).

5.2.7.4 Decreased KIAA0644 expression leads to less TNF-α, IL-6 and IL-1β cytokine release in response to LPS

Decreased expression of KIAA0644 clearly affects the IκB degradation and phosphorylation of p38 in response to LPS. In order to further highlight KIAA0644's involvement in the LPS signalling pathway I wanted to examine additional readouts. Therefore I choose to look at the cytokine release from PBMC in response to LPS.

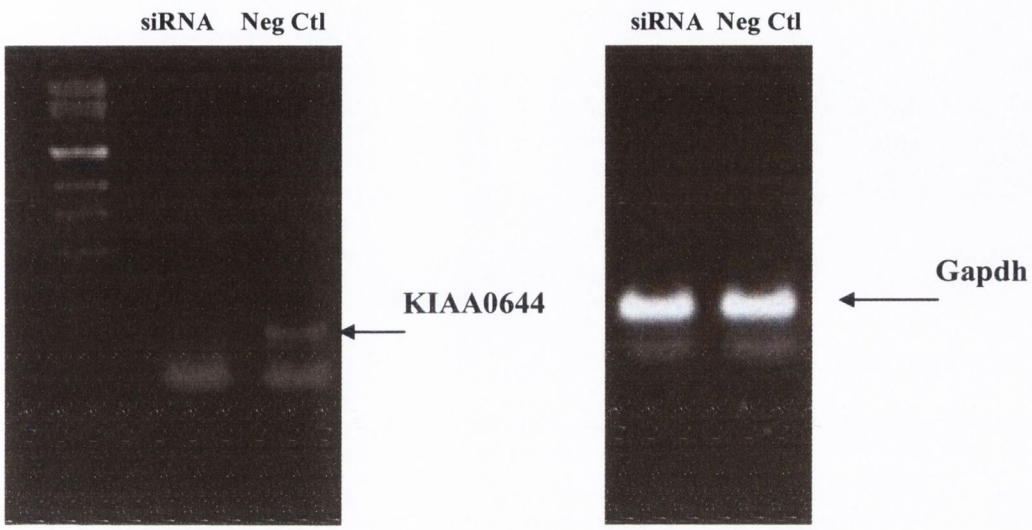


Figure 5.21 RT-PCR confirms knockdown of KIAA0644 in human PBMC. Blood was obtained from the blood bank, PBMC were isolated using a ficoll gradient. Cells were set up at a concentration of 2×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was transfected into the cells using the amaxa program S-019. Subsequently the samples were added to complete RMPI medium in 24 well plates and incubated at 37°C for 72 h. Cells were harvested and lysed in buffer R1. Primers specific to KIAA0644 were designed and RT-PCR was performed. This result corresponds to two independent experiments.

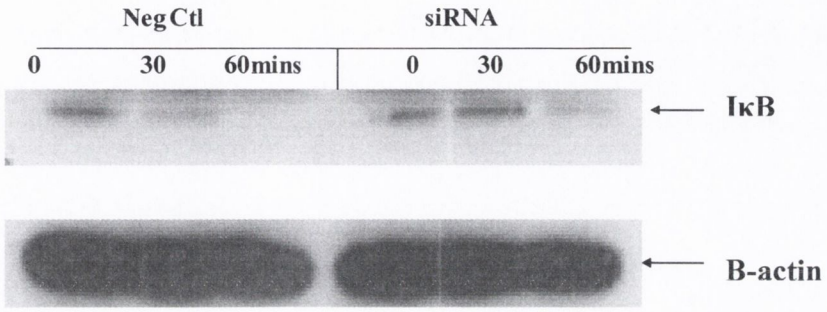


Figure 5.22 Knockdown of KIAA0644 in PBMC affects IκB degradation in response to LPS stimulation. PBMC were isolated from whole blood using a ficoll gradient. Cells were set up at a concentration of 2×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was tranfected into the cells using the amaxa program S-019. Following electroporation the samples were added to complete IMDM media in 24 well plates and incubated at 37°C for 72 h. Samples were stimulated with 100 ng/ml LPS for 24 h. Samples were harvested, lysed and sonicated in sample buffer, resolved on a 10% SDS gels, transferred onto PVDF membrane, and immunoblotted with anti-IκB and β-actin respectively. This result represents three independent experiments.

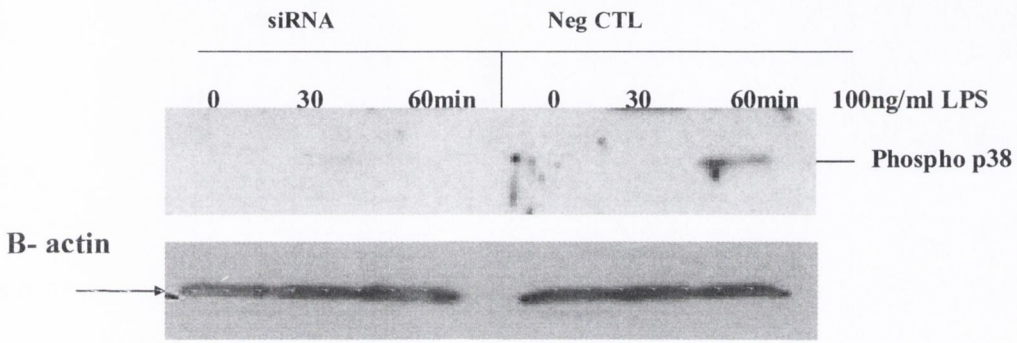


Figure 5.23 Knockdown of KIAA0644 affects phosphorylation of p38 in response to LPS stimulation. PBMC were isolated from whole blood using a ficoll gradient. Cells were set up at a concentration of 2×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was tranfected into the cells using the amaxa program S-019. Subsequently the samples were added to complete IMDM media in 24 well plates and incubated at 37°C for 72 h. Samples were stimulated with 100 ng/ml LPS for 24 h. Samples were harvested, lysed and sonicated in sample buffer, resolved on a 10% SDS gels, transferred onto PVDF membrane, and immunoblotted with anti-phospho p38 and β-actin respectively. This result represents two independent experiments.

PBMCs were electroporated with siRNA or a scrambled negative control siRNA using program S-019 on the amaxa machine. After 72 h cells were stimulated with 100 ng/ml LPS for an additional 24 h. Supernatants were removed from the cells and cytokine elisas were carried out. Fig 5.24 shows that when KIAA0644 is knocked down using siRNA there is a decrease in TNF-α, IL-6 and IL-1β release in response to LPS.

5.2.7.5 Knockdown of KIAA0644 expression does not affect the TNF-α signalling Pathway

As outlined previously using the U373/CD14 cell line, KIAA0644 did not appear to be involved in the TNF-α signalling pathway. I wanted to confirm this again in a primary cell line and therefore choose human PBMC. I had also shown in Fig 4.23 that KIAA0644 does not get induced following TNF-α stimulation. Therefore it would be assumed that knocking down KIAA0644 expression would not have any affect on the TNF- α signalling pathway.

PBMCs were electroporated with siRNA or a scrambled negative control siRNA using program S-019 on the amaxa machine. After 72 h cells were stimulated with 20 ng/ml LPS for 30 and 60 min. Samples were harvested and IκB degradation and phosphorylation of p38 were used as TNF-α signalling read outs. Fig 5.25 shows that when KIAA0644 is knocked down using siRNA it has no affect on the TNF-α signalling pathway. When PBMC are stimulated with TNF-α, it leads to IκB degradation after 30 min and phosphorylation of p38 after 60 min. This also occurs when KIAA0644 is knocked down therefore this suggests that it is not involved in the TNF-α signalling pathway.

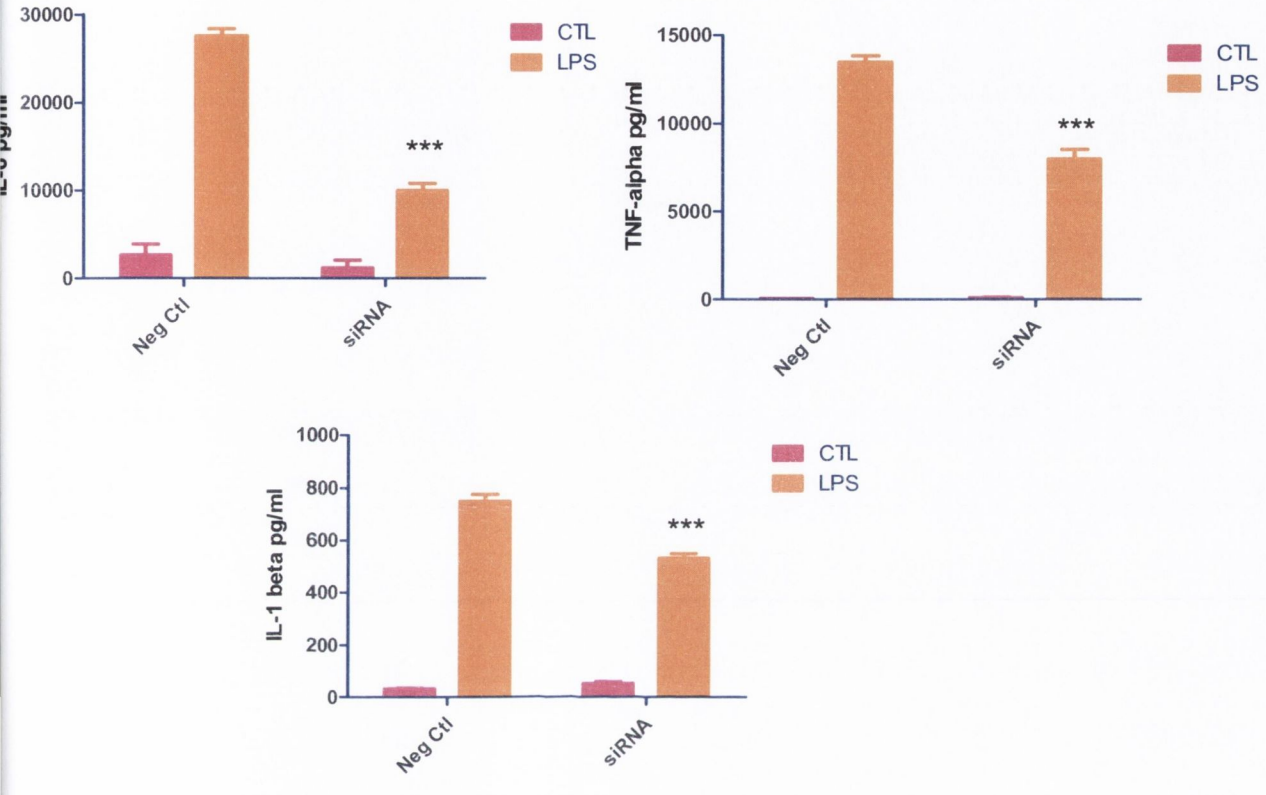
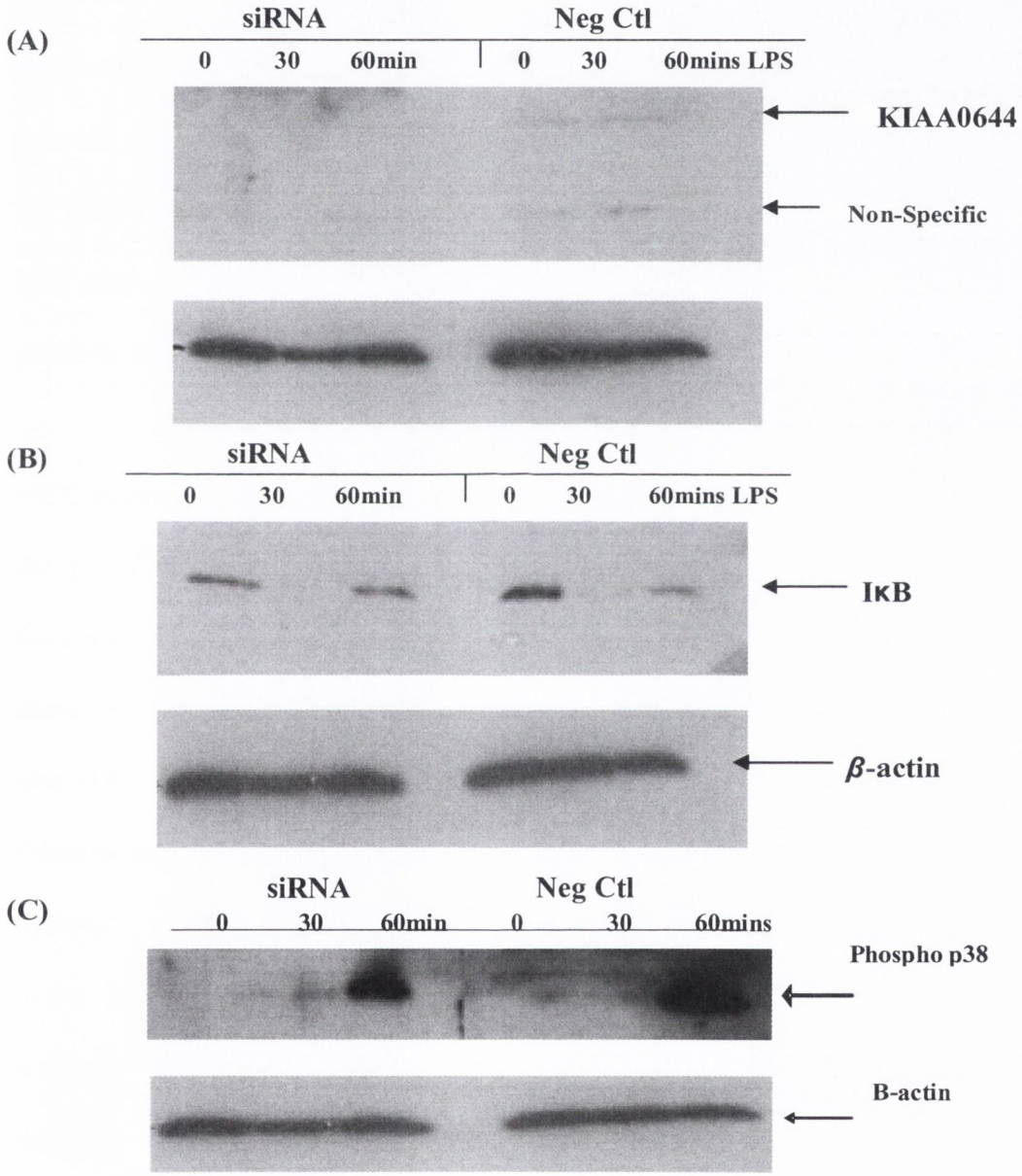


Figure 5.24 Knockdown of KIAA0644 in PBMC causes a decrease in IL6, TNF-α and IL-1β release in response to LPS stimulation. PBMC were isolated from whole blood using a ficoll gradient. Cells were set up at a concentration of 2×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was tranfected into the cells using the amaxa program S-019. Following electroporation, the samples were added to complete IMDM media in 24 well plates and incubated at 37 ^{o}C for 72 h. Samples were stimulated with 100 ng/ml LPS for 24 h. They were removed and centrifuged at 1000 $\times$ *g* for 5 min. Supernatants were removed, TNF-α, IL-6 and IL-1β elisas were carried out. A Tukey-Kramer multiple comparisons test was performed on all data.This represents four separate experiments.

Figure 5.25 Knockdown of KIAA0644 in PBMC does not affect the TNF-α signalling pathway. PBMC were isolated from whole blood using a ficoll gradient. Cells were set up at a concentration of 2×10^6 cells per point. siRNA from qiagen or a scrambled control of the siRNA was transfected into the cells using the amaxa program S-019. Following electroporation the cells were added to complete IMDM media in 24 well plates and incubated at 37 ^{o}C for 72 h. Samples were stimulated with 20 ng/ml TNF-α for 30 and 60 min. Samples were harvested, lysed and sonicated in sample buffer, resolved on a 10 % SDS gels, transferred onto PVDF membrane. They were immunoblotted with anti-KIAA0644 and β-actin (A), anti-IκB and β-actin (B) and with anti-phospho p38 and β-actin (C) respectively.


(A)
siRNA
Neg Ctl
0
30
60min
0
30
60mins LPS
KIAA0644
Non-Specific
(B)
siRNA
Neg Ctl
0
30
60min
0
30
60mins LPS
IκB
β-actin
(C)
siRNA
Neg Ctl
0
30
60min
0
30
60mins
Phospho p38
B-actin

5.3 Discussion

Bacterial LPS is recognised by the receptor complex consisting of TLR4, MD2 and CD14. Signal transduction from this complex occurs when the adaptor molecules MyD88, MAL, TRIF and TRAM are recruited to the complex and ultimately lead to inflammatory gene expression. This is a highly complex signalling cascade involving a number of critical proteins. Recently there is an increasing amount of data suggesting that there are more proteins involved in the LPS signalling pathway which possess distinct and novel roles. Classically the LPS signalling cascade proceeds in the following manner: LPS bind LBP, which transfers monomers to CD14, which in turn passes LPS to the TLR4/MD2 complex leading to the recruitment of adaptor proteins and subsequent gene expression.

MD2 provides the duel role of binding LPS and enhancing TLR4 expression levels at the membrane surface. Another endoplasmic reticulum chaperon protein gp96 has been shown to be required for the association of MD2 and TLR4. Cells lacking gp96 show that TLR4 is unable to associate at the cell surface (Randow *et al.*, 2001). They also show that gp96 is involved in the cellular distribution of TLR2 as well as TLR4. Discovery of this protein has introduced another level of complexity to the TLR4 pathway, indicating that gp96 is needed to allow association of TLR4 and MD2 to occur. MD2 can exist in a membrane associated form bound to TLR4 but it is mainly a soluble protein. Several studies have been carried out on a human kidney cell line that does not express MD2. In these cells TLR4 is still expressed on the cell surface, suggesting that there are other proteins present within the immune system that may compensate for the lack of MD2 (Kennedy *et al.*, 2004, JIA *et al.*, 2004, Gioannini *et al.*, 2004). It is also possible that soluble MD2 could facilitate membrane expression

of TLR4 in these cells. A recently identified novel protein PRAT4A has been shown to have a role in the cell surface expression of TLR4 in a manner analogous to MD2 (Wakabayashi *et al.*, 2006). It has also been shown that PRAT4A is required for the subcellular distribution of the number of TLRs (Takahashi *et al.*, 2007).

The role of CD14 has been under investigation for a number of years. Clearly it is very important for TLR4 signalling, as mice lacking CD14 are insensitive to LPS challenge (Moore *et al.*, 2000, Haziot *et al.*, 1996). All studies on CD14 agree that its main function is to concentrate LPS and increase the sensitivity of TLR4, although this may be an important function it doesn't appear to be essential. Based on this hypothesis cell lacking CD14 can still respond to LPS albeit with delayed kinetics. As mentioned previously CD14 has been reported to be required for responses to smooth LPS only and not to rough LPS (Jiang *et al.*, 2005).This paper suggests that CD14 is required for the MyD88 dependent pathway in response to smooth LPS, however CD14 is needed for the MyD88 independent pathways in response to both rough and smooth LPS. This result indicated that TLR4/MD2 can bind to rough but not smooth LPS in the absence of CD14. Although there have been over 1500 publications on the role of CD14 in LPS signalling, there still remains a number of unanswered questions surrounding its apparent non essential role. It is unclear why rough LPS is capable of binding to the TLR4/MD2 complex without CD14 and how it can lead to differential downstream responses. Data from this chapter suggests that cells that do not express CD14 are capable of responding to rough or smooth LPS, indicating that there may be another receptor in the complex which has not yet been identified. β2 integrins has been shown to be able to partially compensate for the role of CD14 (Ingalls *et al.*,

1995). This would suggest that there could be other proteins present in the immune system capable of compensating for CD14 in cells lacking the protein.

Results in chapter four confirmed that KIAA0644 is a functional protein, expressed in a wide variety of cells, astrocytes, microglia and macrophages and tissues, brain, lung, ovary etc. KIAA0644 is inducible of LPS at both the RNA and protein level. The protein is mainly localised to the cytosol, when over-expressed it is present in the membrane. We predict that it is present in undefined vesicles throughout the cytosol. There is also a secreted form of the protein. The next step in the study of this protein was to carry out a biochemical analysis and try to determine the biological function of the protein

Following on from the data indicating that KIAA0644 is inducible by LPS, the next step was to elucidate if over-expressing KIAA0644 would have any affect on LPS signalling. Two approaches were used, NFκB luciferase based assay and cytokine read outs in response to LPS. The best characterised regulation of NFκB following stimulation with LPS occurs via the release of NFκB from the inhibitory protein IκBα, subsequently allowing NFκB to translocate to the nucleus and bind its target genes. For the NFκB luciferase based assay U373/CD14 cells were chosen as the experimental model. I demonstrated that KIAA0644 can enhance activation of NFκB in response to LPS. Over-expression of KIAA0644 alone was capable of increasing the NFκB-linked luciferase expression, and this was considerably enhanced following LPS stimulation. These results along with the knockdown results suggest a role for KIAA0644 in the process of IκBα degradation and hence activation of NFκB.

The next step was to determine what affect over-expression of KIAA0644 would have on cytokine secretion following LPS stimulation. I choose to carry these experiments out on U373 parental cells and also on MEFs. These cell lines were chosen as they do not express membrane bound CD14. It is interesting to note that in our hands these cells are still capable of responding to LPS, both rough and smooth, therefore indicating that there must be another receptor present compensating for the lack of CD14. U373 parental cells respond with slower kinetics than U373/CD14 cells, further confirming the role of CD14 in enhancing TLR4 sensitivity to LPS. However it cannot be overlooked that although U373 parental cells do not express CD14 they are still capable of producing cytokines in response to LPS. When the U373 cells are cultured in media containing 10 % FCS it could be the presence of soluble CD14 in the serum which allows responses to LPS. However I have demonstrated that when U373 parental cells are cultured in serum free media they are still capable of responding to LPS and lead to cytokine production. This could be due to the expression of β2 integrins or perhaps KIAA0644 is involved. I have shown that when KIAA0644 is over-expressed in U373 parental cells it leads to a considerable increase in IL-6, TNF-α and Rantes production in response to LPS. In the same experiment cells were reconstituted with CD14 and they showed the same increase in cytokine production in response to LPS as the cells over-expressing KIAA0644. This result was also observed when cells are cultured overnight in serum free media. This data suggests that KIAA0644 is capable of compensating for the lack of CD14 expressed in these cells and would appear to have a similar role in the LPS signalling pathway as CD14. Over-expressing KIAA0644 causes an increase in sensitivity to LPS therefore leading to an increase in cytokine production. When KIAA0644 and CD14 were co-expressed no additional increase in cytokine production was observed. This indicates

that the cells are responding at their maximum to the ligand and that either protein alone is sufficient to supply a full signal to the complex. These results were also repeated in MEFs, which showed increased cytokine release in response to both rough and smooth LPS when KIAA0644 or CD14 was over-expressed. Crucially over-expression of KIAA0644 in U373 parental cells did not lead to an increase in cytokine production following TNF-α stimulation, providing even more evidence that KIAA0644 is specific to the LPS signalling pathway and not involved in the TNF-α signalling pathway.

CD14 functions to transfer LPS monomers to the MD2/TLR4 signalling pathway. LPS is composed of O-antigen repeats, the core region, and lipid A. The O-antigen structures vary by strain and are recognized by the adaptive immune system, resulting in the production of specific antibodies. Lipid A is the target for the innate immune system and consists of conserved structures (Raetz *et al.*, 1990). Both MD2 and CD14 have been shown to bind directly to LPS. However it has been suggested that MD2 binding of LPS is more specific and stronger towards pathogenic LPS and that CD14 possess a broader spectrum ability to bind a variety of LPS from various bacteria (Koraha *et al.*, 2005). KIAA0644 like CD14 and MD2 was shown to be capable of binding LPS. Biotinylated LPS was used to precipitate KIAA0644 from cell lysates and unlabelled LPS was used to compete with the interaction between LPS and KIAA0644. This data again confirms that KIAA0644 is part of the LPS signalling complex. Since it appears that KIAA0644 has the ability to increase TLR4s sensitivity to LPS stimulation to the same extent as CD14, it is not surprising that KIAA0644 can bind LPS in a manner analogous to CD14.

In this chapter I have established that KIAA0644 can make physical contact with both CD14 and TLR4. I have showed that KIAA0644 binds with greater affinity to wild type TLR4 than to the mutant form of TLR4. The TLR4 (P/H) mutant is unable to respond to LPS due to its inability to homodimerise and when it is over-expressed it out competes wild type TLR4 and therefore acts as a dominant negative on the TLR4 signalling pathway. KIAA0644 may be binding with greater affinity to the wild type TLR4 as it may require the receptor to dimerise in order to form a complex, therefore it binds with a lower affinity to the mutant TLR4 which is unable to dimerise. Over-expression of leucine rich repeat containing proteins can often lead to false positive interactions as the proteins interact artificially via their LRR domains. It was important that I could show that Flag tagged TLR4 can interact with endogenous KIAA0644..therefore removing the possibility that the interactions previously observe was due to the over-expression of the two leucine rich repeating containing proteins. A very interesting finding that emerged from these interaction studies was that KIAA0644 interacted more with TLR4 following LPS stimulation. I was also able to demonstrate that endogenous KIAA0644 is capable of binding to CD14 and that this interaction weakened with LPS stimulation. Since I was able to show that KIAA0644 can bind LPS these interaction observations would suggest that perhaps KIAA0644 is required to initially bind CD14 and then transfer LPS onto TLR4. KIAA0644 could have a different role to play such as allowing for the association between LPS bound CD14 and the TLR4/MD2 receptor complex. Either way this data strengthens our argument that KIAA0644 is a key component of the LPS signalling pathway. Many questions still remain around the CD14 TLR4 receptor complex. Although it is clear that CD14 can bind LPS, CD14 is only a GPI anchored protein which does not possess any a transmembrane region and therefore cannot signal within the cell. Also

studies carried out using blocking antibodies to CD14 only partially impaired LPS binding, suggesting that there are other proteins capable of binding LPS (Lynn *et al* 1993, Troelstra *et al*,. 1997, Triantafilou *et al* 2000). It has also remained unclear how CD14-LPS binds to TLR4 to allow the initiation of a signal. Are CD14, TLR4 and MD2 solely required for LPS signalling or are there other proteins involved. Could KIAA0644 be the missing link in the pathway? KIAA0644's role may be to transfer LPS from CD14 onto TLR4 and allow downstream signalling to occur. It has been believed that LPS binds to CD14 which leads to the recruitment of the signalling receptors to the interaction site, CD14 is then believed to release LPS which then binds to TLR4/MD2 complex. This appears to be quite a non specific action within the immune system; therefore it would be more likely that there is another protein present within the complex that allows for the transfer of LPS from CD14 onto the TLR4/MD2 complex.

In order to determine the biological function of KIAA0644 RNAi technology was used. This is an excellent technique to determine the functional importance of a gene. In the absence of knockout cells it is the next best approach. It can also be carried out in human cells and therefore may provide a more realistic view of the proteins function. A number of criteria can be adopted when choosing siRNA oligos such as choosing ones with low G/C content (Reynolds *et al.*, 2004). Some oligos work much better than others, and different transfection approaches may also lead to greater knockdown. I tried a number of oligos from Dharmacon, Ambion and Qiagen. I also tested a number of the oligos in combination to try and obtain the most consistent knockdown, however the danger with using more than one oligo is that it increases the chances of off target effects. I optimised two oligos, one from Dharmacon and one

from Qiagen. In the end the oligo from Qiagen gave the most consistent knockdown at the lowest concentration, therefore limiting any off target effects that may occur. In all experiments a non-targeting negative control or a scrambled version of the siRNA oligo was used as a negative control to ensure that KIAA0644 was not being non-specifically knocked down. Knockdown of the gene was confirmed both at the RNA and protein level.

Initial siRNA experiments were optimisied in U373/CD14 cells as I had previously determined that KIAA0644 is inducible by LPS in these cells. Knockdown of KIAA0644 in these cells has a dramatic affect on the LPS signalling pathway. When KIAA0644 expression is abolished IκB degradation and Phosphorylation of P38 no longer occurs in response to LPS. These results were also repeated in the human monocytic cell line THP1 and importantly in the human primary cells PBMCs. Both THP1 cells and PBMCs are semi adherent and hence are difficult to tranfect using conventional lipid based transfection reagents. Therefore the newly developed amaxa transfection system was optimised and used on these cells. Cytokine data from these cells also revealed that when KIAA0644 expression is knocked down there is less IL-6, TNF-α and Rantes secreted from cells in response to LPS. Importantly knockdown of KIAA0644 did not have any affect on the TNF-α signalling pathway. This confirmed previous results showing that KIAA0644 expression was not inducible by TNF-α and therefore provides evidence that KIAA0644 is specific to the LPS signalling pathway. As an additional control expression levels of TLR4 were assayed and shown to be unaffected by the transfection of siRNA oligos specific to KIAA0644. This is an important additional control to ensure that the results observed are specific to KIAA0644 and not the result of off target effects leading to decreased

expression of KIAA0644. Overall the results obtained from the gene silencing experiments established KIAA0644 as an essential protein involved in the LPS signalling pathway.

Many other LPS binding proteins have been identified over the years. LPS is the causative agent of sepsis syndrome and intensive studies have been carried out on the pathway to try and determine a therapeutic target to treat conditions such as sepsis. Due to the fact that this pathway involves a host of receptors within a signalling complex, targeting a single protein as a therapeutic remains futile. Various cell types have been shown to possess a variety of LPS binding proteins including heat shock proteins (Hsp) 70 and 90, chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (GDF5) (Triantafilou et al., 2001). Another highly complex LPS signalling complex was proposed consisting of CD14, TLR4, CD55, CD16a, CD11b/CD18, Fcγ-receptors CD32 and CD64, FcγRIIIa, CD36 and CD81 (Pfeiffer *et al.*, 2001). All these LPS complexes may form to allow a broader range of ligand recognition from various microbes. It is also possible that not all LPS binding proteins are expressed in particular cell types. Therefore in order for the immune system to function optimally a variety of LPS signalling complexes are capable of forming depending on the pathogen. Overall TLR4 is a common component present in most models of LPS recognition. However many gaps still remain in our understanding of exactly how LPS binds it's signalling apparatus leading to proinflammatory gene expression. This study examined KIAA0644 as a novel component of the TLR4-LPS signalling pathway. It is widely expressed throughout the body with highest expression levels in the brain. This localisation of the protein could be important functionally. Like all the

other proposed LPS binding proteins, KIAA0644 may have a specific expression pattern required for responding most efficiently to particular microbial challenge.

Chapter Six

Final Discussion and Future Perspectives

Chapter 6

6.1 Final Discussion and Future Perspectives

Within the innate immune system there are hundreds of LRR proteins which all play important and distinct functions. Many still remain uncharacterised and of the known proteins research is showing that some play more than one role within innate immunity. This study has focused on a novel LRR containing protein KIAA0644. This protein appears to be brain enriched as well as being more globally expressed throughout the body. The work to date indicates that it may have a role within TLR signalling. TLRs are essential initiators of innate immunity, which are capable of recognising a wide range of microbial products. Once activated the TLR signalling pathways ultimately lead to the production of inflammatory proteins which can trigger innate and adaptive immunity. Although these pathways have been under intense scrutiny for the past ten years many unanswered questions still remain around ligand binding and subsequent receptor activation and the proteins involved in these processes. The initial goal of this project was to identify novel LPS inducible genes through use of a microarray experiment carried out on wild type and MAL knockout embryonic stem cells. The results from this array led to the identification of KIAA0644.

Initial work carried out on KIAA0644 was all computer based using web based prediction programs which allowed for the identification of 13 leucine rich repeats, a fibronectin domain and a transmembrane domain within the protein. Structural homology searches showed that KIAA0644 is quite similar to the TLR family and also to the brain enriched protein Lingo-1. A recent paper published by Dolan *et al* also suggested that through LRR homology studies KIAA0644 is more closely linked to the TLR family than to other LRR containing families. RNA and protein expression

studies confirmed that this protein is widely expressed throughout tissues in the body and is inducible by LPS. Transient over-expression of KIAA0644 enhanced LPS signalling in a number of different cell types. Knockdown of KIAA0644 expression greatly affected the LPS signalling pathway. These results were confirmed in astrocytoma cell line, a monocytic cell line and also in primary human monocytes. These cells showed decreased IκB degradation and phosphorylation of p38 following LPS stimulation when KIAA0644 was knocked down. There were also less cytokines released from cells when expression of KIAA0644 was reduced. All these results indicated that KIAA0644 is an essential component of the LPS signalling pathway.

Although all work in this thesis suggests that KIAA0644 plays a role within the LPS signalling pathway the exact function of the protein is still unknown. The TLR4 signalling pathway is highly complex and a number of proteins are required to allow TLR4 to function. As described previously LPS initially binds LBP and transfers LPS monomers to CD14 which then delivers the LPS to the MD2/TLR4 complex. MD2 has been shown to be essential for TLR4 signalling and it is required for cell surface expression of TLR4 (Nagai *et al.*, 2002). TLR4 is unable to signal without MD2 (Schromm *et al.*, 2001) however the role for CD14 is still under much investigation. Although it is clear that CD14 expression allows for a greater response to LPS its role does not appear to be essential. Work by Jiang *et al* has suggested that CD14 is required for binding smooth LPS and delivering it to TLR4 whereas they show that rough LPS can bind TLR4 without CD14. There is some controversy over the role for CD14 and work I have carried out on cells transiently expressing CD14 show no differences when stimulated with rough or smooth LPS. The presence of CD14 in these experiments enhances the responses of the cells to both rough and smooth LPS.

The ultimate question of this thesis is what is the function of KIAA0644 and what role does it have within the TLR signalling complex? KIAA0644 shares a number of common features with the components of the TLR4 complex. CD14 can exist in two forms, GPI anchored and in a secreted form and it is made up of leucine rich repeats. MD2 is mainly found as a soluble protein but it is membrane associated when bound to TLR4. KIAA0644 like MD2 and CD14 exists as a membrane protein and also in a secreted form and it also contains leucine rich repeats. Many questions still remain around the exact functioning of each protein within the TLR4 complex. I found that cells that do not possess CD14 are still capable of responding to LPS except with slower kinetics. MD2 has been shown to be required for cell surface expression of TLR4, however some cells have been identified that do not possess MD2 and still express TLR4 at the cell surface. It could be argued that the presence of soluble forms of both CD14 and MD2 can help compensate for the lack of membrane bound forms. However it is also possible that there are other proteins within the immune system that can compensate for these proteins. Newly identified proteins such as PRAT4A have been shown to share roles with MD2. I have shown that KIAA0644 is capable of binding to both TLR4 and CD14. More importantly I have shown that endogenous KIAA0644 can bind TLR4 and this interaction increased upon LPS stimulation. I have also shown that endogenous KIAA0644 binds CD14 and this interaction decreases following LPS stimulation. Therefore it is possible that KIAA0644 is facilitating the movement of LPS from CD14 onto TLR4 as it has also been shown that KIAA0644 can bind LPS (Fig 6.1). Another possible function is that KIAA0644 can compensate for a lack of CD14 in cells. I have showed that over-expression of KIAA0644 in cells lacking CD14 leads to an enhancement in LPS signalling similar to results observed when CD14 is over-expressed. If as Jiang et al suggest CD14 is

only required for binding to smooth LPS perhaps KIAA0644 functions as the co-receptor for rough LPS and is therefore responsible for the activation of MyD88 dependent responses to rough LPS (Fig 6.2). Further experimental work is needed to confirm these hypotheses.

In order to fully understand the function of KIAA0644 within the immune system it is essential that work is carried out on knockout and transgenic animals. Knocking out the gene in mice should reveal a phenotype which will help us to determine the exact function of the protein. Such animal studies are required to confirm function. Experiments such as RNAi, although very useful do possess their own limitations. There is a high risk of off target affects which may lead to incorrect interpretation of function. All the components and their functions within the TLR signalling pathway have been confirmed through the use of knockout and transgenic animals. For years the receptor for LPS was unknown and the studies on animal models finally revealed the much sought after receptor to be TLR4. The key breakthrough in LPS signalling came from animal studies on a mutant strain of mice C3H/HeJ which were hyporesponsive to LPS due to a mutation in their TLR4 gene (Poltorak *et al.*, 1998). Studies on TLR4 transgenic mice showed that increased expression levels of TLR4 resulted in increased cytokine production, neutrophil recruitment and lung damage following intranasal administration of LPS (Togbe *et al.*, 2006). Functions for the other components of the TLR4 signalling pathway were also confirmed through work on knockout and transgenic studies. LPS lethality studies on CD14 deficient mice revealed that CD14 plays an important role in the LPS signalling pathway as these mice were resistant to toxic levels of LPS (Haziot *et al.*, 1996, Moore *et al.*, 2000). Work on CD14 transgenic mice have shown that these mice are hyperesponsive to

LPS and display increased sensitivity to endotoxic shock (Ferrero *et al.*, 1993). Further work on the CD14 transgenic mice indicate that these mice are more resistant to specific LPS lethality models in which an initial injection of LPS into the footpad of mice is administered prior to an intravenous injection of LPS (Tamura *et al.*, 1999).

All these results highlight the importance of work on animal models to the determination of a proteins function. Therefore it is essential that work is carried out on KIAA0644 knockout and transgenic mice in order to fully understand the function of this gene. LPS lethality models on KIAA0644 knockout and transgenic models would help confirm that this protein has a role in the LPS signalling pathway. Work from this thesis has indicated that KIAA0644 is a brain enriched protein therefore brain specific assays on KIAA0644 knockout mice should further reveal the exact role of this protein in neuroimmunology. TLRs are believed to be involved in the inflammatory response following stroke in the brain. This role was confirmed when the TLR4 knockout mice were put through a stroke disease model (Middle cerebral artery occlusion (MCAO)). These mice displayed lower infarct volumes and lower expression of proteins associated with brain damage following stroke such as IRF-1, nitric oxide synthase, cyclooxygenase-2 and matrix metalloproteinase 9 (Caso *et al.*, 2007; Caso *et al.*, 2008). Putting KIAA0644 knockout mice through disease models of stroke and other inflammatory related disease models such as arthritis and multiple sclerosis should reveal if this protein has a function surrounding these conditions. Earlier expression studies on wild type and experimental autoimmune encephalomyelitis (EAE) mice indicated that the protein expression level of KIAA0644 may be increased in the EAE mice model (Fig 4.13). EAE mice are an experimental model of multiple sclerosis. This model could be tested in the

KIAA0644 knockout mice and this would help reveal if this protein has a role in the pathogenesis of multiple sclerosis.

In conclusion this study has identified a novel leucine rich repeat containing protein expressed in brain, lung, kidney and spleen. It is expressed on astrocytes, microglia and macrophages and is induced by LPS. It is clearly capable of enhancing LPS signalling and inhibition of KIAA0644 expression greatly affects the LPS signalling pathway. The localisation and specific expression pattern of this protein may be important functionally. KIAA0644 may have a role within the TLR4/CD14/MD2 signalling complex or KIAA0644 may be capable of substituting for a co-receptor when it is not expressed. Either way this study shows that KIAA0644 is a functional protein, which is a key molecule within the LPS signalling pathway. Further work must be carried out to determine the exact role of this protein in TLR4 signalling. Studies on transgenic and knockout mice will provide valuable insight into the specific role of KIAA0644 in the immune system.

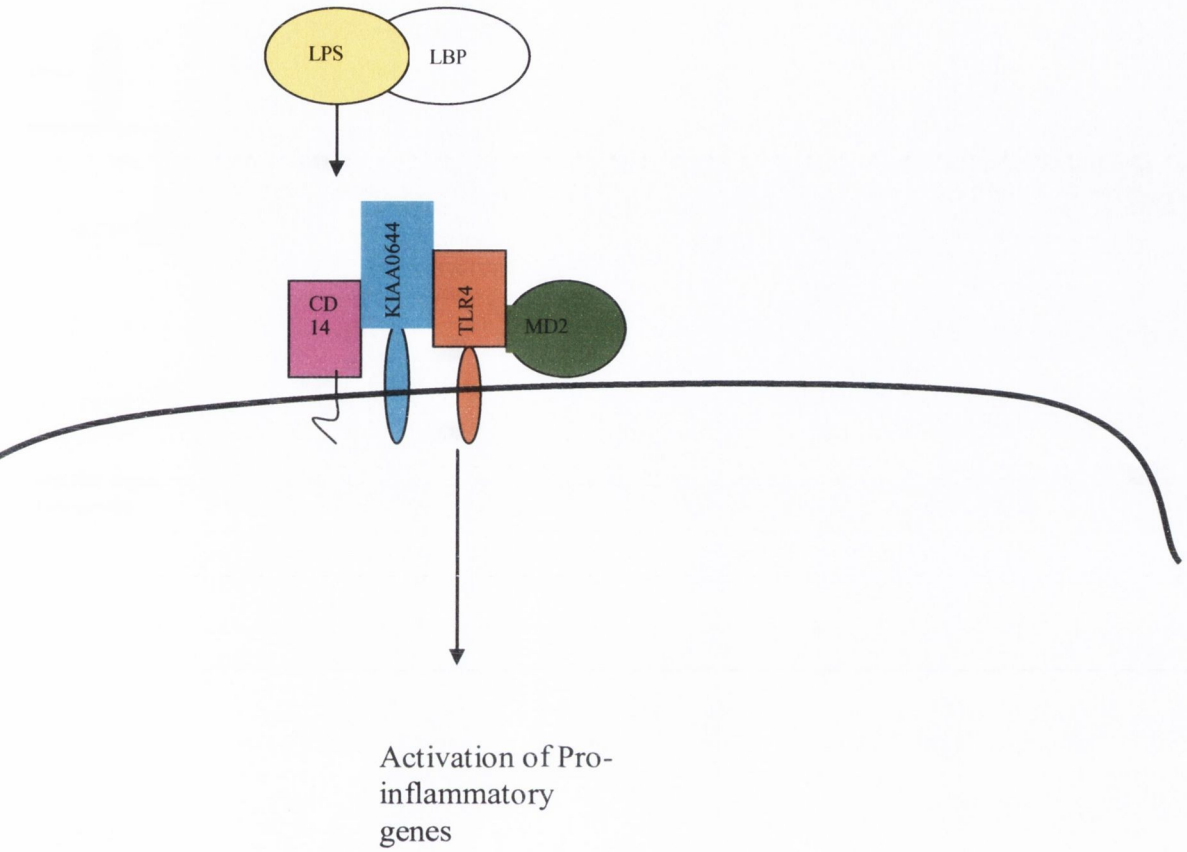


Fig 6.1 KIAA0644 may play a role in transferring LPS from CD14 to the TLR4/MD2 complex. KIAA0644 has been shown to bind CD14, TLR4 and LPS. The interaction between KIAA0644 and CD14 decreases following LPS stimulation where as its interaction with TLR4 increased post LPS stimulation. Therefore it is possible that KIAA0644 plays a role in the movement of LPS from the CD14/LPS complex onto the TLR4/MD2 complex leading to the activation of downstream signalling pathways.

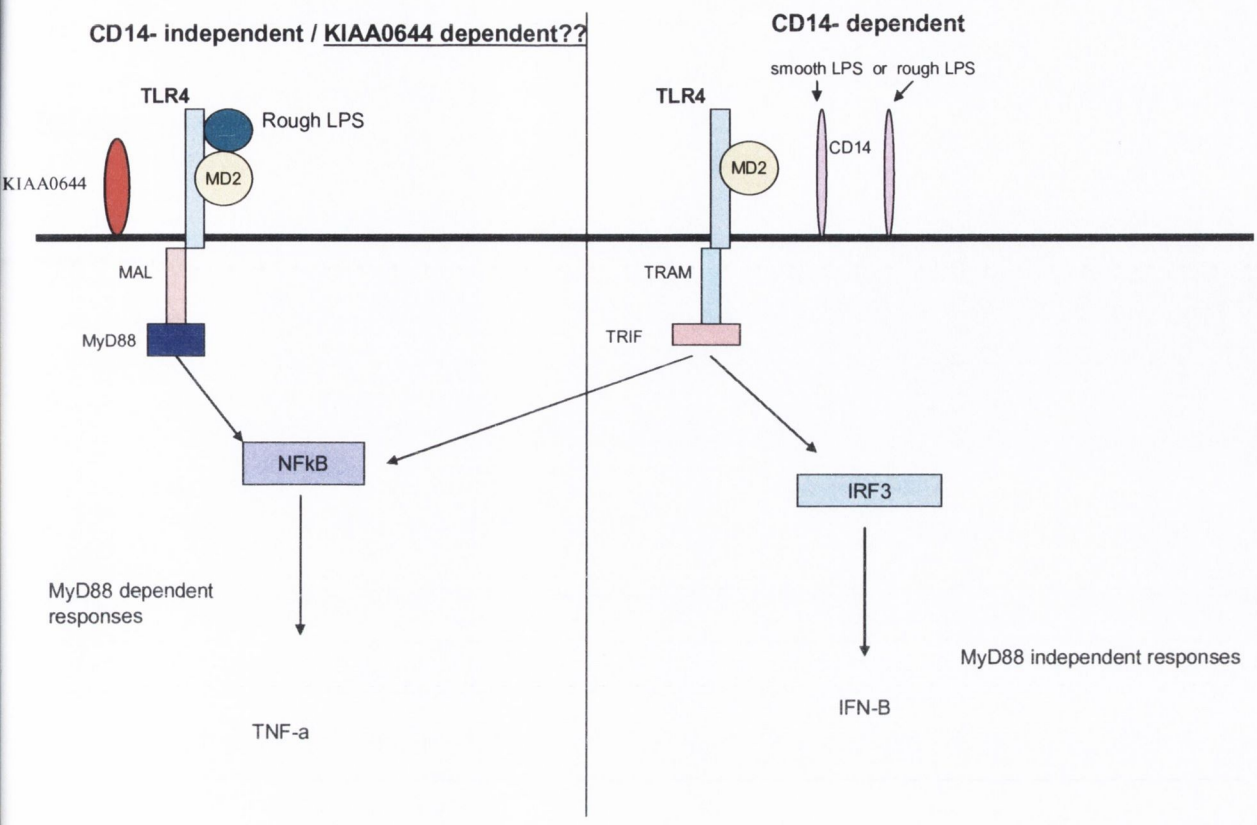


Fig 6.2 KIAA0644 may be the co-receptor for rough LPS and MyD88 dependent responses. CD14 has been reported to be mainly required for binding smooth LPS and not rough LPS (Jiang *et al.*, 2005). This paper suggests that CD14 is required for the MyD88 dependent pathway in response to smooth LPS, however CD14 is needed for the MyD88 independent pathways in response to both rough and smooth LPS. These result indicated that TLR4/MD2 can bind to rough but not smooth LPS in the absence of CD14. It is possible that KIAA0644 can bind to rough LPS and deliver it to the TLR4/MD2 complex as I have demonstrated that KIAA0644 is capable of binding LPS. Another possibility is that KIAA0644 can compensate for a lack of CD14 by performing functions similar to CD14.

Chapter Seven

References

Chapter 7

References

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Chapter Eight

Appendices

A NOVEL LEUCINE RICH REPEAT CONTAINING PROTEIN INVOLVED IN THE TLR4 SIGNALLING PATHWAY

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Toll-like receptors are a family of proteins that act as the primary sensors of microbial products. These receptors recognise a range of ligands and activate a series of signalling pathways that lead to the induction of immune and inflammatory genes. TLR4 is the most characterised of all the TLRs, it recognises lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria. In order for TLR4 to recognise LPS it requires a number of accessory proteins, or co receptors. TLRs and the accessory proteins associated with them are characterised by the presence of extracellular leucine rich repeats. Here we identified a novel leucine rich repeat containing protein which is annotated as KIAA0644. The KIAA0644 gene maps to human chromosome 7p15. We have found that the protein is expressed and highly conserved across species. It contains 12 leucine rich repeats, a signal sequence and a putative transmembrane region. Expression is highest in brain, lung and ovary and this expression is enhanced by microbial products (e.g. LPS) suggesting that it may function as an immuno-modulator. We have found that overexpression of KIAA0644 enhances LPS induced signalling. In addition, knockdown of KIAA0644 using specific siRNAs abolishes LPS-induced signalling. The preliminary data obtained therefore demonstrates that KIAA0644 is a functional protein which may be acting as a co-receptor in TLR4 signalling.

et al., 2004). TLR3 is predominantly expressed in the brain, heart lung and muscle. Within the brain it has been suggested that TLR3 is involved in mediating the entry of West Nile virus across the blood brain barrier which can lead to lethal encephalitis (Wang *et al.*, 2004). This was demonstrated in TLR3$^{-/-}$ mice, which showed greater resistance to West Nile virus infection.

TLR5

TLR5 is predominantly expressed on epithelial cells, monocytes and immature cells. It is localized to the basolateral surface of intestinal epithelial cells where it is capable of recognizing flagellin from bacteria which have invaded the epithelia (Gewirtz *et al.*, 2001). Flagellin is a 55 kDa monomeric component of bacterial flagella which are used for motility (Hayashi *et al.*, 2001). Salmonella possess two flagellin proteins FliC and FljB, which are capable of activating NFκB-dependant genes (Simon and Samuel, 2007). A study on TLR5 knockout mice highlighted redundancy within the TLRs. It appears that TLR4 can function to induce antimicrobial responses in TLR5-deficient mice challenged by *S. typhimurium* and *Pseudomonas aeruginosa* (Feuillet *et al.*, 2006). A recent study has shown that some bacteria are capable of evading recognition by TLR5 by possessing flagellin which has no immunostimulatory properties; these include *Helicobacter pylori* and *Campylobacter jejuni*. TLR5 knockout mice have been shown to be susceptible to *Escherichia coli*-induced urinary tract infection (Andersen-Nissen *et al.*, 2007). This is the first evidence to implicate TLR5 in host protection in the urinary tract.

TLR7/8

TLR7 and TLR8 are structurally quite similar and are both localized to endosomal compartments. TLR8 is non-functional in mice. Murine TLR7 and human TLR8 both respond to a range of synthetic antiviral compounds including resiquimod (R848) and loxoribine (Hemmi *et al.*, 2002). It has recently been shown that TLR7 and TLR8 respond to guanosine (G)- and uridine (U)-rich single-stranded RNA (ssRNA) from the human immunodeficiency virus-1 (HIV-1) (Heil *et al.*, 2004). TLR7 and TLR8 respond to ssRNA viruses such as *Dengue* and *Influenza*. TLR7-deficient dendritic cells showed impaired responses to ssRNA and failed to release IFN-α or TNF-α (Lund *et al.*, 2004).

TLR9

Studies carried out on TLR9-deficient mice have revealed that unmethylated CpG is the natural ligand for TLR9 (Hemmi *et al.*, 2000). TLR9-deficient mice did not produce any inflammatory cytokines in response to CpG, their splenocytes failed to proliferate and there was no maturation of dendritic cells. TLR9, like TLR7 and TLR8, is localized to endosomal compartments which may allow them to discriminate between self and non-self, as host DNA and RNA tend not to enter into endosomal compartments (Barton *et al.*, 2006). TLR9 has been shown to be able to respond to MCMV and herpes simplex virus (HSV), presumably through the presence of unmethylated CpG within these viral genomes (Krug *et al.*, 2004). It was reported that TLR9 can respond to haemozoin from *Plasmodium falciparum* (Coban *et al.*, 2005); however, more recent data argue that it is malarial DNA bound to the haemozoin which activates TLR9 (Parroche *et al.*, 2007).

TLR11

TLR11 is only functional in mice. This is due to the presence of a stop codon in the human TLR11 gene (Zhang *et al.*, 2004). TLR11 knockout mice are highly susceptible to infection by uropathogenic *E. coli*. TLR11 also responds to profilin-like proteins from *Toxoplasma gondii* (Yarovinsky *et al.*, 2005). The recent finding that TLR5 also has a function within the urinary tract is very important as it could be functioning in humans as the key receptor to mount host responses to uropathogenic *E. coli* as TLR11 is non-functional.

The adaptors used in TLR signalling

Once TLRs are activated by a ligand they recruit adaptor molecules to initiate downstream signalling. There are five adaptor proteins in the TLR system and they are myeloid differentiation factor 88 (MyD88), MyD88 adaptor like (MAL), TIR-related adaptor protein inducing interferon (TRIF), TRIF-related adaptor molecule (TRAM) and (SARM).

MyD88

MyD88 was the first adaptor protein identified and it is used by all TLRs except TLR3. The main evidence for the role of MyD88 in TLR signalling came from the study of MyD88-deficient mice. These mice were unresponsive to PAMPs detected by TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 (Takeuchi *et al.*, 2000a). They were hyporesponsive to LPS and were susceptible to infection by a number of bacteria including *S. aureus* (Takeuchi *et al.*, 2000b), *T. gondii* (Scanga *et al.*, 2002), *Listeria monocytogenes* (Edelson and Unanue, 2002) and *M. tuberculosis* (Ryffel *et al.*, 2005). There were no reported developmental defects in the MyD88 knockout mice. Despite the fact that MyD88 knockout mice are susceptible to a number of bacterial infections, they have been shown to still be

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capable of mounting an adaptive immune response (Ryffel *et al.*, 2005). Other results have shown that MyD88 is critical for mounting both innate and adaptive immunity in response to the virus infection, Lymphocytic choriomeningitis virus (LCMV) (Zhou *et al.*, 2005). Therefore, the role of MyD88 in initiating an adaptive immune response is dependent on the infection.

Like TLRs, MyD88 possesses a TIR domain and it is believed that it interacts with TLRs through a TIR–TIR interaction. MyD88 recruits interleukin 1 receptor-associated kinase 4 (IRAK-4) which in turn phosphorylates IRAK1 (Suzuki *et al.*, 2002; Khor *et al.*, 2007). IRAK1 can activate TRAF6. Both proteins leave the receptor complex and interact with TGF-β-activated kinase 1 (TAK1) and two TAK1 binding proteins TAB1, TAB2 (Jiang *et al.*, 2002). TAK1 becomes phosphorylated and activates the IκB kinase (IKK) complex (Wang *et al.*, 2001). IκB is then phosphorylated allowing NFκB to translocate to the nucleus and induce expression of proinflammatory cytokines (Fig. 1). TAK1 is also capable of phosphorylating MKK6 and 7 which leads to the activation of p38 and JNK.

MAL

The second adaptor protein to be identified was MAL, also known as TIRAP (TIR domain containing adaptor protein) (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). MAL has been shown to have a role in TLR4 and TLR2 signalling leading to NFκB activation (Fig. 1). This was confirmed in MAL knockout mice which showed impaired responses to TLR2 and TLR4 ligands (Horng *et al.*, 2002; Yamamoto *et al.*, 2002). These mice have also been shown to be at a greater risk to infection by *E. coli* (Jeyaseelan *et al.*, 2005) and *Klebsiella pneumoniae* (Jeyaseelan *et al.*, 2006). It is now believed that MAL acts as a bridging adaptor to facilitate the movement of MyD88 to TLR4 and possibly TLR2 to initiate signalling (Kagan and Medzhitov, 2006).

TRIF

The third adaptor to be identified is TRIF. TRIF is utilized by both TLR4 and TLR3 to activate the transcription factor Interferon response factor 3 (IFR3). TRIF recruits the kinases Tank binding kinase-1 (TBK1) and the IκB kinase epsilon to activate IRF3 (Yamamoto *et al.*, 2002) (Fig. 1). TRIF knockout mice were impaired in their ability to activate IRF3 or induce IFN-β following stimulation with TLR3 or TLR4 ligands. They were also more susceptible to MCMV infection (Hoebe *et al.*, 2003).

TRAM

The fourth adaptor to be identified is TRAM. Studies carried out on TRAM knockout mice confirmed that it interacts with TRIF and is involved in TLR4 signalling (Fitzgerald *et al.*, 2003). It is thought to act as a bridging adaptor for TRIF (Fig. 1).

SARM

The fifth adaptor to be identified is SARM (Khor *et al.*, 2007). It contains two sterile alpha motifs and HEAT/Armadillo repeats. The function of SARM in TLR signalling has recently been discovered and that is as a negative regulator of NFκB and IRF3 activation (Carty *et al.*, 2006). The target for SARM is TRIF, which it appears to sequester for the signalling pathway. The phenotype of SARM-deficient mice is yet to be defined.

Toll-like receptor and adapter knockout mice are all susceptible to a variety of microbial infections. In summary, the results obtained from studies on these mice confirm the importance of TLRs in innate host defence *in vivo*. When a knockout mouse is generated a certain phenotype is often predicted based on known ligands; however, often this phenotype is not observed. We can conclude that this could be due to redundancy among PRRs. This could be the case with the TLR3 knockout studies which conclude that TLR3 is not needed for the initial detection of viral PAMPs. In these mice there could be redundancy with the RLRs which are capable of recognizing viral infections. Other PRRs such as the NLRs recognize similar PAMPs as the TLRs, which is the case with some TLR2 ligands and also flagellin. Perhaps under normal conditions there can be cooperation between the PRRs to induce a rapid antimicrobial challenge, but when one receptor is removed from the system the others present are still capable of mounting a challenge to ensure survival of the host.

The proof is in the polymorphism?

Tightly controlled TLR signalling is required for the host to respond to microbial challenge. If TLR signalling is impaired it can lead to susceptibility to infection. If there is excessive signalling it can lead to septic shock and possibly to autoimmune diseases and allergies. TLRs have been implicated in numerous disease conditions. Several single nuclear polymorphisms (SNP) within individual TLRs have been identified and some are linked with disease progression.

Individuals possessing a cosegregating polymorphism in TLR4 (Asp299Gly and Thr399Ile) are hyporesponsive to LPS and they are more susceptible to Gram-negative bacterial infections (Arbour *et al.*, 2000). These TLR4 mutations Asp299Gly and Thr399Ile individually have been shown to predispose African children to severe malaria (Mockenhaupt *et al.*, 2006).

A polymorphism in the TLR2 gene (Arg753Gln) has been linked with an increased susceptibility to tuberculo-

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sis (Pandey and Agrawal, 2006) and reduced responses to Gram-positive bacteria especially streptococci (Lorenz *et al.*, 2000). There is another mutation in TLR2 (Arg677Trp) which has been associated with an increased risk of lepromatous leprosy in a Korean population (Malhotra *et al.*, 2005).

A point mutation (392STOP) resulting in the introduction of a stop codon into TLR5 has been described. This stop codon severely impairs the signalling function of TLR5. Individuals with this mutation show increased susceptibility to Legionnaire's disease (Hawn *et al.*, 2003). The results from this study are quite controversial as this work was repeated on a much larger scale with Vietnamese typhoid patients and no disease association was observed (Dunstan *et al.*, 2005).

A number of polymorphisms have been identified within the adaptor molecules and downstream kinases used in the TLR signalling cascades. There is a disorder in which patients are deficient in IRAK4. These patients are highly susceptible to pyogenic Gram-positive bacteria; however, they show resistance to viral, fungal and parasitic infections (Picard *et al.*, 2003). The results obtained from this IRAK4 study helps to confirm that TLRs are essential for host defence in humans although IRAK4 is also involved in IL-1 and IL-18 signalling.

A study carried out on individuals from Gambia, Kenya, UK and Vietnam found a SNP in the adaptor MAL (S180L). This polymorphism is associated with a protective effect against infectious diseases such as malaria, bacteraemia, tuberculosis and pneumococcal bacteraemia (Khor *et al.*, 2007). Another MAL polymorphism has been identified (C558T) and it is linked with an increased susceptibility to tuberculosis (Hawn *et al.*, 2006).

There are a number of hypomorphic mutations present in the NFκB essential modulator (NEMO) one of which is C417R. All mutations lead to the X-linked recessive disorder XR-EDA-ID. Patients suffering from this are highly susceptible to pyogenic bacterial such as *S. pneumonia* and *S. aureus* and viral infections like HSV and cytomegalovirus. These patients produce little cytokines in response to LPS or TNF-α. A missense mutation in the IκBA gene (S32I) which encodes IκBα can lead to the autosomal dominant condition AD-EDA-ID. Patients with this condition much like the patients with XR-EDA-ID show impaired responses to pyogenic infections. These patients show impaired TLR signalling as NFκB cannot be properly activated in response to TLR stimuli (Ku *et al.*, 2005).

Results obtained from genetic analysis of various polymorphisms must be interpreted with caution. Large-scale case-controlled studies are essential to obtain statistical significance to link a polymorphism with infectious disease susceptibility. It is believed by some that the observed polymorphisms in human TLRs indicate that this receptor family is completely redundant in protective immunity (Ku *et al.*, 2005). However, it has also been suggested that certain polymorphisms may be maintained within a population as they are protective against specific pathogens and infectious diseases (Khor *et al.*, 2007). Overall the evidence provided by polymorphisms highlight the role that TLRs play in various infections and diseases in humans.

Therapeutic targeting of TLRs in infectious diseases

It is clear that TLRs play important roles in a number of disease conditions including sepsis syndrome and inflammatory diseases. By targeting components of the TLR signalling pathways, it may be possible to develop drugs to treat infectious diseases and inflammatory disorders. Most TLR pathways once stimulated ultimately lead to the activation of the transcription factor NFκB and the MAP kinase family. There are already clinical trials underway to try and block NFκB and p38 MAP kinase; however, to date there is no drug that has been found to be clinically efficacious in the treatment of inflammatory conditions (Khor *et al.*, 2007). It is estimated that over 1400 people die each day worldwide from sepsis. LPS is the known causative agent of Gram-negative sepsis. Once TLR4 was identified as the receptor for recognizing LPS, it became a viable drug target in the treatment of sepsis. A synthetic TLR4 antagonist (CRX-526) has been developed and it has shown anti-inflammatory effects when used in two murine models of inflammatory bowel disease (IBD) (Fort *et al.*, 2005). The TLR4 antagonist is a lipid A analogue. It functions by blocking LPS interaction with TLR4 therefore inhibiting the release of proinflammatory cytokines. A specific role for TLR4 in the progression of IBD in humans remains to be identified; however, elevated levels of TLR4 have been reported in patients with IBD (Hausmann *et al.*, 2002). Therefore, targeting TLR4 in human IBD may be beneficial.

It has been shown that both Gram-negative and Gram-positive bacterial infections can lead to severe sepsis, therefore TLR2 may also be involved. A TLR2-neutralizing antibody has been developed and it has shown great therapeutic potential for the treatment of sepsis in a murine model. Mice treated with the antibody were protected from a lethal challenge with *Bacillus subtilis* (Meng *et al.*, 2004).

Eritoran (E-5564) is a new drug which is an analogue of the non-toxic lipid A from *Rhodobacter sphaeroides*. It has been shown to act as an inhibitor of LPS-induced proinflammatory cytokine release. It is currently in a phase II trail for patients undergoing cardiac surgery as most complications of this surgery are due to LPS-induced inflammation, pulmonary and renal dysfunction (Bennett-Guerrero *et al.*, 2007).

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In recent years there has been a lot of interest among vaccinologists in developing vaccine adjuvants to help the host in generating a long-lasting immune response to particular antigens. The problem with most vaccines is that in order to get the immune system to elicit an appropriate response they require a vaccine adjuvant. CpG DNA has shown promising results as a potential vaccine adjuvant (Daubenberger *et al.*, 2007). Some lipid A analogues are being used as vaccine adjuvants and most of them function as agonists for TLR4 (Persing *et al.*, 2002). A lot of work is currently underway to try and understand the functioning of dendritic cells and TLRs for use in vaccine development.

Possibly the most successful TLR therapeutic to date is Imiquimod. Imiquimod is a synthetic agonist which activates TLR7. Topical Imiquimod cream (5%) is routinely used for the treatment of genital warts. Human papillomavirus (HPV) is one of the most common sexually transmitted diseases and its clinical manifestation is usually genital warts. If HPV is left untreated it can lead to cervical cancer. Therefore, obtaining a simple topical cream to treat this infection is a major therapeutic breakthrough. Imiquimod is also used to treat focal epithelial hyperplasia or Heck's disease, which is a rare skin disease, caused by HPV (Maschke *et al.*, 2004). Imiquimod has proven effective in the treatment of a number of inflammatory dermatologic disease and skin cancers. This therapeutic targeting of TLR7 has been a great success story and provides hope that targeting other TLRs may produce similar results in relation to a number of serious disease conditions.

Conclusions

The discovery of PRRs has lead to a greater understanding of innate immunity and how we deal with invading microbes. TLRs have been studied intensely for more than 10 years and our knowledge of the functioning of this family has expanded greatly. We have learned that each TLR responds to specific pathogens leading to activation of unique signalling pathways. The generation of knockout mice has allowed us to investigate the complex components of the signalling pathways of each TLR. The identification of SNPs in TLRs has lent further evidence to the important role they play in host defence. The therapeutic manipulation of TLRs has shown great promise for the future treatment of infectious diseases. It is clear that TLRs are of extreme importance within the innate immune system; however, they are not the only innate PRRs, the NLRs and RLRs are present also. It is the interplay between these three families which provide us with a ubiquitous antimicrobial protection. The NLRs respond to bacterial products; however, they require cooperative signalling with the TLRs to function, therefore highlighting the importance of the TLRs in host defence. The RLRs are essential for antiviral protection. They are more widely expressed and protect all virally infected cells compared with the TLRs which are only essential for antiviral responses in plasmacytoid dendritic cells. In conclusion, we believe that these families of PRRs are all essential to provide global antimicrobial responses. Each family of PRRs is differentially expressed to optimize their ability to respond to wide varieties of pathogen. Further studies into PRRs will provide a greater understanding of innate immunity and may also yield a number of therapies for human immunological disorders.

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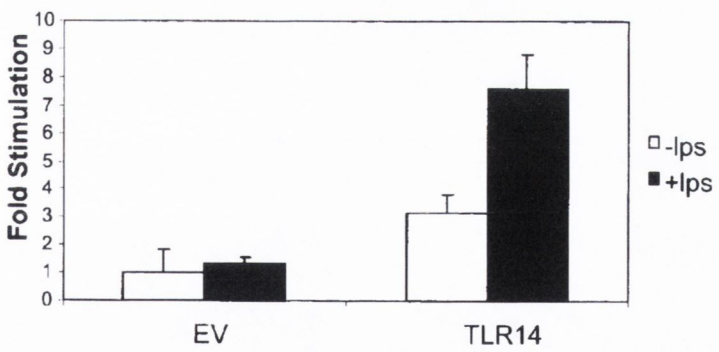
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(57) Abstract: The present invention relates to compositions and methods for use in the treatment of conditions such as septicaemia and septic shock. The invention further provides compositions and methods for the suppression Toll-like Receptor 4 mediated activation of the immune system. The invention further provides screening assays to identify compounds which have utility in the foregoing compositions and methods.

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Glucocorticoids Inhibit IRF3 Phosphorylation in Response to Toll-like Receptor-3 and -4 by Targeting TBK1 Activation*

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Phosphorylation of the transcription factor interferon regulatory factor 3 (IRF3) is essential for the induction of promoters which contain the interferon-stimulated response element (ISRE). IRF3 can be activated by Toll-like receptor 3 (TLR3) in response to the double-stranded RNA mimic poly(I-C) and by TLR4 in response to lipopolysaccharide (LPS). Here we have analyzed the effect of the glucocorticoid dexamethasone on this response. Dexamethasone inhibited the induction of the ISRE-dependent gene RANTES (regulated on activation normal T cell expressed and secreted) in both U373-CD14 cells and human peripheral blood mononuclear cells and also an ISRE luciferase construct, activated by either TLR3 or TLR4. It also inhibited increased phosphorylation of IRF3 in its N terminus in response to LPS and in its C terminus on Ser-396 in response to either poly(I-C) or LPS. Several dexamethasone-induced phosphatases were tested for possible involvement in these effects; MKP1 did not appear to be involved, although MKP2 and MKP5 both partially inhibited induction of the ISRE, pointing to their possible involvement in the effect of dexamethasone. Importantly, we found that dexamethasone could inhibit TBK1 kinase activity and TBK1 phosphorylation on Ser-172, both of which are required for IRF3 phosphorylation downstream of TLR3 and TLR4 stimulation. Our study, therefore, demonstrates that TBK1 is a target for dexamethasone, common to both TLR3 and TLR4 signaling.

Interferon-regulatory factor 3 (IRF3)[2] is a transcription factor that is activated through recognition of viral double-stranded RNA by receptors such as Toll-like receptor 3 (TLR3) or by intracellular receptors such as retinoic acid-inducible gene I (RIG-I) (1–5). Recognition of bacterial components such as lipopolysaccharide (LPS) by TLR4 also leads to IRF3 activation (6–8). In response to these stimuli, IRF3 becomes readily phosphorylated, resulting in IRF3 dimerization, association with co-factor cAMP-response element-binding protein (CREB)-binding protein, and subsequent translocation to the nucleus (1, 9). Entry to the nucleus allows IRF3 to bind to consensus DNA sequences known as the interferon (IFN)-stimulated response element (ISRE) found in the promoter regions of genes such as those encoding IFN-β, IFN-α1, CXC-chemokine ligand 10 (CXCL10), and RANTES (10–12). These IRF3-dependent genes play an important role in both the anti-viral and anti-bacterial innate immune response (13).

Multiple phosphorylation sites have been identified on IRF3. Phosphorylation of Ser-396, which lies in a cluster of 5 serine/threonine residues (Ser-396, Ser-398, Ser-402, Thr-404, Ser-405) located in the C terminus is particularly important for IRF3 activation as mutation of this site alone to a phosphomimetic aspartic acid generates a constitutively active form of IRF3 which can strongly induce the ISRE promoter element of genes encoding IFN-β, IFN-α1, and RANTES (9). Phosphorylation of this site is also critical for IRF3 dimerization and association with cAMP-response element-binding protein (CREB)-binding protein as well as nuclear translocation (1, 9, 14). Ser-386, which lies proximal to the C-terminal cluster, also appears to be important as mutation of this residue to an alanine abolishes the ability of IRF3 to dimerize, a function that is critical for the translocation of IRF3 to the nucleus (15). The role for this multitude of IRF3 phosphorylation sites has been clarified in a more recent study detailing that activation of IRF3 appears to be a sequential process of phosphorylation, where phosphorylation of Ser-396 occurs first followed by phosphorylation of Ser-404 and Ser-405, thereby priming IRF3 for phosphorylation on Ser-386, required for dimerization (14). Each of these sites plays a slightly different but equally important role in overall IRF3 activation and transcriptional activation of IRF3-dependent genes. The same study also identified Ser-339 as another important residue that appears to share a redundant role with that of Ser-396 (14). IRF3 can also be phosphorylated within its N terminus by stress inducers such as anisomycin, sorbitol, and DNA-damaging agents such as doxorubicin (4). LPS (but not double-stranded RNA) has also been shown to induce N-terminal phosphorylation (8).

TBK1 and IKKϵ are two serine/threonine kinases that have been shown to lie upstream of IRF3 and are required for phosphorylation of the C-terminal cluster, nuclear translocation and, activation of IRF3-dependent ISRE reporters (16–18). TBK1$^{-/-}$ mouse embryonic fibroblasts were shown to be defective in IRF3 nuclear translocation and IFN-α1, IFN-β, and RANTES gene expression in response to viral infection (both Sendai and Newcastle disease virus), poly(I-C) (a double-stranded RNA mimic), and LPS stimulation (18–20). Solis *et al.* (8) more recently showed that LPS could activate both TBK1

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[2] The abbreviations used are: IRF3, interferon-regulatory factor 3; TLR, Toll-like receptor; ISRE, interferon-stimulated response element; MKP1, MAP kinase phosphatase; RIG-I, retinoic acid-inducible gene I; IFN, interferon; LPS, lipopolysaccharide; RANTES, regulated on activation normal T cell expressed and secreted; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; PBMC, peripheral blood mononuclear cells; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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and IKKε in macrophages but with different kinetics, the effect on TBK1 being much more rapid and pre-dominant. Knockdown of both, however, with small interfering RNA inhibited IFN-β expression in macrophages stimulated with LPS (8). Phosphorylation of IRF3 by TBK1, therefore, appears to be a common component of signaling pathways activated by RIG-I, TLR3, and TLR4. However, evidence has emerged to show that specificity can arise within these pathways. One important observation showed that the nuclear factor κB component p65 can form a complex with IRF3 downstream of TLR4 activation but not TLR3 (21). This observation was supported in an elegant study by Ogawa *et al.* (22), which showed that a subset of ISRE-dependent genes activated by LPS were inhibited by the glucocorticoid dexamethasone through the disruption of this p65/IRF3 complex. The same set of genes when induced by poly(I-C) were, however, insensitive to dexamethasone since p65 is not in the IRF3 complex when activated by this stimulus. In a more recent study, however, Reily *et al.* (23) showed that dexamethasone could in fact inhibit the expression of certain IRF3-dependent genes such as RANTES, IFN-β, IP10, ISG15, and ISG56 downstream of TLR3 stimulation by poly(I-C). They showed that dexamethasone could disrupt the ability of a co-activator protein called glucocorticoid receptor-interacting protein to interact with IRF3, thereby suppressing IRF3-dependent genes (23). The basis for this discrepancy with the study performed by Ogawa *et al.* (22) is unclear.

The fact that IRF3 is a common component to both the TLR4 and TLR3 pathways led us to examine the effect of glucocorticoids on IRF3 phosphorylation. In this study we have found that dexamethasone can inhibit the induction of an ISRE-dependent reporter gene and the IRF3-dependent gene encoding RANTES by both LPS and poly(I-C). Importantly, we suggest that this is due to a decrease of IRF3 phosphorylation due to the inhibition of TBK1 kinase activity. This demonstrates for the first time that TBK1 is a target for glucocorticoids downstream of TLR4 and TLR3 activation.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—The ISRE luciferase plasmid was purchased from Clontech. pcDNA3.1 empty vector was from Invitrogen. pCMV-MKP1 wild-type and in-active $MKP1^{C258S}$ were kind gifts from Andrew Clark (Imperial College London). pEFr-FLAG PAC-1 expression plasmid was obtained from Steve Gerondakis (WEHI, Melbourne). pSG5-myc-tagged DUSP4–7, DUSP8, and DUSP10 plasmids were generous gifts from Stephen Keyse (Ninewells hospital, Dundee, Scotland). Constitutively active Calcineurin pEGFP CN-CaMIA was obtained from Young Jun Kang and Masato Kubo (RIKEN Yokohama Institute) (24). Wild-type GST-IRF3 (380–427), GST-IRF3 (5A), and GST-IRF3 (7A) were kind gifts from Katherine Fitzgerald (University of Massachusetts Medical School). pcDNA3-FLAG kinase dead TBK1 plasmid was obtained from Makoto Nakanishi (Nagoya City University). The following antibodies were used: total IRF3 (Santa Cruz Biotechnology, Inc.), phospho-Ser-396 IRF3, and phospho-p38 MAP kinase (Cell Signaling Technology Inc.), anti-TBK1 (Imgenex), and anti-β-actin (Sigma). Anti-NAK (TBK1) and anti-TBK1 (Ser-172) used for immunoprecipitation assays were from Abcam (Cambridge, UK) and BD Pharmingen, respectively. Non-phosphorylated TBK1 peptide was obtained from Sir Philip Cohen (University of Dundee). LPS from *Escherichia coli*, serotype EH100, was from Alexis (San Diego, CA), poly(I-C) was from Amersham Biosciences, and dexamethasone (D4902) was purchased from Sigma. RANTES enzyme-linked immunosorbent assay kit was from R&D Systems (Abingdon, UK).

Cell Culture and Transient Transfection—Human peripheral blood mononuclear cells (PBMC) were isolated from human blood and maintained in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% penicillin/streptomycin solution (v/v). U373 astrocytoma cells stably transfected with CD14 (U373-CD14) were a kind gift from Katherine Fitzgerald (University of Massachusetts Medical School) and were grown in Dulbecco's modified Eagle's medium supplemented as above with the addition of 250 μg/ml neomycin analog G418 to maintain CD14 expression. For transfections, U373-CD14 cells were seeded in 24-well plates at 3×10^4 cells per well (for ISRE luciferase assays) or in 6-well plates at 1.2×10^5 per well (for Western blot analysis), incubated overnight, and transfected using GeneJuice transfection reagent (Novagen, Madison, WI) according to the manufacturer's instructions. For ISRE luciferase assays, 75 ng of ISRE luciferase plasmid, 30 ng of *Renilla* luciferase, and empty pcDNA3.1 vector made up to a total of 220 ng of DNA were transfected into each well of a 24-well plate. For Western blot analysis varying amounts of pCMV-MKP1 plasmid (100 ng, 1 μg, or 2 μg) and empty pcDNA3.1 vector made up to a total of 2 μg of DNA was transfected into each well of a 6-well plate. In both cases cells were transfected for 24 h before treatment with dexamethasone and stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) as indicated in the figure legends.

ISRE Luciferase Assays—Cells were lysed in 100 μl of passive lysis buffer (Promega, Southampton, UK) for 15 min. Firefly luciferase activity was assayed by the addition of 40 μl of luciferase assay mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 $MgSO_4$, 0.1 M EDTA, 33.3 mM dithiothreitol, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to 20 μl of the lysed sample. *Renilla* luciferase was read by the addition of 40 μl of a 1:1000 dilution of Coelentrazine (Argus Fine Chemicals) in phosphate-buffered saline. Luminescence was read using the Reporter microplate luminometer (Turner Designs). The *Renilla* luciferase plasmid was used to normalize for transfection efficiency in all experiments.

Western Blot Analysis—Human PBMC and U373-CD14 cells were seeded in 6-well plates at 3×10^6 and 1.2×10^5 per well, respectively. Cells were treated with dexamethasone and stimulated with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) as indicated in the figure legends. Cells were washed in ice-cold phosphate-buffered saline before being lysed on ice in 100 μl of low stringency lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 10% glycerol (v/v), 0.5% Nonidet P-40 (v/v), 1 mM EDTA containing 1 mM dithiothreitol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The cell lysates were centrifuged at 13,000 rpm for 15 min after which the supernatants were removed and deter-

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mined for protein concentration using Coomassie Bradford reagent according to manufacturer's instructions (Pierce). Samples containing equal protein concentrations were generated using 5× SDS sample loading buffer (125 mM Tris-HCl, pH 6.8, 15% glycerol (v/v), 2% SDS (v/v), 10 mg bromphenol blue) containing 50 mM dithiothreitol. SDS protein samples (25 μg) were resolved on 8 or 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% (w/v) dried milk in TBS-T (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, and 0.1% (v/v) Tween 20), and incubation of primary antibody was carried out in the same buffer with the exception of phospho-Ser-396 IRF3 and phospho-p38 MAP kinase, which were incubated in 2.5% bovine serum albumin (w/v)/TBS-T. Blots were then incubated with the appropriate secondary antibody in 5% (w/v) dried milk/TBS-T before being developed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Cell Signaling Technology, Inc.). Densitometric analysis of band intensities was determined using Multi Gauge Version 2.2 software.

Native PAGE for Analysis of IRF3 Dimerization—Preparation of cell lysates and native gel analysis was performed as previously described (5).

Immunoprecipitation Assays—U373-CD14 cells were seeded at 8×10^5 per 10-cm plate. Once confluent, cells were treated as indicated in the figure legends, washed in ice-cold phosphate-buffered saline, lysed in 300 μl of low stringency lysis buffer, and determined for protein concentration as described above. For immunoprecipitation kinase assays, 500–800 μg of total protein was incubated with 2 μg of anti-NAK (TBK1) overnight at 4 °C. Additional assay controls such as FLAG-tagged kinase dead TBK1 (KD), which had been transfected into U373-CD14 cells 24 h before stimulation, was immunoprecipitated with 2 μg of anti-FLAG (Sigma). 2 μg of anti-rabbit IgG was used as an internal immunoprecipitation control. In each case, 10 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) were added for 2 h at 4 °C. Beads were washed twice in low stringency lysis buffer followed by one wash in kinase buffer (20 mM Hepes, pH7, 10 mM $MgCl_2$, 50 mM NaCl containing 1 mM dithiothreitol, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 0.1 mM phenylmethylsulfonyl fluoride). The beads were then incubated for 25 min at 30 °C in a kinase reaction with 1 μg of recombinant substrate GST-IRF3 (380–427), GST-IRF3 (5A) or GST-IRF3 (7A), 1 mM ATP and 5 μCi [γ-^{32}P]ATP (Amersham Biosciences) made up to a total volume of 20 μl with kinase buffer. Samples were then resolved by SDS-PAGE. The upper half of the gel was transferred onto polyvinylidene difluoride and blotted for total TBK1 (Imgenex), whereas the bottom half was stained with Coomassie to detect substrate GST-IRF3 and subsequently exposed to an (X-ray film) overnight at −80 °C. For Ser(P)-172-TBK1 assays, 500–800 μg of total protein was incubated with 2 μg of anti-TBK1 (Ser-172) and 2 μg of non-phosphorylated TBK1 peptide overnight at 4 °C after which 10 μl of protein A/G-agarose beads were added for 2 h at 4 °C. The beads were washed 3 times in low stringency lysis buffer and resuspended in 20 μl of 2× SDS sample loading buffer and analyzed for total TBK1 by Western blot analysis.

RESULTS

Dexamethasone Inhibits the Induction of RANTES and an ISRE Reporter Gene by TLR4 and TLR3—We first investigated if dexamethasone could affect the functional outcomes of endogenous IRF3 activity in response to both TLR4 and TLR3 stimulation. We, therefore, analyzed the effects of dexamethasone on RANTES expression in response to LPS and poly(I-C) since phosphorylation of IRF3 is essential for RANTES induction (18). As shown in Fig. 1*A*, stimulation of U373-CD14 cells with LPS (*left-hand panel*) or poly(I-C) (*right-hand panel*) induced a 7–8-fold increase in RANTES expression from a basal 100 pg/ml to 700–800 pg/ml. Dexamethasone dose-dependently inhibited this response, with 10 μM decreasing RANTES induction by 90%. We also investigated whether dexamethasone had the same effect on RANTES expression in primary cells. Human PBMC produced a 7-fold increase in RANTES expression in response to LPS (Fig. 1*B*, *left-hand panel*) and a 2–3-fold increase in response to poly(I-C) (*right-hand panel*). In both cases dexamethasone markedly inhibited this response. We next analyzed the effect of dexamethasone on an ISRE luciferase reporter plasmid, which contains five repeats of the ISRE sequence. Stimulation of cells with LPS and poly(I-C) resulted in a 6- and 4-fold increase of ISRE luciferase activity, respectively (Fig. 1*C*). Dexamethasone dose-dependently inhibited both stimuli with an optimal effect evident at 10 μM. These results indicate that the effect of dexamethasone on IRF3-dependent genes is unlikely to be specific to LPS.

Dexamethasone Inhibits IRF3 Phosphorylation in Response to LPS and Poly(I-C)—We next examined whether dexamethasone had any effect on the phosphorylation status of IRF3. Phosphorylated IRF3 exists in multiple forms that have been previously characterized (4). In a resting cell IRF3 can be visualized as a doublet on SDS-PAGE (Fig. 2*A*, *upper panels*) where the lower and upper band have been named form I and form II, respectively. Form I represents non-phosphorylated IRF3, whereas form II represents a basally phosphorylated IRF3 (Fig. 2*A*, *left side*, *upper panel*, *lane 1*). Here we show that treatment of U373-CD14 cells with LPS over time induces a band shift to form II, which is maximal at 60 min (Fig. 2*A*, *left side*, *upper panel*, *lane 7*). This band shift to form II is indicative of IRF3 N-terminal phosphorylation (4, 18). Pretreating the cells with dexamethasone inhibited this response (compare *lane 6* to *lane 5* for 30 min LPS and *lane 8* to *lane 7* for 60 min of LPS). To establish if LPS could cause phosphorylation of any of the C-terminal residues, the same lysates were immunoblotted for Ser-396, a critical residue for IRF3 activation (9, 14). Phosphorylation of IRF3 on Ser-396 occurred from 30 min after LPS stimulation and was maximal at 60 min, which correlated with the band shift observed with the total IRF3 antibody (Fig. 2*A*, *left side*, *second panel*, *lanes 5* and *7*). Pretreating the cells with 1 μM dexamethasone again inhibited this response (compare *lane 6* to *lane 5* for 30 min of LPS and *lane 8* to *lane 7* for 60 min of LPS).

We next examined the effect of dexamethasone on poly(I-C)-induced IRF3 phosphorylation. Despite repeated attempts we were unable to detect a band shift in IRF3 on SDS-PAGE analysis (Fig. 2*A*, *right side*, *upper panel*), which may be explained by

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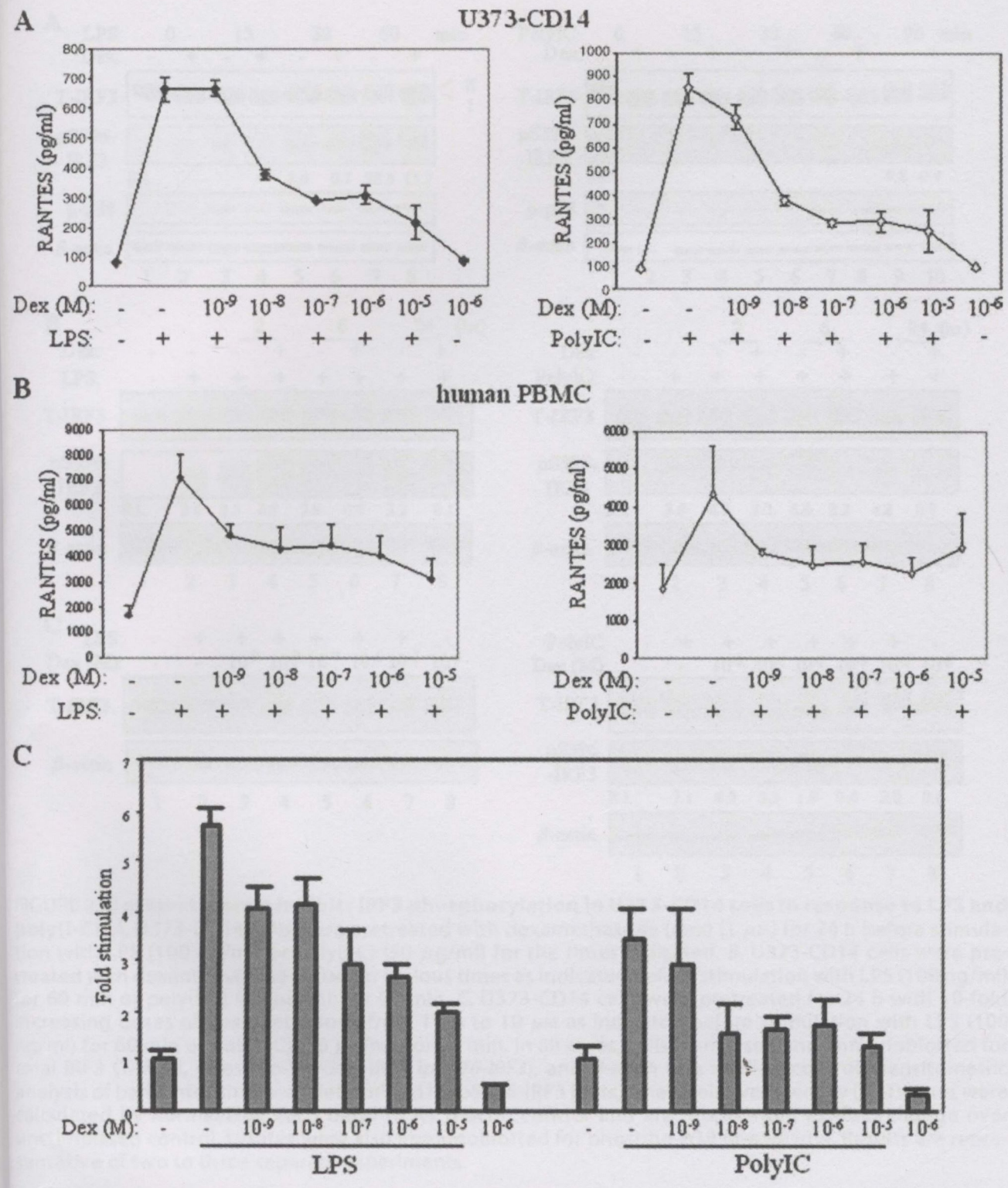


FIGURE 1. **Dexamethasone inhibits the induction of RANTES and an ISRE reporter gene by TLR4 and TLR3.** U373-CD14 cells (*A*) and human PBMC (*B*) were treated with 10-fold increasing doses of dexamethasone (*Dex*) from 1 nM to 10 μM as indicated for 2 h before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for a further 24 h. Cell supernatants were removed and assayed for RANTES expression by enzyme-linked immunosorbent assay. RANTES concentrations were calculated by comparison to a RANTES standard curve and measured as pg/ml. Results are expressed as the mean ± S.D. for triplicate determinations and are representative of two separate experiments. *C*, U373-CD14 cells were transiently transfected with ISRE luciferase (75 ng) and *Renilla* luciferase (30 ng). 24 h after transfection cells were treated with 10-fold increasing doses of dexamethasone from 1 nM to 10 μM for 2 h as indicated before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for 6 h. The cells were lysed, and luciferase activity was subsequently measured. Results were normalized for *Renilla* luciferase activity and represented as -fold stimulation over the unstimulated control. Results are expressed as the mean ± S.D. for triplicate determinations and are representative of three separate experiments.

the fact that poly(I-C) has never been found to induce N-terminal phosphorylation of IRF3. However, we were able to detect increased phosphorylation of Ser-396 after a treatment time of 90 min (Fig. 2*A*, *right side, second panel, lane 9*). Importantly this was inhibited with dexamethasone pretreatment (*lane 10*). As a positive control for dexamethasone, lysates were also immunoblotted for phospho-p38, which has previously shown to be inhibited by dexamethasone (25). Both LPS and poly(I-C) strongly induced p38 phosphorylation where the LPS effect was evident at 15 min (Fig. 2*A*, *left side, third panel, lane 3*), and poly(I-C) caused an effect from 60 min (Fig. 2*A*, *right side, third panel, lane 7*). As expected, dexamethasone could inhibit p38 phosphorylation in response to both stimuli at all time points (*e.g.* compare *lane 4* to *lane 3* for 15 min LPS, and compare *lane 8* to *lane 7* for 60 min poly(I-C)).

The effects of dexamethasone were further analyzed by performing both a time course and a dose response (Fig. 2, *B* and *C*). In the time course, cells were incubated with dexamethasone for either 2, 6, or 24 h before being stimulated with LPS for 60 min (Fig. 2*B*, *left side*) or poly(I-C) for 90 min (Fig. 2*B*, *right side*). At all incubation time points, dexamethasone could reduce the ability of LPS to cause a band shift in IRF3 to form II (Fig. 2*B*, *left side, upper panel*), and this appeared to be maximal when the cells were pretreated with dexamethasone for 24 h (*lane 8*). Optimal inhibition of LPS-induced phosphorylation of Ser-396 was also maximal at a pretreatment time of 24 h (Fig. 2*B*, *left side, second panel, lane 8*). As mentioned previously, poly(I-C) failed to induce an IRF3 band shift; however, activation could be monitored by immunoblotting for Ser-396. Dexamethasone dramatically reduced Ser-396 phosphorylation at each time point, with pretreatment for 24 h causing the greatest effect (Fig. 2*B*, *right side, second panel, lane 8*). For the dose response, cells were treated with increasing doses of dexamethasone (1 nM to 10 μM) for 24 h before stimulation with LPS for 60 min or poly(I-C) for 90 min (Fig. 2*C*). This resulted in a dose-dependent reduction of an LPS-induced band shift (Fig. 2*C*, *left side, upper panel*). Ser-396 phosphorylation was also dose-dependently reduced in response to poly(I-C) (Fig. 2*C*, *right side, second panel*). These results, therefore, suggested that dexamethasone inhibits IRF3 phosphorylation in response to both LPS and poly(I-C).

To corroborate that the effects of dexamethasone on IRF3 phosphorylation were not restricted to one cell type, similar experiments were performed in primary human PBMC (Fig. 3). Dexamethasone could reduce Ser-396 phosphorylation at 30 and 60 min LPS stimulation (Fig. 3*A*, *left side, second panel*, compare *lane 4* to *lane 3* and *lane 6* to *lane 5*) and could reduce Ser-396 phosphorylation at 90 and 120 min of poly(I-C) stimulation (Fig. 3*A*, *right side, second panel*, compare *lane 8* to *lane*

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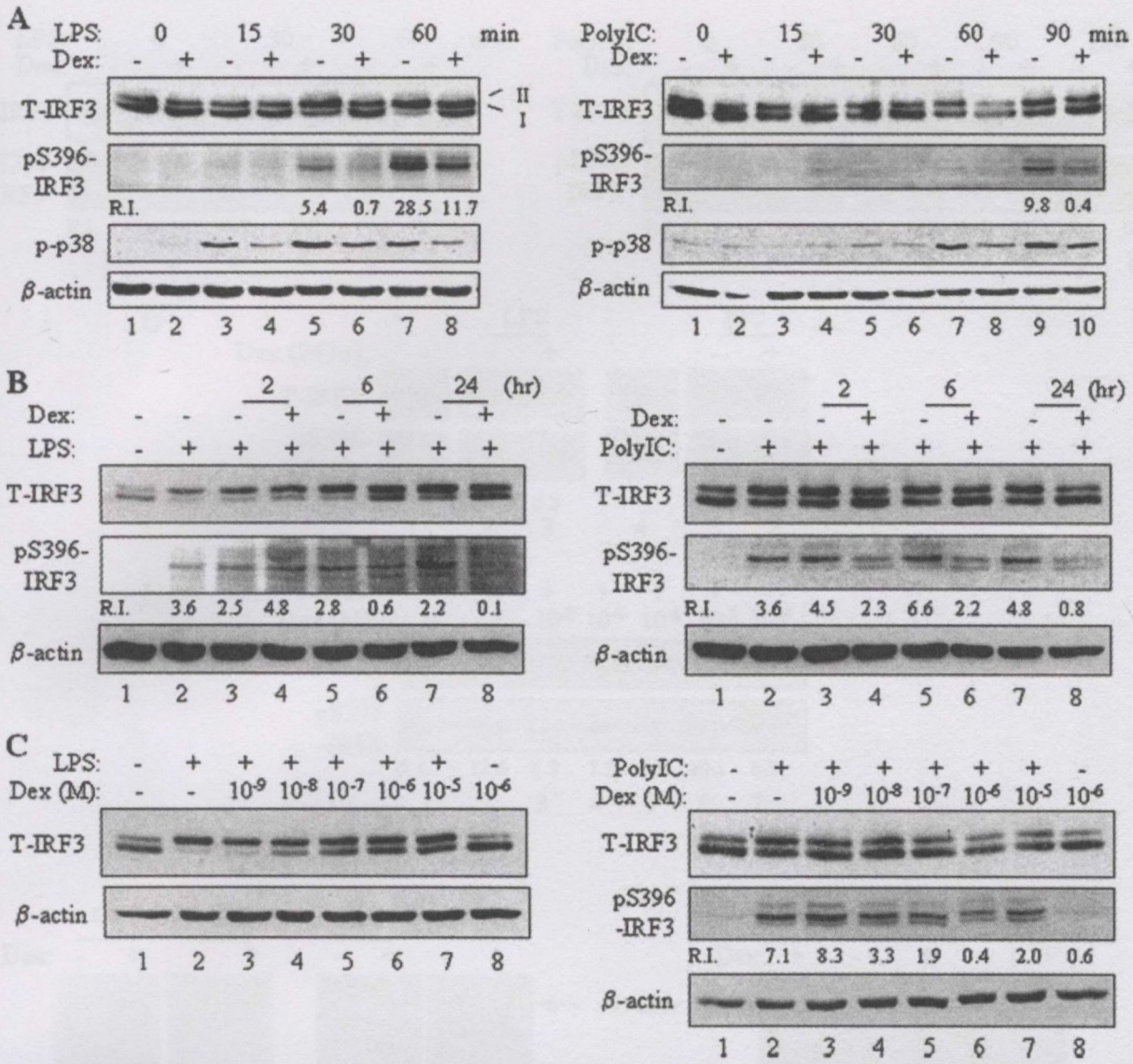


FIGURE 2. **Dexamethasone inhibits IRF3 phosphorylation in U373-CD14 cells in response to LPS and poly(I-C).** *A*, U373-CD14 cells were pretreated with dexamethasone (*Dex*) (1 μM) for 24 h before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for the times indicated. *B*, U373-CD14 cells were pretreated with dexamethasone (1 μM) for various times as indicated before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. *C*, U373-CD14 cells were pretreated for 24 h with 10-fold increasing doses of dexamethasone from 1 nM to 10 μM as indicated before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. In all cases, cells were lysed and immunoblotted for total IRF3 (*T-IRF3*), phospho-Ser-396 IRF3 (*pS396-IRF3*), and β-actin as a loading control. Densitometric analysis of band intensities was determined for pS396-IRF3 blots, where relative intensity (*R.I.*) values were calculated by normalizing each band to its β-actin control and are represented as -fold change over unstimulated control. Lysates were also immunoblotted for phospho-p38 (*p-p38*) in *A*. Results are representative of two to three separate experiments.

7 and *lane 10* to *lane 9*). PBMC were also pretreated with dexamethasone for 2, 6, and 24 h before stimulation with LPS and poly(I-C). Consistent with the U373-CD14 data, pretreatment with dexamethasone for 24 h had the greatest effect, and for simplicity this time point alone is illustrated in Fig. 3*B* (compare *lane 3* to *lane 2* and *lane 6* to *lane 5*). In addition, PBMC were sensitive to a dexamethasone dose response with inhibition evident from 10 nM as shown for poly(I-C) stimulation in Fig. 3*C*.

Dexamethasone Inhibits IRF3 Dimerization in Response to LPS and Poly(I-C)—The phosphorylation of IRF3 at its C terminus, particularly on Ser-386 and Ser-396, is required for its dimerization and subsequent translocation to the nucleus (9, 14, 15). The fact that dexamethasone can reduce IRF3 phosphorylation on Ser-396 led us to examine whether dexamethasone could affect IRF3 dimerization. As shown in Fig. 3*D*, pretreatment of both U373-CD14 cells and human PBMC with dexamethasone could abolish the induction of IRF3 dimerization in response to LPS (compare *lane 4* to *lane 3* for U373-CD14 and *lane 3* to *lane 2* for PBMC) and poly(I-C) (compare *lane 8* to *lane 7* for U373-CD14 and *lane 5* to *lane 4* for PBMC).

MKP1 Overexpression Does Not Affect ISRE Luciferase Activity or IRF3 Phosphorylation—To understand how dexamethasone affects IRF3 phosphorylation, we turned our attention *DUSP1*, a gene that encodes the dual-specificity phosphatase MAP kinase phosphatase 1 (MKP1). MKP1 has the ability to dephosphorylate serine/threonine and tyrosine residues and is the phosphatase responsible for dephosphorylating both the threonine and tyrosine residues within the activation loop of p38 MAPK (26). Interestingly, dexamethasone has been shown to strongly induce MKP1 expression, which subsequently resulted in p38 dephosphorylation (25). Therefore, we tested whether MKP1 might be responsible for dephosphorylating IRF3 by first determining the effect of MKP1 on the ISRE luciferase system. Transfection of cells with increasing amounts of plasmid encoding MKP1 had no effect on ISRE luciferase activity in U373-CD14 cells stimulated with either LPS or poly(I-C), whereas dexamethasone alone could dramatically reduce luciferase activity in response to both stimuli (Fig. 4*A*). To analyze the effect of MPK1 overexpression in more detail, IRF3 phosphorylation was examined (Fig. 4*B*). MKP1 expression had no effect on the LPS-induced band shift (Fig. 4*B*, *upper panel*) or Ser-396 phosphorylation (*second panel*). In contrast, increasing amounts of MKP1 expression could reduce LPS-induced phosphorylation of p38 (Fig. 4*B*, *third panel*, compare *lanes 4* and *5* to *lane 2*), whereas an inactive mutant of MKP1 had no effect (*lane 7* and *lane 8*). This implies that MKP1 is unlikely to be targeting IRF3.

To investigate the potential roles of other candidate phosphatases, we turned our attention to other dual-specificity phosphatases encoded by DUSP genes, PAC-1 (DUSP2), MKP2 (DUSP4), VH3 (DUSP5), MKP3 (DUSP6), PYST2 (DUSP7), VH5 (DUSP8), and MKP5 (DUSP10). Of particular interest were the phosphatases PAC-1, MKP2, and MKP5, whose expression has also been shown to increase upon dexamethasone treatment (25). We also focused on calcineurin, a serine/threonine phosphatase that has recently been shown to negatively regulate TLR pathways by inhibiting the adaptor proteins MyD88 and TRIF (24). To determine whether any of these phosphatases could play a negative role in the IRF3 pathway, increasing concentrations (1, 10, and 100 ng) of plasmids encoding these phosphatases were tested in the ISRE luciferase

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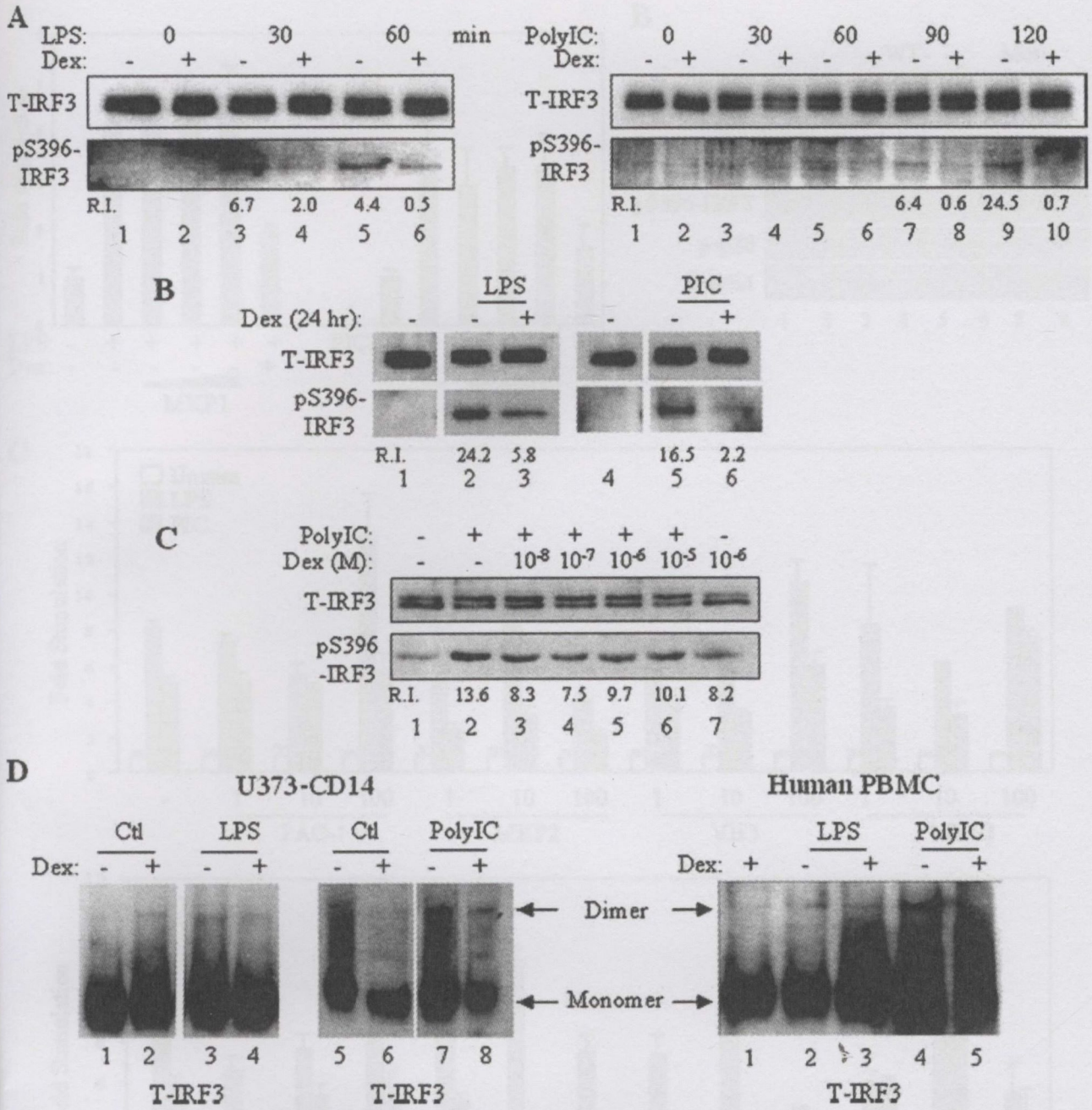


FIGURE 3. **Dexamethasone inhibits IRF3 phosphorylation in human PBMC and IRF3 dimerization in response to LPS and poly(I-C).** *A*, human PBMC were pretreated with dexamethasone (*Dex*, 500 nM) for 24 h before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for the times indicated. *T-IRF3*, total IRF3; *pS396*, phospho-Ser-396. *B*, human PBMC were pretreated with dexamethasone (500 nM) for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. *PIC*, poly(I-C). *C*, human PBMC were pretreated for 24 h with 10-fold increasing doses of dexamethasone from 10 nM to 10 μM as indicated before stimulation with poly(I-C) (50 μg/ml) for 90 min. In all cases cells were lysed and immunoblotted for T-IRF3 and pSer-396-IRF3. Densitometric analysis of band intensities was determined for pS396-IRF3 blots, where relative intensity (*R.I.*) values were calculated by normalizing each band to T-IRF3 and are represented as -fold change over unstimulated control. *D*, U373-CD14 cells and human PBMC were pretreated with dexamethasone (500 nM) for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. Lysates were run on native gels and immunoblotted with T-IRF3 to visualize IRF3 monomers and dimers. Results are representative of two to three separate experiments. *Ctl*, control.

system upon LPS and poly(I-C) stimulation (Fig. 4*C*). Both LPS and poly(I-C) alone could increase ISRE luciferase activity 8- and 6-fold, respectively, and this -fold change did not dramatically alter when increasing concentrations of any of the phosphatase plasmids were transfected into this system. However, of potential interest were the phosphatases MKP2 and MKP5, which could reduce ISRE activity by half when transfected at the highest plasmid concentration (Fig. 4*C*, *upper* and *lower graph*). These may, therefore, play a role in the effects of dexamethasone.

Dexamethasone Can Inhibit TBK1 Kinase Activity—Finally we examined the effect of dexamethasone on TBK1 activation. To test this we immunoprecipitated TBK1 from LPS- or poly(I-C)-stimulated U373-CD14 cells and examined its kinase activity by observing the amount of ^{32}P-labeled ATP incorporated into the wild-type recombinant GST-IRF3 (380–427) substrate in the absence or presence of dexamethasone. We first performed a time course of activation, demonstrating that a treatment time of 60 min for LPS resulted in maximal ^{32}P incorporation, whereas poly(I-C) required 90 min of treatment time (Fig. 5*A*). These time points correlated with phosphorylation of endogenous IRF3 (Fig. 2*A*). Dexamethasone dose-dependently inhibited the response to both LPS and poly(I-C), the effect being optimal at 500 nM for LPS (Fig. 5*B*, *upper panel, upper blot, lane 5*) and 100 nM for poly(I-C) (Fig. 5*B*, *lower panel, upper blot, lane 5*). To ensure that equal amounts of TBK1 were being immunoprecipitated, the samples were also analyzed for total TBK1 (Fig. 5*B*, *upper panel, lower blot* and *lower panel, lower blot*). The specificity of TBK1 to phosphorylate IRF3 in this assay was confirmed when it was shown that TBK1 failed to phosphorylate a GST-IRF3 (5A) substrate where the 5 C-terminal residues responsible for IRF3 activation Ser-396, Ser-398, Ser-402, Thr-404, and Ser-405 were mutated to an alanine, and this was further confirmed by a GST-IRF3 (7A) mutant (5A plus S385A/S386A) (Fig. 5*B*, *lane 6* and *7*, *upper panel* and *lane 7* and *8*, *lower panel*). Immunoprecipitation of a FLAG-tagged kinase dead TBK1 (KD) also failed to phosphorylate GST-IRF3 (Fig. 5*B*, *lane 9*, *upper panel* and *lane 10*, *lower panel*).

Phosphorylation of TBK1 on Ser-172 within its activation loop is essential for its kinase activity (27). We, therefore, decided to investigate if dexamethasone had any effect on Ser-172 phosphorylation in response to both LPS and poly(I-C) (Fig. 5*C*). LPS and poly(I-C) both induced phosphorylation of TBK1 on Ser-172, and this phosphorylation was completely abolished in the presence of dexamethasone (Fig. 5*C*, *upper panel*, compare *lane 4* to *lane 3* and *lane 6* to *lane 5*). A fraction of the same lysates was also used to immunoprecipitate total TBK1 to ensure equal TBK1 protein levels (Fig. 5*C*, *second panel*). Samples were also blotted for phospho-p38 to ensure that dexamethasone was active (*third panel*).

Taken together these data, therefore, confirm that TBK1 is the kinase responsible for IRF3 phosphorylation. In addition, it predicts that dexamethasone inhibits the phosphorylation of IRF3 by LPS and poly(I-C) via the inhibition of TBK1 kinase activity.

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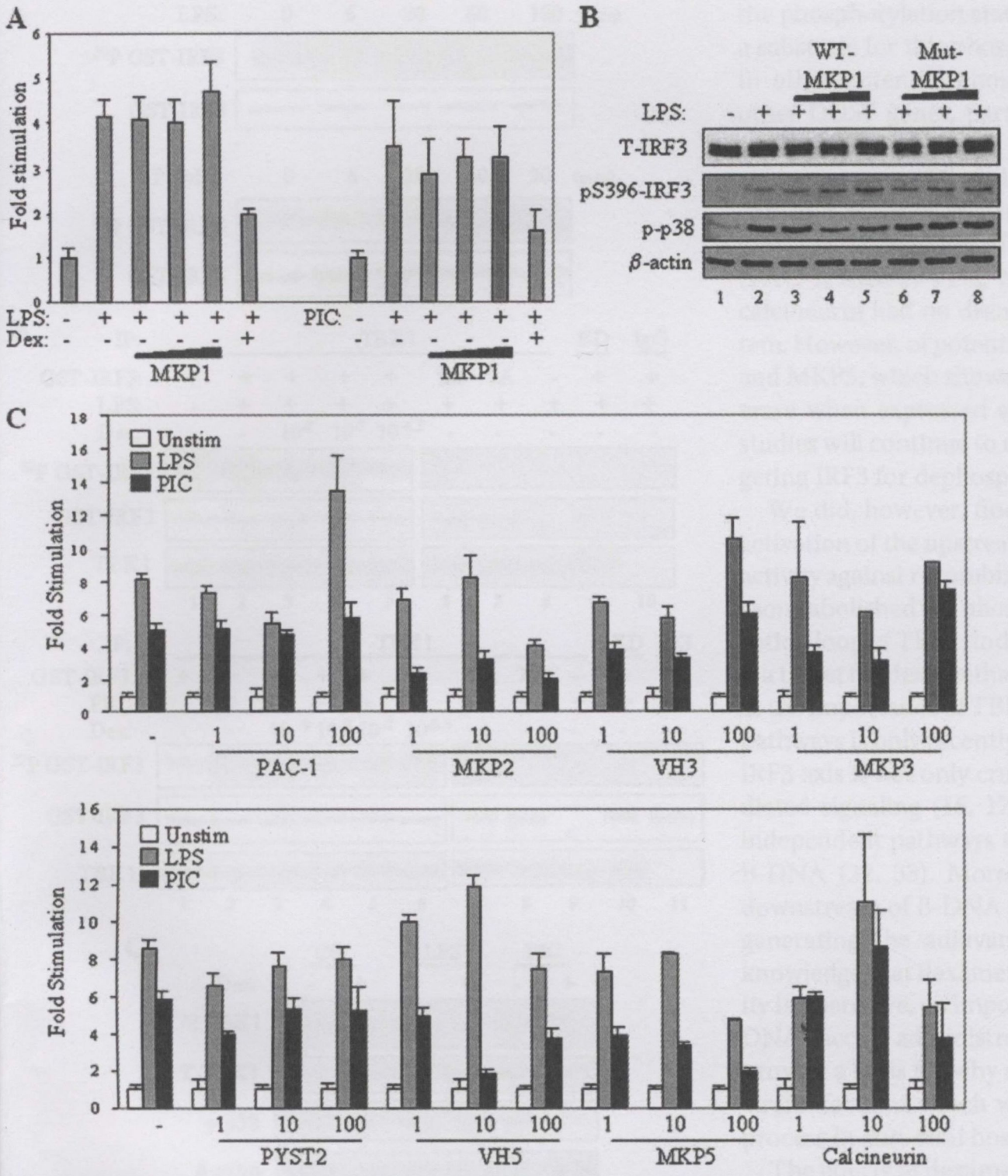


FIGURE 4. **Analysis of various phosphatases on ISRE luciferase activity and IRF3 phosphorylation.** *A*, U373-CD14 cells were transfected with ISRE luciferase (75 ng) and *Renilla* luciferase (30 ng) along with increasing amounts of plasmid expressing wild-type (*WT*) MKP1 (25, 100, or 200 ng). 24 h after transfection cells were stimulated with LPS (100 ng/ml) or poly(I-C) (*PIC*, 50 μg/ml) for 6 h. Pretreatment of cells with dexamethasone (*Dex*, 1 μM) for 2 h was used as a control. ISRE luciferase activity was subsequently measured. Results were normalized for *Renilla* luciferase activity and are represented as -fold stimulation over the unstimulated control. *B*, U373-CD14 cells were transfected with increasing amounts (100 ng or 1 or 2 μg) encoding wild-type MKP1 or inactive (*Mut*)-MKP1 before stimulation with LPS (100 ng/ml) for 60 min. Cells were lysed and immunoblotted for total IRF3 (*T-IRF3*), phospho-Ser-396 (pS396)-IRF3, phospho-p38 (*p-p38*), and β-actin as a loading control. *C*, as in *A*, except that increasing amounts of phosphatase plasmids expressing PAC-1, MKP2, VH3, MKP3, PYST2, VH5, MKP5, and constitutively active calcineurin (1, 10, and 100 ng) were co-transfected with ISRE luciferase (75 ng) and *Renilla* luciferase (30 ng).

DISCUSSION

In this study we demonstrate that dexamethasone targets IRF3 in response to both TLR4 and TLR3 stimulation mediated by LPS and poly(I-C), respectively. We propose that this is due to the ability of dexamethasone to reduce IRF3 phosphorylation via inhibition of TBK1 activation. This in turn inhibits activation of the ISRE and thereby blocks induction of IRF3-dependent gene expression, as exemplified by decreased expression of the chemokine RANTES. The effect on IRF3 phosphorylation was first indicated from the observation that dexamethasone can reduce a band shift in IRF3 from form II to form I, an event that has been characterized as N-terminal phosphorylation in previous studies (4). In addition, dexamethasone can reduce C-terminal phosphorylation as indicated by a reduction of Ser-396 phosphorylation in response to both LPS and poly(I-C) in both U373-CD14 cells and human PBMC. This reduction in phosphorylation could have been due to two mechanisms. Either dexamethasone induces a phosphatase, which could de-phosphorylate IRF3, or it targets an upstream kinase such as TBK1, again possibly via induction of a phosphatase.

To date very few phosphatases have been identified that may target components of the TLR signaling cascades. Instead, polyubiquitination and proteasomal degradation of these components have been studied in far greater detail (28, 29). Indeed, IRF3 itself has been shown to become phosphorylated by an unknown kinase on Ser-339 and Thr-440 in response to poly(I-C), resulting in the recruitment of a peptidyl-prolyl isomerase called Pin1. This association with Pin1 results in polyubiquitination and proteasome-dependent degradation of IRF3, thereby terminating the IRF3 signal (30). The polyubiquitination of IRF3 in response to Sendai virus infection, mediated by Cullin-based ligases, has also been demonstrated where phosphorylation of the C-terminal residues by TBK1 is critical (31). These results highlight that IRF3 is not only positively regulated by phosphorylation but that phosphorylation is also required for negative regulation, a fact that makes IRF3 a likely target for phosphatases. Indeed it has been shown that treatment of cells with a general phosphatase, calf intestinal phosphatase, has the ability to abolish IRF3 phosphorylation on the C-terminal cluster but also on the N terminus in response to various stimuli, illustrating that IRF3 phosphorylation sites are sensitive to phosphatase treatment (1, 4). However, to date no effort has been made to try and identify specific phosphatases that may be responsible. The fact that dexamethasone can reduce an LPS-induced band shift and can also reduce Ser-396 phosphorylation in response to both LPS and poly(I-C) provides us with a tool to explore and identify a phosphatase which dexamethasone may induce to down-regulate IRF3 phosphorylation.

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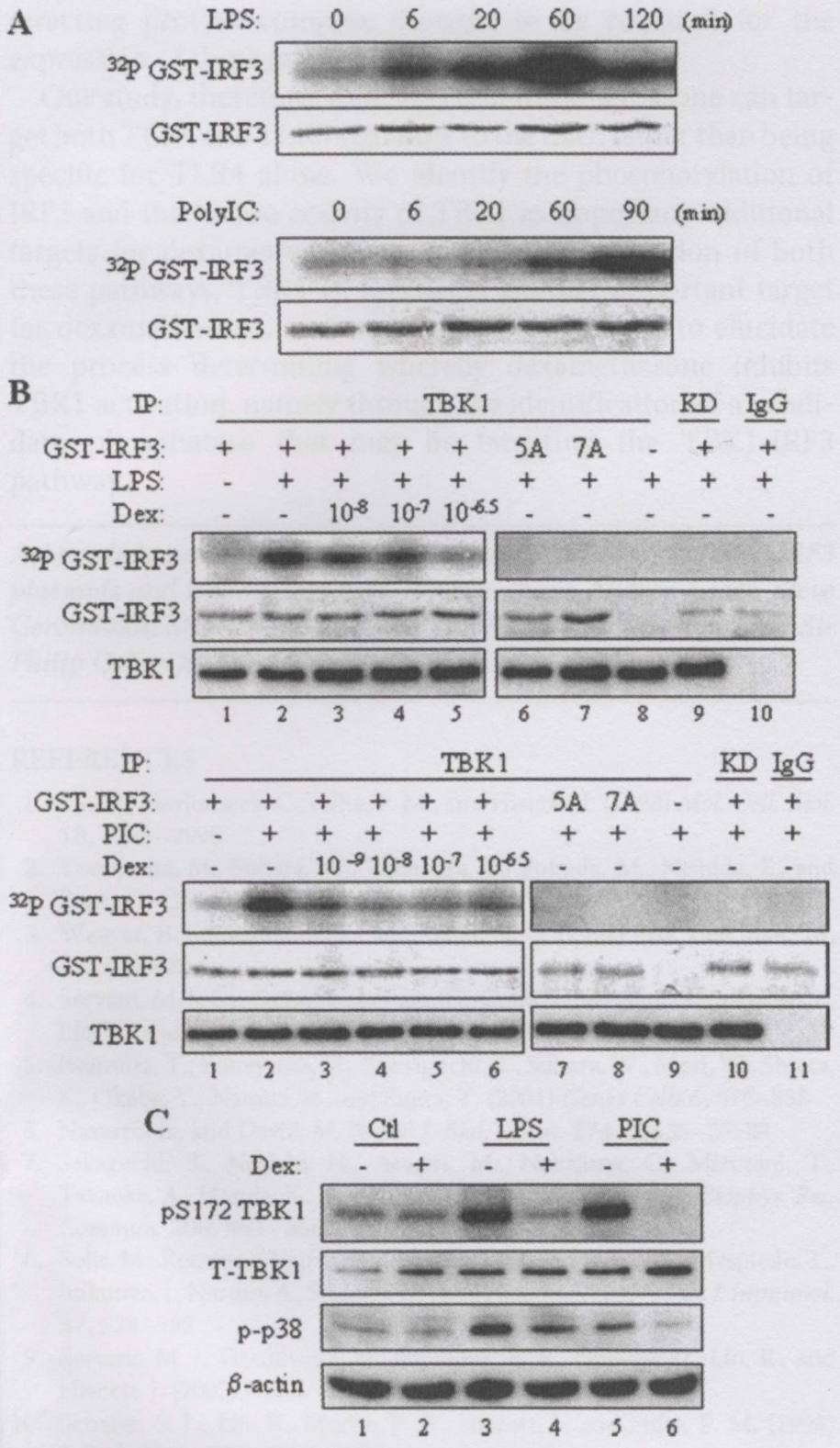


FIGURE 5. **Dexamethasone inhibits TBK1 kinase activity.** *A*, U373-CD14 cells were stimulated for various times as indicated with LPS (100 ng/ml) or poly(I-C) (50 μg/ml). *B*, cells were pretreated with 10-fold increasing doses of dexamethasone (*Dex*) from 1 nM to 10 μM for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min as indicated. In both cases, TBK1 was immunoprecipitated and assayed for kinase activity against recombinant GST-IRF3 (380–427), GST-IRF3 (5A), or GST-IRF3 (7A) as indicated. Additional assay controls such as FLAG-tagged kinase dead TBK1 (*KD*) and anti-rabbit IgG were assayed for kinase activity against recombinant GST-IRF3 (380–427). The *upper blots* illustrate ^{32}P incorporation into GST-IRF3, *middle blots* were stained with Coomassie Blue to demonstrate equal loading of GST-IRF3 (*A* and *B*) and *lower blots* were analyzed for total TBK1 to ensure equal immunoprecipitation (*IP*, *B*). *C*, U373-CD14 were pretreated with dexamethasone (500 nM) for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (*PIC*, 50 μg/ml) for 90 min. 55% of the lysate was immunoprecipitated with anti-phospho-Ser-172 (*pS172*) TBK1, and 35% was immunoprecipitated with total-TBK1 (*T-TBK1*). The immunoprecipitation samples were then blotted for total-TBK1. The remaining 10% of the lysate was used to blot for phospho-p38 (*p-p38*) and β-actin. Results are indicative of two to three separate experiments. *Ctl*, control.

We, therefore, first turned our attention to MKP1, a phosphatase expressed by DUSP1, because it has been shown to become induced by dexamethasone (25). However, overexpression of MKP1 had no effect on the ISRE luciferase system or on the phosphorylation status of IRF3, suggesting that IRF3 is not a substrate for this phosphatase. We next turned our attention to other potential phosphatases such as those expressed by other DUSP genes, particularly PAC-1, MKP-2, and MKP-5, which were also shown to become induced upon dexamethasone treatment (25). Calcineurin was also an interesting candidate and has been shown to negatively regulate TLR pathways (24). Overexpression of seven DUSP expressed phosphatases (PAC-1, MKP2, VH3, MKP3, PYST2, VH5, and MKP5) and calcineurin had no dramatic effect on the ISRE luciferase system. However, of potential interest are the phosphatases MKP2 and MKP5, which showed a significant reduction of ISRE luciferase when expressed at the highest concentration. Ongoing studies will continue to examine if these phosphatases may targeting IRF3 for dephosphorylation.

We did, however, find that dexamethasone could block the activation of the upstream kinase, TBK1, as judged by its kinase activity against recombinant GST-IRF3. In addition, dexamethasone abolished the phosphorylation of Ser-172 within the activation loop of TBK1, indicating for the first time that this kinase is a target for dexamethasone. This is an interesting observation as the importance of TBK1 as a central player in innate immune pathways is only recently becoming more apparent. The TBK1-IRF3 axis is not only crucial for TLR3-, TLR4-, and RIG-I-mediated signaling (16, 17, 19, 20) but is also critical for TLR-independent pathways such as those activated by intracellular B-DNA (32, 33). More recently, this dependence for TBK1 downstream of B-DNA signaling was shown to be essential for generating the adjuvant effect of DNA vaccines (33). The knowledge that dexamethasone can abolish TBK1 kinase activity is, therefore, an important observation that could impact on DNA vaccine administration. In addition, this observation may provide a basis for why steroids are contraindicated for certain viral infections which would trigger TBK1 activation as a key process in anti-viral host defense.

The effects of dexamethasone as an anti-inflammatory agent are highly complex, and many molecular targets have been identified to date (34). Ogawa *et al.* (22) analyzed the effects of dexamethasone on a range of 543 and 572 genes that were induced by LPS and poly(I-C), respectively. Interestingly they identified a subset of identical genes (~80) which contained ISRE sites that were sensitive to dexamethasone in response to LPS but were resistant in response to poly(I-C). The fact that p65 exists in a complex with IRF3 downstream of the TLR4 pathway but not the TLR3 pathway led them to examine whether dexamethasone could disrupt an IRF3·p65 complex by sequestering p65 out of the complex, which they demonstrated to be the case (21, 22). Because IRF3 activation downstream of TLR3 is p65-independent, they concluded that the targeting of the IRF3·p65 complex was the basis for the specific effect of dexamethasone. However, in our hands dexamethasone can affect both LPS and poly(I-C) pathways, indicating that TBK1 and IRF3 may be the common target. This was supported by the study where Reily *et al.* (23) could show that certain IRF3-dependent genes are in fact sensitive to dexamethasone in response to poly(I-C). The mechanism involved the ability of dexamethasone to disrupt an IRF3-glucocorticoid receptor-in-

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teracting protein complex thought to be required for the expression of these genes (23).

Our study, therefore, indicates that dexamethasone can target both TLR4 and TLR3 signaling to the ISRE rather than being specific for TLR4 alone. We identify the phosphorylation of IRF3 and the kinase activity of TBK1 as important additional targets for dexamethasone in response to activation of both these pathways. TBK1 is, therefore, another important target for dexamethasone, and ongoing studies will aim to elucidate the process determining whereby dexamethasone inhibits TBK1 activation, namely through the identification of a candidate phosphatase that may be targeting the TBK1-IRF3 pathway.

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Characterisation of the Novel Leucine Rich Repeat containing protein KIAA0644

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By

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Abstract

This thesis reports the existence of a novel leucine rich repeat containing protein annotated as KIAA0644 in the Kazusa DNA Research Institute database (www.Kazusa.or.jp), which was discovered in a study for proteins induced by Lipopolysaccaharide (LPS). This protein is expressed and highly conserved across species. It contains thirteen leucine rich repeats, a signal sequence, a fibronectin domain and a single spanning transmembrane region. It shows sequence similarity to Toll-like receptor, Nogo receptor and the Fibronectin leucine-rich transmembrane families. KIAA0644 is highly expressed in the brain, lung and ovary. RT-PCR results demonstrate that at the mRNA level KIAA0644 is induced by Lipopolysaccaharide (LPS). An antibody to the C terminus of the protein was raised and expression of KIAA0644 in the brain of human tissue samples was confirmed at the protein level. The protein was induced by LPS in the brain derived cell lines U373 and 1321-N1. KIAA0644 was also shown to be induced by LPS in the monocytic cell line THP1, in primary human peripheral blood mononuclear cells (PBMC), primary murine bone marrow derived macrophages (BMDM) and also in primary murine mixed glial cells.

Further investigation into the localisation of the protein revealed that endogenous KIAA0644 is mainly present in the cytosol whilst over-expressed KIAA0644 localises to the membrane fraction. Confocal imaging of KIAA0644 in A172 and U373/CD14 cells revealed the presence of KIAA0644 in undefined vescicles throughout the cytosol of the cell.

Biological assays were carried out to try and determine a function for this novel protein. siRNA experiments were carried out on U373/CD14 cells, THP1 cells and

human PBMCs. siRNAs specific to KIAA0644 were obtained and shown to reduce KIAA0644 expression at both the RNA and protein level. When KIAA0644 expression is knocked down in cells there is no IκB degradation or phosphorylation of p38 in response to LPS stimulation. There is also a decrease in proinflammatory cytokine release from cells in response to LPS. Further biological assays indicate that over-expression of KIAA0644 can enhance LPS signalling. KIAA0644 can bind LPS and physical interaction between KIAA0644 and TLR4 and KIAA0644 and CD14 was observed. The interaction between KIAA0644 and TLR4 increases upon LPS stimulation and the interaction between KIAA0644 and CD14 decreases post LPS stimulation.

This study therefore demonstrates that KIAA0644 is a functional protein which may be acting as a co-receptor within the LPS signalling pathway.