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**Modulation of Interleukin-1 and Toll-Like
Receptor Signalling by the Vaccinia Virus
Protein A52R**

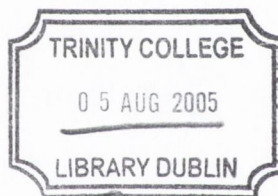
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For the degree of
Doctor of Philosophy

By
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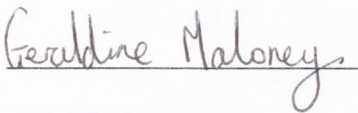
Abstract

Vaccinia virus (VV) has many mechanisms to suppress and modulate the host immune response. Given the role of Toll-like receptors (TLRs) and interleukin 1 (IL-1) in immunity, it was possible that VV would have mechanisms to antagonise these host proteins. Indeed, the VV protein A52R was previously shown to act as an inhibitor of NF κ B signalling by IL-1 and TLR4. In this present study A52R is shown to inhibit the activation of NF κ B by IL-1 and multiple TLRs, including TLR3, a receptor for viral RNA. Co-immunoprecipitation studies had previously revealed that A52R interacted with both TRAF6 and IRAK2. Here, the interaction between A52R and IRAK2 was shown to be sufficient for maximal NF κ B inhibition. In addition, data is also shown for a role for IRAK2 in TLR3 signalling. Evidence is shown for a functionally distinct role for A52R from another VV TLR antagonist, A46R. A52R had no inhibitory effect on TLR3-induced interferon regulatory factor (IRF) 3 activation or signaling by IRF5 or IRF7 activation in comparison with the potent inhibition of these signals by A46R.

Furthermore, A52R does not inhibit TLR-induced p38 or c-jun amino N-terminal kinase (JNK) mitogen activating protein (MAP) kinase activation. Rather, A52R could in fact drive activation of these kinases. Two lines of evidence suggested that the A52R:TRAF6 interaction was critical for these effects. Firstly, A52R-induced p38 MAP kinase activation was inhibited by a dominant negative version of TRAF6. Secondly, a truncated version of A52R, which interacted with IRAK2 and not TRAF6, was unable to activate p38. Since IL-10 production is strongly p38-dependent, the effect of A52R on IL-10 gene induction was examined. A52R was found to be capable of inducing the IL-10 promoter through a TRAF6-dependent mechanism. Furthermore, A52R enhanced LPS induced IL-10 production, while inhibiting the LPS-induced NF κ B dependent genes IL-8 and RANTES. These results highlight the ability of A52R to differentially modulate TLR signalling. Deletion of *A52R* has been shown to attenuate VV in a murine intranasal model. The inhibitory effects of A52R on TLR-NF κ B activation and its stimulatory effects on MAP kinase activation, leading to enhanced IL-10 production may both be important to its role in virulence.

Declaration

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



Geraldine Maloney

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Doing this Ph.D. would not have been possible without the support of my parents, Mary and Jim and my brother, Kevin (to whom I dedicated this thesis).

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Abbreviations

Ab	antibody
APC	antigen presenting cell
ARM	Armadillo repeat motif
BSA	bovine serum albumin
⁰ C	degrees Celsius
CMV	cytomegalovirus
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DMEM	Dulbecco's modified Eagle medium
DN	dominant-negative
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetra-acetic acid
EEV	extracellular enveloped virus
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
Fig.	figure
g	gram
h	hour(s)
HCV	hepatitis C virus

HIV	human immunodeficiency virus
HSV	herpes simplex viurs
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
ISG	IFN-stimulated gene(s)
ISRE	IFN stimulation response element
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
k	kilo
kDa	kilo Dalton(s)
LPS	lipopolysaccharide
LPB	LPS binding protein
MAP	mitogen-activated protein
MEF	murine embyronic fibroblast(s)
MHC	major histocompatibility complex
min	minute(s)
μ	micro
m	milli
mRNA	messenger RNA
N/C terminus	amino/carboxyl terminus
NFκB	nuclear factor kappa B
ORF	open reading frame

PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDCs	plasmacytoid DCs
PKR	protein kinase R
poly(I:C)	polyinosine-polycytidylic acid
PRR	pattern recognition receptor
RANTES	regulated on activation normal T cell expressed and secreted
RNA	ribonucleic acid
rpm	revolutions per minute
RSV	respiratory syncytial virus
s	second(s)
SAM	sterile α motif
SARM	SAM and ARM protein
siRNA	small inhibitory RNA
TBK1	TRAF family member associated NF κ B activator-binding kinase 1
TCR	T cell receptor
TEMED	N, N, N', N'-tetramethylethylenediamine
Th	T helper cell
TICAM	TIR-containing adaptor molecule
TM	trademark
TNF	tumour necrosis factor
TNFR	TNF receptor
TRAF	TNFR-associated factor

TRIF	TIR domain-containing adaptor inducing IFN- β
Tween-20	polyoxyethylenesorbitan monolaurate
V	volts
v/v	volume/volume
VV	vaccinia virus
w/v	weight/volume
WR	Western reserve
TIR	Toll-IL-1 receptor resistance domain
TLR	Toll-like receptor(s)

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Chapter 1

Introduction

1.1 Innate and Adaptive Immunity

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and non-self. In mammals the immune system can be divided into two branches: "innate immunity" and "adaptive immunity." Adaptive immunity detects non-self through recognition of peptide antigens using antigen receptors expressed on the surface of B and T lymphocytes. In order to respond to a wide range of potential antigens, B and T cells rearrange their immunoglobulin and T cell receptor genes to generate over 10^{11} different species of antigen receptors. Engagement of antigen receptors by the cognate antigen triggers clonal expansion of the lymphocyte and further production of antigen-specific antibodies.

B cells, the precursors of antibody-secreting cells, can directly recognise native antigen through their B-cell receptors. T lymphocytes, however, need the antigen to be processed and presented to them by an antigen presenting cell (APC). The T-cell antigen receptors (TCRs) recognise fragments of antigens bound to the major histocompatibility complex (MHC) on the surface of an APC. The peptide-binding proteins are of two types MHC class I and MHC class II, which then stimulate cytotoxic T lymphocytes (CTLs) and helper T cells (T_H). Naive T_H cells, when stimulated with cognate antigens by APCs, differentiate into two cell subsets: T_H 1 and T_H 2. T_H 1 cells secrete $IFN\gamma$ and promote mainly cellular immunity, whereas T_H 2 cells produce IL-4, IL-5, IL-10 and IL-13 and primarily promote humoral immunity (Abbas *et al.*, 1996). This highly sophisticated system is observed only in vertebrates and is a potent defence against infection.

However, the adaptive immune response has two main limitations. Firstly, randomly generated antigen receptors are unable to determine the source and the biological context of the antigen for which they are specific. Secondly, a clonal distribution of antigen receptors requires that specific clones expand and differentiate into effector cells before they can contribute to host defence. As a result, primary adaptive immune responses are delayed by 4-7 days, which is too much of a lag period to combat quickly replicating microbial invaders. However, the adaptive immune system does not function independently. Almost every aspect of the adaptive immune response is controlled by the innate immune system (Reviewed in Iwasaki *et al.*, 2004).

The innate immune system, which was first described over a century ago, is phylogenetically conserved and is present in almost all multicellular organisms. Whereas the system of adaptive immunity has been the subject of considerable study in the past century, innate immunity has been less well appreciated. Innate immunity plays a primary role in acute host defence. It critically contributes to destroying the pathogen, determining the localization and extent of the challenge, and facilitating the adaptive immune response. Innate immunity primarily serves to restrict the infectious challenge during the lag period required for adaptive immunity to develop (Janeway *et al.*, 2005). The innate component of immune recognition is based on a germ line-encoded recognition system. Pattern recognition receptors (PPR) selectively recognise membrane-derived structures in pathogenic microorganisms. The recent identification of Toll-like receptors (TLRs) as pattern recognition receptors in mammals has made immunologists aware that innate immunity plays an important role in the detection of invading pathogens.

Recent evidence shows that TLRs recognise specific patterns of microbial components, especially those from pathogens, and regulates the activation of both innate and adaptive immunity (Reviewed in Hallman *et al.*, 2001).

1.1.1 Toll-Like Receptors – Linking Innate and Adaptive Immunity

Dendritic cells (DCs) comprise a family of APCs that act like “conductors” of the immune response in their capacity to orchestrate signals derived from different parts of the immune system (Banchereau *et al.*, 2000). In humans, two types of DCs have been described: myeloid and plasmacytoid DCs. Myeloid DCs include epidermal DCs and dermal or interstitial DCs which can also be found in other peripheral tissues. Plasmacytoid DCs (pDCs) are also known as type I IFN-producing cells owing to their capacity to release type I IFNs after viral infection (Reschigno 2002). DCs are able to interact via several pattern recognition receptors (e.g. TLRs) with pathogen associated molecular patterns (e.g. LPS) which activate DCs. This activation is called maturation and results in the capacity of DCs to initiate an effective adaptive immune response. DC maturation is characterised by the production of proinflammatory cytokines (IL-12 and TNF α), upregulation of costimulatory molecules (CD40, CD80 and CD86) and altered expression of chemokine receptors (CCR2, CCR5 and CCR7). Mature DCs show increased antigen-presenting capacity and migrate from the peripheral tissues to draining lymph nodes, where they instruct the adaptive immune response by stimulating T lymphocytes. The DCs in the lymphoid tissue are no longer able to engulf antigens by phagocytosis. However, they now express very high levels MHC class II molecules; this enables them to stably present peptides from proteins acquired from the infecting pathogens. They express very high levels of adhesion molecules, including cell-specific adhesion molecule called DC-SIGN, as well as high levels

of B7 molecules. They also secrete a chemokine that specifically attracts naïve T cells. This chemokine, called DC-CK, is expressed only in DCs in lymphoid tissues. These properties help to explain the DC's ability to stimulate strong naïve T-cell responses. Thus, DC maturation, which is mediated through the TLR family, is a critical link between innate and adaptive immunity (Fig. 1.1) (Akira *et al.*, 2001).

1.2 TLRs - Discovery and Structure

Toll was initially identified in *Drosophila* as a receptor essential for dorso-ventral polarity during embryogenesis (Morisato and Anderson 1994). Subsequent studies revealed that it also plays an essential role in insects in the innate immune response to fungal infection. *Toll* mutant *Drosophila* rapidly succumb to fungal infection, due to a failure to induce the antifungal peptide Drosomycin (Lemaitre *et al.*, 1996). The cytoplasmic signalling domain of Toll was found to be conserved in the mammalian type 1 interleukin-1 receptor (IL-1R) (Gay and Keith 1991). Mammalian homologues of Toll were subsequently identified through EST and genomic sequence database searches. To date thirteen members of the TLR family have been identified. TLRs 1-9 are common to mouse and human, while TLR10 is only functional in humans, and TLRs 11, 12, and 13 are unique to the mouse. The TLRs are type I integral membrane glycoproteins and they are members of a larger superfamily that includes the interleukin-1 receptors. This family share significant homology in their cytoplasmic regions. The region of homology is known as the Toll-IL-1R Resistance (TIR) domain (Reviewed Akira 2003) (Fig 1.2). This domain mediates homotypic interactions between TLRs and also recruitment of TIR domain containing adaptor molecules. Five cytosolic adaptor molecules

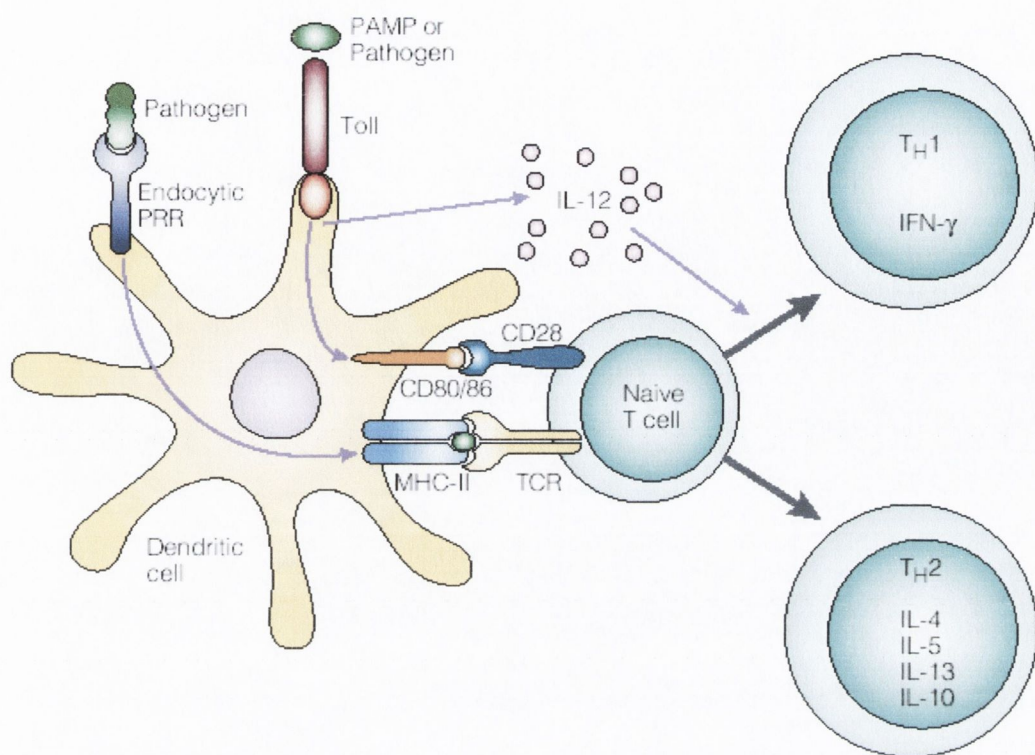


Fig. 1.1 Role of TLRs in control of adaptive immunity

TLRs sense the presence of infection through recognition of PAMPs. Recognition of PAMPs by TLRs expressed on APCs, such as DCs, upregulates cell-surface expression of co-stimulatory (CD80 and CD86) molecules and MHC class II molecules. TLRs also induce expression of cytokines, such as IL-12. Induction of CD80/CD86 on APCs by TLRs leads to the activation of T cells specific for pathogens that trigger TLR signalling. IL-12 induced by TLRs also contributes to the differentiation of activated T cells into T_H1 effector cells. (Taken from Medzhitov, 2001).

MyD88, Mal (MyD88 adaptor like) also known as TIRAP (TIR domain containing adaptor molecule), TRIF (TIR domain containing adaptor inducing interferon β), TRAM (TRIF related adaptor molecule) and SARM (sterile α and HEAT-Armadillo motifs) also have TIR domains (Fig. 1.3).

The region of homology in the TIR motif is most conserved within three boxes that contain amino acids crucial for signalling. Box 1 is the signature sequence of the TIR domain. Box 2 forms an important loop in the structure (called the BB loop) and most TIR box 2 sequences have a conserved proline in the BB loop which when mutated to a histidine renders the protein unable to signal. The function of box three is not fully understood, although it contains residues important in signalling based on mutational analysis of the Type 1 IL-1 receptor (Reviewed in O'Neill *et al.*, 2003). The crystal structures of the TIR domains of TLR1 and TLR2 have been obtained and analysed. They contain a central five stranded parallel β -sheet, which is surrounded by five α -helices on each side. These two secondary structural elements are connected by loops, for example, the BB loop connects a β -strand and an α -helix (Xu *et al.*, 2000). The conserved boxes 1 and 2 and the BB loop are adjacent and display most of their side chains for interaction.

In contrast to the similarity between the IL-1/TLR superfamily in their intracellular regions the extracellular portion of the TLRs contain a leucine rich repeat (LRR) motif whereas that of the IL-1s contain three immunoglobulin domains (Fig. 1.2). Each leucine rich repeat consists of 24-29 amino acids and contains the conserved sequences XLXXLXLXX and XOXXOX₄FXXLX, where X denotes any amino acid and O a hydrophobic amino acid. The LRR domains of TLRs form a

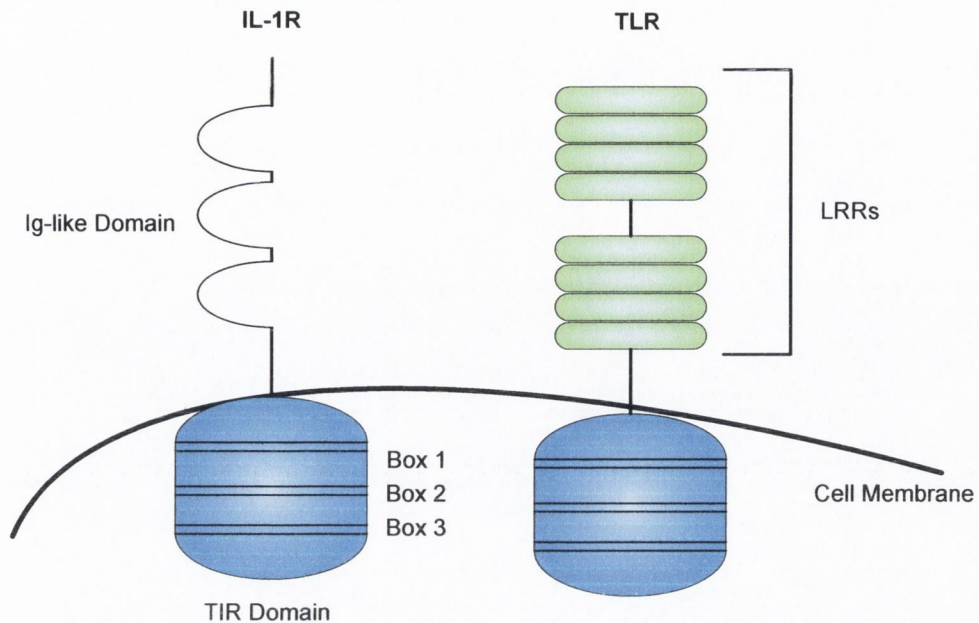


Fig. 1.2 IL-1R/TLR structure

TLRs and IL-1Rs have a conserved cytoplasmic domain, known as the Toll-IL-1R Resistance (TIR) domain. The TIR domain is characterised by the presence of three highly homologous regions (known as boxes 1, 2 and 3). Despite the similarity of the cytoplasmic domains of these molecules, their extracellular regions differ markedly: TLRs have tandem repeats of leucine rich regions (known as leucine rich repeats, LRR), whereas IL-1Rs have three immunoglobulin (Ig)-like domains. (Adapted from Akira, 2004).

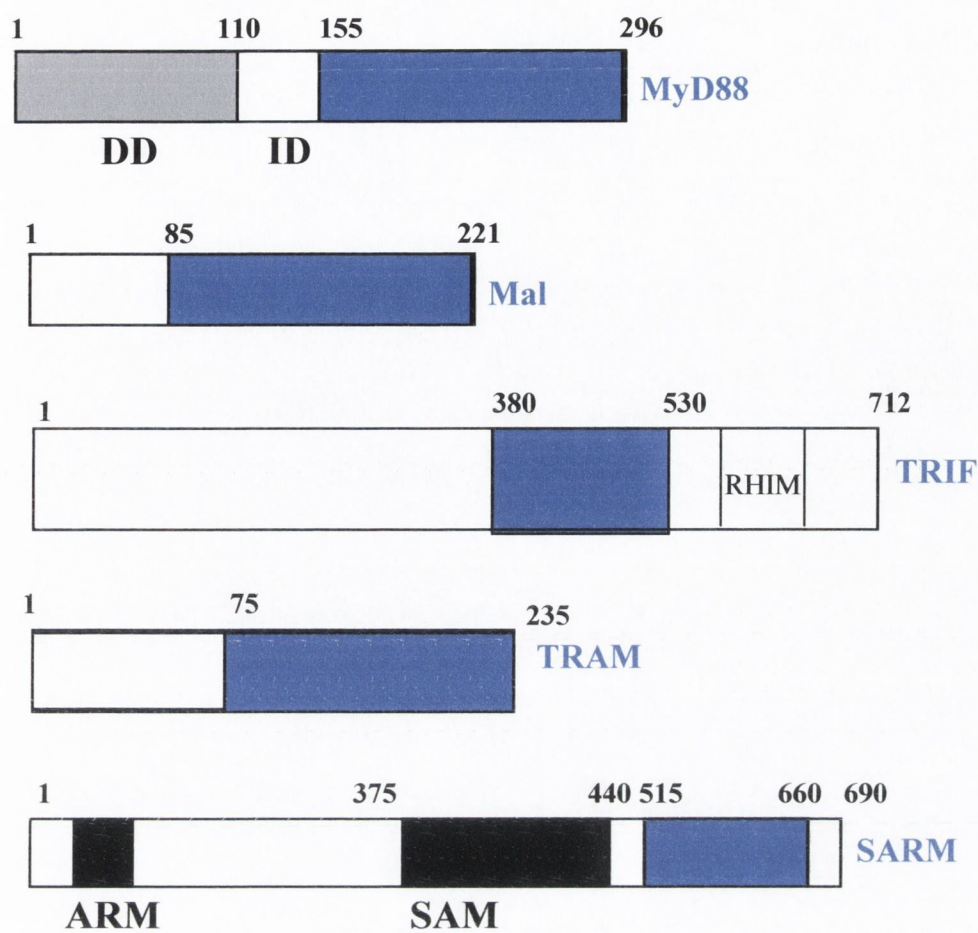


Fig. 1.3 Schematic representation of TIR adaptors (MyD88, Mal, TRIF, TRAM and SARM)

DD: Death Domain, ID: Intermediary Domain, TIR: Toll-IL-1R Resistance homology domain, RHIM domain: RIP interaction motif, ARM: Armadillo motif, SAM: Sterile and HEAT Armadillo motif

horseshoe structure and it is thought that the concave surface of the LRR domains is involved directly in the recognition of various PAMPs (Bell *et al.*, 2003). Despite the conservation among LRR domains, different TLRs recognise several structurally unrelated agonists. Each TLR is described in greater detail in the following section. The known TLR agonists are depicted in Fig. 1.4.

1.3 TLR Agonists

Numerous PAMPs have been shown to activate particular TLRs, while activators for some TLRs, for example, TLR 10 remain unknown (Fig. 1.4). Recognition of PAMPs by TLRs triggers the activation of signalling cascades that lead to the activation of both the innate and adaptive immune responses. The innate immune response is characterised by, for example, the production of inducible nitric oxide synthase and the pro-inflammatory cytokines IL-6 and TNF whereas the adaptive immune response induces IL-12 and the co-stimulatory molecules CD80 and CD86, which are involved in T-cell activation (Section 1.1) (Kaisho *et al.*, 2001). Engagement of TLRs with their ligands leads to the activation of several intracellular signal transduction pathways. Among the most prominent and best characterised of these are those leading to the activation of the transcription factor NF κ B and mitogen activated protein (MAP) kinases. Triggering of TLRs to activate an anti-viral response requires the activation of the transcription factors interferon regulatory factor (IRF) 3, IRF5 and IRF7 which induce the production of type I interferons (IFNs).

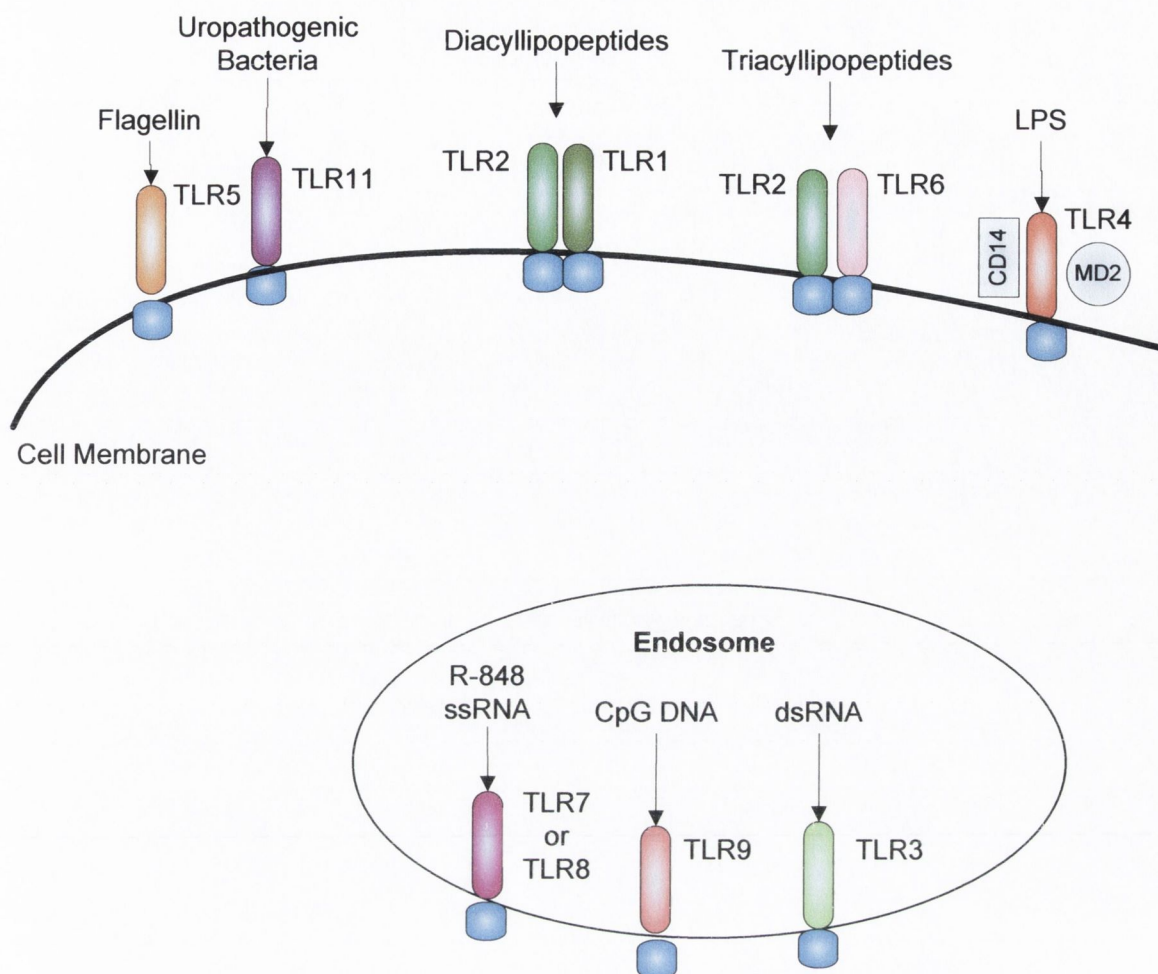


Fig. 1.4 Schematic diagram of activators of TLRs

TLRs respond to specific PAMPs. TLR4 requires CD14 and MD2 in order to respond to LPS. TLR2 can dimerise with either TLR1 or TLR6 in order to respond to diacyllipoproteins or triacyllipoproteins respectively.

1.3.1 TLR4

Human TLR4 was the first characterized mammalian Toll (Medzhitov *et al.*, 1997). It is expressed in a variety of cell types but is most predominantly found on the cells of the immune system, for example macrophages and dendritic cells (Medzhitov *et al.*, 1997). TLR4 recognises lipopolysaccharide (LPS). The identification and characterisation of two mouse strains, C3H/HeJ and C57BL10/ScCr, led to this discovery as both of these mouse strains were known to be hypo-responsive to LPS. The C3H/HeJ mouse strain has a point mutation in the intracellular region (TIR domain) of the *Tlr4* gene leading to the replacement of a highly conserved proline in box 2 with histidine. This point mutation results in the generation of a dominant negative allele, which abrogates downstream signalling through TLR4 rendering the C3H/HeJ mice unresponsive to LPS (Poltorak *et al.*, 1998, Qureshi *et al.*, 1999, Hoshino *et al.*, 1999). Further evidence to confirm that TLR4 is an essential receptor for the recognition of LPS came from studies using another LPS hypo-responsive strain, C57BL10/ScCr, which has a null mutation in the *Tlr4* gene (Poltorak *et al.*, 1998)

Recognition of LPS by TLR4 is complex and requires several accessory molecules. LPS is first bound to a serum protein termed LPS binding protein (LBP), which functions by transferring LPS monomers to CD14 (Wright *et al.*, 1989). CD14 is a high affinity LPS receptor that can either be secreted into serum or expressed as a glycoprophosphoinositol (GPI-linked) protein on the surface of macrophages (Wright *et al.*, 1990). CD14 deficient mice have a profound defect in responsiveness to LPS showing the importance of CD14 in LPS recognition (Hazirot *et al.*, 1996). Another component of the LPS receptor complex is MD2. It was identified as a molecule

that associates with the extracellular portion of TLR4 and enhances LPS responsiveness (Shimazu *et al.*, 1999, Akashi *et al.*, 2000) (Fig. 1.4).

TLR 4 also recognises a range of other PAMPs. Table 1.1 shows a list of the other known PAMPs that signal through TLR4.

1.3.2 TLR2

TLR2 has the ability to recognise a broad range of microbial products. Table 1.1 highlights the diverse array of ligands recognised by TLR2. The ability of TLR2 to response to a wide variety of PAMPs maybe partly attributed to the fact that TLR 2 forms heterodimers. Both TLR1 and TLR6 have been shown to signal co-operatively with TLR2, and this co-operation can affect the specificity of the receptor pair. For instance a heterodimer between TLR2 and TLR6 recognises mycoplasmal lipopeptide which is a diacylated form, whereas the TLR2 and TLR1 pair preferentially recognises a triacylated lipopeptide for example Pam₃Csk₄ (Ozinsky *et al.*, 2000). Evidence for direct binding of a PAMP to a TLR2 was reported by Vasselon *et al.*, 2004. This report showed that the extracellular domain of TLR2 interacted directly with synthetic bacterial lipopeptide (Vasselon *et al.*, 2004). It is also possible that a TLR other than TLR1 and TLR6 pairs with TLR2 too to recognise other TLR2 ligands. TLR10 may be a partner for TLR2 given the fact that its amino acid sequence is most similar to TLR1 and TLR6 (Reviewed in Akira and Hemmi 2003). Indeed, recent studies have shown that TLR10 can heterdimerise with TLR1 and TLR2 (Hasan *et al.*, 2005). Although TLR2 in cooperation with TLR1 and TLR6 can respond to a variety of TLR agoinsnts (Table 1.1) K_D values of these interactions have not yet been established.

Table 1.1 TLRs and their agonists

TLR Family	Ligands (origin)
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria)
TLR2	Lipoprotein/lipopeptides (a variety of pathogens) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) A phenol-soluble modulins (Staphylococcus epidermidis) Glycoinositolphospholipids (Trypanosoma Cruzi) Glycolipids (Treponema maltophilum) Porins (Neisseria) Zymoan (fungi) Atypical LPS (Leptospira interrogans) Atypical LPS (Porphyromonas gingivalis) HSP70 (host)
TLR3	Double Stranded RNA (virus) DsRNA from Schistosoma mansoni (Parasite) West Nile Virus (virus) CMV (virus) Influenza A (virus)
TLR4	LPS (Gram-negative bacteria) Taxol (plant) Fusion protein (RSV) Envelope proteins (MMTV) HSP60 (Chlamydia pneumoniae) HSP60 (Host) HSP70 (Host) Type III repeat extra domain A of fibronectin (Host) Oligosaccharides of hyaluronic acid (Host) Polysaccharide fragments of heparan sulfate (Host) Fibrinogen (Host)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopeptides (mycoplasma)
TLR7	Imazoquinoline (synthetic compound) Loxoribine (synthetic compound) Bropiramine (synthetic compound) Influenza A (virus) VSV (virus) HIV-1 (virus)
TLR8	Imazoquinoline (synthetic compound)
TLR9	CpG DNA (bacteria) HSV-1 (virus) HSV-2 DNA (virus) CMV (virus)
TLR10	?
TLR11	Uropathogenic bacteria

Adapted Takeda *et al.*, 2002

1.3.3 TLR3

Viral replication within infected cells results in generation of dsRNA, which can stimulate immune cells. dsRNA binds and activates the protein kinase PKR, initiating downstream signalling that inhibits viral replication and upregulation of inflammatory cytokine production. Cells from PKR deficient mice still showed a response to polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue, suggesting the existence of another receptor which recognises dsRNA. Indeed, TLR3 deficient mice showed decreased responses to poly(I:C) indicating a role for TLR3 in viral recognition (Alexopoulou *et al.*, 2000). Recently, studies have shown that TLR3 recognises viruses and induces an anti-viral response. This is discussed in greater detail in section 1.8.4.

Interestingly, dsRNA from the helminth parasite *Schistosoma mansoni* has been reported to activate the NF κ B luciferase reporter and the IFN β promoter via TLR3 in dendritic cells (Aksoy *et al.*, 2005). This study is the first to show a non-viral pathogen acts as an inducer of the innate immune system through TLR3. It has also been proposed that host mRNA is an endogenous ligand for TLR3, possibly through regions of double-stranded secondary structure (Kariko *et al.*, 2004).

1.3.4 TLR5

The only known ligand for TLR5 is flagellin, a component of bacterial flagella. Flagellin is a polymeric rod like appendage extending from the outer membrane of Gram-negative bacteria that propels the organism through its aqueous environment. The recognition of flagellin mediated by TLR5 can induce NF κ B activation and inducible nitric oxide synthetase expression in transformed human epithelial cells

and murine macrophages (Hayashi *et al.*, 2001). A further study mapped the TLR5 recognition site on flagellin to a cluster of 13 amino acid residues that participate in intermolecular interactions with the flagellar protofilaments that are required for bacterial motility (Smith *et al.*, 2003). In addition, this study reported that flagellin coprecipitated with TLR5, suggesting a close physical interaction between the molecules (Smith *et al.*, 2003).

1.3.5 TLR7/8

Imiquimod (Aldara, R-837) and R-848 (resiquimod) are low molecular weight drugs of the imidazoquinoline family that have potent anti-viral and anti-tumour properties in animal models. TLR7/8 has been shown to be essential for the response to imidazoquinolines. However, direct binding of R-837 and R-848 to TLR7/8 has not yet been shown. The generation of TLR7 knockout mice revealed no detectable amounts TNF- α , IL-6 and IL-12 and splenocytes did not proliferate in response to imiquimod or R-848. Furthermore TLR7 deficient bone marrow derived dendritic cells did not show upregulation of co-stimulatory molecules in response to R-848 and the activation of NF κ B and JNK by TLR7 was completely abolished in TLR7 knockout macrophages (Hemmi *et al.*, 2002).

1.3.6 TLR9

Bacterial DNA and certain oligonucleotides containing unmethylated CpG dinucleotides can stimulate immune cells, whereas eukaryotic DNA and methylated oligonucleotides cannot. CpG DNA directly stimulates B cells and a subtype of dendritic cells, known as plasmacytoid DCs (pDC) through TLR9 (Hemmi *et al.*, 2000). Unlike most other TLR receptors, which are present on the cell membrane,

TLR9 is located in and signals from intracellular endosomal compartments (Ahmad-Nejad *et al.*, 2002, Latz *et al.*, 2004). No direct binding of CpG to TLR9 has been demonstrated, but confocal microscopy studies show that TLR9, MyD88 and CpG co-localise in late endosomes and endosomal maturation is necessary for TLR signalling (Verthelyi and Zeuner, 2003).

Herpes Simplex Virus 2 (HSV2) has been shown to activate IFN- α in pDCs through recognition of TLR9. Furthermore, this report provides evidence that the ligand recognised by TLR9 is the genomic DNA of HSV2, this raises the possibility of TLR9 mediated recognition of other DNA viruses (Lund *et al.*, 2003). Indeed, recently further evidence for the recognition of DNA viruses rich in CpG motifs by TLR9 has been reported, and is discussed in section 1.8.6.

1.3.7 TLR10

Despite the fact the PAMP for TLR10 is unknown it has recently been shown that TLR10 expressed by B cells and plasmacytoid dendritic cells (pDCs) activates gene transcription through a MyD88-dependent pathway (Hasan *et al.*, 2005). This study demonstrated that TLR10 can homodimerise and also heterodimerise with TLR1 and TLR2. In addition, using recombinant CD4-TLR10 it was shown that TLR10 directly associated with MyD88 (but not Mal, TRIF or TRAM) and was capable of activating NF κ B in a luciferase reporter gene assay (Hasan *et al.*, 2005).

1.3.8 TLR11

TLR11 was first detected as an expressed sequence tag (EST) from a mouse liver database. Sequential searches using this EST led to the discovery of the mouse

genomic sequence encoding TLR11. TLR11 sequences are present in the genomes of many mammalian species including humans. However, there is a stop codon in the human TLR11 sequence thus rendering the human TLR11 non-functional. TLR11 has been shown to have a distinct pattern of expression in mouse macrophages of liver and kidney and bladder epithelial cells. TLR11 specifically responds to uropathogenic bacteria. Mice lacking TLR11 were shown to be highly susceptible to infection of the kidneys by uropathogenic bacteria (Zhang *et al.*, 2004). Hence, TLR11 may be critically important in preventing infection of internal organs of the urogenital system.

1.4 IL-1/TLR-Mediated NF κ B and IRF3 Activation

1.4.1 NF κ B Activation

NF- κ B is a transcription factor originally identified by its ability to bind the promoter of the kappa light chain gene in B cells (Sen and Baltimore, 1986) but has since been shown to be present and functional in many other cell types. At present five mammalian members of the NF- κ B family have been identified, p65, c-Rel, RelB, p50/p105 and p52/p100 (Reviewed in Viatour *et al.*, 2005). p65, c-Rel and RelB are all transcribed as transcriptionally active molecules from their genes, however p50 and p52 are made as larger precursor molecules that require proteolytic cleavage in order to become transcriptional regulators (Reviewed in May and Ghosh 1998). Each member of this family contains a rel-homology domain (RHD), which acts as the dimerization and DNA binding domain, the nuclear localization signal and also is the binding site for the inhibitory molecule of NF- κ B (Thanos and Maniatis, 1995). Members of the NF- κ B family are able to form both hetero- and homodimers to produce the NF- κ B complex. However, the term NF-

κ B is generally used to describe p50/p65 homodimers, the predominant form of NF- κ B (Thanos and Maniatis, 1995). In unstimulated cells NF κ B-family proteins exist as hetero- or homodimers that are sequestered in the cytoplasm by virtue of their association with a member of the I κ B family of inhibitor proteins (Reviewed in Ghosh *et al.*, 1998). Like NF- κ B, the I κ Bs are also members of a multigene family containing seven known mammalian members including I κ B α , I κ B β , I κ B γ , I κ B ϵ , I κ B ζ , Bcl-3 and the precursor Rel-proteins p105 and p100. Each member has a multiple ankyrin repeat domain that mediates interaction with the RHD of NF- κ B molecules. The best characterised of the I κ Bs is I κ B α , which is responsible for the transitory activation of NF- κ B in response to cytokines (Sun *et al.*, 1993). Activation of NF κ B involves the phosphorylation and proteolysis of the I κ B α and concomitant release and nuclear translocation of the NF κ B factors, p50/p65. This process is mediated by the I κ B kinase (IKK) complex. The mechanism of NF κ B activation is similar to another transcription factor called Nrf2. Nrf2 activity is primarily repressed by Keap1-mediated sequestration of Nrf2 in the cytosol. Activation of Nrf2 requires Keap1-mediated degradation of the protein by the proteasome (Kato *et al.*, 2005).

Interestingly, It has been recently shown that transcription of I κ B ζ is rapidly induced by stimulation with IL-1 and the TLR ligands, LPS, CpG and R-848 (Yamamoto *et al.*, 2004). This report also demonstrated that I κ B ζ was indispensable for TLR induction of IL-6. I κ B ζ -deficient cells showed severe impairment of IL-6 production in response to a variety of TLR ligands as well as IL-1, but not in response to TNF α (Yamamoto *et al.*, 2004). Endogenous I κ B ζ

specifically associated with the p50 subunit of NF κ B and it is recruited to the NF κ B binding site of the IL-6 promoter on stimulation (Yamamoto *et al.*, 2004).

IKK is composed of three subunits, the catalytic subunits IKK α and IKK β (also known as IKK1 and IKK2) and the regulatory subunit IKK γ (also known as NF κ B essential modulator, NEMO). IKK α and IKK β have very similar primary structures (52% identity), with protein kinase domains near their N-termini and leucine zipper and helix-loop-helix motifs near their C-termini. IKK γ lacks a kinase domain but contains long stretches of coiled-coil sequence which function in protein-protein interactions (Rothwarf and Karin 1999).

The IKK α and IKK β subunits preferentially form heterodimers, and both can directly phosphorylate the critical S32 and S36 residues of I κ B α (Brown *et al.*, 1995). The phosphorylated I κ B α is then ubiquitinated at Lys21 and Lys22 which targets it for degradation by the 26S proteasome, thereby releasing NF- κ B dimers from the cytoplasmic NF- κ B-I κ B complex and allowing them to translocate to the nucleus (Chen *et al.*, 1995). I κ B α is quickly resynthesised to allow inhibition of the pathway (Krappmann and Scheidereit, 1997).

1.4.2 IL-1-Mediated NF κ B Activation

Activation of the transcription factor NF κ B in response to IL-1 has been extensively documented and the pathway leading to its release from the cytoplasm is well characterised. In general, the IL-1/TLR superfamily share downstream signalling molecules. These include the adaptor molecule MyD88, IL-1R1

associated protein kinases (IRAKs), the tumour necrosis factor receptor associated factor 6 (TRAF6), the TGF- β activated kinase (TAK1), TAK1 binding protein 1 (TAB1) and 2 (TAB2). The generally accepted scenario of the IL-1-mediated pathway to NF κ B is shown in Fig. 1.5.

Upon binding, IL-1 induces a structural change that allows dimerisation of the IL1RI with the IL1RAcP (Greenfeder *et al.*, 1995) to form the active signalling complex. Subsequently, the adaptor protein MyD88 is recruited to the receptor complex via its TIR domain (Burns *et al.*, 1998, Muzio *et al.*, 1997, Wesche *et al.*, 1997). This association promotes the recruitment of the IRAKs (IRAK1 and IRAK4) (Cao *et al.*, 1996, Wesche *et al.*, 1997). During the formation of this complex, IRAK4 is activated, leading to the hyperphosphorylation of IRAK1, which then induces the interaction of TRAF6 with the complex (Li *et al.*, 2001). The association of IRAK4/IRAK 1/TRAF6 causes disengagement from the receptor complex and leads to an interaction of this complex with another preformed complex consisting of TAK1, TAB1 and TAB2 at the cell membrane (Cao *et al.*, 1996). Subsequent phosphorylation of TAB2 and TAK1 occurs and causes the complex to disengage from IRAK1 at the membrane and translocate to the cytosol (Deng *et al.*, 2000). TRAF6 interacts with TRAF6 regulated I κ B kinase activator 1 (TRIKA1) which is a dimeric ubiquitin conjugated enzyme complex composed of Ubc13 and Uev1A (Wang *et al.*, 2001). Phosphorylated TAK1 in the cytosol leads to the activation of the IKK complex, which then phosphorylate the I κ Bs. The transcription factor NF κ B is sequestered in the cytoplasm by I κ Bs, but activation leads to phosphorylation and degradation of the inhibitory molecule I κ B and consequently the release of NF κ B (Chen *et al.*, 2005). NF κ B is then free to

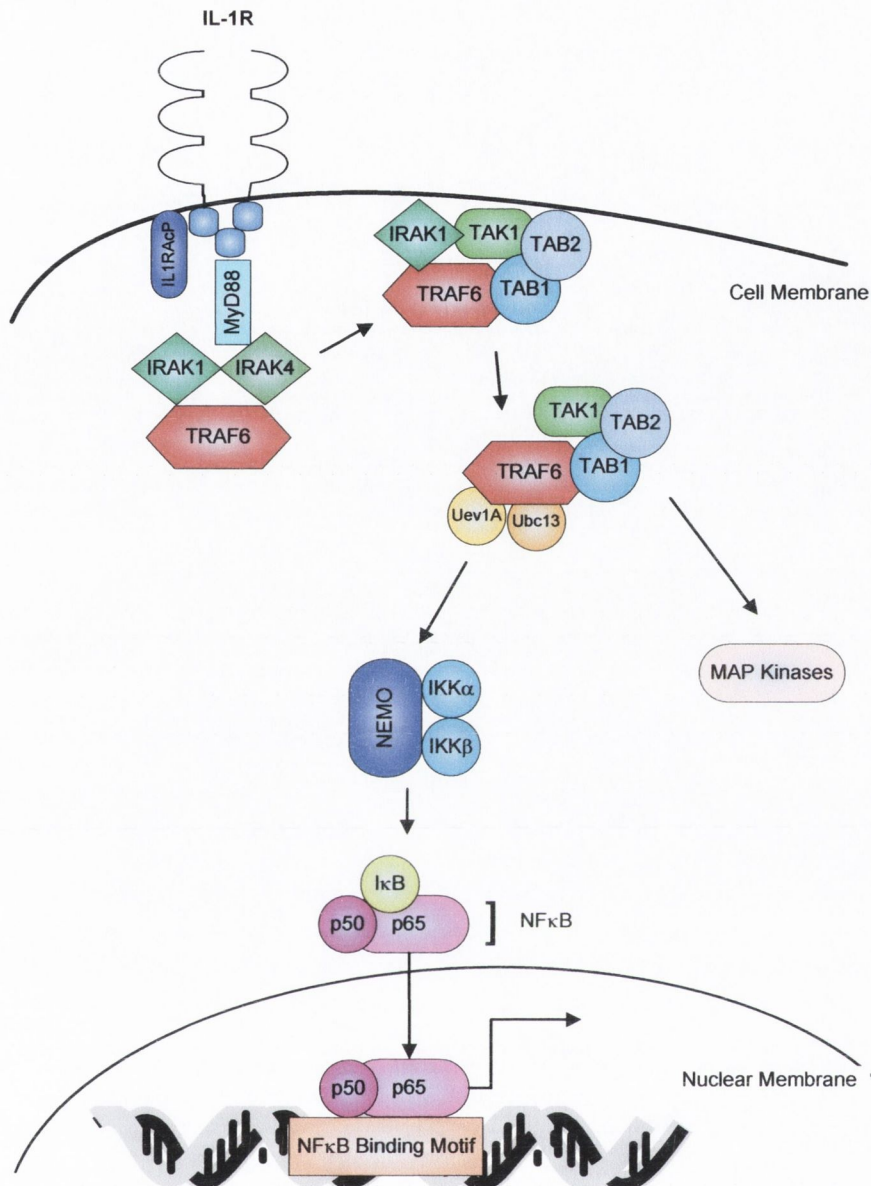


Fig. 1.5 IL-1R-mediated NFκB activation

Activated IL-1R, associates with a cytoplasmic adaptor molecule MyD88, through the homophilic interaction between their TIR domains. MyD88 also possesses a death domain, which mediates the association with the serine-threonine kinases, IRAK1 and IRAK4. Subsequently, TRAF6 is activated and in turn activates the IKK complex and MAP kinases. The IKK complex induces phosphorylation of IκB, which renders IκB competent for being ubiquitinated and degraded. IκB degradation liberates NFκB and allows it to translocate to the nucleus where it can induce target gene expression.

enter the nucleus and induce target gene expression. Activation of TAK 1 also results in the activation of MAP kinases (p38 and JNK MAP kinase) (Reviewed Akira 2003).

In addition to TAK1, mitogen-activated extracellular signal regulated kinase activating kinase (MEKK) 3 has also been implicated in IL-1/TLR mediated NF κ B activation. It has been shown that MEKK3 forms a complex with TRAF6 in response to IL-1 and this interaction is crucial for IL-1-induced NF κ B activation (Huang *et al.*, 2004a).

The signalling pathway leading to NF κ B activation by TLR family members is very similar to IL-1-mediated NF κ B activation. However, layers of complexity have been added to this simple scheme in recent years as novel signalling components have been identified, and differences have emerged for different TLRs. The divergence of the pathways appears to emanate from the initial recruitment of a TIR adaptor. Each of the TIR adaptors and the key signalling molecules IRAKs and TRAF6 are discussed in greater detail in the following sections 1.5 - 1.7.

1.4.3 IRF3 Activation

IRF3 is a member of the IFN regulatory factor (IRF) family, which play important roles in host defences against pathogens. IRF3 is found in an inactive cytoplasmic form that upon serine/threonine phosphorylation results in homodimerisation. The homodimer then translocates to the nucleus and forms a complex with coactivator p300/CBP. This complex activates the transcription of type I IFNs and IFN stimulated genes (ISGs) (Reviewed in Servant *et al.*, 2002). IRF3 is known to be

activated in response to virus infection, for example, infection of cells with HCMV, Newcastle disease virus or Sendai virus all caused activation of IRF3 (Navarro *et al.*, 1998, Lin *et al.*, 1998, Yoneyama *et al.*, 1998). In addition, recent studies indicated that IRF3 is a phosphorylation target following engagement of certain TLR receptors. For example, LPS, via TLR4, was shown to trigger the nuclear translocation and DNA binding of IRF3, and subsequent induction of interferon regulatory genes (Navarro and David, 1999). Furthermore, poly(I:C) has been reported to induce IRF3 through TLR3, and activation of cells by poly(I:C) led to phosphorylation of Ser396 of IRF3 (Servant *et al.*, 2003). Recently, the IKK-related kinases IKK ϵ (also known as inducible IKK, IKKi) and TBK1 (TRAF-family-member-associated NF κ B activator (TANK)-binding kinase 1) have been implicated in the phosphorylation and activation of IRF3 (Fitzgerald *et al.*, 2003a, Sharma *et al.*, 2003).

1.4.4 TLR3-Mediated NF κ B and IRF3 Activation

It is now well established that TLR3 uses a MyD88-independent pathway to activate NF κ B and IRF3. Indeed, the TIR adaptor used by TLR3 to mediate these signals is TRIF (Reviewed in Section 1.6.3). TLR3-mediated NF κ B activation has also been reported to be IRAK1 and IRAK4-independent (Jiang *et al.*, 2002). Instead, TRIF interacts with TRAF6 via its TRAF6 binding sites which ultimately mediates downstream activation of NF κ B (Sato *et al.*, 2003). Recently, receptor interacting protein (RIP) 1 has also been implicated in TLR3/TRIF-mediated NF κ B activation (Myelan *et al.*, 2004). However, the exact mechanism of RIP1 activation and participation in the TLR3 pathway remains to be elucidated. Poly(I:C) can also induce the activation of IRF3 via the TLR3 pathway. TRIF can also interact with

TBK1, the kinase responsible for IRF3 activation (Fitzgerald *et al.*, 2003, Sharma *et al.*, 2003), and thus mediate IRF3 activation and subsequent production of type I IFNs and ISGs (Fig. 1.6). Activation of IRF3 by poly (I:C) can also requires the recruitment of PI3 kinase to TLR3. Activated TLR3 recruits PI3 kinase through its phosphotyrosine residues and activates the PI3 kinase-Akt pathway, which, by an unknown mechanism causes complete phosphorylation of IRF3 making it transcriptionally active (Sen *et al.*, 2005).

1.4.5 TLR3-Independent Signalling Pathways Mediated by dsRNA

PKR is the prototypic member of a family of dsRNA-binding proteins and this cytoplasmic serine/threonine kinase has been shown to mediate dsRNA-signalling in certain cell types in a TLR3-independent manner. Biochemical analyses have shown that the MAP kinase MKK6 is specifically regulated by PKR-mediated phosphorylation and this can activate the MAP kinase p38 (Silva *et al.*, 2004). Interestingly, PKR may play a role in NFκB activation. A recent study showed that PKR has TRAF binding motifs and facilitates in the activation of NFκB (Gil *et al.*, 2004). Another TLR3-independent but dsRNA-dependent mechanism of IRF3 activation involves the RNA helicase retinoic acid-inducible gene I (Yoneyama *et al.*, 2004). This cytoplasmic protein has a helicase domain in the carboxyl terminus, which recognises dsRNA. In its N-terminus is a caspase recruitment domain (CARD), which transmits downstream signals that result in the activation of NFκB and IRF3. The RIG-I pathway only responds to dsRNA that has been transfected into the cell or from viruses replicating inside the cell.

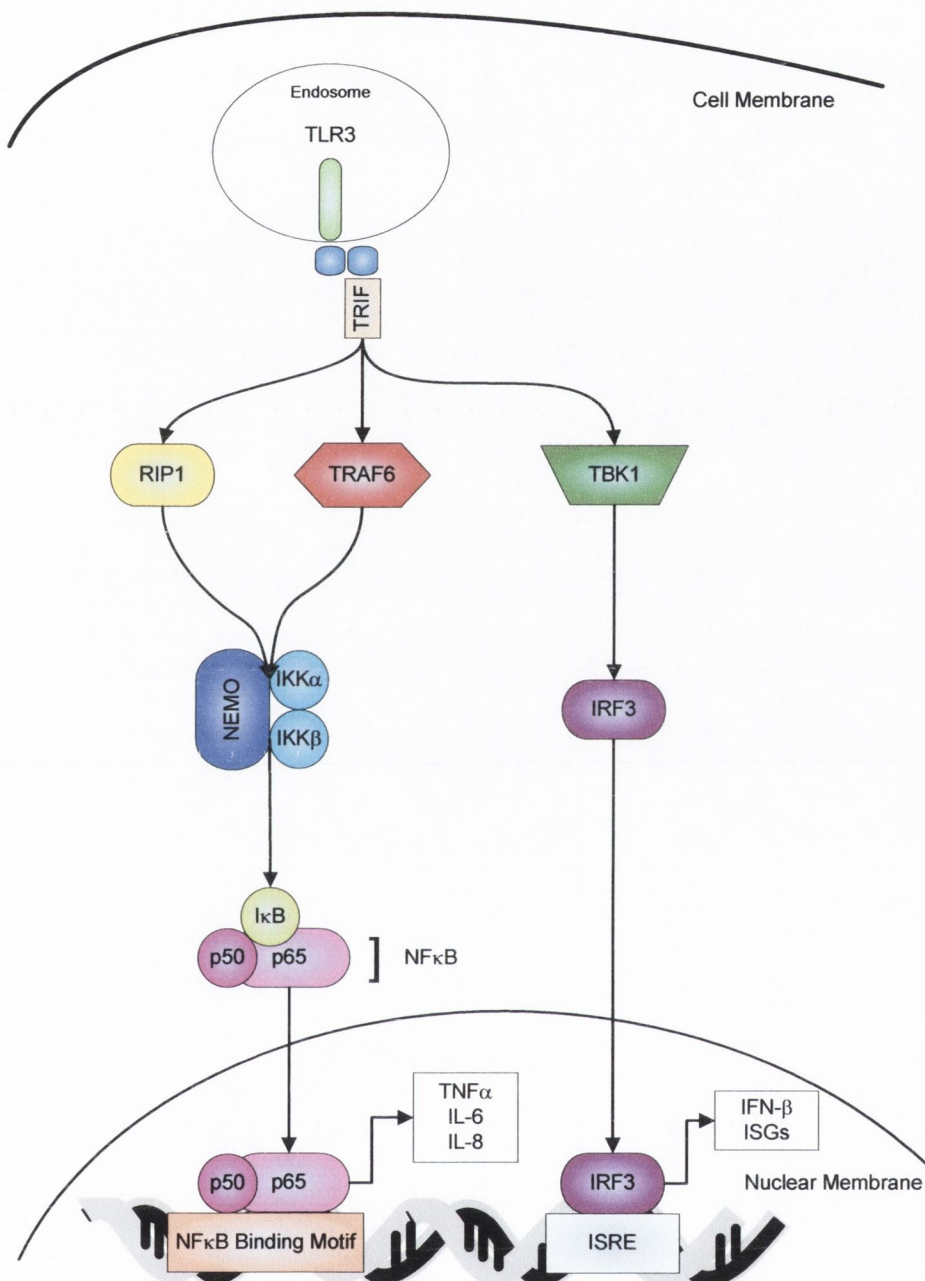


Fig. 1.6 TLR3 Signalling to NF κ B and IRF3

TLR3 mediates downstream signalling pathways through the TIR adaptor molecule TRIF. Association of either RIP1 or TRAF6 with TRIF results in NF κ B activation. TLR3-mediated activation of IRF3 and subsequent induction of IFN- β also requires TRIF. TRIF interacts with TBK1, the kinase involved in phosphorylation and activation of IRF3.

1.5 TIR Adaptors

1.5.1 MyD88

MyD88 was originally isolated as one of 12 different mRNA transcripts that were induced in M1 myeloblastic leukaemia cells upon activation with lung conditioned medium or recombinant IL-6. At that time, the MyD88 sequence did not show any homology to other available sequences in the databases and contained no recognisable protein motifs (Reviewed in Janssens and Beyaert 2002). In 1994, the C-terminal portion of MyD88 was found to be similar to a conserved stretch of ~ 200 amino acids in the intracellular regions of the *Drosophila* toll receptor and the mammalian IL-1 receptor (Hultmark, 1994). This stretch of sequence was thus referred to as the TIR domain (Slack *et al.*, 2000). The N-terminal of MyD88 encodes a death domain (DD), a motif of ~ 90 amino acids that was originally identified in apoptosis-promoting proteins. However, several DD containing proteins have no obvious apoptotic function and the DD seems to function as a general protein-protein interaction motif (Feinstein *et al.*, 1995) (Fig. 1.3).

Evidence that MyD88 was involved in IL-1R/TLR signalling was first demonstrated by overexpression studies. Overexpression of deletion mutants of MyD88 were shown to act as dominant negatives and inhibit IL-1 and TLR 4 induced activation of NF κ B and JNK (Wesche *et al.*, 1997, Medzhitov *et al.*, 1998, Burns *et al.*, 1998). Final proof for the essential function of MyD88 in IL-1R/TLR mediated induction of NF κ B and MAPKs was obtained after the generation of MyD88 deficient mice (Adachi *et al.*, 1998). It was subsequently shown that cells from these mice did not respond to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9 in terms of TNF and IL-12 production (Reviewed Janssens and Beyaert

2002). Intriguingly, in contrast to IL-1- or CpG-induced effects, which were completely abolished in MyD88 knockout mice (Adachi *et al.*, 1998, Schnare *et al.*, 2000), very little effects were observed with poly (I:C) treatment while some LPS responses were not abrogated (Kaisho *et al.*, 2001, Alexopoulou *et al.*, 2001). MyD88 knockout mice were highly resistant to LPS or poly (I:C)-induced shock and showed no increase in cytokine or nitric oxide production. However, NF κ B and JNK were still activated in MyD88 deficient cells, although the kinetics were delayed compared to wild-type controls (Kaisho *et al.*, 2001, Alexopoulou *et al.*, 2001). Furthermore, LPS and poly(I:C) still triggered dendritic cell maturation, as reflected by the upregulated expression of co-stimulatory molecules (e.g. CD80, CD86) (Kaisho *et al.*, 2001, Alexopoulou *et al.*, 2001). These results imply the existence of a MyD88-independent pathway that emanates from TLR3 and TLR4.

1.5.2 Mal (MyD88 Adaptor Like)

The first TIR adaptor molecule discovered that had sequence homology to MyD88 was designated Mal (MyD88 adaptor like) or TIRAP (TIR domain containing adaptor protein) (Fitzgerald *et al.*, 2001, Horng *et al.*, 2001). Mal unlike MyD88 does not have a DD, however initial studies identified a role for Mal in TLR4 signalling. The generation of Mal (TIRAP) deficient mice determined that Mal was also involved in TLR2 signalling (Horng *et al.*, 2002, Yamamoto *et al.*, 2002a). The TIR domains of Mal and MyD88 can form stable heterodimers, while docking studies revealed that Mal and MyD88 bind to different regions in TLR2 and TLR4 thus suggesting a cooperative role of the two adaptors in signalling (Dunne *et al.*, 2003). Interestingly, the production of inflammatory cytokines in response to LPS was defective in TIRAP knockout mice, but the expression of IFN-inducible genes

and the delayed activation of NF κ B were still observed (Fitzgerald *et al.*, 2001, Yamamoto *et al.*, 2002a). This phenotype was similar to that of MyD88 knockout mice, and it indicated that Mal is essential for the TLR4-mediated, MyD88-dependent signalling pathway, but not the MyD88-independent pathway. Therefore, this suggests the existence of another TIR adaptor that would be essential for LPS-induced activation of the IRF3 pathway leading to the production of IFNs, also known as the MyD88-independent pathway.

1.5.3 TRIF (TIR Domain Containing Adaptor Molecule Inducing IFN- β)

Two groups reported on a third TIR domain containing adaptor, called TRIF or TICAM-1 (Yamamoto *et al.*, 2002b, Oshiumi *et al.*, 2003a). One group found TRIF in a search for sequences with predicted TIR domains (Yamamoto *et al.*, 2002b) whereas the other identified it in a yeast two hybrid screen using TLR3 as bait (Oshiumi *et al.*, 2003a). TRIF does contain the conserved proline (P434) in box 2 of its TIR domain, although its box 1 appears to be quite divergent compared to other TIR domains (O'Neill *et al.*, 2003). *In vitro* studies demonstrated that TRIF could activate NF κ B and IFN- β in response to poly(I:C) via TLR3 (Yamamoto *et al.*, 2002b, Oshiumi *et al.*, 2003a). The generation of TRIF deficient mice and the identification of Lps2 mice (mutant mice in which the gene encoding *Trif* is disrupted) confirmed these results (Yamamoto *et al.*, 2003a, Hoebe *et al.*, 2003a). Macrophages from TRIF deficient mice showed impaired expression of IFN- β and IFN inducible genes in response to poly(I:C). In TRIF deficient mice, poly(I:C)-induced NF κ B activation was severely impaired, thus indicating that TRIF is essential for the TLR3 mediated signalling pathway (Yamamoto *et al.*, 2003a). TRIF deficient mice also identified a role for TRIF in TLR4 mediated

signalling. The mice displayed normal LPS-induced MyD88-dependent activation of NF κ B and MAP kinases, suggesting that TRIF is not involved in the LPS-induced activation of the MyD88-dependent pathway. However, IFN-inducible genes were completely abolished in TRIF/MyD88 double deficient cells (Yamamoto *et al.*, 2003a). These findings demonstrate that TRIF is essential for TLR4-mediated activation of the MyD88-independent signalling pathway.

Another study identified a germline mutation in mice called *Lps2*, which abolished cytokine responses to dsRNA and severely impaired responses to LPS. The *Lps2* mutation is a distal frameshift error in *Trif*. These studies implicate TRIF in both the TLR3 and TLR4 pathways proving that *Trif/Lps2* homozygotes are markedly resistant to the toxic effects of LPS and are hypersusceptible to mouse cytomegalovirus failing to produce type I interferons. In addition, an alternative MyD88-dependent “adaptor x” pathway was proposed due to the observation that some macrophages reveal a distinct *Trif*-independent cell population when stimulated with LPS (Hoebe *et al.*, 2003a). This “adaptor x” is now believed to be TRAM (TRIF related adaptor molecule) (Fitzgerald *et al.*, 2003) (See section 1.5.4).

The mechanism of TLR3-induced NF κ B and IRF3 activation is mediated by two distinct regions of TRIF. The N-terminal of TRIF harbours the TRAF6 binding motif (Pro-X-Glu-X-Aromatic/acidic residue) and associates with TRAF6 in yeast. This binding motif is required for activation of NF κ B (Sato *et al.*, 2003). Recently, investigation into the downstream signalling events that regulate TLR3-dependent TRIF-induced NF κ B activation revealed that RIP1 is recruited to TRIF and

interacts with TRIF via a RIP homotypic interaction motif in the C-terminal region of TRIF (Meylan *et al.*, 2004).

Two IKK-related proteins – IKK- ϵ and TBK1 have been demonstrated to activate IRF3 downstream of TRIF (Fitzgerald *et al.*, 2003a, Sharma *et al.*, 2003). Sharma *et al.*, 2003, showed that only cells expressing IKK ϵ or TBK1 (but not IKK α or IKK β) could phosphorylate IRF3. In addition, they showed using siRNA that blocking IKK ϵ or TBK1 prevented Sendai virus-induced IRF3 phosphorylation and subsequent activation of the IFN promoter. Another group showed a requirement for IKK ϵ and TBK1 in Sendai-virus- and poly(I:C)-induced IRF3 activation (Fitzgerald *et al.*, 2003). Recently, it has been shown that TRIF-induced IRF3 activation was prevented in 1 *Tbk1*^{-/-} fibroblasts (McWhirter *et al.*, 2004), furthermore, TBK1 can associate with a distinct region from TRAF6 in the N-terminal of TRIF (Sato *et al.*, 2003).

1.5.4 TRAM (TRIF Related Adaptor Molecule)

Searches of the human genome revealed additional sequences predicted to encode an additional two TIR domain containing adaptors. One group designated one of these molecules TIRP (TIR containing adaptor protein) and proposed a role for this protein in IL-1R signalling (Bin *et al.*, 2003). TIRP interacted with IL-1R, IL-1RAcP, kinase inactive IRAKs and TRAF6. Overexpression of TIRP activated NF κ B but not the IFN- β promoter. A dominant negative mutant of TIRP (TIRP 78-171) inhibited IL-1-induced but not TNF-induced NF κ B activation. This study also suggested that TIRP functions upstream of IRAK, IRAK2, TRAF6 and IKK β upon IL-1 induced NF κ B activation (Bin *et al.*, 2003).

Two other groups identified the same TIR adaptor. One group named this adaptor molecule TRAM (Fitzgerald *et al.*, 2003b) while the other study called it TICAM-2 (Oshiumi *et al.*, 2003b). TRAM/TICAM-2 is a small TIR domain containing protein, which like TRIF, induces both NF κ B and IRF3 activation when overexpressed. Dominant negative and siRNA studies demonstrated that TRAM functions downstream of TLR4. Further, it could also interact with TLR4 but not TLR3 (Fitzgerald *et al.*, 2003b, Oshiumi *et al.*, 2003b).

Analysis of the TRAM knockout mouse established that TRAM has an essential role in the MyD88-independent cascade of TLR4-induced signals (Yamamoto *et al.*, 2003b). The knockout studies demonstrated that TRAM was not involved in IL-1R signalling, contrary to other findings (Bin *et al.*, 2003). Macrophages from TRAM-deficient mice showed impaired LPS-induced production of pro inflammatory cytokines TNF- α and IL-6, compared to TLR2, TLR7 and TLR9 ligands. Interestingly, TLR4 but not TLR3 mediated IFN- β and IFN inducible gene expression was severely impaired. Furthermore, IRF3 activation was impaired in TRAM deficient mice for LPS in comparison to normal IRF3 activation exhibited by poly(I:C). Therefore, this study confirmed a role for TRAM in the MyD88-independent pathway leading to activation of IRF3 and IFN- β induction for TLR3 but not TLR4 (Yamamoto *et al.*, 2003b). TRAM acts as a bridging molecule between TLR4 and TRIF and therefore participates in the late phase NF κ B activation by TLR4 (Yamamoto *et al.*, 2003b, Oshiumi *et al.*, 2003b, Seya *et al.*, 2005).

1.5.5 SARM (Sterile- α and HEAT-Armadillo Motif)

A second putative new adaptor was described in 2001 as sterile- α and HEAT-Armadillo motifs containing protein (SARM), and was subsequently annotated as having a TIR domain. An alignment of the TIR domains of TLR4, MyD88, Mal, TRIF, TRAM and SARM clearly shows that box 1 is particularly conserved in SARM (O'Neill *et al.*, 2003). An orthologue of mammalian SARM, the *Caenorhabditis elegans* TIR-domain-containing protein (TIR1) has recently been shown to mediate the expression of genes that encode antimicrobial peptides (Couillault *et al.*, 2004). Another study has been demonstrated that in contrast to the other TIR adaptors, MyD88, Mal, TRIF and TRAM, SARM failed to drive the NF κ B activation in a reporter gene assay (Liberati *et al.*, 2004). In addition, overexpression of TRIF and TRAM activated the interferon stimulated response element (IRSE) reporter gene while overexpression of SARM failed to drive this reporter (Liberati *et al.*, 2004). Therefore, a specific role for mammalian SARM in TLR signalling remains to be elucidated.

1.5.6 Differential Use of TIR Adaptors

The identification and characterisation of the TIR adaptors has established roles for these adaptors in TLR signalling. As discussed earlier, MyD88 is essential for all TLR-mediated production of inflammatory cytokines, except for TLR3 which uses TRIF. Stimulation of TLR3 or TLR4 results in induction of type I IFNs in a MyD88-independent manner. This MyD88-independent response is entirely dependent on TRIF. In addition, Mal is a specific adaptor for TLR2 and TLR4-mediated activation of the MyD88-dependent pathway and TRAM is a specific adaptor in the TLR4-mediated, MyD88-independent/TRIF-dependent pathway.

Therefore, Mal and TRAM provide specificity for different TLR signalling pathways. For example, MyD88 and Mal are involved in the early phase NF κ B activation through TLR4 and TRIF and TRAM are required for late phase NF κ B activation. Examination of cells from MyD88^{-/-} mice revealed the existence of MyD88-dependent and MyD88-independent pathways, both of which mediate signalling in response to LPS. For example, the activation of NF κ B in response to mycoplasmal lipopeptide, a TLR2 agonist, is completely abolished in MyD88^{-/-} macrophages, whereas NF κ B activation still occurs in response to LPS, a TLR4 agonist, although with delayed kinetics. The specific TIR adaptors that mediate the delayed activation of NF κ B (also known as late phase NF κ B activation) are TRIF and TRAM (Sections 1.5.3 and 1.5.4). Both early phase NF κ B activation and late phase NF κ B activation lead to the production of inflammatory cytokines such as IL-6, IL-8 and TNF α . The cytoplasmic portion of TLR4 binds directly to TRAM and not TRIF as shown in *in vitro* studies. Hence TRAM might act as a bridging molecule between TLR4 and TRIF and therefore facilitate the robust production of type I IFNs by TLR4 (Oshiumi *et al.*, 2003b, Seya *et al.*, 2005) (Fig. 1.7).

1.6 IRAKs (Interleukin 1 Receptor Associated Kinases)

There are at least four members of human IRAK family that have been identified to date: (IRAK1, 2, M and 4) (Fig 1.8). However, the expression pattern of these members differs. IRAK1 and IRAK 4 are expressed in all tissues tested so far (Cao *et al.*, 1996, Li *et al.*, 2002). IRAK2 has a narrower cellular distribution, being found mainly in the spleen and thymus (Muzio *et al.*, 1997), and IRAKM expression is mainly restricted to monocytes and macrophages (Kobayashi *et al.*, 2002). IRAKs contain an N-terminal death domain (DD) and a central

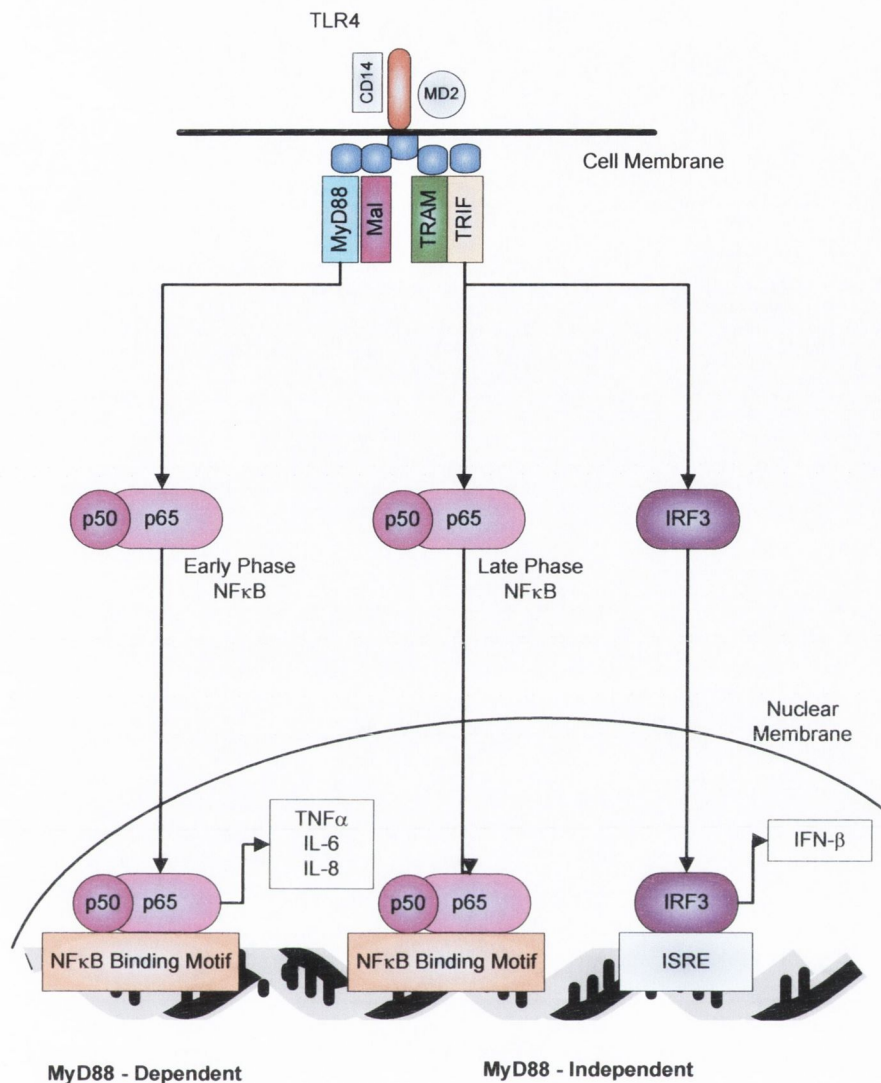


Fig. 1.7 TLR4 signalling: MyD88-dependent and -independent pathways

Stimulation of TLR4 facilitates the activation of two pathways: the MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the TIR adaptors MyD88 and Mal and facilitates the early phase of NFκB activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway requires the TIR adaptors TRIF and TRAM and activates IRF3 and involves the late phase of NFκB activation, both of which lead to the production of IFN-β and the expression of IFN-inducible genes.

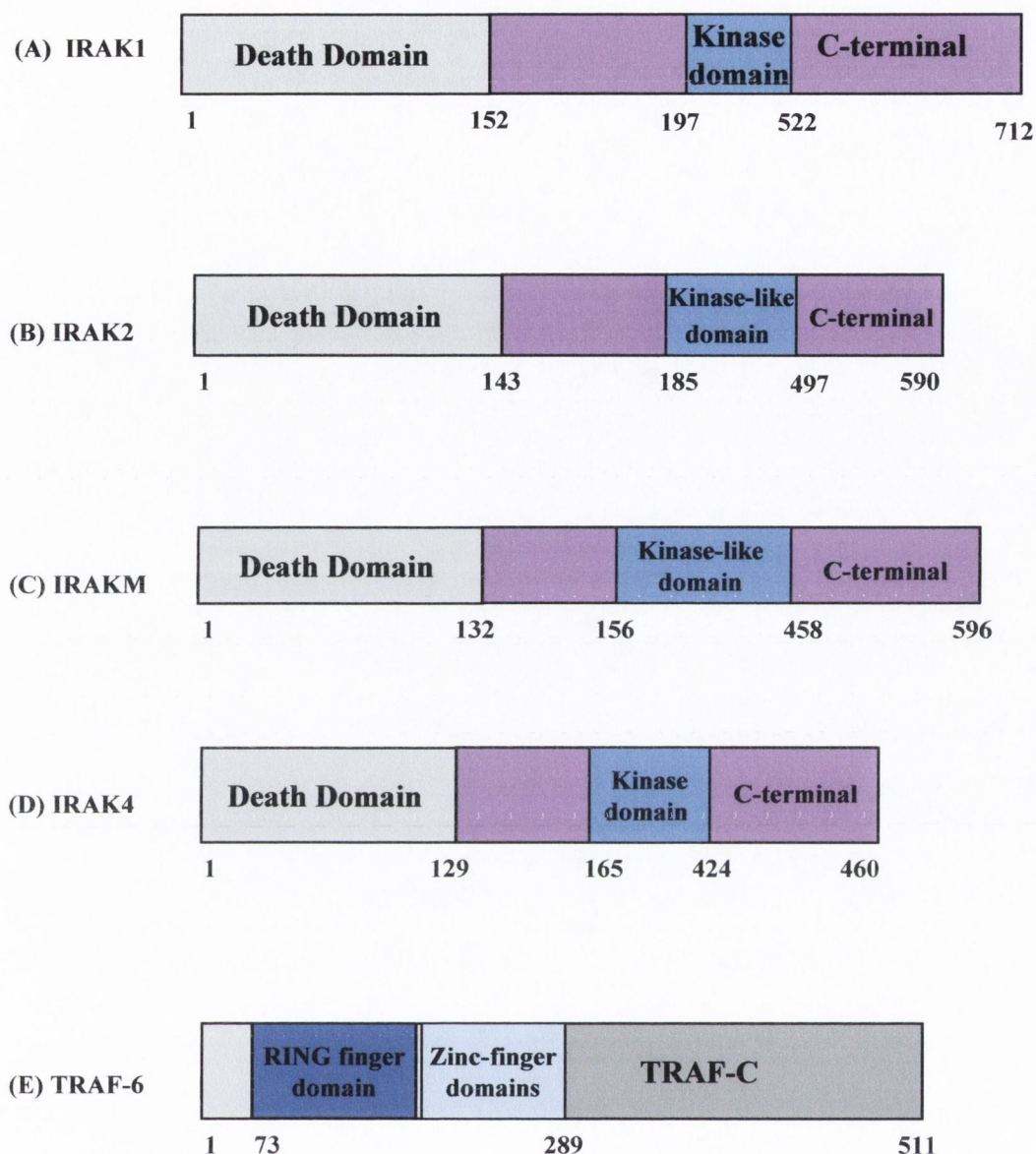


Fig. 1.8 Schematic representation of IRAK family members (IRAK1, 2, M and 4) and TRAF6. All the IRAK family members have a death domain and a C-terminal domain. IRAK1 and IRAK4 have a kinase domain whereas IRAK2 and IRAKM have a kinase-like domain. TRAF6 contains a TRAF-C domain, a RING finger and a zinc-finger domain.

serine/threonine-kinase domain or kinase-like domain. IRAK1 and IRAK4 have intrinsic kinase activity, whereas IRAK2 and IRAKM have no detectable kinase activity (Reviewed in Janssens and Beyaert 2003).

The kinase activity of IRAK1 increases strongly following IL-1/TLR stimulation, and its kinase domain is essential for mediating NF κ B activation. Upon stimulation, IRAK1 is recruited to the receptor through a homophilic interaction with the DD of MyD88 (Cao *et al.*, 1996). MyD88 also binds to IRAK4 and thereby facilitates IRAK4 phosphorylation of critical residues in the kinase activation loop of IRAK1, triggering IRAK1's own kinase activity. Once activated IRAK1 autophosphorylates residues in its NH₂ terminus (Li *et al.*, 2002).

IRAK1 has recently been shown to be essential for TLR7 and TLR9-mediated IRF7 activation (Uematsu *et al.*, 2005). The study demonstrated IRAK1 directly interacted with IRF7 and phosphorylated IRF7 in vitro. Furthermore, the kinase activity of IRAK1 was shown to be necessary for transcriptional activation of IRF7. IRAK1 has also been shown to be critically involved in LPS-induced IL-10 production (Dean *et al.*, 2004).

Tollip is another protein that has been shown to have a role in recruiting IRAK1 to the IL-1R complex (Burns *et al.*, 2000) and it may also have a similar role in TLR2 and TLR4 signalling (Bulut *et al.*, 2001). Both IRAK1 and IRAK4 associate with TRAF6 in the cytoplasm to initiate downstream signals (Medzhitov *et al.*, 1998, Wesche *et al.*, 1997, 1999, Li *et al.*, 2002).

IRAK1 activity might also be regulated by a newly described family of molecules called pellinos. Pellino was originally identified in a yeast-two-hybrid screening with *Drosophila pelle* as bait (Grosshans *et al.*, 1999). The human Pellino family consists of at least three different members (Rich *et al.*, 2000, Resch *et al.*, 2001, Jensen and Whitehead 2003a). Both Pellino1 and Pellino2 have been described as interacting with IRAK1 in an IL-1-dependent manner and downregulation of their expression levels interferes with IL-1- and LPS-induced NF κ B activation (Jiang *et al.*, 2003b, Yu *et al.*, 2002). Pellino3 has been described as a scaffolding protein and has been shown to interact with IRAK1, TRAF6 and TAK1 (Jensen and Whitehead 2003b). In contrast to Pellino1 and Pellino2 which activate NF κ B, Pellino3 has been shown to activate the MAP kinases, ERK and JNK while having not effect on NF κ B activation in a reporter assay system (Jensen and Whitehead 2003b).

IRAK1 deficiency has a relatively mild effect on IL-1R and TLR signalling. IL-1 induced cytokine responses, NF κ B and MAP kinase activation are all reduced but not abolished in IRAK1 deficient cells (Kanakaraj *et al.*, 1998, Thomas *et al.*, 1999). Similar partial defects in IL-18 and LPS signalling are also observed in mice lacking IRAK1 (Thomas *et al.*, 1999, Kanakaraj *et al.*, 1999, Swantek *et al.*, 2000). These results suggest that IRAK1 is required for optimal TLR signalling but that there is considerable redundancy of IRAK function. Consistent with this, the IRAK family members, IRAK2 and IRAK4 could both genetically replace IRAK1 in a mutated HEK293 cell line (Wesche *et al.*, 1999). Dominant negative versions of IRAK2 have been reported to inhibit IL-1-induced and IL-1 receptor accessory protein –induced NF κ B activation (Muzio *et al.*, 1997). In addition, dominant

negative IRAK2 constructs have been shown to block TRAF6-mediated NF κ B activation, implicating IRAK2 upstream of TRAF6 in the signalling pathway (Muzio *et al.*, 1997). Indeed recently, direct evidence for binding of IRAK2 to both MyD88 and TRAF6 has been reported (Boch *et al.*, 2003). IRAK2 has also been shown to interact with Mal in co-immunoprecipitation studies (Fitzgerald *et al.*, 2001).

Recently, four isoforms of murine Irak2 (designated Irak2a-Irak2d) were cloned and have been shown to differentially regulate TLR signalling. These isoforms were generated by alternative splicing at the 5' end of the gene, however, no evidence of similar splicing was found in the human IRAK2 gene. When overexpressed, Irak2a and Irak2b potentiate activation by LPS whereas Irak2c and Irak2d were inhibitory (Hardy and O'Neill 2004). So far, there is no evidence that hIRAK2 has any inhibitory role.

IRAKM, however, might have a role in negatively regulating TLR signalling. Mice and cells lacking IRAKM have enhanced responses to bacterial challenges and TLR stimulation. Furthermore biochemical analysis revealed that IRAKM prevents the dissociation of IRAK1/IRAK4 from MyD88 thereby inhibiting the formation of the IRAK1/TRAF6 interaction (Kobayashi *et al.*, 2002).

IRAK4 deficient mice have also been generated and these showed almost complete unresponsiveness to IL-1, LPS and other bacterial components, demonstrating that IRAK4 is a key player in the IL-1/TLR signalling cascades (Suzuki *et al.*, 2002). Furthermore, two studies have revealed patients with inherited IRAK 4 deficiency

(Picard *et al.*, 2003, Medvedev *et al.*, 2003). In one study the cells from these patients failed to respond to IL-1, IL-18 or stimulation of at least five TLRs (TLR2, 3, 4, 5, 9) (Picard *et al.*, 2003). In contrast the other investigation revealed a failure to respond only to IL-1, IL-18 and LPS. Interestingly the patient had a normal response to viral infections (Medvedev *et al.*, 2003).

1.7 TRAF6

TRAFs, the tumour necrosis factor (TNF) receptor associated factors, constitute a family of evolutionally conserved adaptor proteins. To date, seven members of the family have been identified in mammals (Chung *et al.*, 2002, Xu *et al.*, 2004). The TRAF proteins are characterised by the presence of a TRAF domain at the C-terminus, which consists of a coiled-coil domain followed by a conserved TRAF-C domain. The TRAF domain plays an important role in TRAF function by mediating self-association and upstream interactions with receptors and other signalling proteins. The N-terminal portion of most TRAF proteins contains a RING finger and several zinc finger motifs, which are important for downstream signalling events (Chung *et al.*, 2002).

TRAF6 was independently cloned by a search against a DNA database for TRAF2-like sequences followed by cDNA library screening (Cao *et al.*, 1996) and by a yeast two-hybrid screen using CD40 as bait (Ishida *et al.*, 1996). Unlike other TRAFs, which only mediate signalling from the TNF receptor superfamily, TRAF6 also participates in the signal transduction of the IL-1R/TLR superfamily. One study reported no role for TRAF6 in TLR3 signalling (Gohda *et al.*, 2004). TRAF6 has no role in TNF signalling but it directly interacts with CD40 and RANK

(receptor activator of NF κ B also known as TRANCE-R), which are members of the TNF receptor superfamily (Ye *et al.*, 2002). The importance of TRAF6 in signal transduction outside the TNF receptor superfamily was first shown by its participation in IL-1 signalling (Cao *et al.*, 1996) and subsequently in TLR signalling (Medzhitov *et al.*, 1998). Interestingly, TRAF6 exhibits close homology to dTRAF2, which has been implicated as the intracellular adaptor for the *Drosophila* Toll receptor involved in anti-microbial responses and in dorsal-ventral patterning (Shen *et al.*, 2001). Evolutionary analysis showed that TRAF6 is one of the most divergent mammalian TRAFs in both sequence homology in the TRAF-C domain and in its gene structure (Chung *et al.*, 2002).

The unique biological function of TRAF6 is largely a product of its distinct specificity for upstream receptors and signalling proteins, which is determined by its unique TRAF-C domain. For example, TNF receptor family members CD40 and RANK associate with TRAFs 1-3, 5 and 6 via their cytoplasmic tails. While TRAF 1-3 and 5 share common binding sites on CD40 and RANK the binding sites of TRAF6 are distinct. Structural studies of TRAF6 in complex with CD40 and RANK peptides revealed striking differences between receptor recognition by TRAF6 and TRAF2 (Ye *et al.*, 2002). Peptides based on the sequences of the upstream receptor that bind TRAF6 were generated. When these peptides bound on the surface of TRAF6 they showed a 40° difference in the peptide directions relative to TRAF2-binding peptides. Structure-based sequence alignment suggested that TRAF6 recognises a conserved Pro-X-Glu-X-X-(aromatic/acidic residue) motif. Further sequence inspection revealed that IRAK1 (Cao *et al.*, 1996), IRAK2 (Muzio *et al.*, 1997), IRAKM (Wesche *et al.*, 1999), TRIF (Sato *et*

al., 2003) and Mal (Mansell *et al.*, 2004) all contain at least one TRAF6-binding motif, providing a structural basis for the participation of TRAF6 in IL-1/TLR signalling.

TRAF6 participates in the activation of the transcription factors NF κ B and AP-1 through interaction and subsequent activation of the MAP3 kinase known as TAK1 (Wang *et al.*, 2001). TAK1 also interacts with two other proteins known as TAB 1 and TAB2 (Wang *et al.*, 2001). When ectopically co-expressed, TAB1 augments the kinase activity of TAK1, indicating that TAB1 functions as an activator of TAK1. TAB2 associates with the cell membrane in unstimulated cells, but upon stimulation translocates to the cytosol, where it functions as an adaptor linking TAK1 and TRAF6 (Deng *et al.*, 2000). A ubiquitination step involving a dimeric ubiquitin conjugating enzyme complex known as TRKA2 interacts with TRAF6 and catalyses the formation of a Lys63 linked polyubiquitin chain, which triggers TAK1 activation through a unique proteasome independent mechanism (Wang *et al.*, 2001). Recently, the coiled-coil domain of TRAF6 has been shown to be essential for its auto-ubiquitination and in the activation of NF κ B (Yang *et al.*, 2004).

The unique and diverse molecular characteristics of TRAF6 are reflected in the physiological functions of TRAF6. For instance, the RANK signal regulating osteoclast development and function is dependent on TRAF6. Mice lacking RANK exhibit osteopetrosis (increased bone mass) due to defective development and function of osteoclasts (Dougall *et al.*, 1999). Among TRAF knockout mice (TRAF1-6), only TRAF6 knockout mice showed osteopetrosis even though other

TRAFs, including TRAFs 1-3 and 5, can also associate with RANK. These findings imply that TRAF6 is essential in regulating osteoclast development and function (Lomaga *et al.*, 1999).

TRAF6 is also a critical factor involved in dendritic cell maturation and development. Indeed, dendritic cells from TRAF6 knockout mice exhibited a severe cytokine production defect (IL-6 and IL-12) compared to wild-type controls. Administration of LPS or anti-CD40 antibody *in vivo* induced DC maturation in wild-type mice but not in TRAF6 knockout mice. In addition, LPS treatment of TRAF6 deficient DCs failed to augment their T cell stimulatory capacity (Kobayashi *et al.*, 2003, Koyabashi *et al.*, 2004). These results indicate that TRAF6 regulates the critical processes required for DC maturation, activation and development, the primary cellular bridge linking innate and adaptive immunity.

1.8 TLRs and Viruses

1.8.1 Interferons

The discovery and elucidation of TLR-mediated signalling pathways leading to the production of type I IFNs is crucial in the understanding of exactly how viral infection leads to the IFN response. As mentioned, strong evidence for a role for both TLR3 and TLR4 in the anti-viral response was shown by Doyle *et al.*, (2002). This study found that, like TLR4, TLR3 activation leads to the activation of the anti-viral transcription factor IRF3. Agonists that activate TLR2 or TLR9 did not activate IRF3. In addition, microarray experiments showed that activation of TLR3 and TLR4 led to induction of a subset of genes that were not induced by TLR2 or TLR9 activation (Doyle *et al.*, 2002). Furthermore, pre-treatment of cells with

poly(I:C) or lipid A, the moiety of LPS recognised by TLR4, inhibited the replication of murine herpesvirus in macrophages (Doyle *et al.*, 2002).

IFNs are a family of cytokines that were discovered by their ability to render cells resistant to viral infection. IFNs are classified into two types based on amino acid sequence, recognition of specific receptors and, to a lesser extent, the producing cell. Type I IFNs consist of 14 IFN- α subtypes, a single IFN- β , IFN- ω , IFN- τ , IFN- κ and limitin, which act through receptor subunits designated IFNAR1 and IFNAR2. Type II IFN, IFN- γ , has no amino acid similarity to type I IFNs and acts through distinct receptor subunits IFNGR1 and IFNGR2 (Reviewed Hertzog *et al.*, 2003).

IFN- β can be produced by several cells of the innate immune system, including macrophages and DCs, induced by activation of several TLRs that are present on these cells. This activation of IFN- β is mediated through the anti-viral transcription factor IRF3 (Section 1.4.3). The TIR adaptor TRIF has been shown to induce activation of IRF3 by TLR3, 4 (Fitzgerald *et al.*, 2003, Yamamoto *et al.*, 2003). Recently, TLR7, 8 and 9 have been implicated in activating IRF5 and IRF7 through a MyD88-dependent pathway (Schoenomeyer *et al.*, 2005, Takaoka *et al.*, 2005).

1.8.2 TLR4 and Viruses

Respiratory syncytial virus (RSV), a paramyxovirus that infects the upper respiratory tract and causes an acute infection in children, was the first virus shown to activate cells via TLRs. Incubation of human and mouse peripheral blood

mononuclear cells with the viral attachment F protein elicited IL-6, IL-8 and TNF- α secretion at levels comparable to that seen after LPS stimulation. F protein induced cytokine expression was dependent on both CD14 and TLR4, since mice deficient in either protein failed to produce IL-6 (Haynes *et al.*, 2001).

TLR 4 has also been shown to be important for murine retrovirus activation of B cells. Mouse mammary tumour virus (MMTV) was unable to activate B cells from C3H/HeJ mice (Rassa *et al.*, 2002). Additionally, the envelope proteins from both MMTV and Moloney murine leukaemia virus could be co-immunoprecipitate with TLR4. It is still unclear whether MMTV exploits TLR4 to achieve higher virus infection and increased pathogenicity, or whether TLR4 mediated host defence helps the pathogenesis of MMTV (Otten *et al.*, 2002).

1.8.3 TLR2 and Viruses

MMTV can also utilise TLR 2 to activate cells. A recent review by Rassa *et al.*, (2003) highlights the fact that MMTV can activate B and DCs from C57BL/10ScN mice. These mice have a genomic deletion of the *Tlr4* gene and thus have no TLR4 receptor protein, rather than the dominant negative molecule produced in C3H/HeJ mice. However, MMTV failed to activate B cells or DCs from doubly null animals that were generated by crossing C57BL/10ScN with TLR2 knockout mice. This data indicates that MMTV also utilises the TLR2 pathway. Furthermore, the MMTV Env protein co-immunoprecipitates with TLR2 (Rassa *et al.*, 2003). Measles virus (MV) is another virus that may use TLR activation to create a cellular environment more permissive to infection. MV hemagglutinin protein (H) activates cells through either human or murine TLR2 (Bieback *et al.*, 2002).

Recent studies indicate that human cytomegalovirus (hCMV) may also activate TLR2, as UV-inactivated hCMV particles induced a dose-dependent production of IL-6 and IL-8 in wild type, but not TLR2 deficient macrophages (Compton *et al.*, 2003).

1.8.4 TLR3 and Viruses

dsRNA is a molecular pattern produced by most viruses, and as such can be considered a viral PAMP. TLR3 has been shown to sensitise cells to poly (I:C), a synthetic dsRNA analogue, in terms of NF κ B activation and induction of type I interferons (Alexopoulou *et al.*, 2001). Further, TLR3^{-/-} mice were significantly resistant to poly(I:C)-induced shock compared to wild-type controls (Alexopoulou *et al.*, 2001). Confirmation of a role for TLR3 in the anti-viral response was shown by Tabeta *et al.*, 2004. Using TLR3^{-/-} mice this study demonstrated that TLR3 contributes to murine CMV-induced type I IFN production, and to overall protection against virus (Tabeta *et al.*, 2004).

Since this first publication confirming a role for TLR3 in the anti-viral response many more reports have shown that viruses signal through TLR3. Indeed, it has been demonstrated that TLR3 is involved in the immune response to Influenza A virus (Guillot *et al.*, 2005). Another study showed that viral replication of RSV could evoke elevated production of the chemokines IL-8 and RANTES in a TLR3-dependent manner (Rudd *et al.*, 2005). Surprisingly, one study showed that TLR3 plays a role in West Nile virus entry into the brain (Wang *et al.*, 2004). Using TLR3^{-/-} mice this study reported that these mice were more resistant to lethal WNV infection and had impaired cytokine production (IL-6, TNF α , IFN α and IFN β)

compared to wild-type controls (Wang *et al.*, 2004). However, some negative stranded RNA viruses do not recognise TLR3. Lopez *et al.*, (2004) demonstrated that TLR3 is not essential for the induction of costimulatory molecule expression or secretion of cytokines by mature DCs in vitro or in vivo infection by Sendai virus (Lopez *et al.*, 2004).

Recently, TLR3 has been implicated in cross priming of DCs (Schulz *et al.*, 2005). Cross-priming is a mechanism by which extracellular proteins are taken up by DCs and can give rise to peptides presented by MHC class I molecules. In this way, viruses that are unable to infect DCs are still able to stimulate effective immune responses (Janeway *et al.*, 2005). Schulz *et al.*, (2005) investigated the effect of immunisation of cells with encephalomyocarditis virus (EMCV) or Semliki Forest Virus (SFV), both of which have only limited capacity to infect DCs, or cells containing synthetic dsRNA on cross-priming of DCs. This report revealed a striking increase in cross-priming following immunisation with virus-infected cells or poly(I:C), which was largely dependent on TLR3 expression by the DCs (Schulz *et al.*, 2005).

Interestingly, cells such as fibroblasts express TLR3 on the cell surface; however, in dendritic cells (Matsumoto *et al.*, 2003) or human alveolar and bronchial epithelial cells (Guillot *et al.*, 2005), TLR3 expression is restricted to intracellular compartments. An intracellular location is consistent with a role in responding to viral nucleic acid, which would be expected to be a lot more abundant intracellularly during viral infection. In addition, dendritic cells can phagocytose

and therefore can take up dsRNA. Hence, the intracellular location of TLR in DCs facilitates a robust response to the dsRNA uptake by these cells.

Further experimental evidence has revealed that TRIF is the sole TIR adaptor used by TLR3 to mediate downstream signalling events (Yamamoto *et al.*, 2002b, Oshiumi *et al.*, 2002a) and TRIF has been shown to be required for TLR3-induced IRF3 activation (Fitzgerald *et al.*, 2003a, Yamamoto *et al.*, 2003a). Thus there may be a central role for TRIF in the anti-viral response. Indeed, this hypothesis was strengthened by the observations in mice with a mutation in the *Trif* gene known as *Lps2*. These mice were shown to be hypersusceptible to mouse cytomegalovirus, failing to produce type I interferons when infected (Hoebe *et al.*, 2003a). In addition, macrophages from *Lps2* mice supported replication of Vaccinia virus (VV) to a significantly higher titre than wild-type controls (Hoebe *et al.*, 2003a).

Recently, a viral immune strategy specifically targeting the TLR3 pathway has been identified. The hepatitis C virus protein NS3/4, a serine protease, has been shown to cleave TRIF and thus inhibits TLR3-induced signalling (Li *et al.*, 2005).

1.8.5 TLR7/8 and Viruses

TLR7/8 also have a role in responding to viral infection. Two imidazoquinoline compounds termed imiquimod and resiquimod (R-848) that are known to have potent anti-viral properties were shown to activate macrophages. An analysis of these compounds in cells from various knockout mice showed that their ability to activate signalling pathways depended on TLR7 (Hemmi *et al.*, 2002). It was subsequently shown that human TLR8 could also confer cells with responsiveness

to R-848 (Jurk *et al.*, 2002). Recently, the PAMP for TLR7 and TLR8 was identified as ssRNA. Influenza A virus, vesicular stomatitis virus (VSV) and human immunodeficiency virus type 1 (HIV-1) have been implicated as being detected by TLR7 (Diebold *et al.*, 2004, Heil *et al.*, 2004, Lund *et al.* 2004). Purified influenza virus genomic RNA elicits IFN- α from wild-type but not TLR7^{-/-} mouse dendritic cells (Diebold *et al.*, 2004). GU-rich synthetic oligonucleotides derived from HIV-1 RNA have been shown to stimulate costimulatory molecule expression as well as inflammatory cytokine and IFN- α secretion in a TLR7-dependent manner (Heil *et al.*, 2004).

1.8.6 TLR9 and Viruses

TLR9 has now been added to the repertoire of TLRs that have a role in responding to viral infections. Herpes simplex virus type 1 (HSV-1), HSV-2 and murine cytomegalovirus (MCMV), all of which contain genomes rich in CpG DNA motifs have been shown to activate inflammatory cytokine and IFN- α secretion via TLR9 (Krug *et al.*, 2004, Lund *et al.*, 2004, Tabeta *et al.*, 2004). Intriguingly, TLR9-mediated IFN- α secretion in response to HSV-1, HSV-2 and MCMV occurs in a MyD88-dependent manner. However, one group has recently shown that the transcription factor IRF5 is involved in downstream induction of pro-inflammatory cytokines, such as IL-6, IL-12 and TNF α in response to the TLR9 agonist CpG (Takaoka *et al.*, 2005).

1.8.7 Inhibition of IL-1R/TLR Function by Vaccinia virus

In addition to the NS3/4A protein from Hep C virus that has recently been shown to cleave TRIF and inhibit NF κ B and IRF3 activation by TLR3 other viral inhibitors

of the IL-1/TLR signalling pathways have been described. These inhibitors are encoded by Vaccinia virus (VV), a member of the poxvirus family.

1.9 Poxviruses

Poxviruses have played an amazing role in the development of virology, immunology and vaccinology. Despite VV being the only vaccine to have eradicated a disease (smallpox), its origin and natural host remain unknown. In 1796 Edward Jenner used a virus (probably cowpox virus) taken from the hand of a milkmaid, who had acquired the infection from a cow, to introduce vaccination to the world. In 1939 in Liverpool, England, Alan Downie showed that the smallpox vaccines being used in the 20th century were distinct from cowpox virus and these became known as vaccinia virus (after vacca, latin for cow) (Moss, 1996).

The poxviridae comprise a large family of complex DNA viruses that replicate in the cytoplasm of vertebrate and invertebrate cells. The most notorious member, variola virus is the causative agent of smallpox and consequently had a profound effect on human history. Smallpox was finally eradicated in 1977, nearly two centuries after the introduction of prophylactic inoculations with VV (Moss 1996).

The general properties of poxviridae include:

1. the production of large (approximately 300 nm) complex virions containing enzymes involved in mRNA synthesis
2. a genome composed of a single linear dsDNA molecule of 130-300 kilo base pairs with a hairpin loop at each end
3. a cytoplasmic site of replication

Table 1.2 Poxvirus Genomes

Genome

Chordopoxvirus

Vaccinia virus (Copenhagen)
Vaccinia virus (modified vaccinia virus Ankara)
Vaccinia virus (Tian Tian)
Variola major virus (Bangladesh-1975)
Variola major virus (India-1967)
Variola major virus (Garcia-1996)
Fowlpox virus (virulent challenge virus)
Molluscum contagiosum virus (subtype)
Myxoma virus (Lausanne)
Shope rabbit fibroma virus (Kasza)
Lumpy skin disease virus (Neethling 2490)
Monkeypox virus (Zaire)
Yaba like disease virus (Smith)
Swinepox virus (Brighton Red)
Camelpox virus (Kazakhstan M-96)
Ectromelia virus (Moscow)
Rabbitpox virus (Utrecht)
Sheeppox virus (Turkey; TU-V02127)

Entomopoxviruses

Melanoplus sanguinipes (Tucson)
Amsacta moorci (Moyer)

Taken from Upton *et al.*, 2002

The family is divided into two subfamilies: Chordopoxvirinae and Entomopoxvirinae, based on vertebrate and insect host range respectively. 21 poxvirus genomes have been completed to date (Table 1.2). Vaccinia virus belongs to the orthopoxvirus genera in the Chordopoxvirinae subfamily. The members of a genus are genetically and antigenically related and have similar morphology. Indeed, Variola, Coxsack and Vaccinia all belong to the same genus. (Pastoret and Vanderplasschen 2003).

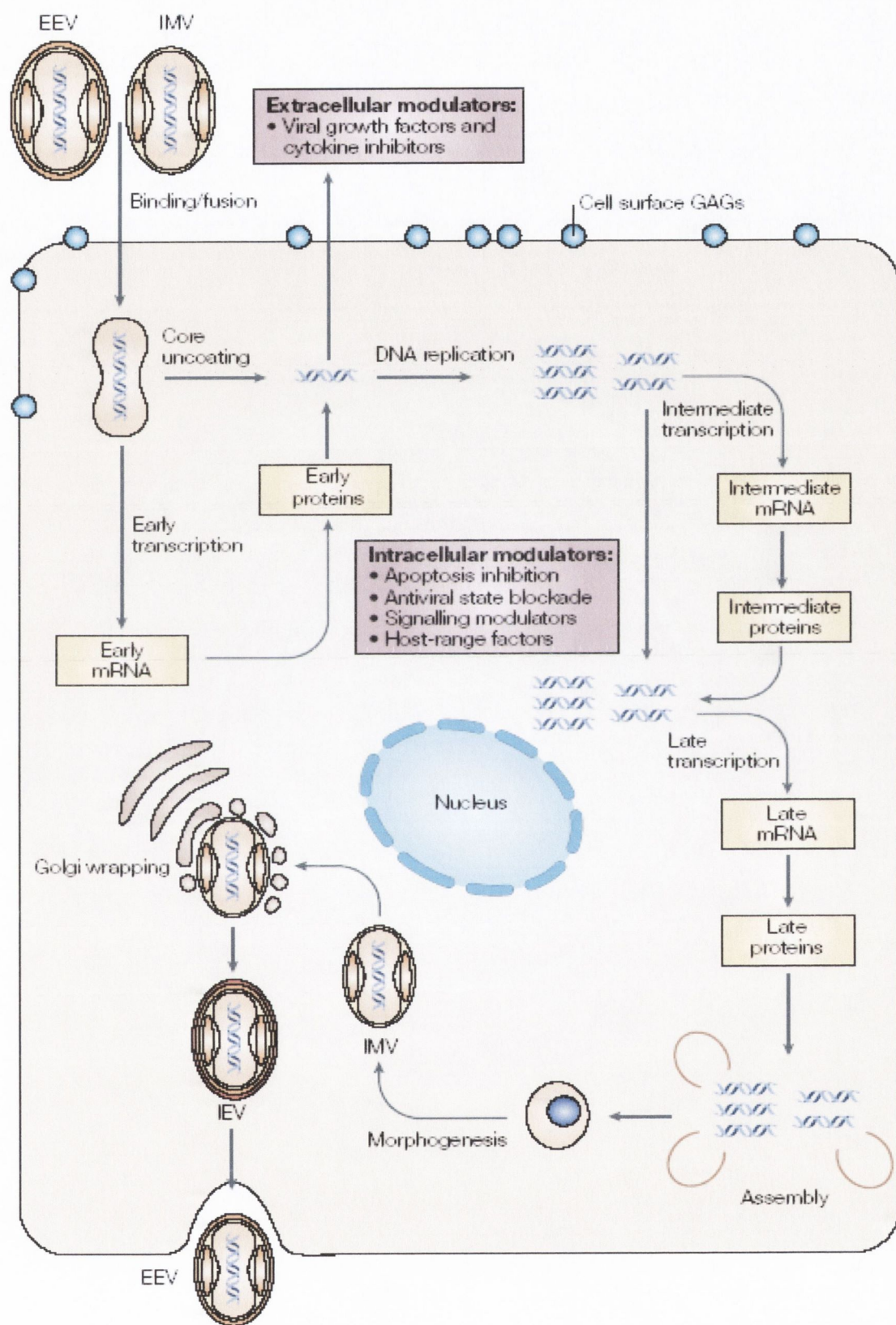
1.9.1 Poxvirus Replication

Vaccinia virus and other poxviruses replicate in the cell cytoplasm and consequently encode many of their own enzymes for transcription and DNA replication. The genome of the VV strain Copenhagen has been sequenced and contains approximately 200 closely packed genes. These are expressed in a strictly regulated cascade that culminates in the formation of new virus particles.

As mentioned, all poxviruses replicate in the cytoplasm of infected cells by a complex, but largely conserved, morphogenic pathway. Two infectious forms of virus are made: the majority of infectious progeny remain with the infected cell until cell lysis and are intracellular mature virus (IMV); the other form of virus is released from the infected cell late during infection and is called extracellular enveloped virus (EEV) (Moss 2001). The IMV and EEV virions differ in their surface glycoproteins and in the number of wrapping membranes (Locker et al., 2000). The binding of the virion is determined by several virion proteins and by glycosaminoglycans on the surface of the target cell or by components of the extracellular matrix. Fully permissive viral replication is characterised by three

Fig. 1.9 Poxvirus Replication

Virions attach to cells, enter and release the virus into the cytoplasm. The cores synthesis early mRNAs that are translated into a variety of proteins including enzymes and factors for DNA replication and intermediate transcription as well as immune defense molecules and growth factors. Uncoating of the core releases the DNA that is replicated to form concatemeric molecules. Intermediate genes are transcribed from the replicated DNA and some encode transcription factors. The late genes are transcribed and encode structural proteins, enzymes and early transcription factors. Assembly begins with the formation of discrete membrane structures. The concatemeric DNA intermediates are resolved into individual genomes and packaged in immature virions. Modified glogi membranes and early endosomes are used to wrap the virions to form IEV, which are transported to the periphery of the cell (Taken from McFadden, 2005).



waves of viral mRNA and protein synthesis (known as early, intermediate and late), which are followed by morphogenesis of infectious particles. The initial IMV is transported via microtubules and is wrapped with golgi-derived membrane, after which it is referred to as an intracellular enveloped virus (IEV). The IEV fuses to the cell surface membrane to form cell-associated enveloped virus, which is either extruded away from the cell by actin-tail polymerisation or is released to form free EEV. EVV might also form by direct budding of IMV, therefore by passing the IEV form (Fig. 1.9) (Reviewed in McFadden, 2005).

1.9.2 VV Immune Evasion

Upon poxvirus infection a complex response is mounted by the immune system using components of the innate immune system such as, complement, interferon, natural killer cells and an inflammatory response. In addition, the adaptive immune system produces antibodies directed against the virus. In response to this mounted attack the viruses over time have developed different mechanisms to evade host immunity. A diverse array of pathways and molecular targets are used by the virus to evade immune detection and destruction. Therefore, the VV genome contains numerous genes encoding proteins involved in immunomodulation and immunoevasion (Tortorella *et al.*, 2000).

Vaccinia virus encodes soluble proteins that are secreted from infected cells and function as soluble interferon gamma (IFN γ) receptors which block IFN γ activity. The virus also encodes proteins that act as decoy receptors for IL-1, IL-18 and TNF. The VV encoded gene B15R encodes an abundant, secretory glycoprotein that functions as a soluble IL-1 β receptor. Intranasal infection of mice with a VV

B15R deletion mutant accelerated the onset of symptoms and increased mortality (Reviewed in Bugert and Darai 1999). A Vaccinia virus encoded secreted protein structurally related to complement control proteins was one of the first soluble microbial proteins postulated to have an active role in the immunomodulation of the host defence (Jha. and Kotwal 2003).

The products of two other Vaccinia virus genes, K3L and E3L, are active at the intracellular level affecting IFN pathways by causing interference with the activity of the double stranded RNA dependent protein kinase PKR (Davies et al., 1992, Davies et al., 1993). However, they cause inhibition by acting in two different ways. Binding of IFNs to cellular membrane receptors induces the expression of several enzymes, including PKR and 2'5'-oligoadenylate (oligoA) synthetase, which are both then activated by dsRNA produced during virus infection. Activated (i.e. autophosphorylated) PKR phosphorylates and thereby inactivates the eukaryotic translation initiation factor 2 α (eIF2 α), blocking initiation of protein synthesis. The activated 2', 5'-oligoA synthetase catalyses the formation of oligoA molecules of various length which in turn activate a latent RNase (RNase L) that prevents protein synthesis by cleavage of RNAs. By binding to dsRNA, E3L inhibits the activation of both IFN-induced enzymes, arresting the translational block. The K3L is a mimic of eIF2 α and competes with eIF2 α for its interaction with PKR, reducing the level of phosphorylated eIF2 α in Vaccinia virus infected cells (Reviewed in Bugert and Darai 1999).

Another VV encoded protein, N1L, has been shown to be among the strongest determinants of VV virulence (Kotwal *et al.*, 1989, Bartlett *et al.*, 2002). Whereas

N1L had modest effects on adaptive immunity, it suppressed initial host defences against VV challenge by a factor of 10^4 (Kotwal *et al.*, 1989, Bartlett *et al.*, 2002), suggesting a mechanism of action that inhibits innate immunity. Recently, it was shown that N1L could inhibit TLR-induced NF κ B activation (DiPerna *et al.*, 2004). This study demonstrated that TLR2-, TLR3- and TLR4-mediated NF κ B were inhibited by N1L. Furthermore, downstream signals (e.g. MyD88, Mal, TRIF, TRAM and TRAF6) leading to NF κ B activation in the TLR pathway were also potentially blocked by N1L. The mechanism of action of N1L was attributed to its interaction with the components of the IKK complex (DiPerna *et al.*, 2004). A further VV encoded protein, K1L has also been reported to inhibit host NF κ B activation through a mechanism of preventing I κ B α degradation (Shisler *et al.*, 2004).

1.10 Discovery of A46R and A52R

During a database search for novel TIR-domain containing proteins, a VV open reading frame (ORF), A46R was identified that was related to the family at a statistically significant level. A BLAST search with A46R then identified a further VV ORF, A52R as the highest scoring sequence producing significant alignment. The names A46R and A52R are based on the standard nomenclature of the Copenhagen strain (Bowie *et al.*, 2000).

The alignment of A46R with the TIR domain from a number of TLRs (1, 2, 3, 4, 5, 6 & 10) revealed that A46R displays sequence similarity with boxes 1 and 2. Box 1 is particularly strong in A46R, the sequence DTFISY being as closely related to

the box 1 consensus as other proven family members. In addition, A46R can fold in a three layer $\alpha\beta\alpha$ sandwich (Bowie 2003) which is the topology adopted by the TIR domain of TLR1 and TLR2 (Xu *et al.*, 2000). Therefore, the evidence suggests that A46R does contain a TIR domain. A46R also displays a high degree of sequence conservation between different strains of VV (Bowie 2003).

In contrast, A52R does not appear to have a TIR domain and is not as conserved across different orthopoxviruses as A46R. For example, the gene encoding A52R in variola is disrupted. However, there is region of strong sequence similarity between A52R and A46R spanning approximately 50 amino acids, in the region of A46R that seems most distantly related to other TIR domains (Fig. 1.10) (Bowie 2003). A blast search with A52R against the human genome revealed that A52R does not share similarity with any proteins in the human genome.

Both A46R and A52R are expressed from a region in the VV genome that is rich in immunomodulatory genes. Given the fact that A46R is extremely similar to the TIR domain and the similarity between A46R and A52R; the question arose if they represent a previously unidentified VV strategy directed against IL-1R/TLR signalling (Bowie *et al.*, 2000). Bowie *et al.*, (2002) demonstrated that A46R partially inhibited IL-1 mediated activation of NF κ B and A52R potently blocked both IL-1 and TLR4 mediated NF κ B activation. A52R mimicked the dominant negative effect of a truncated version of MyD88 on IL-1, TLR4 and IL-18 signalling but had no effect on TNF signalling, which is MyD88 independent (Bowie *et al.*, 2000).

A46R	MAFDISVNASKTINALVYPSTQONKLVIRNEVNDTHYTFEFDKQVVDTFISYNRHNDTIEIRGVLPEETNIGCAVNTIPVSMITVLYNKYSFK
A52R	MDIKIDISISGDKFTVTTRRENEERKKYLPQKEKTTDVIKPDYLEYDDLLDRDEMPTILEE
A46R	LILAEYIRHRTISGNIYSALMTLDDLAIKQYGDIDLLFNEKLKVDSDSGLFDFVNFVKDMICCDSRIVVALSSLVSKHWELTNKKYRCMA
A52R	YPMYRGLLGLRIKYGRLEFNEIKKFONDABEQFGTIEELKQKLRLNSEEGADNFIDYIKVQKQDIVKLTVYDCISMIGLCACVVDVWRNEK
A46R	LAEHISDSIPISLSRLRYNLCKYLRGHTESTEDKFDYPEDDDSSSTCSAVTDRETDV
A52R	LPSEWKYCLRAIKLPINDHMLDKIKSILQNRLVYVEMS

Fig. 1.10 Predicted amino acid sequence of A52R and A46R.

The region of sequence similarity detected in a BLAST search with A46R is boxed. Identical amino acids are indicated by lines and conserved amino acids are indicated by dots. (Taken from Bowie *et al.*, 2000)

1.11 Aims

The aim of this study was to explore how the Vaccinia virus protein A52R acted and to use this protein and derivatives of it as tools to probe the signalling mechanisms whereby IL-1 and TLRs initiate an immune response. The effect of A52R on NF κ B and MAP kinase activation by various members of the IL-1R/TLR superfamily was measured. In addition, the effect of A52R on TLR-induced IL-10, IL-8 and RANTES production was tested. This proved useful in probing and more fully understanding the subtleties in signalling by different family members. The intracellular targets for A52R were characterised using co-immunoprecipitation studies. In order to map the sites of interaction of A52R with its intracellular targets, truncated versions of A52R were generated. These were checked for expression and for their ability to interact or not with the identified targets. Furthermore, a comparison between the mutants and A52R was analysed with regard to NF κ B and MAP kinase signalling induced by the TLRs. Finally, the effect of A52R on the TLR-IFN pathway was assessed with particular focus on a comparison between A52R and another VV TLR antagonist, A46R, with regard to TLR-mediated IRF3, IRF5 and IRF7 activation.

Chapter 2

Materials and Methods

2.1 Materials

Human rIL-1 α was a gift from the National Cancer Institute (Frederick, WA, USA), while human rTNF- α was a gift from Dr. S. Foster (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). The synthetic double-stranded RNA analogue, poly(I:C), was purchased from Amersham Pharmacia. Lipopolysaccharide from *E. coli* (>99.9% pure with respect to protein and DNA) and macrophage-activating lipopeptide 2kDa (MALP2) were purchased from Alexis Biochemicals (Nottingham NG13 8LS, UK). Synthetic tripalmitoyl lipopeptide, Pam₃-Cys-Ser-(Lys)₄ was purchased from Invivogen. Flagellin was a gift from Dr. A. Gerwitz (Emory University, Atlanta, GA 30322, USA). R-848 was a gift from Dr. K. A. Fitzgerald (The University of Massachusetts Medical School, Worcester, MA 01605). Phosphothioate CpG DNA was purchased from Sigma (Poole, Dorset, U.K.).

Anti-TRAF6 polyclonal antibody was purchased from Santa Cruz Biotechnology (2161 Delaware Avenue Santa Cruz CA 95060 USA). Anti-AU1 monoclonal antibody was purchased from Eurogentec (Tilman, Searing, Belgium). Monoclonal anti c-Myc (clone 9E10), monoclonal anti-Flag®, anti-mouse IgG (whole molecule) peroxidase conjugate and anti-rabbit IgG (whole molecule) peroxidase conjugate antibodies were all purchased from Sigma. The A52R antibody was a gift from Dr. Mary Harte and Prof Luke O'Neill. Anti-I κ B α monoclonal antibody was a kind gift from Professor Ron Hay (University of St. Andrews, Scotland). Anti-phospho p38 MAP Kinase (Thr180/Thr182) antibody and anti-p38 MAP Kinase antibody were both purchased from Cell Signalling Technology (Hitchin, Hertfordshire SG4 0TY U.K.).

The competent *E. coli* strain, NovaBlue, the UltraMobius™ 1000 plasmid purification kits and GeneJuice were all purchased from Novagen (Merck Biosciences Ltd. Padge Road Beeston/Nottingham NG9 2JR United Kingdom). Bacto™ Agar, Bacto™ Yeast Extract and Bacto™ Tryptone were purchased from Unitech (United Drug House Belgard Road Dublin 24 Ireland).

All cell culture materials including DMEM, gentamycin, trypsin, l-glutamine and foetal calf serum were obtained from Sigma. Foetal calf serum of a higher grade of purity was used for culture of the RAW264.7 cell line and this was purchased from LabTech (1 Acorn House, The Broyle Ringmer, East Sussex BN8 5NN, United Kingdom). Cell culture plastics were purchased from Greiner GmbH (Maybachstrasse, P.O. Box 1162 D-7443, Frickenhausen, Germany).

The human embryonic kidney cell line 293 (HEK293) and 293 cells stably transfected with IL1R1 (HEK293R1) were gifts from Tularik Inc, San Francisco, CA 94080. The human embryonic kidney cell line 293 stably expressing either pcDNA3.1 or TLR 2 or TLR3 or TLR4 or TLR8 were a kind gift from Dr. Kate Fitzgerald (University of Massachusetts MA). The mouse leukaemic monocyte-macrophage cell line, RAW 264.7, were obtained from the European collection of Animal cell Cultures (Salisbury, U.K.). Wild type and TRAF6 knockout murine embryonic fibroblasts (MEFs) were a kind gift from Professor Tak Mak (University of Toronto, Canada).

General laboratory chemicals were purchased from Sigma, with the exception of Glutathione Sepharose 4B (Amersham, Uppsala, Sweden), coelentrastazine (Argus

Fine Chemicals, Brighton, U.K.), passive lysis buffer (Promega, Madison, WI), broad range prestained protein marker (New England BioLabs Ltd., Beverly, MA), Supersignal® West Pico chemiluminescent substrate (Pierce). ELISA kits were purchased from R & D Biosystems (19 Barton Lane, Abington Science Park, OX14 3NB U.K.). The p38 MAP kinase inhibitor SB203580 was purchased from Alexis (Bingham, Nottingham, NG13 8LS U.K.), the ERK MAP kinase inhibitor PD98059 and the JNK SP600125 MAP kinase inhibitor were purchased from Calbiochem (Boulevard Industrial Park, Padge Road, Nottingham, NG9 2JR U.K.).

2.2 Expression and Reporter Plasmids

The empty vector pcDNA3.1 was purchased from Invitrogen (Groningen, The Netherlands). The NF κ B luciferase reporter construct containing five κ B elements was a gift from R. Hofmeister. The IFN- β luciferase construct was a kind gift from Dr. Taniguchi (University of Tokyo, Japan). The phRL-TK vector was purchased from Promega. The components for the PathDetect™ p38 ERK and JNK assay and the ISRE-luciferase reporter gene were obtained from Stratagene. The human IL-10 reporter construct was a kind gift from Professor L. Ziegler-Heitbrock. The plasmids encoding Gal4-IRF3, Gal4-IRF5, Gal4-IRF7, TRAM, TBK1 and dominant negative TBK1 (K38A) were kind gifts from Dr. K. A. Fitzgerald (The University of Massachusetts Medical School, Worcester, MA 01605). The plasmids encoding AU1-MyD88, Myc-IRAK2 and Myc-kIRAK2 (amino acids 97-590) were a kind gift from M. Muzio (Mario Negri Institute, Milan, Italy). The plasmids encoding CD4TLR1, 2, 4, 5 or 6 were kind gifts from Dr. R. Medzhitov (Yale University, New Haven, CT, USA). The plasmid encoding Flag-hTLR3 was a gift from Dr. D. T. Golenbock (The University of Massachusetts Medical School,

Worcester, MA 01605). While Ha-Mal encoding plasmid was from Professor Luke O'Neill (Trinity College Dublin, Ireland). Flag-TRAF6 domain (Δ TRAF6, amino acids 289-522) and Flag-TRAF2 were provided by Tularik Inc. (San Francisco CA). The plasmid encoding Flag-hTRIF was a gift from Dr. S. Akira (Research Institute for Infectious Diseases, Osaka University, Japan). Plasmids encoding VV A52R, Flag-A52R and A46R were generated by PCR amplification from Western Reserve (WR) DNA and have been described (Bowie *et al.* 2000). The expression plasmid encoding the poxvirus protein NIL was a gift from Professor W. Marshall (University of Massachusetts Medical School, Worcester, MA).

2.3 Methods

2.3.1 Cell Culture

HEK293, HEK293R1, HEK293T HEK293 cells stably expressing TLRs (pcDNA3.1 or TLR2, 3, 4 or 8) and MEF cell lines were cultured in DMEM medium containing 10% (v/v) foetal calf serum (FCS), 100 μ g/ml gentamicin and 2mM L-glutamine. The medium for the HEK 293 cells stably expressing TLRs also contained 500 μ g/ml of the neomycin analogue G-418 as a selection agent. RAW 264.7, a mouse leukaemic monocyte-macrophage cell line, were cultured in DMEM containing 10% (v/v) foetal calf serum, 100 μ g/ml gentamicin and 2 mM L-glutamine also, but the foetal calf serum used was of higher purity (LabTech). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. For use in transfection assays, 200 μ l HEK293, HEK293R1 HEK293T and TRAF6 MEFs were typically seeded at 1×10^5 per ml in 96 well plates 24 hrs prior to transfection, whereas RAW 264.7 were seeded at 2×10^5 per ml 24 hours prior to transfection. For continuing cell culture, both of the cell lines cells were seeded at 1×10^5 per ml

and sub-cultured two or three times a week. HEK293, HEK293RI, HEK293T and MEF cells were removed from the surface of the flask by incubation with 5 ml of trypsin-EDTA (0.05mg/ml) for 1 minute. 10 ml of complete medium was then added to the cells and they were centrifuged at 110 x g for 5 minutes. RAW 264.7 cells were removed from the surface of the flask using a cell scraper. The entire contents of the flask were then transferred to a 30 ml sterilin and centrifuged at 110 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in 1 ml of complete medium. In all cases cell viability was determined using the dye Trypan blue, which is excluded from healthy cells but taken up by non-viable cells. Cells were counted using haemocytometer and a bright light microscope.

2.3.2 Cryo-Preservation of Cells

Cells were grown to a state of sub-confluency and were harvested and counted as previously described (Section 2.3.1). The cells were centrifuged and the pellet was resuspended in the correct volume of FCS and dimethyl sulphoxide (DMSO) (9:1) to give a density of 2×10^6 cells/ml. Aliquots (1ml) were placed at -20°C for 2 hours and then at -80°C for 3 hours before being stored in a liquid nitrogen vessel.

2.4 Cloning

2.4.1 Polymerase Chain Reaction

For generation of ΔN (amino acids 59-190 of A52R) and ΔC (amino acids 1-144 of A52R) full length A52R (amino acids 1-190) was used as a template. ΔN and ΔC were cloned into pRK5 and pCMV-HA. Primers were used incorporating an EcoRI restriction site in the forward primer and a Sal I restriction site in the reverse primer.

Primers used to generate ΔN :

Forward Primer: 5' CGC GAA TTC CTA TGA TTC TAG AGG AAT AT 3'

Reverse Primer: 5' ACG CGT CGA ATG ACA TTT CCA C 3'

Primers used to generate ΔC :

Forward Primer: 5' CGG GAA TTC CCA TGG ACA TAA AGA TAG AT 3'

Reverse Primer: 5' ACG CGT CGA CCT ACA CGC ATG CAC ACA A 3'

PCR reactions were set up with 1X buffer (10 mM Tris-HCL, 50 mM KCL pH 8.3), 1.5 unit KOD Hi polymerase, 200 μ M of each dNTP, 10 ng plasmid DNA, 400 nM of each primer and 1.5 mM $MgCl_2$. Amplification was carried out using the conditions given below:

96°C for 5 minutes

5 cycles of 96°C for 15 seconds

45°C for 15 seconds

72°C for 15 seconds

28 cycles of 96°C for 15 seconds

64°C for 15 seconds

72°C for 15 seconds

2.4.2 Gel Cleaning of PCR Products

PCR products were resolved on 1% (w/v) agarose gels in TAE (4.84 g/L Tris base, 1.142 ml/L glacial acetic acid, 0.744 g/L EDTA pH 8.5) at 90 V for 30 minutes. The gel was stained with ethidium bromide and visualised under UV radiation. The products were then excised from the gel using sterile razor blades and placed in pre-weighed tubes. The tubes were reweighed and the weight of the slice calculated. Using this figure, 3 volumes of binding buffer (Perfectprep Gel Cleanup

kit was used) were added. The mixture was then incubated at 50°C for 10 minutes. After the gel slice was completely dissolved, one volume of isopropanol equal to the original gel size was added and mixed thoroughly by inversion. 800 µl of the sample was added to a spin column and centrifuged at 6000 x g for 1 minute. The filtrate was discarded and the spin column replaced in the collection tube. The spin column was washed with 750 µl of wash buffer by centrifugation for 1 minute at 6000 x g. The filtrate was discarded and the spin column replaced in the same collection tube and centrifuged at 6000 x g for an additional minute to remove any residual wash buffer. The spin column was placed in a new collection tube and 30 µl of molecular biology grade water was added and the column was centrifuged for 1 minute at 6000 x g. The spin column was discarded and the purified DNA was then used for digestion reactions.

2.4.3 Digestion of PCR Products and Plasmids

Approximately 1 µg DNA to be digested was incubated with 1 unit of the appropriate restriction enzyme and 1X buffer, as recommended by the manufacturers, in a final volume of 20 µl. The reaction mix was then incubated at 37°C for 1 hour.

2.4.4 Spin Column Cleaning of Digested Products

250 µl binding buffer was added to the digested products and mixed well before applying to a spin column filter. The filter was inserted into a 1.5 ml microfuge tube, which was spun at max speed for 30 seconds. The flow through was discarded and 250 µl of wash buffer was applied again to the column. Another 100 µl of wash buffer was added and the column centrifuged at maximum speed for 1

minute and the flowthrough discarded. Finally 50 µl of elution buffer was added to the column and centrifuged at maximum speed for 1 minute. The flowthrough containing the DNA was either used immediately or stored at -20°C until required.

2.45 Ligation reaction

PCR product and digested vector were run on an 0.8% (w/v) agarose gel in order to quantify products prior to ligation reaction. Ligations were carried out at a ratio of 4:1 insert:vector. The ligation reaction contained DNA (0.1-1 µM), 1X T4 DNA ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA) and 1 µl (0.5 U) T4 DNA Ligase (New England Biolabs, Hitchin, Herts, UK) in 20 µl final volume. The reaction was carried out at 16°C overnight.

2.5.6 Transformation of Ligation Reactions

Ligation reactions were incubated with 100 µl of competent bacteria (Novablue SinglesTM) on ice for 10 minutes. The mixture was then plated out on pre-warmed Luria Bertani (LB) agar plates, allowed to air-dry and incubated at 37°C overnight. The resulting colonies from the transformation were streaked onto an agar plate with numbered grids, and grown overnight at 37°C, and thereafter stored at 4°C.

2.5.7 Mini-Preparation of Plasmid DNA

DNA from bacterial colonies was isolated using Wizard[®] Plus SV Minipreps DNA Purification System (Promega, Madison WI, USA). Briefly, bacterial cultures were grown in 5 ml LB medium. Bacteria were pelleted and resuspended in 250 µl Cell Resuspension Solution. Cells were incubated for 5 mins with the addition of 250 µl Cell Lysis Solution before addition of 350 µl Neutralization Solution. The mixture

was centrifuged at 13,000 x g for 10 minutes 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 minute at room temperature and then washed with 750 µl and then 250 µl of Column wash solution. The DNA was eluted by addition of 100 µl Nuclease Free H₂O. Insertion of the PCR product into the vector was then confirmed by restriction digest.

2.5 Plasmid Purification for Transient Transfection

2.5.1 Plasmid Transformation

1 µl of plasmid DNA was added to 25 µl of competent *E.coli* cells (Novablue, Novagene) and left on ice for 5 minutes. The cells were then heat shocked at 42°C for 15 seconds and cooled on ice for 2 minutes. SOC medium (125 µl) was added. Aliquots (50 µl) were plated out and grown for 16-18 hours at 37°C. Transformed cells were then single colony purified and used to purify plasmids for transfection.

2.5.2 Plasmid Purification Procedure

Plasmids were purified using Novagene Ultramobius 200 and 1000 plasmid kits. Volumes for Ultramobius 1000 kits are given in brackets. 35 ml (100 ml) L-broth (amp) was inoculated with a single colony from a single colony purified plasmid transformation or a small amount was taken from a plasmid stock in DMSO kept at -80°C. Cells were grown to a density of 3-5 OD₆₀₀ at 37°C in an orbital shaker (300 rpm), which took approximately 16 hours. Cells were harvested by centrifugation at 5000 x g for 10 minutes at 4°C. Pellets were resuspended in 2.1 ml (8 ml) chilled resuspension buffer. Cells were lysed for 5 minutes in 2.1 ml (8 ml) lysis buffer at room temperature and the reaction stopped using 2.1 ml (8 ml)

chilled neutralization buffer, incubating the cells on ice for 5 minutes. The sample was then centrifuged at 10,000 x g for 2 minutes. 1 ml (2.4 ml) of Detox agent was added to the supernatant and incubated on ice for 15 minutes. The lysate was then centrifuged for 10 minutes (200 kit) or for 1000 kits the lysate was placed in a ClearSpin filter and centrifuged at 2000 x g for 3 minutes. The supernatant was removed immediately and applied to a Novagene column, which had been equilibrated with 5 ml (10 ml) equilibration buffer. The column was washed three times with 5 ml (10 ml) wash buffer. The bound plasmid was eluted using 2 ml (5 ml) elution buffer. The DNA was precipitated using 0.7 volumes of room temperature isopropanol and collected by centrifugation at 15,000 x g for 15 minutes. The pellet was washed twice with 70% (v/v) ethanol and the DNA resuspended in 100 ml (250 ml) sterile endotoxin free H₂O. The concentration and the purity of the DNA was determined on a 1%(w/v) agarose gel stained with ethidium bromide, together with A₂₆₀/A₂₈₀ spectrophotometer measurements.

2.5.3 Transient Transfection using GeneJuice™

GeneJuice™ transfection reagent from Novagen™ was used for transfection of HEK293 cells and RAW 264.7 cells. For 96 a well plate transfection, cells were seeded at 2×10^4 ml⁻¹ and grown overnight. Cells were transfected in triplicate with 230 ng DNA per transfection for HEK 293 cells and 200 ng for RAW 264.7 cells. In all cases the amount of DNA used per transfection was kept constant using the appropriate amount of relevant empty vector control. GeneJuice™ (0.8 µl) was mixed with 9.2 µl serum free DMEM per transfection and incubated at room temperature for 5 minutes. 30 µl of this mixture was added to triplicate amounts of DNA and incubated for 15 minutes at room temperature. 10 µl per well was then

added to the cells, which were allowed to recover for 16 hours at 37°C prior to stimulation. For 6 well plate transfection the total DNA used was 1-2 µg, with 8 µl GeneJuice™ and 92 µl serum free medium. For transfection of 100 mm plates, 4-8 µg DNA was used in combination with 35 µl GeneJuice™ and 315 µl serum free DMEM.

2.5.4 Reporter Plasmids Used

To examine activation of NFκB, ISRE, the IFN-β promoter or the IL-10 promoter, 60 ng of κB-luciferase reporter gene, ISRE-luciferase reporter gene, IFN-β promoter or IL-10 luciferase reporter gene were transfected using GeneJuice™ according to the manufacturers instructions. For MAP Kinase activation the Stratagene Pathdetect System™ was used whereby a vector encoding CHOP-Gal4 fusion protein (0.25 ng) (p38) Elk-Gal4 fusion protein (2ng) (ERK) or cJun-Gal4 fusion protein (0.25 ng) (JNK) was used in combination with 60 ng pFR-luciferase reporter. For experiments involving IRF3-, IRF5-, IRF7-Gal4 fusion proteins, 3 ng of each were transfected in combination with 60 ng pFR luciferase reporter. In all cases 20 ng of Renilla-luciferase internal control was used.

2.6 Luciferase Gene Reporter Assay

2.6.1 Preparation of Cellular Lysates

Cells were transfected in 96 well plates as described in section 2.3.1. Six hours before harvesting the cells were stimulated with the appropriate ligands as indicated. The medium was removed and the cells were lysed for 15 minutes at room temperature with 50 µl Passive Lysis Buffer by shaking. 20 µl of the lysed

cells were used to assay Firefly luciferase activity and an equivalent amount used for *Renilla* luciferase assay.

2.6.2 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 (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to the sample and *Renilla* luciferase activity was determined by the addition of 40 µl of 2 µg/ml of Coelenterazine in PBS. Luminescence was read using a Firefly luminescence reader and values were corrected for *Renilla* activity and expressed as fold stimulation over unstimulated empty vector (EV) control.

2.7 Western Blot Analysis

2.7.1 Preparation of Whole Cell Lysates using Sample Buffer

Cells were seeded at a density of 1×10^5 per ml. After appropriate stimulation or transfection, cells were washed with PBS and lysed in 100 µl Laemmli sample buffer (62.5 mM Tris pH 6.8, 2% (w/v) SDS, 50 mM DTT, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue). Samples were then sonicated for 10 seconds and boiled for 5 minutes.

2.7.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were resolved on sodium dodecyl sulphate (SDS) polyacrylamide gel using a constant voltage of 150 volts. Samples were first run through a stacking gel (1 ml 30% (v/v) bisacrylamide mix, 0.06 ml 10% (v/v) SDS, 0.75 ml 1 M Tris pH 6.8, 60 µl 10% ammonium persulphate and 6 µl TEMED made up to 6 ml with

H₂O) to condense protein and then resolved according to size using 10% polyacrylamide gels (required volume of 30% bisacrylamide mix, 0.1 ml 10% (v/v) SDS, 3.75 ml 1.5 M Tris pH 8.8, 150 µl 10% (w/v) ammonium persulphate, 6 µl TEMED made up to 15 ml with H₂O). Samples were then run with pre-stained protein markers (New England Biolabs) as molecular weight standards.

2.7.3 Transfer of Proteins to Membrane

The resolved proteins were transferred to a polyvinylidene difluoride (PVDF membrane) using a wet transfer system with all components soaked beforehand in transfer buffer (25 mM Tris-HCl pH 8.0, 0.2 M glycine, 20% methanol). The membrane was activated by methanol and then washed in water. The gel was placed on a layer of filter paper on a sponge and overlaid with the PVDF. A second piece of filter paper was placed on top followed by a second sponge. The entire assembly was placed in a cassette and constant voltage applied 90 volts for 1 hours.

2.7.4 Antibody Blotting

Membranes were blocked to prevent non-specific protein binding, by incubation in blocking buffer (5% (w/v) non-fat dried milk in 1% (v/v) phosphate buffered saline (PBS)- with 1% (v/v) Tween at 4°C overnight. The membrane was washed for 5 minutes in 1% (v/v) PBS-Tween three times. The membrane was then incubated for 1 hour at room temperature with the antibody of interest at 1:100 to 1:1000 dilutions depending on antibody. Next the membrane was washed three times and incubated with the appropriate secondary horseradish peroxidase linked antibody. Blots were developed by enhanced chemiluminescence (ECL) according to manufacturers instructions (Amersham).

2.7.5 Co-Immunoprecipitation

HEK 293T cells were seeded into 100 mm dishes (1.5×10^6) 24 hours prior to transfections. Transfections were carried out using GeneJuice™ (Section 2.53). Four μg of each construct was transfected. Where only one construct was expressed the total amount of DNA (8 μg) was kept constant by supplementation with vector DNA. Cells were harvested 24 hours post transfection. Cells were washed twice in 1X PBS and lysed in 850 μl lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) NP40 containing 1 mM PMSF and proteases inhibitor cocktail (Sigma) (1/100) and 1 mM sodium orthovanadate). For immunoprecipitations the indicated antibodies were precoupled to either protein A Sepharose (polyclonal and Flag monoclonal antibodies) or protein G Sepharose beads (all other monoclonal antibodies). The antibodies were left to attach to the beads overnight on a roller at 4°C. The beads were then washed twice in lysis buffer and incubated with the cell lysates overnight at 4°C. The immune complexes were then washed twice in lysis buffer by centrifugation at 4000 rpm for 3 minutes and once in lysis buffer without NP40 and glycerol. Thirty μl of 3x sample buffer (62.5 mM Tris, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue) was added to the immune complexes, which were then boiled for 5 minutes, thus the associated proteins were removed. The immune complexes were then analysed by SDS PAGE.

2.8 ELISA**2.8.1 Preparation of Samples**

RAW 264.7 cells (2×10^4 cells per ml) or HEK293-TLR4 (2×10^4 cells per ml) were seeded into a 96 well plate and transfected 24 hours later as described in the

figures legends. Twenty four hours after transfection cells were stimulated as described in the figure legends. Supernatants were then removed for IL-10, IL-8 or RANTES determination.

2.8.2 ELISA Protocol

For IL-10 detection a 96 well plate was coated with rat anti-mouse IL-10 antibody diluted to 2 µg/ml in PBS for IL-10 detection. To assay IL-8, a 96 well plate was coated with mouse anti-human IL-8 diluted to 4 µg/ml in PBS. To determine RANTES production, a 96 well plate was coated with mouse anti-human RANTES diluted to 1 µg/ml in PBS. The plates were covered and incubated overnight at 4°C and then washed three times with PBS/0.5% (v/v) Tween. Plates were patted dry on paper towels as a final step. The plates were blocked with 1% (w/v) BSA and 5% (w/v) sucrose in PBS, for 1 hour at room temperature. The plates were washed twice and dried as before. IL-10 or IL-8 standards (in the range of 62.5 – 2000 pg/ml), or RANTES standards (in the range of 15.3 – 1000 pg/ml) and samples were added at 100 µl per well and incubated at room temperature for 2 hours. Plates were then washed as before. Biotinylated goat anti-mouse IL-10 monoclonal antibody was diluted to 400 ng/ml in PBS or biotinylated goat anti-human IL-8 was diluted to 20 ng/ml or biotinylated goat anti-human RANTES was diluted to 10 ng/ml. In each case 100 µl of the antibody solution was added to each well. The plates were incubated at room temperature for 2 hours and then washed three times as before. 100 µl of streptavidin conjugated to horseradish peroxidase (1:200 diluted in PBS) was added to each well and incubated for 20 minutes in the dark at room temperature. This was removed and plates were washed as before. 100 µl of freshly prepared substrate (0.11 M sodium acetate pH 5.5, 167 µl

tetramethylbenzidin (TMB, 6 mg/ml in DMSO) 3% (v/v) hydrogen peroxidase) was added to each well and incubated for 20 minutes in the dark at room temperature. Addition of 50 μ l H_2SO_4 was added in order to stop this reaction. The optical density of each well was read at 450 nm using a Dynatech MR5000 plate reader. A standard curve was constructed relating IL-10, IL-8 or RANTES concentration to absorbance at 450 nm and was used to determine IL-10, IL-8 or RANTES concentration.

Chapter 3

A52R Inhibits IL-1R/TLR-Induced NF κ B Activation by Targeting IRAK 2

3.1 Introduction

The discovery of the TLR family of proteins has revolutionised our understanding to how the innate immune system recognises pathogens and initiates an effective and appropriate response (Takeda *et al.*, 2003). TLRs are pattern recognition receptors that recognise PAMPs. Bacterial PAMPs such as peptidoglycan, LPS and flagellin are detected by TLR2, 4 and 5 respectively (Takeda *et al.*, 2003). DsRNA is molecular pattern associated with viral infection and TLR3 has been shown to sensitise cells to activation by poly(I:C) a synthetic dsRNA analogue (Alexopoulou *et al.*, 2001). Other TLRs involved in sensing viral infection include TLR7, TLR8 and TLR9 (Reviewed in Boehme and Compton 2004).

Triggering of the TLRs with the appropriate TLR agonist leads to the activation of several intracellular signal transduction pathways. Among the most prominent and best characterised of these pathways is that leading to the activation of the transcription factor NF κ B. TLRs, like the IL-1R, mediate downstream signalling through their TIR domain, which recruits TIR containing adapter proteins for which MyD88 is a prototypical example (Reviewed in Akira 2004). MyD88 can subsequently recruit IRAK4 to the receptor complex through a homotypic death domain interaction. The binding of MyD88 to IRAK4 facilitates IRAK4-mediated phosphorylation of a crucial residue in the kinase activation loop of IRAK1, which induces the kinase activity of IRAK1. IRAK2 can compensate for a lack of IRAK1 in a mutated 293 cell line (Li *et al.*, 1999b). The IRAK1 then engages with TRAF6 which ultimately leads to activation of the IKK complex. This facilitates phosphorylation of the inhibitory molecule I κ B allowing NF κ B to become free to enter the nucleus and induce target gene expression (Reviewed in Akira 2004).

It has previously been shown that the VV protein A52R potentially blocked both IL-1 and TLR4-mediated NF κ B activation. A52R mimicked the dominant-negative effect of a truncated version of MyD88 on IL-1, TLR4 and IL-18-induced NF κ B activation but had no effect on TNF-induced NF κ B signalling (Bowie *et al.*, 2000). The aim of this chapter was to examine the ability of A52R to antagonise other TLR pathways to NF κ B activation. The results show that A52R blocked NF κ B activation induced by TLR 3, 4, 5, 7, and 9. Therefore A52R can act as a global inhibitor of TLR-dependent activation of NF κ B. A52R was shown to target two key signalling molecules IRAK2 and TRAF6. Further, a truncated version of A52R was found to interact with IRAK2 and not TRAF6. This truncated version potentially blocked IL-1/TLR-induced NF κ B activation, thus highlighting that the interaction with IRAK2 may be more fundamental for inhibition of TLR-mediated NF κ B signalling than the interaction with TRAF6.

3.2 Ectopic Expression of A52R

A52R was cloned from the laboratory VV strain Western Reserve, where it was previously called SalF15R, into the mammalian expression vector pRK5 (Bowie *et al.*, 2000). The name A52R is based on the standard VV nomenclature of the Copenhagen strain (Goebel *et al.*, 1990). To confirm that A52R could be expressed in mammalian cells, increasing amounts of the expression plasmid encoding A52R was transfected into HEK293 cells. Fig. 3.1 shows that subsequent immunoblotting after 24 hours with an antibody raised to A52R revealed dose-dependent expression of A52R at the predicted molecular weight 21.5 kDa.

3.3 A52R Blocks IL-1R/ TLR but not TNF-Mediated NFκB Activation

A single dose of plasmid encoding A52R had previously been shown to block the activation of NFκB by IL-1, with little inhibitory effect on the activation of NFκB by TNF in a reporter gene assay (Bowie *et al.*, 2000). An NFκB-dependent reporter gene assay was used to compare the effect of A52R on IL-1- and TNF-induced NFκB more closely. Fig. 3.2 shows that the inhibitory effect of A52R on IL-1 was dose-dependent in HEK293R1 cells (Fig. 3.2a). Consistent with published work, A52R had no effect on TNF-mediated NFκB activation; Fig. 3.2b shows that at the same range of plasmid concentrations where IL-1 was inhibited there was no inhibition of TNF-induced NFκB activation.

The effect of A52R on IL-1 and TNF signalling to NFκB at the level of IκBα degradation was next assessed. In order to do this an expression construct of IκBα with a C-terminal tag was transfected into cells, so that the transfected (i.e. A52R containing) population of cells could be examined in isolation. Fig. 3.3

shows a Western blot of cytokine induced degradation of I κ B. Both I κ BcTag (upper band) and endogenous I κ B (lower band) can clearly be seen. Treatment of cells for 30 minutes with IL-1 or TNF led to degradation of both endogenous and overexpressed I κ B α (compare lanes 3 and 5 to lane 1). When a mammalian expression vector encoding A52R was co-transfected it had no effect on the control level of I κ B (compare lanes 1 and 2) but inhibited IL-1-induced degradation (compare lane 4 to lane 3) while not preventing TNF-induced degradation (compare lane 6 to lane 5). This inhibition of I κ B α -degradation by A52R likely accounts for the suppression of IL-1-induced NF κ B-dependent reporter gene expression seen in Fig. 3.2.

3.4 A52R Inhibits NF κ B Activation by Multiple TLRs

Given the emerging role of TLRs in antiviral responses, the effect of A52R on NF κ B activation by TLRs was tested. NF κ B reporter gene assays were carried out in HEK293 cells using chimeric versions of murine CD4 extracellular domain fused to the cytoplasmic domain of a given human TLR, which renders them constitutively active (Hayashi *et al.*, 2001, Ozinsky *et al.*, 2000, Medzhitov *et al.*, 1997). This therefore eliminates the need to use an exogenous activator. Some TLR cytoplasmic domains can induce gene expression as homodimers (TLR4 and TLR5), while others signal as heterodimers (TLR2 with TLR1 or 6) (Hayashi *et al.*, 2001, Ozinsky *et al.*, 2000). Overexpression of either CD4-TLR4 or CD4-TLR5 led to activation of NF κ B, whereas CD4-TLR6 and CD4-TLR2 were only active when co-expressed with CD4-TLR2 (Fig 3.4a and not shown). The activation of NF κ B in each of these cases was inhibited by co-expression of A52R (Fig.3.4a).

DsRNA is a molecular pattern associated with viral infection, and TLR 3 has been shown to sensitise cells to activation by polyinosine-polycytidylic acid (poly (I:C), a synthetic dsRNA analogue (Alexopoulou *et al.*, 2001), which has been extensively used to mimic viral infection. Therefore, the effect of A52R on TLR3 dependent NF κ B activation induced by poly (I:C) was examined. Transfection of HEK293 cells with TLR3 led to a strong dose dependent activation of NF κ B by poly (I:C), which was not seen in the absence of TLR 3 (Fig. 3.4b). This TLR3 dependent induction of NF κ B was completely blocked by A52R (Fig. 3.4b) in a dose-dependent manner (Fig. 3.4c). Stimulation of cells with poly(I:C) in the absence of TLR3 also lead to activation of NF κ B (Fig. 3.4b). The activation of NF κ B observed here may be due to TLR3-independent pathways but dsRNA-dependent pathways, for example RIG-1 or PKR activation (Yoneyama *et al.*, 2004, Gil *et al.*, 2004).

Having identified that A52R was capable of inhibiting NF κ B activation induced by transient overexpression of TLRs in HEK293 cells I next sought to confirm these results for exogenous addition of TLR agonists. For this, HEK293 cells stably expressing pcDNA3.1, TLR2, TLR3, TLR4 and TLR8 were used. These cells have been shown to specifically respond to the IL-1R/TLR agonists IL-1, Malp-2, Poly (I:C), LPS and R-848 respectively (Fitzgerald *et al.*, 2003b). In each case the activation of NF κ B was measured using a reporter gene assay. Consistent with published work, IL-1 (Fig. 3.5a), Malp-2 (Fig. 3.5b), poly (I:C) (Fig. 3.5c), LPS (Fig. 3.5d) and R-848 (Fig.5e) all activated the NF κ B reporter. In each case, co-transfection of a plasmid encoding A52R dose-dependently inhibited the induction of NF κ B by the TLR agonist tested.

Furthermore, as well as the effects in human cells above, the inhibition of NF κ B caused by A52R was also seen in the immunologically relevant murine monocyte cell line RAW 264.7. These are a much used model system for TLR activation in macrophages (Fig. 3.6), which have been reported to express most TLRs (Applequist *et al.*, 2002). TLR agonists added to these cells will therefore induce the activation of signalling cascades. The TLR agonists Pam₃Csk₄, Malp-2, poly (I:C), LPS, flagellin, R-848 and CpG DNA which activate TLR2&1, TLR2&6, TLR3, TLR4 TLR5, TLR7 and TLR9 respectively were examined. In each case the activation of NF κ B was inhibited by the co-transfection of a plasmid encoding A52R. Thus, A52R acts as a broad intracellular inhibitor of NF κ B signalling by TLRs, in both human and murine cell lines.

3.5 A52R Interacts with TRAF6 and IRAK2

The activation of NF κ B by different TLRs is mediated by a common set of signalling molecules (Akira *et al.*, 2001). The ability of A52R to inhibit NF κ B activation by multiple TLRs and IL-1 suggested that its effect might be due to its interaction with a molecule, or molecules, whose function is critical to signalling by all TLRs. It has previously been demonstrated that A52R could interact with both TRAF6 and IRAK2 (Harte *et al.*, 2003) and these results have been repeated here. Fig. 3.7 shows an experiment attempting to co-immunoprecipitate A52R with TRAF6 (Fig 3.7) or IRAK2 (Fig 3.8). It is clear that A52R forms a complex both with TRAF6 (this corresponds to a band in lane 3, top panel, above the heavy chain) and IRAK2 (top panel, lane 3). The difference in the amounts of A52R present in lanes 1 & 3 is due to unequal loading of samples. Although there appears to be less A52R in lane 3 than in lane 1 the interaction between A52R and

TRAF6 was clearly detected (top panel lane 3). In contrast, no interaction was detected between A52R and TRAF2 (Fig. 3.9). TRAF2 is a TRAF family member with a role in TNF signalling (Hsu *et al.*, 1996). Consistent with the lack of interaction with TRAF2, A52R had no inhibitory effect on the activation of NF κ B by TNF (Fig. 3.2b) or TRAF2 overexpression (Fig. 3.11b).

3.6 The Effect of A52R on Downstream Signalling Components Leading to NF κ B Activation

Given that MyD88 is a key adaptor in signalling by TLRs, which is upstream of TRAF6 and IRAK2, the effect of A52R on the ability of MyD88 to activate NF κ B was examined. Overexpression of MyD88 activated NF κ B, and this activation was inhibited by the co-expression of A52R, in a dose-dependent manner (Fig. 3.10a). The fact that IRAK2 is a target for A52R prompted studies to investigate the effect on the ability of IRAK2 to activate NF κ B. Fig. 3.10b shows that overexpression of IRAK2 led to NF κ B activation, and this was inhibited dose-dependently by the co-expression of A52R. A52R was also found to inhibit NF κ B activation induced by its other target, TRAF6 (Fig. 3.11a). Consistent with the lack of effect of A52R on TNF-mediated NF κ B activation (Fig. 3.2b), activation of NF κ B by overexpression of TRAF2 was not blocked by co-expression of increasing amounts of a plasmid encoding A52R (Fig. 3.11b). TRAF6 and IRAKs ultimately activate NF κ B via stimulation of the kinase activity of the IKK complex (Reviewed in Akira and Takeda 2004). The IKK complex contains IKK α , IKK β and IKK γ (Ghosh *et al.*, 1998). Fig. 3.12 highlights the ability of overexpressed IKK α and IKK β to activate NF κ B. No inhibition of these signals was observed with co-expression of A52R, consistent with the fact that they function downstream of IRAK2 and TRAF6.

3.7 A Role for TRAF6 and IRAK2 in TLR Signalling

A role for TRAF6 in the TLR pathways inhibited by A52R was confirmed by testing the effect of dominant-negative TRAF6 (containing only the TRAF domain), here termed Δ TRAF6, on these pathways. Fig. 3.13a shows that activation of NF κ B by each CD4-TLR combination was inhibited by Δ TRAF6. Further, Δ TRAF6 dose-dependently inhibited TLR3-dependent poly (I:C)-induced NF κ B activation (Fig. 3.13b). This is consistent with previous published work showing inhibition of TLR3-dependent NF κ B activation by dominant negative TRAF6 (Alexopoulou *et al.*, 2001)

Similar to TRAF6, there was a correlation between the inhibition of TLR-induced NF κ B activation by A52R and a role for IRAK2 in these pathways. Figure 3.9a shows that each CD4-TLR induced signal that was sensitive to A52R was also blocked by dominant-negative IRAK2 (Δ IRAK2), which lacks the death domain. Further, IRAK2 has a role in TLR3-dependent poly (I:C)-induced NF κ B activation, as dominant negative IRAK2 dose-dependently inhibited this signal (Fig. 3.14b).

3.8 Inhibition of TLR-Induced NF κ B Activation by A52R Requires Interaction with IRAK2

Inhibition of TLR-induced NF κ B activation could conceivably be due to its ability to interact with either IRAK2 or TRAF6 (Fig 3.7 and 3.8, Harte *et al.*, 2003). In order to determine whether this was the case, I began to generate truncated versions of A52R in order to map the sites of interaction between A52R and TRAF6 and IRAK2. Fig. 3.15 shows a schematic representation of the truncated versions of

A52R that were constructed. These were generated by PCR from full length A52R and cloned into pRK5. ΔC encodes amino acids 1-144 of A52R; ΔN begins at the region of similarity with A46R and encodes amino acids 59-190 of A52R. A further truncated version of A52R lacking 46 amino acids at the C-terminal was also constructed. This was generated by PCR from full length A52R and cloned into pRK5 but includes an extra 27 amino acids derived from the vector sequence. This truncated A52R protein, here termed $\Delta A52R$, was the most easily detectable by the anti-A52R antibody and was expressed at similar levels to A52R (Fig. 3.17). To determine if $\Delta A52R$ was still capable of interacting with TRAF6 and/or IRAK2, co-immunoprecipitations were carried out. $\Delta A52R$ was unable to form a complex with TRAF6 but retained its ability to interact with IRAK2. A co-immunoprecipitation with anti-A52R antibody pulled down TRAF6 when A52R was present (Fig. 3.16a top panel: a band in lane 3 corresponding to TRAF6 can be seen above the antibody heavy chain). In contrast, under the same conditions $\Delta A52R$ was not detected in complex with TRAF6 (Fig. 3.16b top panel: no band is present above the heavy chain in lane 6). However, a clear interaction between $\Delta A52R$ and IRAK2 was observed (Fig. 3.17b). The ability of $\Delta A52R$ and A52R to form a complex with IRAK2 appeared equal (Fig. 3.17 top panels: compare lanes 3 & 6).

Given the fact that $\Delta A52R$ interacted with IRAK2 and not TRAF6, it was possible to examine if this interaction was sufficient for maximal NF κ B inhibition. $\Delta A52R$ was therefore used as a tool to determine the contribution of the interaction of A52R with IRAK2 compared to TRAF6 on NF κ B inhibition. The effects of $\Delta A52R$ on TLR signalling, in parallel with A52R, were examined. Addition of IL-

1, overexpression of CD4-TLR4, or overexpression of TLR3 together with poly(I:C) stimulation, led to activation of NF κ B (Fig 3.18). Δ A52R was capable of inhibiting IL-1-induced NF κ B activation to the same extent as A52R over a range of plasmid doses (Fig 3.18a). A single dose of both A52R and Δ A52R was sufficient for maximal CD4-TLR4-induced NF κ B inhibition (Fig. 3.18b). Furthermore, Δ A52R was actually a more potent inhibitor of TLR3/poly(I:C)-induced NF κ B activation than A52R (Fig. 3.18c). Thus, the ability of A52R to inhibit NF κ B induction by various TLRs is defined by its interaction with IRAK2. Preliminary investigation into the effects of Δ C and Δ N on NF κ B activation showed that both of these constructs are also capable of inhibiting LPS- and poly (I:C)-induced NF κ B activation (Fig. 3.19).

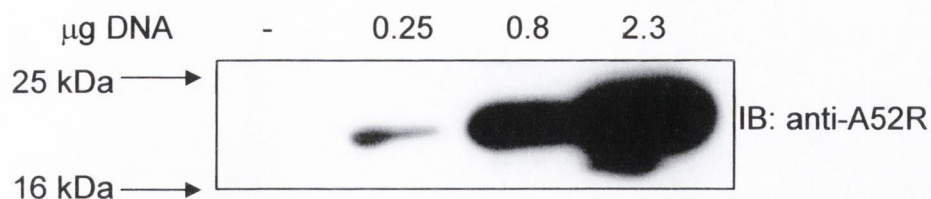


Fig. 3.1: Ectopic Expression of A52R

HEK293 cells (2ml) were seeded at 1×10^5 cells per ml in 6 well plates 24 hours prior to transfection.. Increasing amounts of a plasmid encoding A52R was transfected, as indicated. The total amount of DNA (2.3 µg) was kept constant by supplementation with pcDNA 3.1. Cells were harvested 24 hours post transfection, lysates resolved by SDS-PAGE and probed with an antibody specific for A52R. Data is a representative of one experiment from a minimum of three separate experiments.

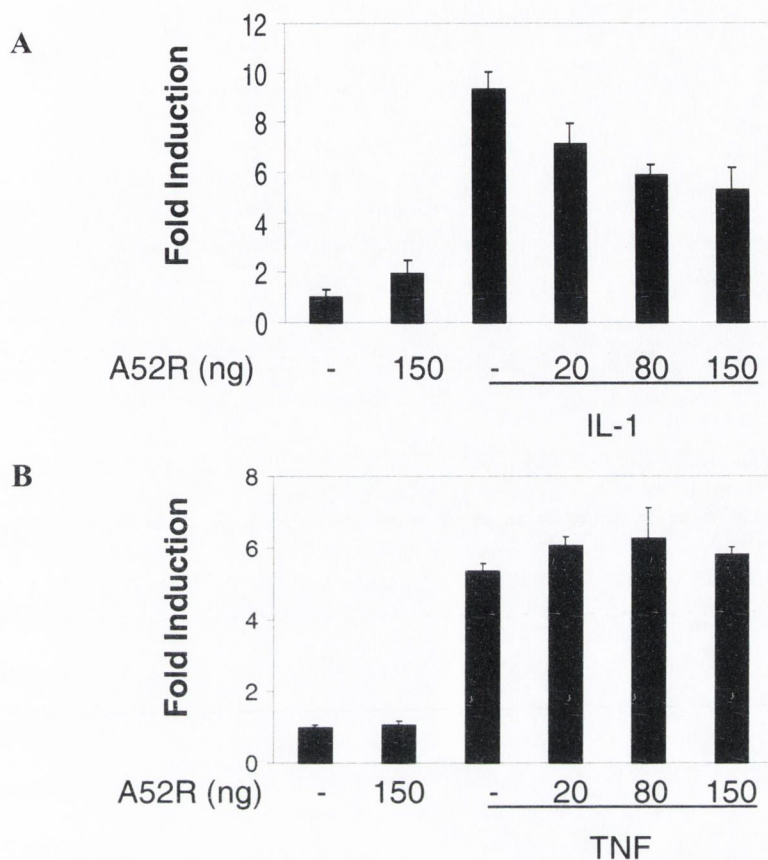


Fig. 3.2: A52R blocks IL-1R mediated NFκB activation

HEK293R1 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κB-luciferase reporter gene and Renilla-luciferase internal control. Cells were transfected with increasing amounts of plasmid encoding A52R (20, 80, 150 ng). Six hours prior to harvesting cells were stimulated with 100 ng/ml IL-1 (A) or 50 ng/ml TNF (B). After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

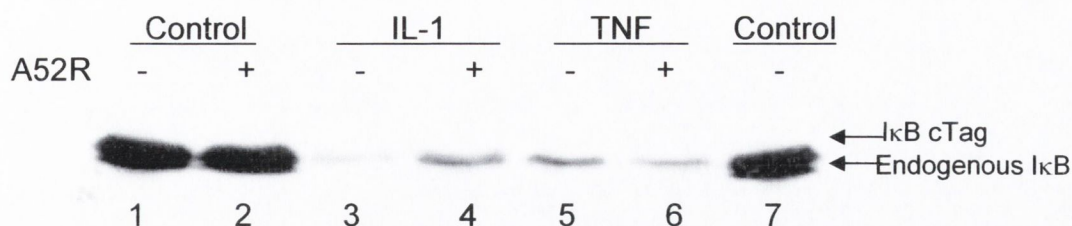


Fig. 3.3: A52R blocks IL-1R/ TLR but not TNF mediated IkBα Degradation

HEK293R1 cells were seeded at 1×10^5 cells/ml in 6 well plates 24 hours prior to transfection. The cells were co-transfected with 1.15 μ g of plasmid encoding IkB with a C-terminal tag (“IkBcTag”). Some cells were transfected with 1.15 μ g of plasmid encoding A52R where indicated (+). The total amount to DNA was kept constant at 2.3 μ g by addition of pcDNA 3.1. Thirty minutes before harvesting cells were stimulated with 100 ng/ml IL-1 and 50 ng/ml TNF were indicated. Cells were harvested 24 hours post transfection and resolved by SDS-PAGE. The blot was probed with an antibody specific for IkB. Data is a representative of one experiment from a minimum of three separate experiments.

Fig. 3.4: A52R inhibits NFκB activation by multiple TLRs

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κB-luciferase reporter gene and Renilla-luciferase internal control as described in Methods (A) Cells were transfected with constitutively active CD4-TLRs (50 ng TLR4 or TLR5, or 25 ng each of TLR2 and TLR6 or TLR2 and TLR1) in the presence of 80 ng empty vector (EV), or plasmid encoding A52R. (B) HEK293 cells were transfected with empty vector (EV), 0.5 ng TLR3 or 0.5 ng TLR3 plus 150 ng A52R. Six hours prior to harvesting cells were stimulated with 5–25 μg/ml poly (I:C). (C) Cells were transfected with 0.5 ng TLR 3 and stimulated with 25 μg/ml poly (I:C), in the presence of increasing amounts of A52R (20, 80, 150 ng). After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

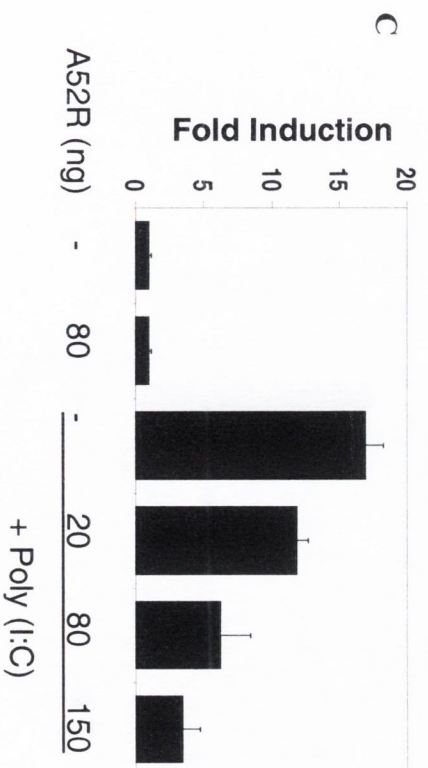
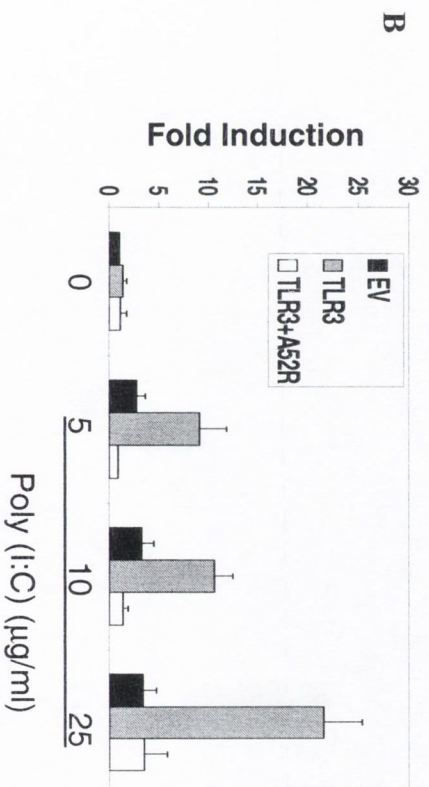
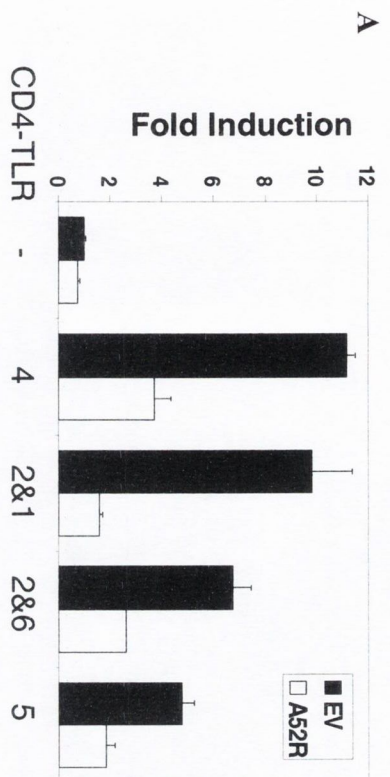
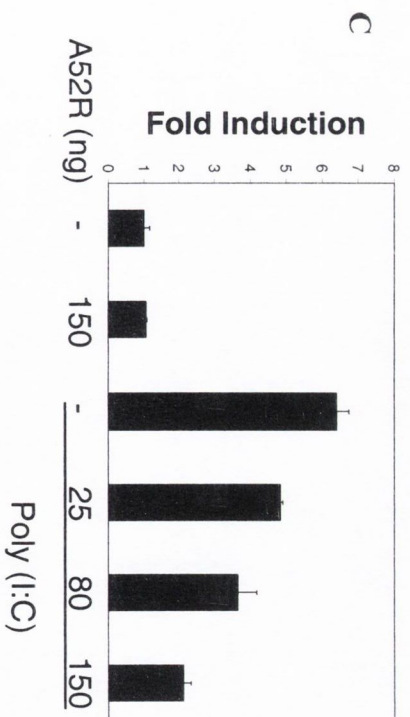
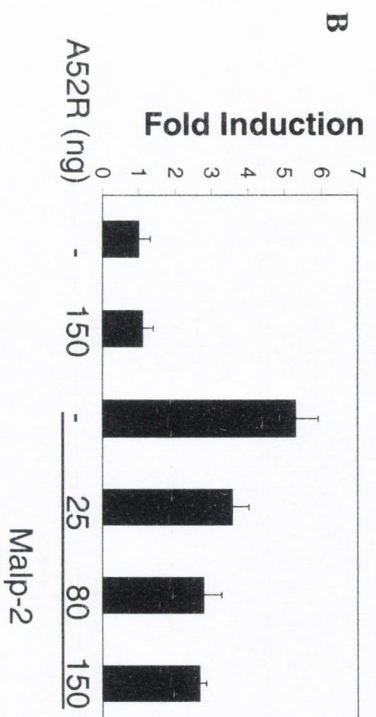
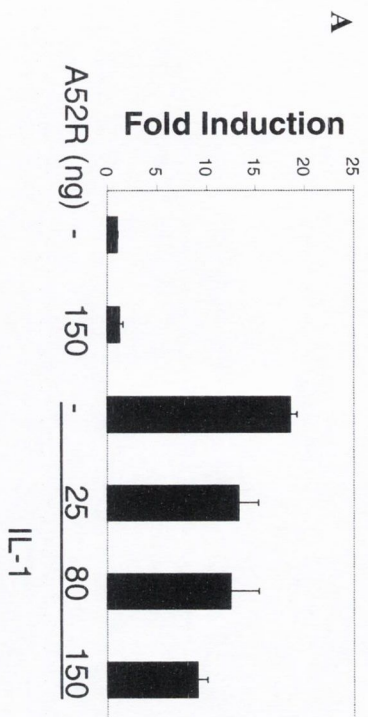
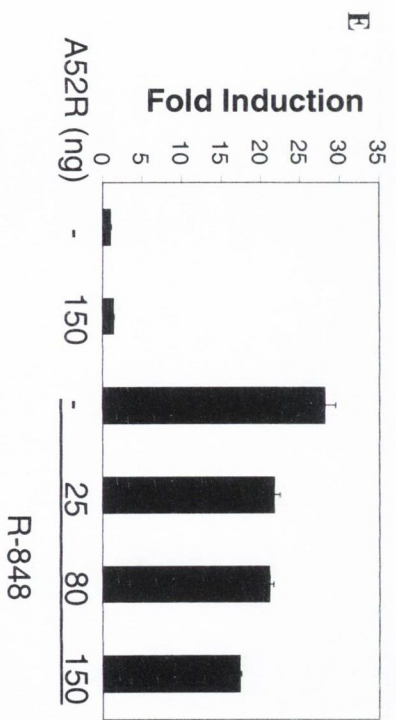
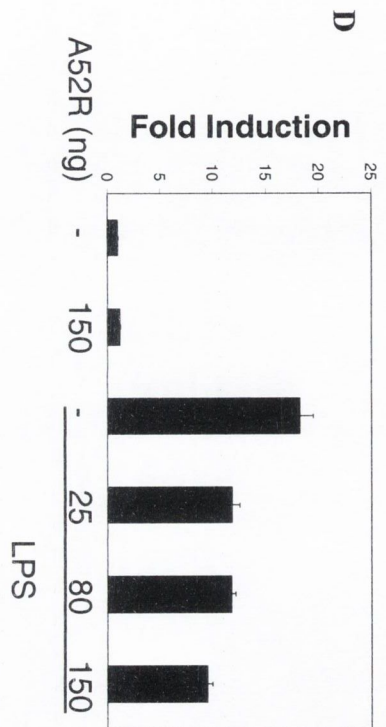


Fig. 3.5: A52R antagonises TLR ligand induced NFκB activation

HEK293 cells (2×10^4 cells per well) stably transfected with pcDNA (A) or TLR2 (B) TLR3 (C) TLR4 (D) TLR8 (E) were seeded into 96-well plates and transfected the next day with expression vectors, κB-luciferase reporter gene and Renilla-luciferase internal control. Cells were transfected with increasing amounts of a plasmid encoding A52R as indicated. Six hours prior to harvesting cells were stimulated with 50 ng/ml IL-1 (A) 1nM Malp-2 (B) 25 µg/ml poly (I:C) (C) 1 µg/ml LPS (D) or 10 µM R-848 (E). After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.





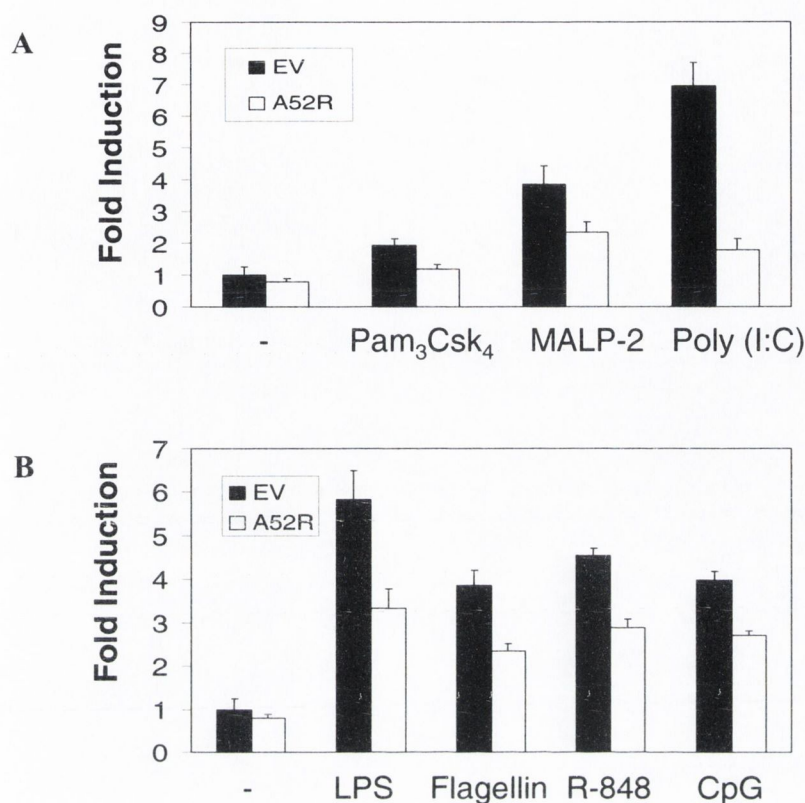


Fig. 3.6: A52R inhibits NFκB activation by multiple TLRs in RAW 264.7 cells

Murine macrophage RAW 264.7 cells were transfected with an NFκB luciferase construct and a Renilla-luciferase internal control, together with 100 ng empty vector (EV), or plasmid encoding A52R. Cells were stimulated six hours before harvesting with the TLR agonists (A) 1nM Pam₃Csk₄, 1 nM Malp-2 or 25 µg/ml poly (I:C) (B) 1 µg/ml LPS, 250 ng/ml Flagellin, 1 nM R-848 or 3 nM CpG. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction +/- s.d. relative to control levels, for minimum of two separate experiments each performed in triplicate.

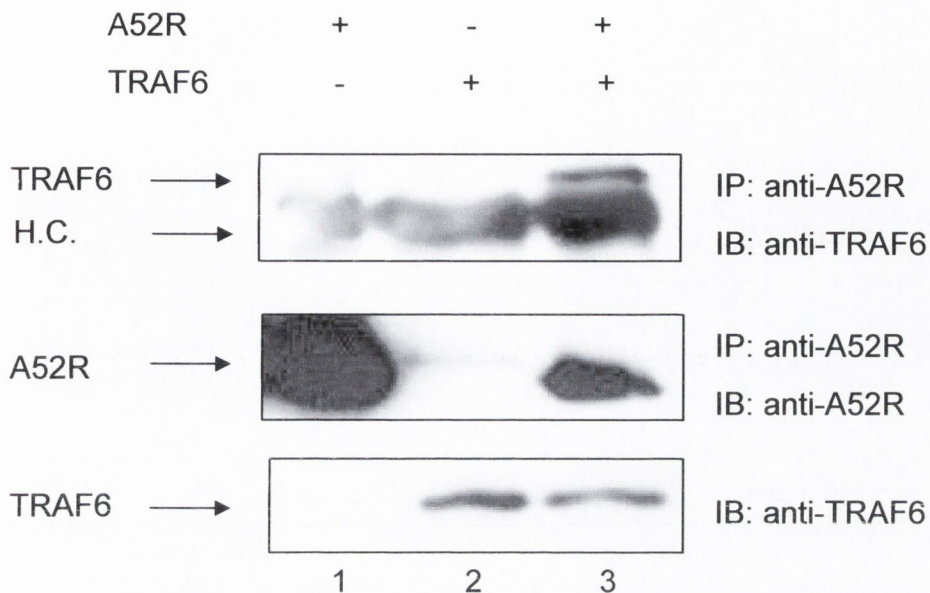


Fig. 3.7: A52R interacts with TRAF6

HEK293T cells (1.5×10^6) were seeded into 100 mm dishes 24 hours prior to transfection. Four μ g of each construct was transfected where indicated (+). The total amount of DNA (8 μ g) was kept constant by supplementation with pcDNA 3.1. Cells were harvested 24 hours post-transfection and immunoprecipitation experiments carried out as described (Section 2.7.5). The top panel corresponds to lysates immunoprecipitated with anti-A52R and blotted for the presence of TRAF6. The appearance of a band in lane 3 that is not detected in lanes 1 and 2 is indicative of an interaction. H.C., antibody heavy chain. The second panel corresponds to lysates immunoprecipitated with anti-A52R and blotted for the same antibody. The lower panel shows lysates probed for TRAF6. Data is a representative of one experiment from a minimum of three separate experiments.

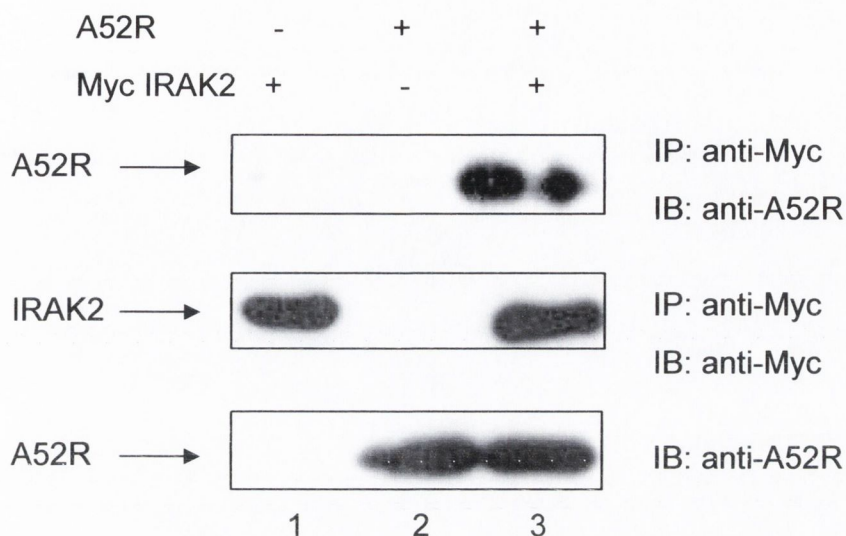


Fig. 3.8: A52R interacts with IRAK2

HEK293T cells (1.5×10^6) were seeded into 100 mm dishes 24 hours prior to transfection. Four μg of each construct was transfected where indicated (+). The total amount of DNA (8 μg) was kept constant by supplementation with pcDNA 3.1. Cells were harvested 24 hours post-transfection and immunoprecipitation experiments carried out as described (Section 2.7.5). The top panel corresponds to lysates immunoprecipitated with anti-myc and blotted for the presence of A52R. The appearance of a band in lane 3 that is not detected in lanes 1 and 2 is indicative of an interaction. The second panel corresponds to lysates immunoprecipitated with anti-myc and blotted for the same antibody. The lower panel shows lysates probed for A52R. Data is a representative of one experiment from a minimum of three separate experiments.

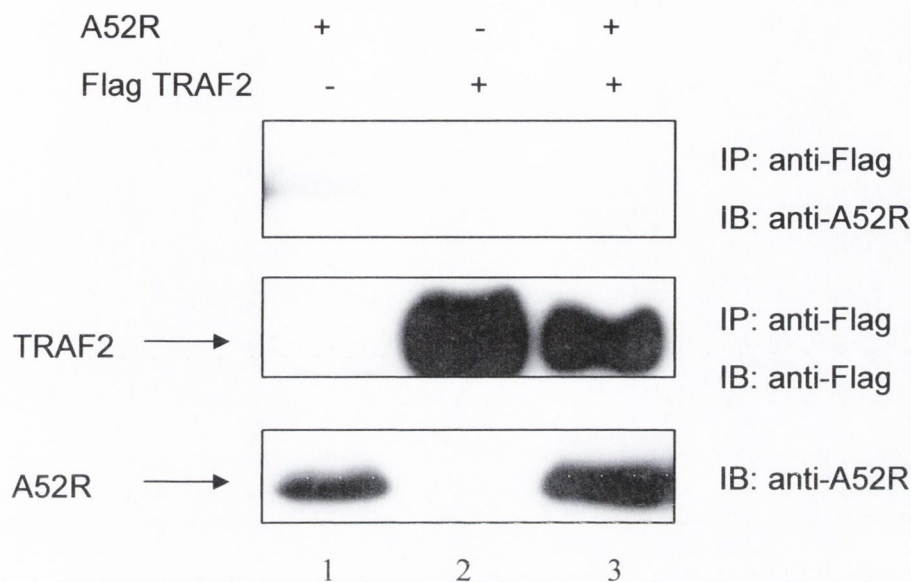


Fig. 3.9: A52R does not interact with TRAF2

HEK293T cells (1.5×10^6) were seeded into 100 mm dishes 24 hours prior to transfection. Four μg of each construct was transfected where indicated (+). The total amount of DNA (8 μg) was kept constant by supplementation with pcDNA 3.1. Cells were harvested 24 hours post-transfection and immunoprecipitation experiments carried out as described (Section 2.7.5). The top panel corresponds to lysates immunoprecipitated with anti-flag and blotted for the presence of A52R. The appearance of a band in lane 3 that is not detected in lanes 1 and 2 is indicative of an interaction. The second panel corresponds to lysates immunoprecipitated with anti-flag and blotted for the same antibody. The lower panel shows lysates probed for A52R. Data is a representative of one experiment from a minimum of three separate experiments.

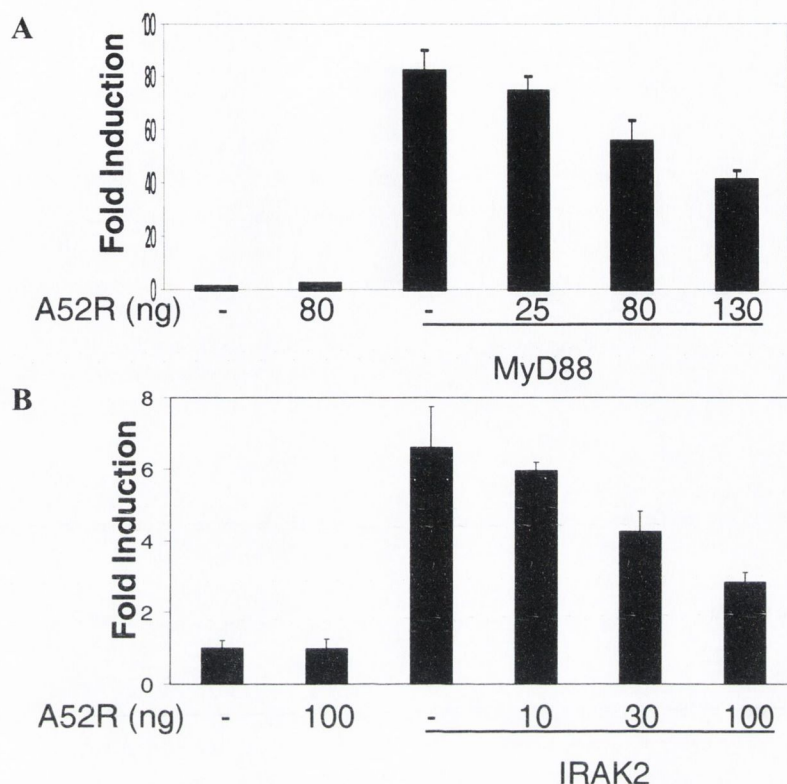


Fig. 3.10: A52R antagonises MyD88 and IRAK2 mediated NFκB activation

HEK293R1 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κB-luciferase reporter gene and Renilla-luciferase internal control. Cells were transfected with increasing amounts of plasmid encoding A52R, as indicated. 25 ng of plasmid encoding MyD88 (A) or 50 ng of a plasmid encoding IRAK 2 (B) was also transfected where indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

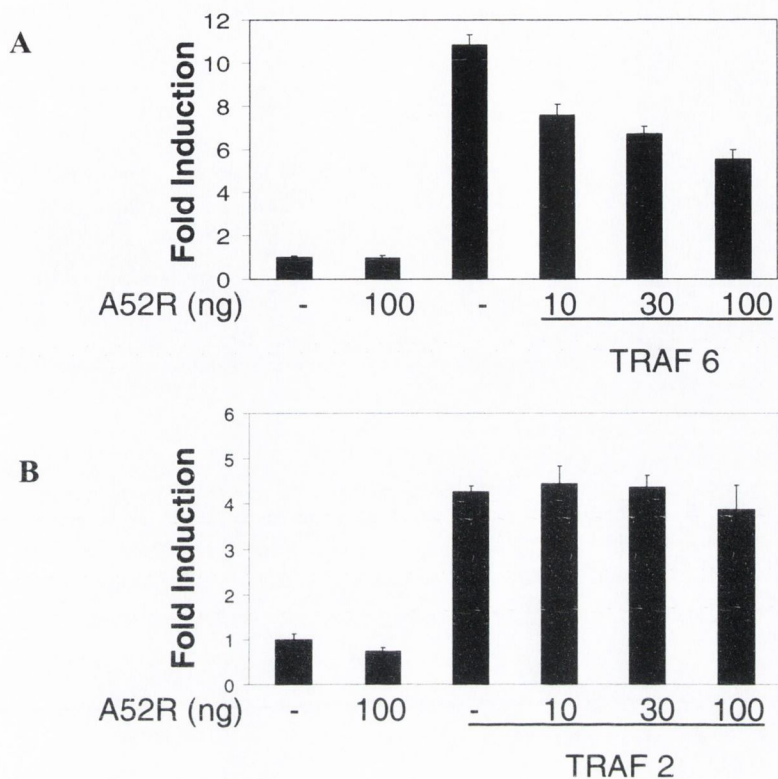


Fig. 3.11: A52R antagonises TRAF6 but not TRAF2 mediated NFκB activation

HEK293R1 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κB-luciferase reporter gene and Renilla-luciferase internal control. Cells were transfected with increasing amounts of plasmid encoding A52R as indicated. Fifty ng of plasmid encoding TRAF 6 (A) or TRAF 2 (B) was also transfected where indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

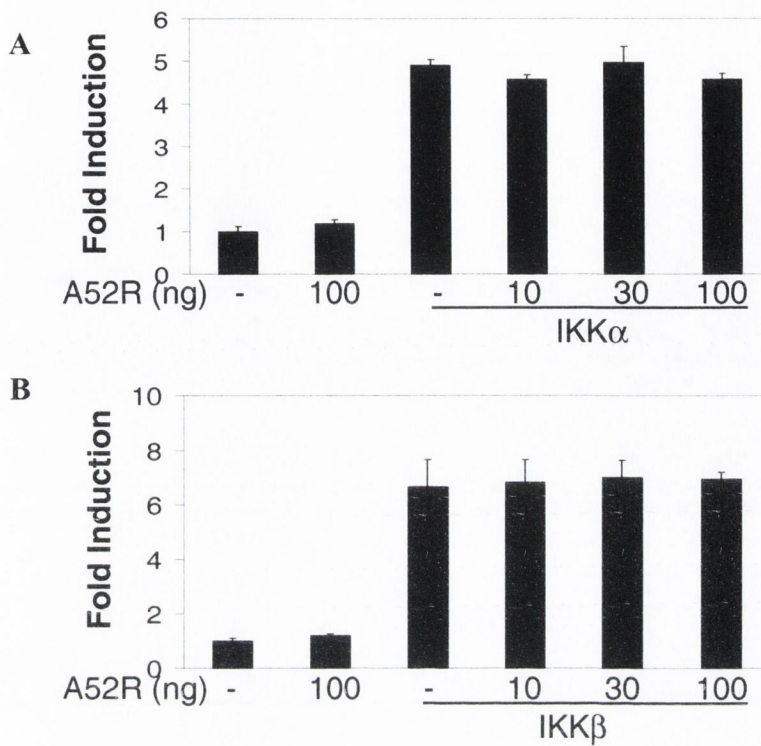


Fig. 3.12: A52R can not antagonise IKK α or IKK β induced NF κ B activation

HEK293R1 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κ B-luciferase reporter gene and Renilla-luciferase internal control. Cells were transfected with increasing amounts of plasmid encoding A52R as indicated. Fifty ng of plasmid encoding IKK α (A) or 50 ng of a plasmid encoding IKK β (B) was also transfected where indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

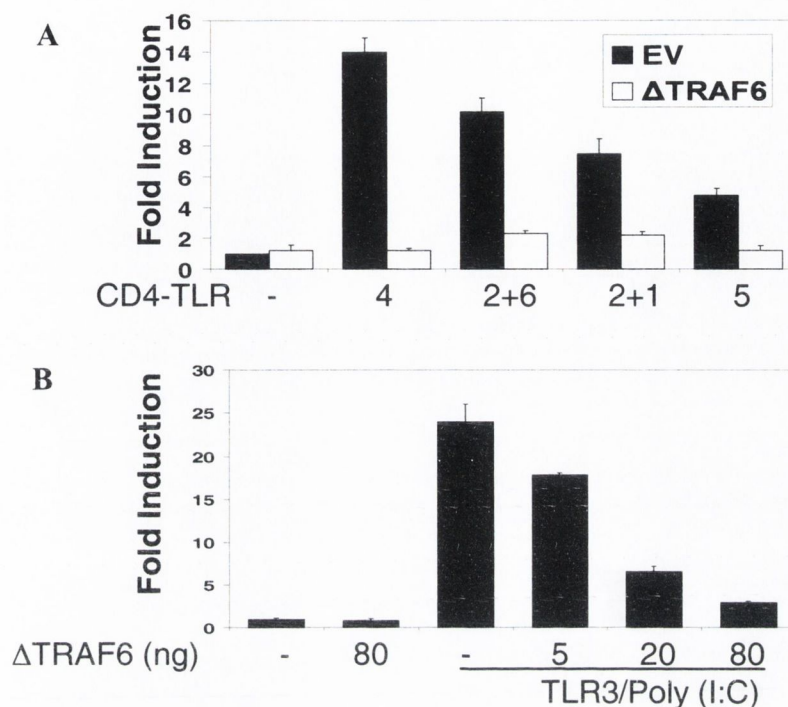


Fig. 3.13: A role for TRAF6 in activation of TLR-induced NFκB activation

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κB-luciferase reporter gene and Renilla-luciferase internal control. (A) Cells were transfected with constitutively active CD4-TLRs (50 ng TLR 4 or TLR 5, or 25 ng each of TLR 2 & TLR 6 or TLR 2 & TLR 1) in the presence of 80 ng empty vector (EV), or plasmid encoding ΔTRAF 6, together with NFκB reporter plasmid. (B) HEK 293 cells were transfected with empty vector (EV), 0.5 ng TLR 3 or 0.5 ng TLR 3 plus increasing amounts of plasmid encoding ΔTRAF 6. Six hours prior to harvesting cells were stimulated with 25 μg/ml poly (I:C) were indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

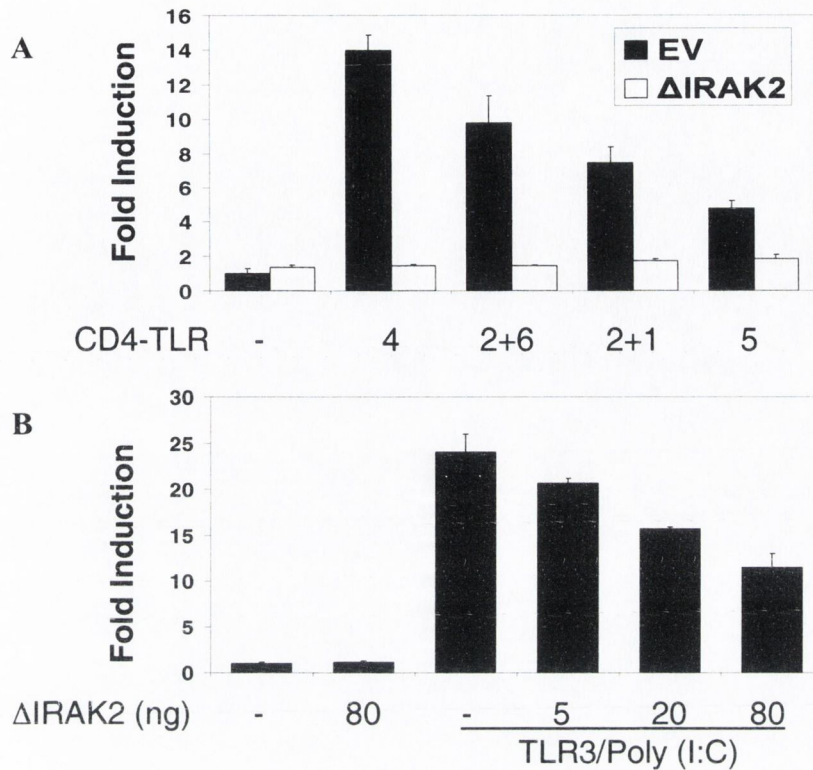


Fig. 3.14: A role for IRAK2 in activation of TLR-induced NF κ B activation

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κ B-luciferase reporter gene and Renilla-luciferase internal control. (A) Cells were transfected with constitutively active CD4-TLRs (50 ng TLR 4 or TLR 5, or 25 ng each of TLR 2 & TLR 6 or TLR 2 & TLR 1) in the presence of 80 ng empty vector (EV), or plasmid encoding Δ IRAK2, together with NF κ B reporter plasmid. (B) HEK 293 cells were transfected with empty vector (EV), 0.5 ng TLR 3 or 0.5 ng TLR 3 plus increasing amounts of plasmid encoding Δ IRAK2. Six hours prior to harvesting cells were stimulated with 25 μ g/ml poly (I:C) were indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

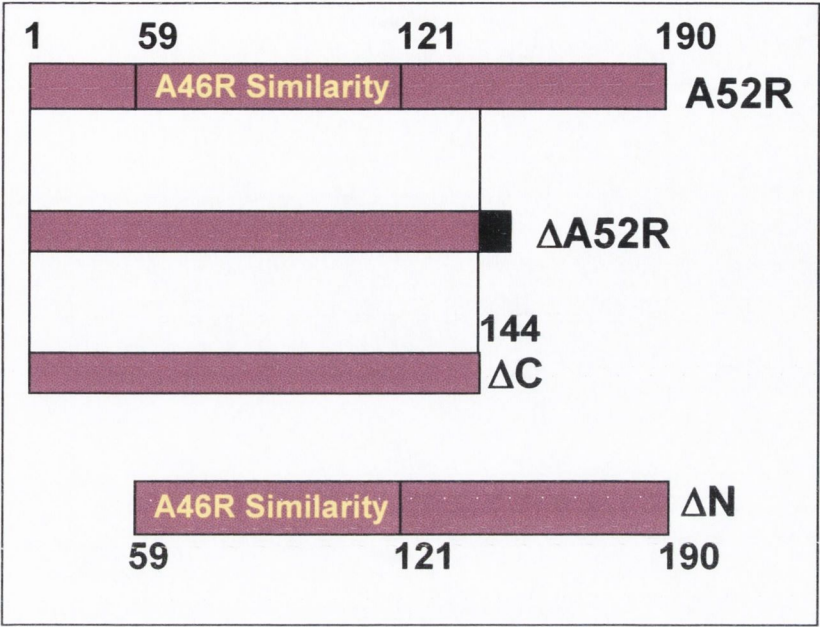


Fig. 3.15: Schematic representation of constructs of A52R

The region of similarity between A52R and A46R is highlighted in yellow. The deletions were generated by PCR from full length A52R and cloned into pRK5. Δ A52R encodes amino acids 1-144 of and includes an extra 27 amino acids derived from the vector sequence. Δ C encodes amino acids 1-144 of A52R and Δ N encodes amino acids 59-190.

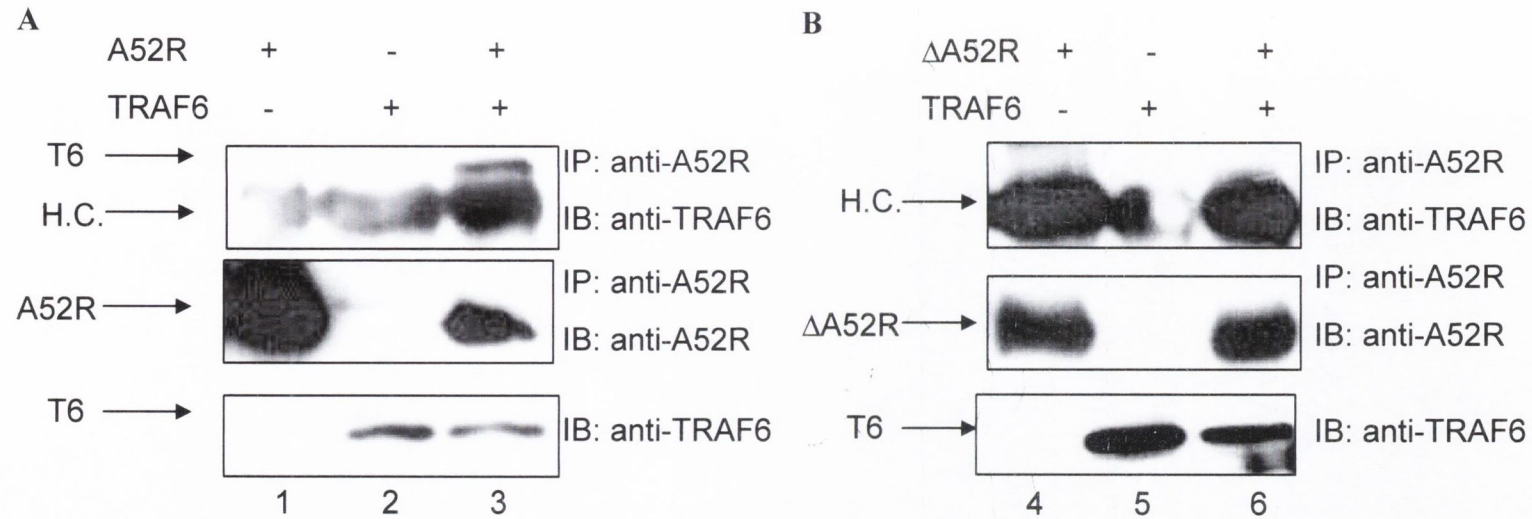


Fig. 3.16 Δ A52R no longer interacts with TRAF6

HEK293T cells (1.5×10^6) were seeded into 100 mm dishes 24 hours prior to transfection. Four μ g of each construct was transfected where indicated (+). The total amount of DNA (8 μ g) was kept constant by supplementation with pcDNA 3.1. Cells were harvested 24 hours post-transfection and immunoprecipitation experiments carried out as described (Section 2.7.5). The top panel corresponds to lysates immunoprecipitated with anti-A52R and blotted for the presence of TRAF6. The appearance of a band in lane 3 that is not detected in lanes 1 and 2 is indicative of an interaction. H.C., antibody heavy chain. The second panel corresponds to lysates immunoprecipitated with anti-A52R and blotted for the same antibody. The lower panel shows lysates probed for TRAF6. Data is a representative of one experiment from a minimum of three separate experiments.

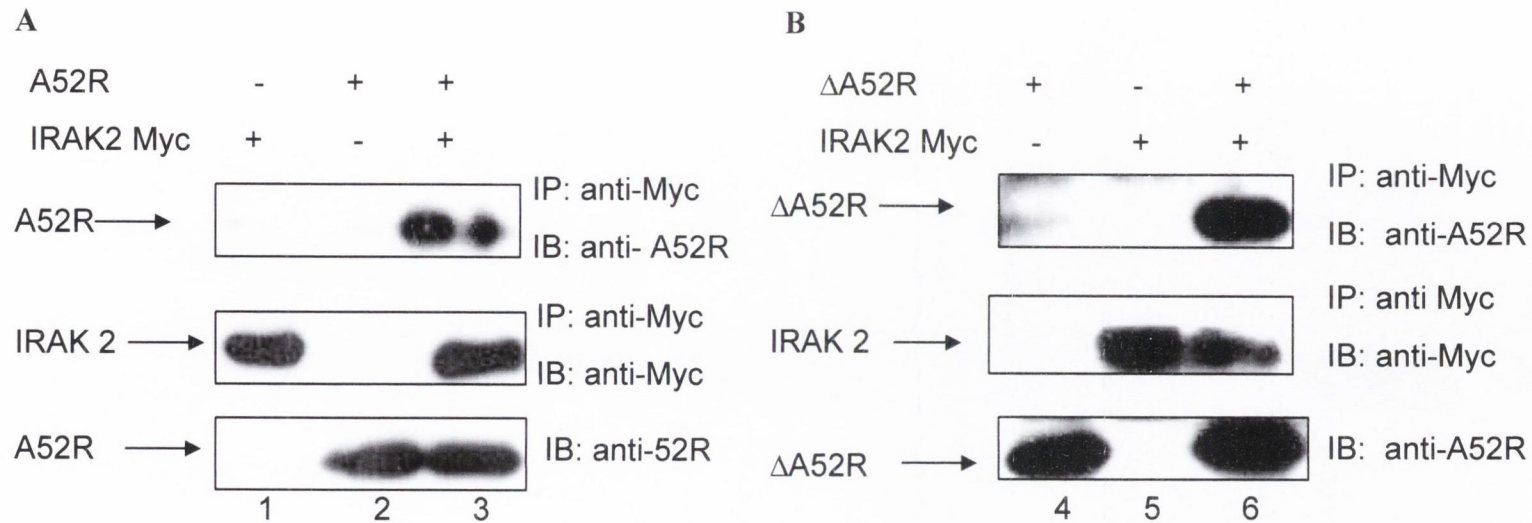
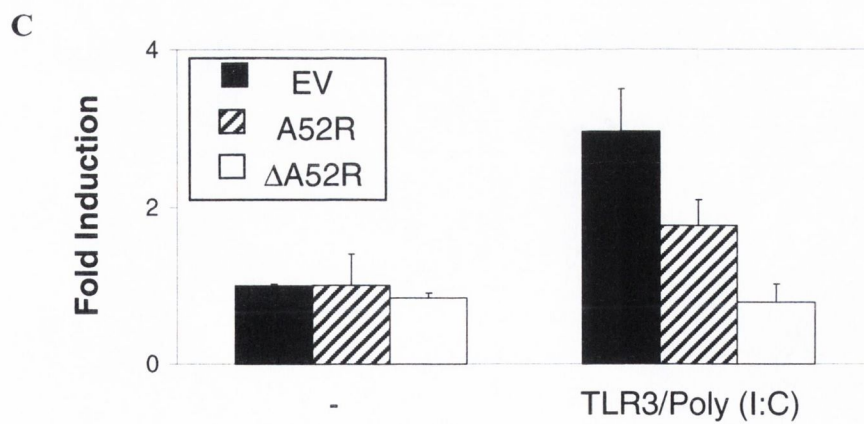
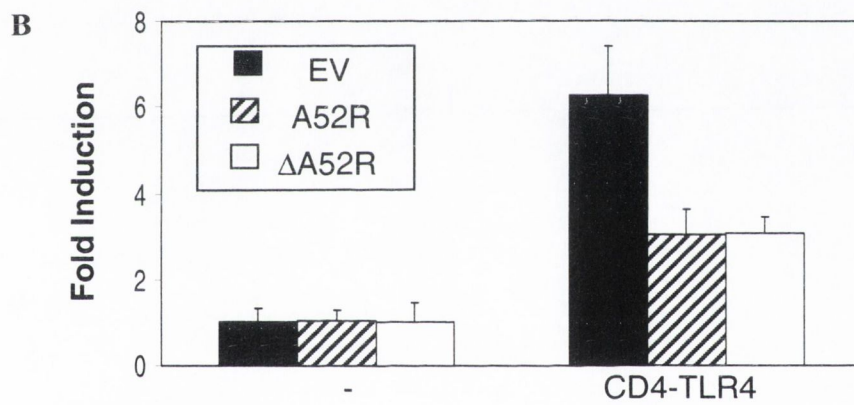
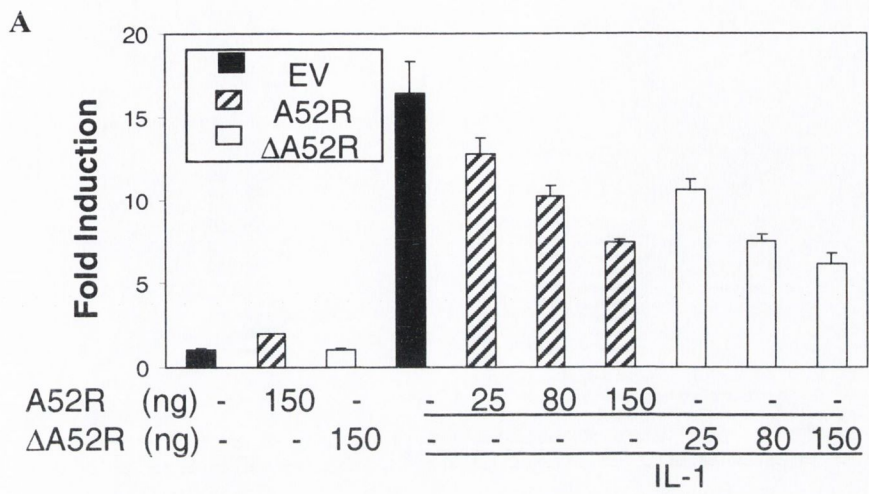


Fig. 3.17 Δ A52R interacts with IRAK2

HEK293T cells (1.5×10^6) were seeded into 100 mm dishes 24 hours prior to transfection. Four μ g of each construct was transfected where indicated (+). The total amount of DNA (8 μ g) was kept constant by supplementation with pcDNA 3.1. Cells were harvested 24 hours post-transfection and immunoprecipitation experiments carried out as described (Section 2.7.5). The top panel corresponds to lysates immunoprecipitated with anti-myc and blotted for the presence of A52R. The appearance of a band in lane 3 that is not detected in lanes 1 and 2 is indicative of an interaction. The second panel corresponds to lysates immunoprecipitated with anti-myc and blotted for the same antibody. The lower panel shows lysates probed for A52R. Data is a representative of one experiment from a minimum of three separate experiments.

Fig. 3.18 Δ A52R antagonises multiple IL-1R/TLR induced NF κ B activation

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κ B-luciferase reporter gene and Renilla-luciferase internal control as previously described. (A) Cells were transfected with increasing amounts of plasmid encoding A52R or Δ A52R as indicated and stimulated with 100 ng/ml IL-1 six hours before harvesting. (B) Cells were transfected with 50 ng of constitutively active CD4-TLR4 in the presence of 100 ng empty vector (EV), A52R or Δ A52R, together with NF κ B reporter plasmid. (C) Cells were transfected with 0.5 ng TLR 3 and stimulated with 25 μ g/ml poly (I:C) where indicated, in the presence of 100 ng of empty vector (EV) , A52R or Δ A52R. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.



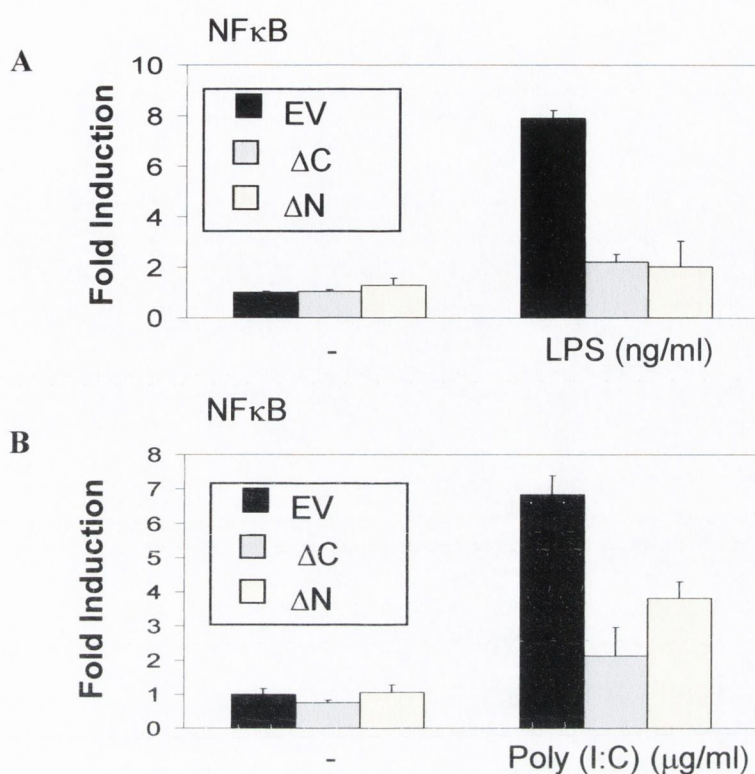


Fig. 3.19 Δ C and Δ N antagonise TLR3 and TLR4 induced NF κ B

HEK-TLR4 cells (A) or HEK-TLR3 (B) (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κ B-luciferase reporter gene and Renilla-luciferase internal control. Cells were transfected with 100 ng of plasmid encoding Δ C or Δ N as indicated and stimulated with 100 ng/ml LPS or 25 μ g/ml poly (I:C) were indicated six hours before harvesting. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

3.9 Discussion

In the present study the Vaccinia virus protein A52R has been shown to act as a broad intracellular inhibitor of NF κ B activation by IL-1R/TLR family members. When overexpressed in mammalian cells, A52R was shown to specifically interfere with IL-1-induced NF κ B signal transduction and have no inhibitory effect on TNF-induced NF κ B activation. A52R also inhibited NF κ B induction by multiple TLRs, including TLR3, TLR7 and TLR9, which have been implicated in anti-viral innate immunity. A52R associated with IRAK2 and TRAF6, two key proteins important in TLR signal transduction. Interestingly, a truncated version of A52R that only interacted with IRAK2 was found to potently block IL1/TLR-induced NF κ B activation. Therefore, this study reveals that the interaction between A52R and IRAK2 is sufficient for maximal NF κ B inhibition.

Members of the IL-1/TLR superfamily are key mediators in innate and adaptive immunity (Akira *et al.*, 2001). During the course of viral infection the body mounts several lines of host defence involving members of the IL-1R/TLR superfamily. For example, IL-1 is responsible for inducing fever, and IL-18 is a potent inducer of IFN- γ (Tanaka-Kataoka *et al.*, 1999). To circumvent the host immune response, the VV genome contains numerous genes encoding proteins involved in immunomodulation and immunoevasion. For example, VV secretes a decoy receptor for IL-1 called B15R. Furthermore, administration of IL-18 has been shown to elicit antiviral effects in VV-infected mice (Tanaka-Kataoka *et al.*, 1999), while deletion of the VV IL-18 binding protein caused virus attenuation in vivo (Symons *et al.*, 2002).

Although the majority of work on TLRs has focused on detection of bacteria, it is becoming increasingly apparent that viruses are also subject to innate sensing by TLRs. Recent work has implicated TLR2, 3, 4, 7, 8 and 9 as crucial mediators of an innate immune response to viral infection (Section 1.). Cell-surface TLR2 and TLR4 may recognise viral glycoproteins on virions (Vaidya and Cheng 2003, Rassa and Ross, 2003). For example, the viral attachment F protein from RSV has been shown to be TLR4 dependent (Haynes *et al.*, 2001), whereas measles virus haemagglutinin activates murine and human cells via TLR2 (Bieback *et al.*, 2002). In terms of viral nucleic acids, TLR3 has been shown to be activated in response to poly(I:C), a synthetic dsRNA analogue (Alexopoulou *et al.*, 2001) while TLR7 and TLR8 have been shown to recognise ssRNA (from influenza, HIV and vesicular stomatitis virus) (Diebold *et al.*, 2004, Heil *et al.*, 2004, Lund *et al.*, 2004). TLR9 recognises genomic DNA of herpes simplex virus-1 and 2 and murine CMV (Krug *et al.*, 2004, Lund *et al.*, 2003, Tabeta *et al.*, 2004). However, no TLR has yet been shown to recognise VV.

An indicator of the importance of any host antiviral strategy is the ability of viruses to counteract that particular arm of the host defence. Therefore if the TLR family is truly important in antiviral host defence, mechanisms must exist to antagonise this family. The VV proteins A52R and A46R were the first viral proteins shown to specifically target the IL1/TLR pathway (Bowie *et al.*, 2000). These two proteins were identified during a database search for novel TIR domain containing proteins. A46R was identified as being related to the IL1/TLR family at a statistically significant level. A BLAST search with A46R then recognised A52R as the highest scoring sequence producing significant similarity (Bowie *et al.*, 2000).

A52R had previously been shown to inhibit IL-1R, IL-18 and TLR4-induced NF κ B activation (Bowie *et al.*, 2000). Recently, A46R has been shown to interact with MyD88, Mal, TRIF and TRAM and thereby interfere with downstream TLR signalling events (Bowie *et al.*, 2001, Stack *et al.*, 2005). Another VV protein, N1L, has been shown to suppress NF κ B activation by IL-1R/TLRs by targeting the IKK complex to achieve maximal NF κ B inhibition (DiPerna *et al.*, 2004).

The role of A52R in the VV life cycle has also been investigated by the construction of a deletion mutant lacking the A52R gene and by the comparison with wild type and revertant controls. The loss of the A52R gene did not affect the replication of the virus in cell culture. However, in a murine intranasal model the deletion mutant caused reduced weight loss and milder signs of illness compared to controls. Thus the A52R protein contributes to virus virulence and this is likely to be due to the inhibition of IL-1R/TLR signalling (Harte *et al.*, 2003). Importantly, deletion of both *A46R* and *N1L* genes from VV also affects virus virulence (Stack *et al.*, 2005, Bartlett *et al.*, 2002). Therefore, the fact that VV encodes three TLR antagonists truly signifies the importance of TLRs in antiviral host defence and indicates that TLR responses pose a realistic threat to viruses.

Recently, a second virus has been shown to specifically target TLR3 signalling pathways. Hepatitis C virus (HCV) has serine protease, NS3/4A which caused specific proteolysis of TRIF. This study showed that NS3/4A-mediated cleavage of TRIF reduced the abundance of TRIF and inhibited poly(I:C) activated signalling through TLR3 to IRF3 and NF κ B (Li *et al.*, 2005).

Although IL-1 and TNF lead to similar pro-inflammatory pathways being activated, TNF-induced NF κ B activation was unaffected by A52R (Fig 3.2b, 3.11b). Furthermore, A52R was unable to form a complex with TRAF2 (Fig 3.9). TNF activates a signal transduction pathway distinct from IL-1, and does not use IRAK2 or TRAF6 (Hsu *et al.*, 1996). A52R was capable of blocking IL-1-induced, but not TNF-induced I κ B α degradation, providing a rationale for its specific effect on IL-1R/TLR-dependent NF κ B reporter gene activity (Fig 3.3).

A52R likely acts as a global inhibitor of TLR-induced NF κ B signalling, since it was shown to inhibit NF κ B activation by TLR3, CD4-TLR4, CD4-TLR5 CD4-TLR2&1 and CD4-TLR 2&6 (Fig. 3.4). Furthermore the addition of TLR agonists to a murine macrophage cell line RAW 264.7, a much used model system for TLR activation, confirmed the ability of A52R to inhibit TLR-induced NF κ B activation (Fig 3.6). Interestingly A52R was unable to inhibit NF κ B activation by IKK α and IKK β , (Fig 3.12), two downstream signalling components of the NF κ B pathway (Ghosh *et al.*, 1998). This observation is consistent with the fact that A52R could be co-immunoprecipitated with either TRAF6 (Fig 3.7) or IRAK2 (Fig. 3.8) since these two signalling molecules are found upstream of the IKK complex. No interaction was detected between A52R and MyD88, Mal or IRAK (Harte *et al.*, 2003).

The strength of the interaction with each partner suggests that each interaction is direct, rather than A52R interacting with one protein via a “bridge” with the other. This is theoretically possible since IRAK2 and TRAF6 interact (Muzio *et al.*, 1997, Boch *et al.*, 2003). It has been demonstrated that the death domain of IRAK2 is

required for its association with A52R (Harte *et al.*, 2003). This domain is not thought to be responsible for interacting with TRAF6 (Boch *et al.*, 2003). The region of IRAK2 responsible for the association with TRAF6 is contained within the amino acids 98-282 sequence (Boch *et al.*, 2003). It has also been shown that Mal and IRAK2 interact (Fitzgerald *et al.*, 2003) but it is unclear how this interaction occurs since Mal does not contain a death domain. However, the interaction of A52R with IRAK2 was able to disrupt IRAK2/Mal interactions (Harte *et al.*, 2003). Therefore it is likely that IRAK2 somehow binds Mal via its death domain. Furthermore, the interaction of A52R with TRAF6 disrupted TRAF6/TAK1 interactions (Harte *et al.*, 2003). Therefore, the disruption of signalling complexes by A52R provides a mechanistic explanation for its ability to inhibit TIR dependent signalling.

Further evidence is presented in this chapter that the IRAK2 interaction may be more important than the TRAF6 interaction for NF κ B inhibition. Indeed, a truncated version of A52R which only interacted with IRAK2 was capable of inhibiting NF κ B activation by IL-1, TLR4 and TLR3 (Fig. 3.18). Recently, a peptide derived from the A52R protein was characterised and shown to inhibit cytokine production in response to both bacterial and viral PAMPs (McCoy *et al.*, 2005). In addition, this peptide was shown to inhibit the phosphorylation of I κ B α in LPS-activated cells (McCoy *et al.*, 2005). Interestingly, all of the truncations of A52R that were made, Δ A52R, Δ C and Δ N, contain the amino acid sequence of the inhibitory peptide and these truncations were all capable of blocking TLR3 and TLR4-induced NF κ B activation (Fig. 3.18, 3.19). This suggests that the inhibition observed by the peptide is mediated by the IRAK2 interaction of A52R.

A52R is the first viral protein identified to target IRAK2. The targeting of IRAK2 by VV together with the evidence from the use of IRAK2 dominant-negative for a role for IRAK2 in TLR2, 3, 4 and 5, suggests an important role for IRAK2 in anti-viral immunity and TLR signalling in general. Signalling between MyD88 and TRAF 6 is mediated by members of the IRAK family (Muzio, M., *et al.*, 1997, Cao, Z., *et al.*, 1999, Wesche, H., *et al.*, 1999, Li, S., *et al.*, 2002) however; the exact function of each IRAK protein remains controversial. Very little is known about IRAK2 and its particular function in IL-1R/TLR signalling. When overexpressed it can associate with MyD88 (Muzio *et al.*, 1997), Mal (Fitzgerald *et al.*, 2001) and TRAF6 (Muzio *et al.*, 1997, Boch *et al.*, 2003). In contrast to IRAK1 it does not seem to have kinase activity, is not hyperphosphorylated and is not degraded after LPS stimulation (Hu *et al.*, 2001). IRAK2, like IRAKM, can compensate for lack of IRAK1 in mutated 293 cell line in response to IL-1 stimulation (Li *et al.*, 1999b). The observation that IRAK2 is involved in TLR3 signalling (Fig. 3.14) is interesting since another study suggested that poly(I:C)-induced TLR3-mediated NF κ B activation is through an IRAK1 independent pathway (Jiang *et al.*, 2003b). This study demonstrated that IRAK1 deficient cells are responsive to poly(I:C) in terms of NF κ B activation, although IRAK2 deficient cells were not tested. The generation of an IRAK2 knockout mouse will be required to determine the exact role of IRAK2 in anti-viral immunity and TLR signalling. Further investigations into the specific role of IRAK2 in IL-1R/TLR signalling are currently being carried out in our laboratory.

The targeting of a TRAF protein by a virus is not unique to Vaccinia virus. Hepatitis C virus non-structural 5A protein (Pork *et al.*, 2002) and the rotavirus

capsid protein VP4 (La Monica *et al.*, 2001) are known to affect intracellular signalling by targeting TRAF2. The oncogenic latent membrane protein 1 (LMP1) of the Epstein-Barr virus harbours the TRAF binding motif PxQxT in its C-terminal and can interact with TRAF6. This protein affects intracellular signalling pathways, such as NFκB and p38 MAP kinase activation (Schultheiss *et al.*, 2001). Although A52R does not have a specific TRAF6 binding consensus sequence (Pro-X-Glu-X-X-aromatic/acidic residue), it clearly forms an interaction with TRAF6 (Fig 3.7). Interestingly, although TRAF2 shares 29% sequence identity with TRAF6 in its TRAF domain (Wajant *et al.*, 2001), and A52R interacts with the TRAF domain of TRAF6 (Chapter 5, Fig. 5.5), it does not associate with TRAF2 (Fig. 3.9). The functional significance of the A52R-TRAF6 interaction is examined in Chapter 5.

The targeting of two key signalling molecules involved in a diverse range of TLR signalling pathways is likely to confer the Vaccinia virus with a selective advantage in the presence of the host immune system. Apart from NFκB activation, another important anti-viral transcription factor activated by TLRs is IRF3. The next chapter examines the effect of A52R on TLR-IFN pathways. It points to the functional differences between the VV proteins A52R and A46R. Furthermore, it highlights the fact that both the effects of A52R and A46R on TLR signalling are quite distinct and therefore these two TLR antagonists are not functionally redundant.

Chapter 4

A52R does not block IRF activation by TLRs

4.1 Introduction

Infection of cells by viruses elicits a potent and immediate response characterized by the induction and secretion of type I IFNs (IFN- α and IFN- β) and the subsequent transcriptional upregulation of ISGs (Goodbourn *et al.*, 2000, Stark *et al.*, 1998). Virus-mediated IFN production occurs in a biphasic manner (Taniguchi *et al.*, 2001) and involves several proteins from the IRF family, a growing collection of transcription factors that play distinct roles in many biological responses (Reviewed in Taniguchi *et al.*, 2001). Constitutively produced IRF3, along with other transcription factors such as NF κ B (Mamane *et al.*, 1999) weakly activate the IFN- β promoter. Secreted IFN- β binds to the IFN receptor on surrounding cells initiating the JAK/STAT IFN signal transduction cascade (Levy 1995) and resulting in the induction of IRF7. In the late phase, IRF3 and IRF7 collectively amplify IFN- α/β gene induction, leading to full ISG stimulation through the IFN signalling pathway.

Doyle *et al.*, 2002 clearly showed that TLR signalling can induce an anti-viral state in that pre-treatment of cells with poly(I:C) or lipid A, the moiety of LPS recognised by TLR, inhibited the replication of a murine herpesvirus in macrophages. Since then it has been shown that triggering of the TLR3 and TLR4 pathways activates the anti-viral transcription factor IRF3 in a MyD88-independent manner. The TIR adaptor molecule TRIF is essential to this MyD88-independent signalling via TLR3 and TLR4 (Yamamoto *et al.*, 2003a, Hoebe *et al.*, 2003a, Yamamoto *et al.*, 2002b). TRIF probably associates directly with TLR3, but indirectly with TLR4, via a bridging interaction with TRAM (Fitzgerald *et al.*, 2003b, Yamamoto *et al.*, 2003b). Two IKK-related kinases, TBK1 and IKKi, are

involved in phosphorylation and activation of IRF3 downstream of TRIF (Fitzgerald *et al.*, 2003a, Sharma *et al.*, 2003). Another important dsRNA-signalling pathway is initiated by RIG-1 (Yoneyama *et al.*, 2004). RIG-1 can activate IRF3 in a TLR3-independent but dsRNA-dependent manner.

Recently, the role of IRF5 and IRF7 in TLR signalling leading to the production of type I IFNs (IFN- α and IFN- β) has been investigated. Unlike IRF3, which is ubiquitously expressed, expression of IRF5 and IRF7 is restricted to B cells and dendritic cells, although their expression is inducible in other cell types by type I IFN stimulation (Barnes *et al.*, 2001, Izaguirre *et al.*, 2003). Plasmacytoid DCs constitutively express IRF5 and IRF7 and rapidly produce type I IFNs in response to viral infection or stimulation with the TLR7 and TLR8 agonist R-848 or the TLR9 agonist CpG DNA (Gibson *et al.*, 2002, Siegal *et al.*, 1999). Since TLR7, 8 and 9 only use one TIR adaptor to signal, MyD88, this implies that activation of the genes encoding type I IFNs in plasmacytoid DCs occurs in a MyD88-dependent manner. Recently, two studies showed that induction of type I IFNs through TLR7, 8 and 9 involves a direct interaction of IRF7 and MyD88 (Kawai *et al.*, 2004, Honda *et al.*, 2004). Other groups demonstrated that IRF5 interacts with and is activated by MyD88 and TRAF6 and that type I IFN signalling by TLR 7 (Schoenomeyer *et al.*, 2005) or TLR 9 (Takaoka *et al.*, 2005) is mediated by IRF5 activation.

Recent evidence has shed light on the complex mechanism employed by TLRs to elicit an appropriate type I IFN response. This chapter explores the effect of A52R on TLR3 and TLR4 signalling in greater detail, with particular focus on the IFN

pathway. I show that A52R has no effect on TLR3- TLR4-, TRIF-or TRAM-mediated IRF3 activation. In addition, A52R was unable to block MyD88- or TRAF6-induced IRF5 or IRF7 activation, in contrast to inhibition of these signals by the VV TLR antagonist A46R. Therefore, the effects of A52R and A46R on TLR-signalling are functionally distinct.

4.2 The Effect of A52R on TLR3-Induced Signalling

In the last chapter A52R was shown to be capable of interacting with IRAK2 and TRAF6 and to block every IL-1R/TLR pathway to NF κ B activation tested. This strongly supports the emerging role of TLRs in the host response to viral infection. The interaction between A52R and IRAK2 was shown to be fundamental for inhibition of NF κ B by various TLRs. It was also shown by Harte *et al.*, (2003) that A52R can disrupt the formation of active signalling complexes that contain either TRAF6 or IRAK2 thus, providing a mechanistic explanation for the ability of A52R to inhibit TIR dependent signalling to NF κ B (Harte *et al.*, 2003). However, the effect of A52R on other transcription factors was not examined.

Here, the effect of A52R on TLR induction of the IFN- β promoter was examined. Fig. 4.1a shows that poly(I:C) treatment of cells that had been transfected with TLR3 led to activation of the IFN- β promoter. Consistent with the fact that activation of the IFN- β -promoter is NF κ B dependent, A52R dose-dependently inhibited poly(I:C)/TLR3 induction of this promoter (Fig. 4.1a). The induction of IFN- β also involves that transcription factor IRF3. IRF3 is a member of the IFN regulatory transcription factor family. It is found in an inactive cytoplasmic form that upon serine/threonine phosphorylation forms a complex with CBP. This complex translocates to the nucleus, binds to the interferon stimulated response element (ISRE) and activates the transcription of type I IFNs (e.g. IFN- β) and ISGs. Poly(I:C) can induce IRF3 activation through TLR3 (Doyle *et al.*, 2002, Servant *et al.*, 2003). To determine the effect of A52R on TLR3-induced IRF3 activation a transactivation reporter gene assay was carried out. For this, IRF3 activation by TLR3 was assessed by using an IRF3-Gal4 fusion protein together with a Gal4-

dependent reporter plasmid, which requires IRF3 phosphorylation in order to induce the reporter gene (Fitzgerald *et al.*, 2003a). HEK-TLR3 cells were stimulated with poly(I:C) and this resulted in a 3.5-fold induction of this IRF3-dependent reporter. However, co-expression of a plasmid encoding A52R had no effect on poly(I:C) stimulated IRF3 activation (Fig. 4.1b). Next, the effect of A52R on TLR3 induction of an ISRE-dependent reporter was tested. Consistent with the inability of A52R to block TLR3-induced IRF3 activation, A52R could not inhibit TLR3-induced ISRE activation (Fig. 4.1c). In similar experiments another VV TLR antagonist, A46R, was capable of blocking both TLR3-mediated IRF3 and ISRE activation (Fig. 4.2 a, b). In contrast, Fig. 4.2c shows that unlike A52R, which potently inhibits TLR3-induced NF κ B activation (Fig. 3.4c), A46R had little effect on this signal. Hence, A52R and A46R are not functionally redundant and both are probably required to effectively shut down TLR3 signalling (Stack *et al.*, 2005).

During the course of this study the specific TIR adaptor for TLR3 signalling was identified. Two studies showed that TRIF (Yamamoto *et al.*, 2002b) also called TICAM-1 (Oshiumi *et al.*, 2003a), could activate NF κ B and IFN- β , and suggested that TRIF was specific for TLR3 signalling, being required for both NF κ B and IRF3 activation. The generation of TRIF deficient mice and the identification of a mouse with a mutation in the *Trif* gene termed *Lps*², which was defective in poly(I:C) and LPS induced IFN responses confirmed these results (Yamamoto *et al.*, 2003a, Hoebe *et al* 2003a). In addition these studies revealed a role for TRIF in TLR4-mediated activation of IRF3 and expression of IFN- β . Therefore, the effect of A52R on TRIF-dependent pathways was assessed. Overexpression of TRIF drove the IFN- β promoter and co-expression of a plasmid encoding A52R blocked this signal in a

dose-dependent manner (Fig. 4.3a). Activation of the NF κ B-dependent reporter gene by ectopic expression of TRIF was also blocked by A52R (Fig. 4.3b). Recently, investigation into the downstream signalling events that regulate TLR3-dependent TRIF-induced NF κ B activation revealed that RIP1 is recruited to TRIF and interacts with TRIF via a RIP homotypic interaction motif (Meylan *et al.*, 2004). Ectopic expression of RIP1 induced NF κ B activation (Fig. 4.4), however, no inhibition of this signal was observed with co-expression of A52R (Fig. 4.4), suggesting that if IRAK2 has a role in TLR3 signalling it is not present on the signalling pathway downstream of RIP1. Interestingly, a role for IRAK2 in TRIF-mediated NF κ B activation was also observed. Overexpression of TRIF drove the NF κ B reporter gene and co-expression of a dominant negative IRAK2 construct, Δ IRAK2, inhibited this signal (Fig. 4.4b).

Consistent with the lack of effect of A52R on TLR3-dependent activation of IRF3, A52R was also unable to block TRIF-dependent activation of IRF3 (Fig. 4.5a). As a control, a dominant-negative version of TBK1 (K38A, in which lysine 38 was changed to an alanine) was shown to block TRIF-dependent activation of IRF3 (Fig. 4.5b).

4.3 The Effect of A52R on TLR4-Induced Signalling

Next the ability of A52R to inhibit TLR4 induction of the IFN- β promoter was tested. Similar to the effect of A52R on poly(I:C) stimulated induction of the IFN- β promoter (Fig 4.1a), A52R dose-dependently inhibited LPS-mediated induction of this promoter (Fig 4.6a). The fact that A52R was unable to block TRIF-mediated IRF3 activation (Fig. 4.5a) and that TLR4-mediated activation of IRF3 is TRIF-

dependent suggested that A52R would have no inhibitory effect on TLR4-induced IRF3 activation. Fig.4.6b shows that this was indeed the case, overexpression of CD4-TLR4-induced IRF3 activation and no inhibition of this signal was observed upon co-expression of A52R. Interestingly, CD4-TLR4-mediated ISRE was inhibited dose-dependently by A52R (Fig. 4.6c).

The activation of the MyD88-independent signalling pathway by TLR4 involves the TIR adaptors TRIF (Yamamoto *et al.*, 2003a, Hoebe *et al.*, 2003a) and TRAM (Oshiumi *et al.*, 2003b, Fitzgerald *et al.*, 2003b, Yamamoto *et al.*, 2003b). TRAM is a unique TIR adaptor to the TLR4 signalling pathway (Yamamoto *et al.*, 2003b). In addition to a role in the MyD88-independent activation of IRF3, TRIF and TRAM are also involved in the late phase NF κ B activation by TLR4 (Yamamoto *et al.*, 2003b). Therefore, in order to fully investigate the effect of A52R on the TLR4 pathway, the effect of A52R on TRAM-mediated IRF3 activation was examined. Ectopic expression of TRAM resulted in activation of the IRF3 promoter (Fig. 4.7a). Co-expression of a plasmid encoding A52R was unable to block this signal (Fig. 4.7a). As a control, dominant negative TBK1 is shown to inhibit TRAM-induced IRF3 activation (Fig. 4.7b).

4.3 The Effect of A52R and A46R on IRF5 and IRF7 Activation

Recently, a specific role for IRF5 and IRF7 activation in TLR signalling has been identified (Kawai *et al.*, 2004, Honda *et al.*, 2004, Schoenomeyer *et al.*, 2005, Takaoka *et al.*, 2005). One group showed that IRF5 activation was required for the induction of type I IFNs by TLR7 (Schoenomeyer *et al.*, 2005) and another study demonstrated that IRF5 activation was essential in the induction of proinflammatory

cytokines, such as IL-6, IL-12 and TNF α by TLR9 (Takaoka *et al.*, 2005). Interestingly, both of these studies showed that IRF5 binds to both MyD88 and TRAF6. This is similar to IRF7, which has also been shown to interact with MyD88 and TRAF6 (Kawai *et al.*, 2004, Honda *et al.*, 2004). The interaction between IRF7 and MyD88 and TRAF6 facilitates the robust induction of type I IFNs (Kawai *et al.*, 2004, Honda *et al.*, 2004). Since A52R has no inhibitory effect on TLR3-mediated IRF3 activation (Fig. 4.1b) and A46R potentially inhibits this signal (Fig. 4.2a), I wondered what effect A52R and A46R would have on IRF5 and IRF7 activation. To test this, IRF5-Gal4 and IRF7-Gal4 fusion proteins were used in a transactivation assay as described earlier for IRF3. Ectopic expression of both MyD88 and TRAF6 led to activation of IRF5 (Fig. 4.8) and IRF7 (Fig. 4.9). Interestingly, no inhibition of either signal was observed by co-expression of A52R (Fig. 4.8, 4.9). In contrast, A46R inhibited both MyD88- and TRAF6-induced IRF5 and IRF7 activation (Fig. 4.8, 4.9). Overall, A52R can block TLR-induced NF κ B while exhibiting no effect on IRF activation. In contrast, A46R can block most TLR-induced NF κ B, with the exception of TLR3 but can also inhibit IRF signalling.

Fig. 4.1: The effect of A52R on TLR3-induced IFN- β -promoter, ISRE and IRF3-dependent reporter genes

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, IFN- β promoter reporter and Renilla-luciferase internal control and 0.5 ng of a plasmid encoding TLR3 (A). HEK-TLR3 (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression plasmids, ISRE promoter (B) or IRF3 reporter plasmid together with pFR luciferase reporter plasmid (C) and Renilla-luciferase internal. Cells were transfected with increasing amounts of plasmid encoding A52R (10, 30, 100 ng) as indicated. Six hours prior to harvesting cells were stimulated with 25 μ g/ml poly (I:C). After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

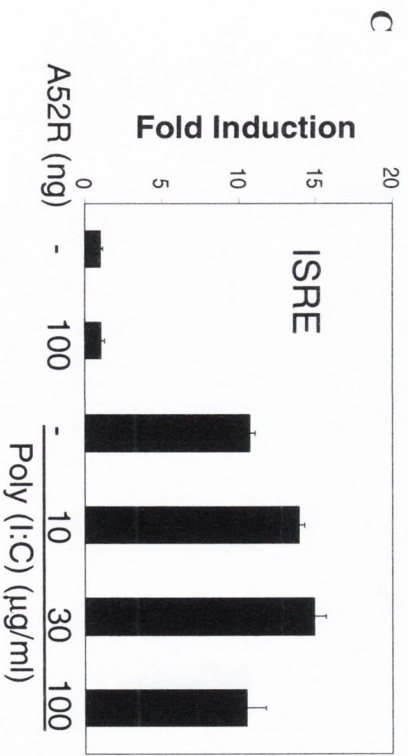
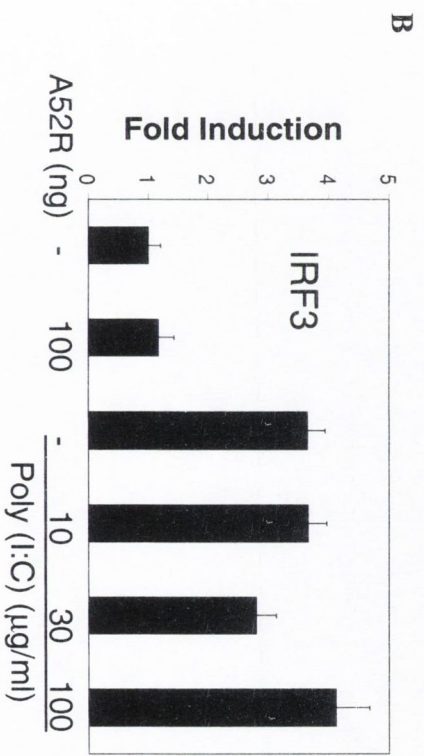
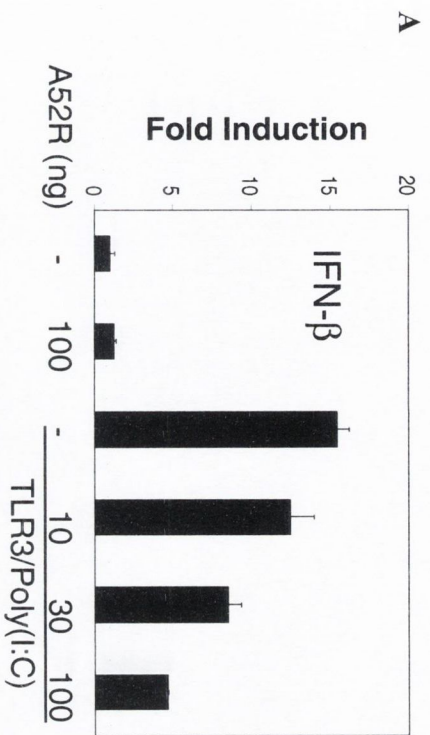
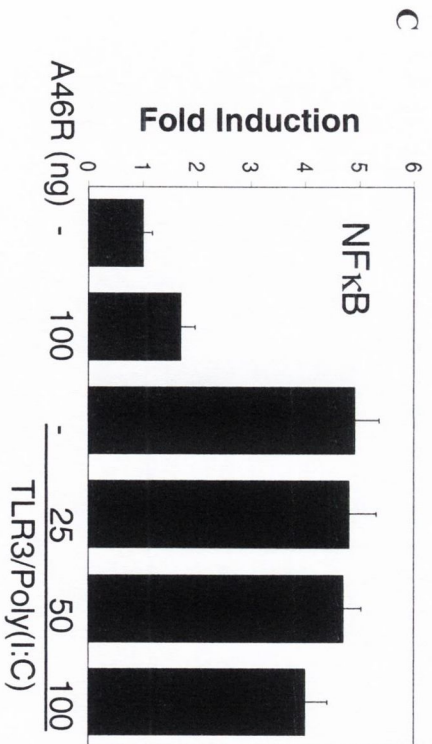
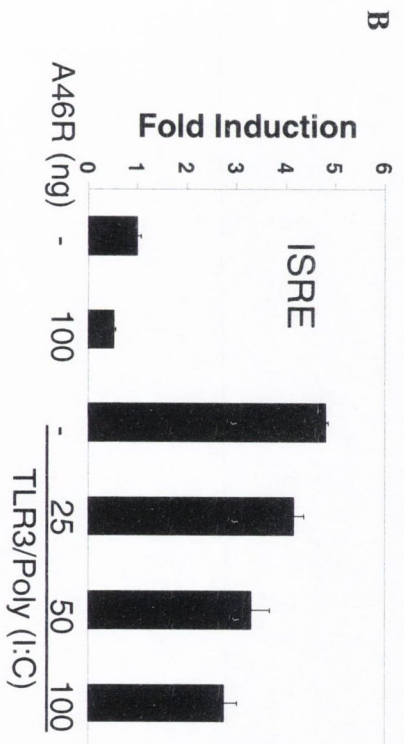
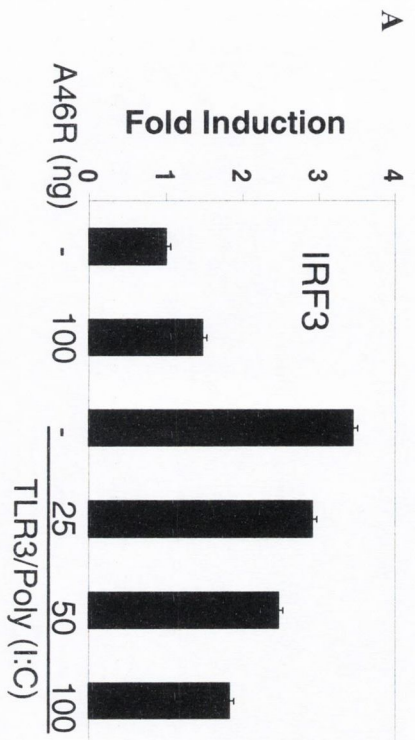


Fig. 4.2: The effect of A46R on TLR3-induced IRF3, ISRE and NFκB signalling

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with 0.5 ng TLR3 as indicated, the IRF3 reporter plasmid together with pFR luciferase reporter plasmid (A) the ISRE promoter (B) or NFκB reporter (C) and Renilla-luciferase internal control. Cells were transfected with increasing amounts of plasmid encoding A46R as indicated. Six hours prior to harvesting cells were stimulated with 25 µg/ml poly (I:C) as indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.



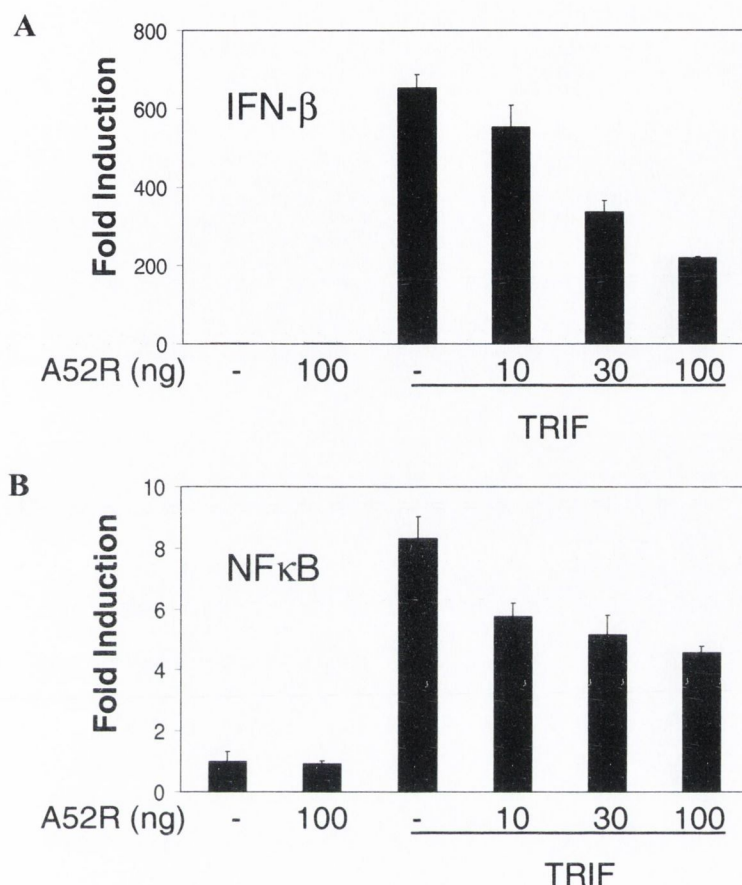


Fig. 4.3 The effect A52R on TRIF-mediated NF κ B and IFN- β signalling

HEK29 R1 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, IFN- β promoter (A) or κ B-luciferase reporter gene (B) and Renilla-luciferase internal control. Cells were transfected with increasing amounts of plasmid encoding A52R and 10 ng of a plasmid encoding TRIF as indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

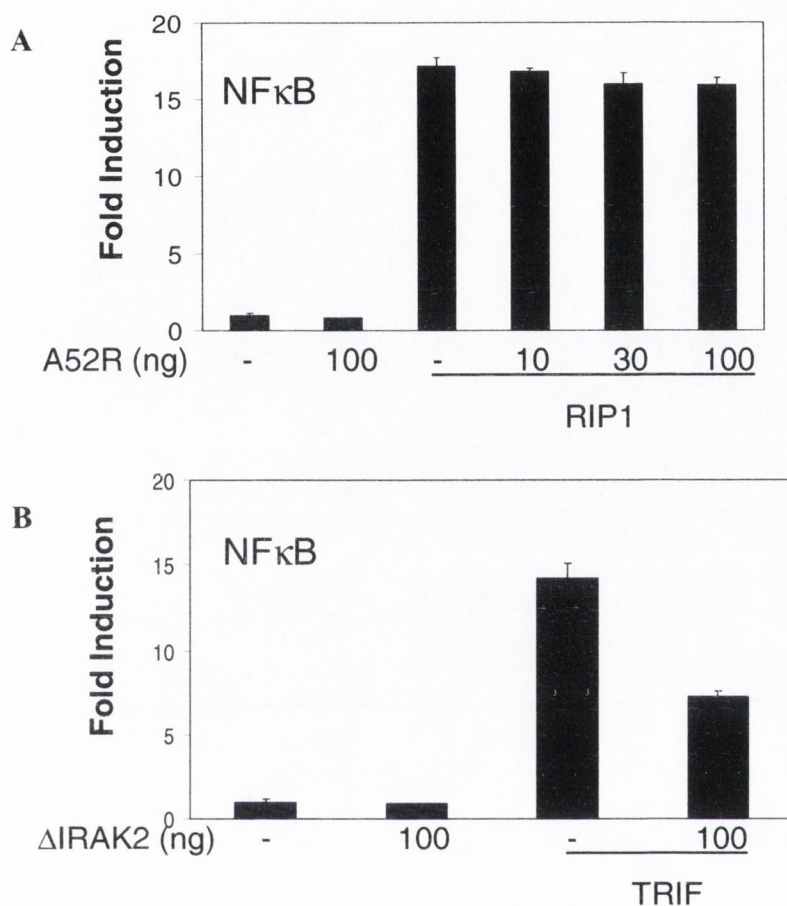


Fig. 4.4 The effect of A52R on RIP1 induced NF κ B activation and a role for IRAK2 in TRIF-induced NF κ B activation

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κ B-luciferase reporter gene and Renilla-luciferase internal control. Cells were transfected with 25 ng RIP1 and increasing amounts of A52R as indicated (A) or 100 ng of Δ IRAK2 (B) After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

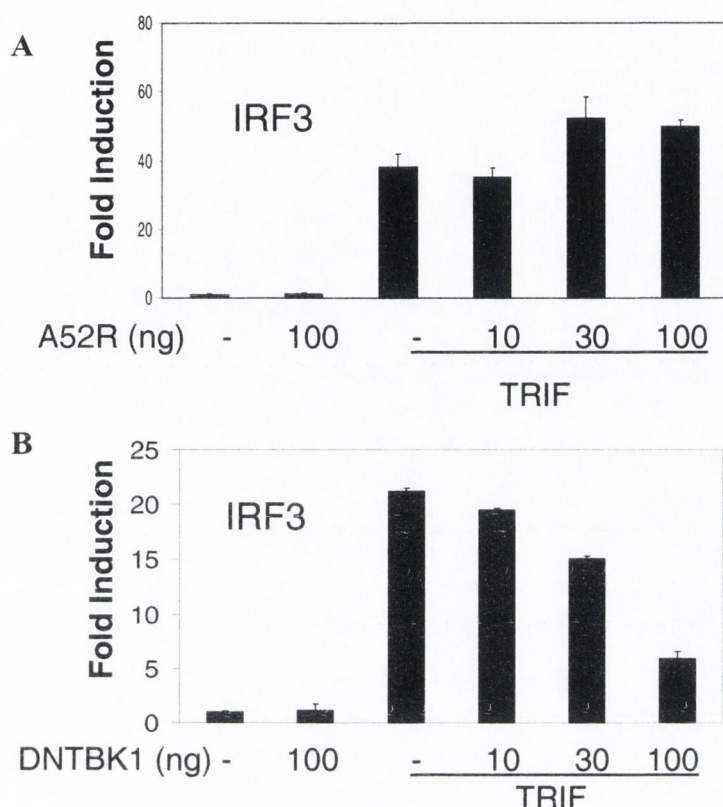
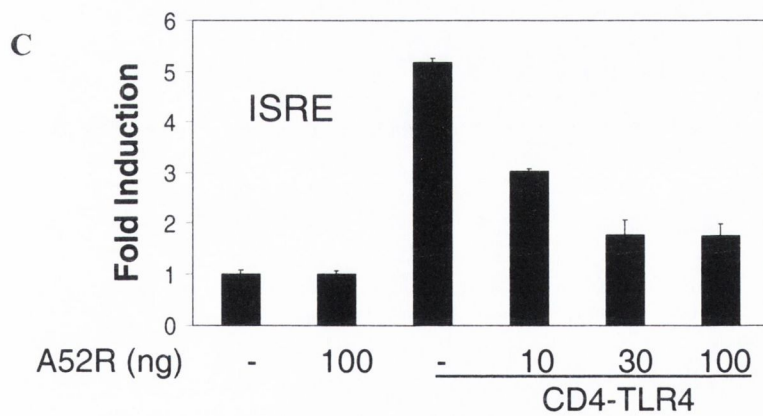
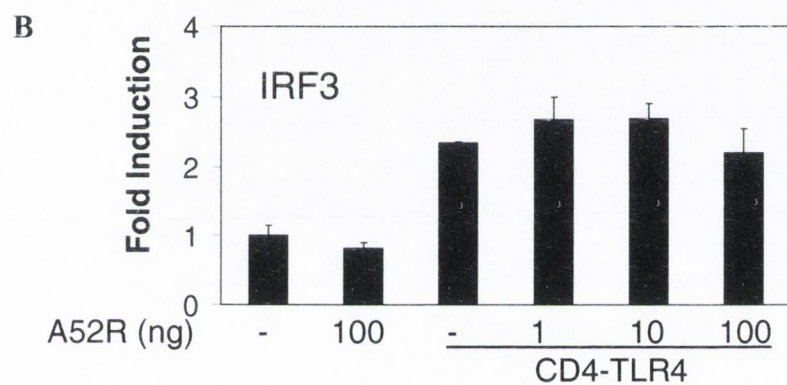
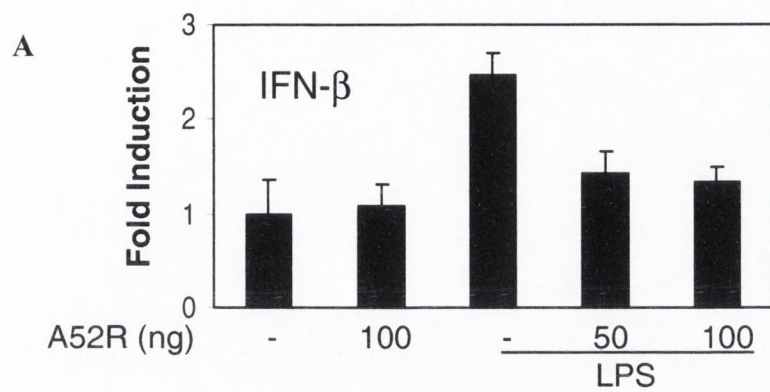


Fig. 4. 5: The effect of A52R on TRIF-induced IRF3 Signalling

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, IRF3 Gal4 fusion reporter gene together with pFR luciferase reporter plasmid and Renilla-luciferase internal control. Cells were transfected with increasing amounts of plasmid encoding A52R (A) or DNTBK1 (B) and 10 ng of a plasmid encoding TRIF as indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Fig. 4.6 : The effect of A52R on TLR4-induced IFN- β promoter, ISRE and IRF3-dependent reporter genes

RAW 264.7 cells were seeded into plates and transfected the next day with expression vectors, IFN- β promoter and Renilla-luciferase internal control. Six hours prior to harvesting cells were stimulated with 1 μ g/ml LPS as indicated. (A). HEK293 cells were seeded into 96-well plates and transfected the next day with expression plasmids, ISRE-dependent promoter (B) or IRF3 reporter plasmid together with pFR luciferase reporter plasmid (C) and Renilla-luciferase internal.. Cells were transfected 50 ng of CD4-TLR4 together with increasing amounts of plasmid encoding A52R as indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.



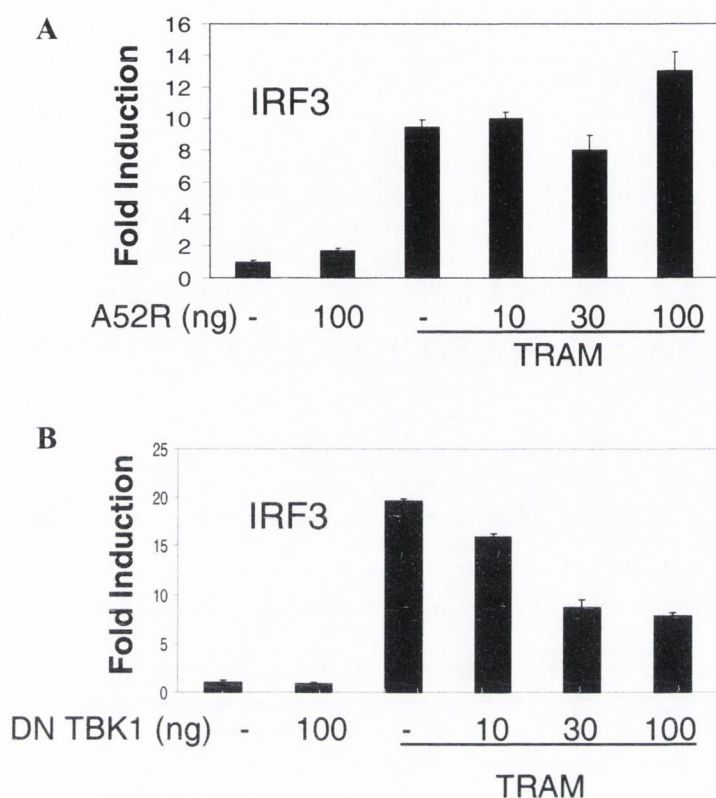


Fig. 4.7 The effect of A52R on TRAM-induced IRF3 activation

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with, IRF3 Gal4 fusion plasmid together with pFR luciferase reporter plasmid, Renilla-luciferase internal control. 25 ng of a plasmid encoding TRAM and increasing amounts of A52R or DN TBK1 as indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

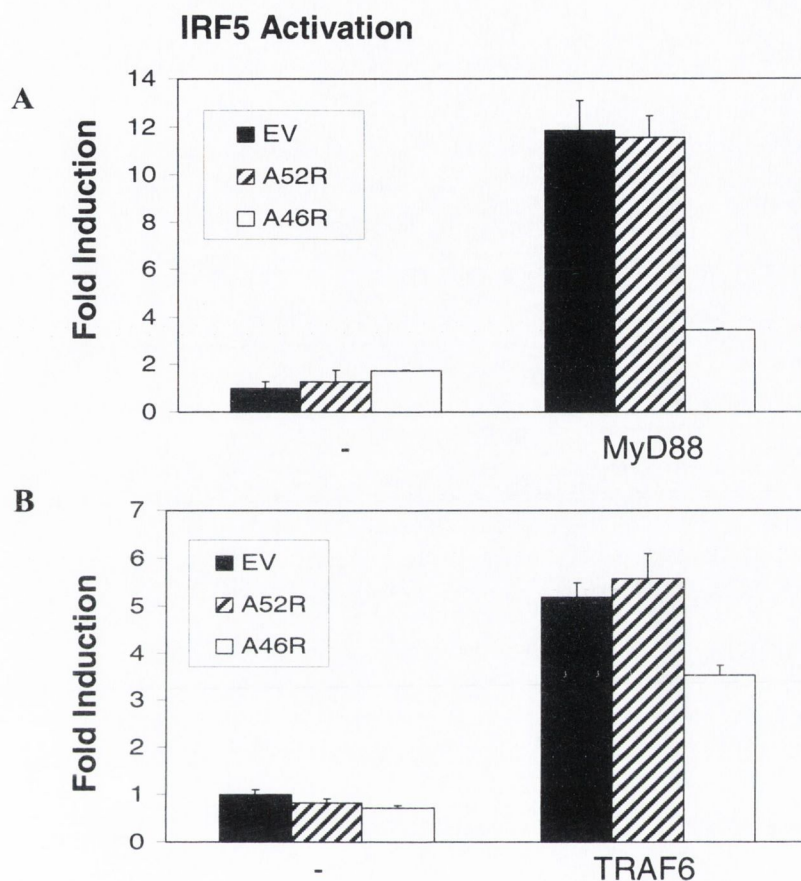


Fig. 4.8: Comparison of the effect of A52R and A46R on MyD88 and TRAF6- mediated IRF5 activation

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with IRF5-Gal4 fusion plasmid together with pFR luciferase reporter plasmid and Renilla-luciferase internal control. Cells were also transfected with 25 ng of a plasmid encoding MyD88 (A) 50 ng of TRAF6 (B) and 100 ng of a plasmid encoding A52R or A46R as indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

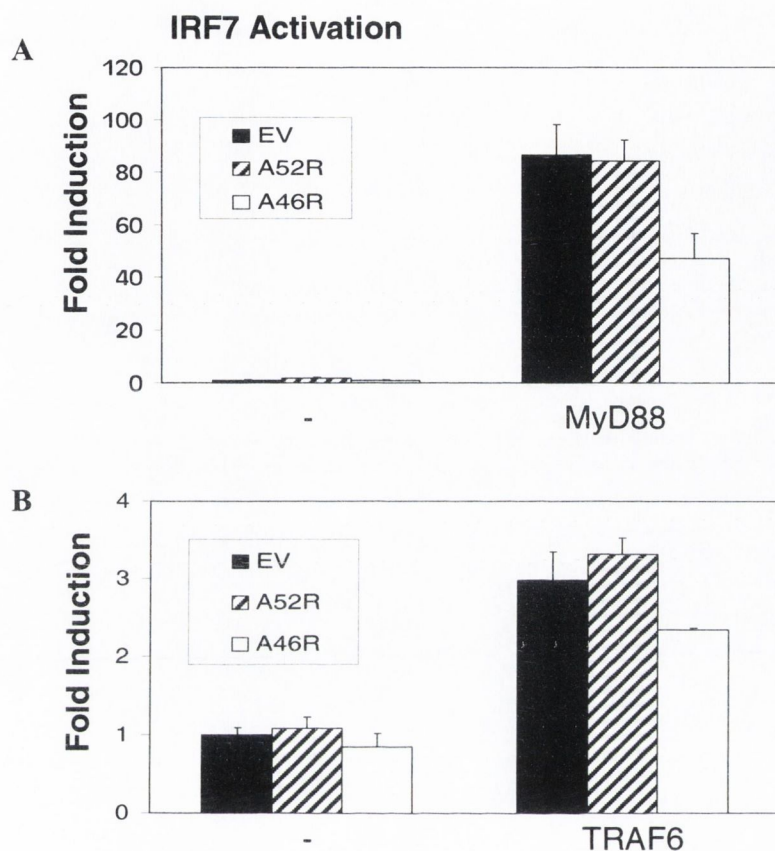


Fig. 4.9: Comparison of the effect of A52R and A46R on MyD88 and TRAF6- mediated IRF7 activation

HEK293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with IRF7-Gal4 fusion plasmid together with pFR luciferase reporter plasmid and Renilla-luciferase internal control. Cells were also transfected with 25 ng of a plasmid encoding MyD88 (A) 10 ng of TRIF (B) and 100 ng of a plasmid encoding A52R or A46R as indicated. After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

4.4 Discussion

In the previous chapter I showed that A52R blocked TLR-induced NF κ B activation and interacted with TRAF6 and IRAK2 (Harte *et al.*, 2003). Furthermore, the interaction with IRAK2 was found to be required for maximal NF κ B inhibition (Chapter 3). Here, I extended the study to examine other TLR signalling pathways to IRF3, 5 and 7. In addition, I also looked at the effect of A52R on TLR3-mediated NF κ B activation in greater detail.

TRIF has an essential role in both TLR3 and TLR4-induced IFN production and thus plays a central role in the anti-viral response (Hoebe *et al.*, 2003a, b, Yamamoto *et al.*, 2003a). Indeed, mice with a mutation in the *Trif* gene (termed *Lps2*) showed an impaired IFN production response to murine CMV infection compared to high levels of type I IFNs detected in the serum of control infected mice (Hoebe *et al.*, 2003a). In addition, the importance of TRIF in controlling VV replication was examined in *Lps2* mice. Macrophages from *Lps2* mice supported replication of VV to a significantly higher titre than wild-type controls (Hoebe *et al.*, 2003a). Thus, one might expect VV to somehow target the TRIF-dependent pathway.

Although TRIF is required for both TLR3 and TLR4-mediated IRF3 activation, key differences are beginning to emerge as to how TRIF regulates IRF3 activation in these two pathways. IRF3-mediated activation of ISRE by TLR4 but not TLR3 requires the p65 subunit of NF κ B (Wietek *et al.*, 2003). Interestingly, A52R had no effect on TLR3-induced ISRE (Fig. 4.1c) but was capable of blocking TLR4-

mediated ISRE activation (Fig. 4.6c) presumably by inhibiting the NF κ B component of this pathway. Consistent with the lack of effect of A52R on TRIF- and TRAM-mediated IRF3 activation (Fig. 4.5a, 4.7a), A52R had no inhibitory effect on CD4-TLR4-induced IRF3 activation (Fig. 4.6b). TRAM is a small TIR domain-containing protein and like TRIF induces both NF κ B and IRF3 activation (Fitzgerald *et al.*, 2003b, Yamamoto *et al.*, 2003b). Activation of NF κ B by TLR4 involves both an early and late phase activation process. MyD88 and Mal are required for early phase NF κ B and TRIF and TRAM are involved in the late phase NF κ B activation by TLR4 (Yamamoto *et al.*, 2003b). Both MyD88 and TRIF-induced NF κ B activation are potently inhibited by A52R (Fig. 3.10a, Fig. 4.3b). Hence, both the early and late phase NF κ B activation by TLR4 is efficiently shut down by A52R.

A recent study indicates that TRIF-dependent NF κ B activation is dependent on RIP1, which associates with the C-terminal region of TRIF. This region of TRIF contains a RIP homotypic interaction motif (Meylan *et al.*, 2004). Interestingly, A52R had no inhibitory effect on RIP1-induced NF κ B activation (Fig. 4.4a). The fact that the interaction between A52R and IRAK2 is fundamental for NF κ B inhibition (Chapter 3) and a dominant-negative version of IRAK2 inhibited TLR3- and TRIF-induced NF κ B activation (Fig. 3.14, 4.4b) implies that IRAK2 may be found upstream of RIP1 in the TLR3 pathway. This hypothesis is currently under investigation in the laboratory. Similarly, the lack of effect of A52R on TLR3-mediated IRF3 and ISRE activation suggests that there is no role for IRAK2 in TLR3-induced IRF3 activation (Fig. 4.1b, c). Interestingly, a recent report

demonstrated that IRAK1 is essential for TLR7 and TLR9-mediated IRF7 activation (Uematsu *et al.*, 2005). Further investigation into the specific role of IRAK2 in TLR-mediated IRF activation is being carried out in the laboratory.

Viral infections of mammalian cells leads to a rapid cellular response aimed at limiting virus replication and spread through the production of IFNs and induction of ISGs. IRF3 and IRF7 play key roles in the anti-viral response and in the induction of IFNs. Therefore, many viral countermeasures involve targeting IRF activation. For example, HSV-1 immediate-early protein ICP0 was shown to block ISG induction by specifically inhibiting IRF3- and IRF7-mediated cellular responses (Eidson *et al.*, 2002, Lin *et al.*, 2004). Kaposi's sarcoma-associated herpes virus encodes four IRF homologues (Moore *et al.*, 1996). One of these homologues encodes vIRF3 and it is hypothesized that vIRF3 inhibits IRF3 and IRF7 activities, resulting in the inhibition of the virus mediated synthesis of IFN- α/β (Lubyora and Pitha 2000). In addition, NS1, the dsRNA-binding protein of influenza has been shown to block IRF3 phosphorylation, and IFN- β mRNA induction was prevented in wild-type influenza virus infected cells but not cells infected with virus lacking the NS1 gene (Talon *et al.*, 2000). Furthermore, the VV protein E3L, which is a dsRNA binding protein and interferes with the binding of PKR to dsRNA has been shown to inhibit IRF3 and IRF7 phosphorylation (Smith *et al.*, 2001). Subsequent investigation revealed that a virus lacking E3L caused phosphorylation and nuclear translocation of IRF3 and induction of IFN- β (Xiang *et al.*, 2002).

Further evidence is presented in this chapter that VV encodes another protein aimed at interfering with IRF activation by TLRs. The VV TLR antagonist A46R, a viral TIR domain-containing protein interacted with MyD88, Mal, TRIF and TRAM has recently been shown to inhibit intracellular signalling by a range of TLRs (Stack *et al.*, 2005). In contrast, A52R targets IRAK2 and TRAF6 but is also an extremely potent TLR-induced NF κ B inhibitor. However, several lines of evidence suggest that these two proteins are not functionally redundant. Firstly, as mentioned they target distinct signalling molecules. Secondly, although they do have some overlapping effects such as inhibition of MyD88-dependent NF κ B activation, their overall effects on TLR signalling are quite distinct. Whereas A52R potently inhibits NF κ B activation, it has no inhibitory effect on TLR3- or TRIF-mediated IRF3 activation (Fig. 4.1b, 4.5a). In addition, A52R had no effect on TLR3-induced ISRE activation (Fig. 4.1c). In contrast, A46R blocks TLR3- and TRIF-mediated IRF3 and ISRE activation (Fig. 4.2a, b, personal communication J. Stack), while having little effect on TLR3-induced NF κ B activation (Fig. 4.2c). Finally, deletion of either A46R or A52R from VV causes attenuation (Harte *et al.*, 2003, Stack *et al.*, 2005) and thus both contribute to virulence and are non-redundant. Therefore, VV likely requires both A46R and A52R to effectively shut down TLR3 signalling.

Although A46R interacts with TRIF it has no effect on TLR3-mediated NF κ B activation. Therefore, the interaction with TRIF may have a greater effect on downstream IRF3 activation compared to NF κ B. Interestingly, these two pathways bifurcate at TRIF and have been shown to be quite distinct (Sato *et al.*, 2003,

Meylan *et al.*, 2004). Indeed, the N-terminal and C-terminal of TRIF can independently activate NF κ B, whereas only the N-terminal region is involved in the activation of IRF3. TRIF has been shown to harbour the TRAF6 interaction motif (P-x-E-x-x- acidic/aromatic residue) in its N-terminal. TRIF can interact with both TRAF6 and TBK1 at two distinct regions in its N-terminal (Sato *et al.*, 2003). TBK1 is a kinase of the IRF3 pathway, which leads to the induction of IFN- β (Sharma *et al.*, 2003, Fitzgerald *et al.*, 2003a). The association between TRIF and TRAF6 is reported to be necessary for activation of NF κ B, while TBK1 mediates the induction of IFN- β . Thus, TRIF appears to act as a scaffold by assembling the two signalling molecules TRAF6 and TBK1, which regulate two distinct pathways leading to the activation of the NF κ B and IFN- β promoters, respectively (Sato *et al.*, 2003). Further comparison between the effects of A52R and A46R on TLR3-mediated signalling should reveal subtle differences in the specific functionality of these two VV TLR antagonists. In addition, mapping the interactions of A52R and A46R with their targeted signalling components should reveal important insights into how specifically TLR3 uses TRAF6, IRAK2 and TRIF.

A46R was shown to inhibit TLR3-induced IRF3 activation (Fig. 4.2a). Given the fact that TLR7, 8 and 9 are sensed by viral nucleic acids it is not surprising that they initiate an anti-viral response mechanism. Recently, two groups demonstrated the TLR7, 8 and 9 activate type I IFNs through the activation of IRF5 and IRF7 in a MyD88-dependent manner (Schoenomeyer *et al.*, 2005, Takaoka *et al.*, 2005). Another study has shown that analysis of genes stimulated by IRF5 and IRF7 in

BJAB cells resulted in upregulation of 14 genes that encode proteins with immunoregulatory and anti-viral functions (Barnes *et al.*, 2004). Here, activation of IRF5 and IRF7 by MyD88 and TRAF6 was completely blocked by A46R, in contrast to A52R which had no inhibitory effect on either of these signals (Fig. 4.8, 4.9).

In conclusion, A52R is a potent inhibitor of TLR-induced NF κ B activation whereas A46R is required to inhibit TLR3-mediated IRF3 activation and MyD88- and TRAF6-induced IRF5 and IRF7 activation. Hence, further investigation into the effects of A52R and A46R on gene induction should reveal important information regarding the ability of these two VV proteins to specifically target certain TLR pathways.

Chapter 5

A52R Activates p38 MAP Kinase and Potentiates LPS-Induced IL-10

5.1 Introduction

The p38 MAP kinases are a family of serine/threonine kinases that play important roles in cellular responses to external stress signals. The p38 MAP kinases have been found to be involved in inflammation, cell growth, cell differentiation, the cell cycle and cell death (Reviewed in Pearson *et al.*, 2001). The four known p38 isoforms share significant amino acid homology with each other and include p38 α , p38 β , p38 γ and p38 δ (Reviewed Pearson *et al.*, 2001). It is well established that the activation of the p38 pathway is regulated by sequential activation of multiple upstream kinases, each of which acts as a substrate for the other. The activation of p38 MAP kinase is regulated by phosphorylation within its Thr-Gly-Tyr motif (Lee *et al.*, 1994). The motif is phosphorylated by a MKK, for example, MKK3, MKK4 and MKK6 (Reviewed in Kyriakis and Avruch, 2001). The activation of MKKs is in turn controlled by other upstream serine/threonine kinases, called MAP3 kinases. Proteins that have been shown to act as MAP3 kinases in the p38 pathway include among others, TAK1 (Yamaguchi *et al.*, 1995, Shibuya *et al.*, 1996, Shirakabe *et al.*, 1997) and ASK1 (Ichijo *et al.*, 1997).

As well as NF κ B, most TLRs tested have been shown to be capable of activating p38 MAP kinase. For IL-1 signalling, the activation of both the IKK complex and MAP kinases is dependent on TRAF6. Downstream of TRAF6 the MAP3 kinase, TAK1 is essential (Wang *et al.*, 2001, Ninomiya *et al.*, 1999). TAK1 is linked to TRAF6 via the adaptor TAB2 (Takaesu *et al.*, 2000). The signalling mechanism requires phosphorylation of IRAK, upstream of TRAF6. Phosphorylated IRAK recruits TRAF6 to the receptor complex and allows TRAF6 to interact with a preformed complex consisting of TAK1, TAB1 and TAB2 at the cell membrane

(Jiang *et al.*, 2002). The TRAF domain of TRAF6 is responsible for mediating oligomerisation of TRAF6 (Baud *et al.*, 1999) which facilitates the interaction of TRAF6 with TRIKA1, a dimeric ubiquitin conjugated enzyme complex composed of Ubc13 and Uve1A (Wang *et al.*, 2001). This interaction causes a novel form of polyubiquitination involving Lys-63 of ubiquitin to occur. TRAF6 is ubiquitinated and TAK1 is activated which in turn activates IKK in the NF κ B pathway and phosphorylates MKK6 in the p38 MAP kinase pathway (Wang *et al.*, 2001).

p38 MAP kinase's can affect gene regulation through its serine kinase activity which mediates phosphorylation of transcription factors, and regulation of downstream gene transcription (Reviewed in Kyriakis and Avruch, 2001), as well as regulating mRNA stability. The activation of the p38 pathway plays an essential role in induction of cytokines, for example LPS induced IL-10 production has been shown to be p38-dependent (Foey *et al.*, 1998, Ma *et al.*, 2001).

IL-10 inhibits inflammatory and cell-mediated immune responses (Moore *et al.*, 2001). TLR-induced IL-10 has been shown to have a role in the generation of T regulatory cell, and to lead to the inhibition of Th1-type responses (Netea *et al.*, 2004a, Netea *et al.*, 2004b and Higgins *et al.*, 2003).

The rationale of this chapter was to investigate if A52R would have an inhibitory effect on MAP kinase activation given the role TRAF6 plays in this pathway. Surprisingly, A52R was shown to activate the MAP kinases p38 and JNK in a TRAF6-dependent manner. In addition, A52R leads to enhancement of the TLR-induced p38-dependent gene IL-10. In contrast, inhibition of the TLR-induced

NF κ B dependent genes IL-8 and RANTES is observed. The ability of A52R to activate p38 and potentiate TLR-induced IL-10 may be important to its role in virulence.

5.1 Introduction

The p38 MAP kinases are a family of serine/threonine kinases that play important roles in cellular responses to external stress signals. The p38 MAP kinases have been found to be involved in inflammation, cell growth, cell differentiation, the cell cycle and cell death (Reviewed in Pearson *et al.*, 2001). The four known p38 isoforms share significant amino acid homology with each other and include p38 α , p38 β , p38 γ and p38 δ (Reviewed Pearson *et al.*, 2001). It is well established that the activation of the p38 pathway is regulated by sequential activation of multiple upstream kinases, each of which acts as a substrate for the other. The activation of p38 MAP kinase is regulated by phosphorylation within its Thr-Gly-Tyr motif (Lee *et al.*, 1994). The motif is phosphorylated by a MKK, for example, MKK3, MKK4 and MKK6 (Reviewed in Kyriakis and Avruch, 2001). The activation of MKKs is in turn controlled by other upstream serine/threonine kinases, called MAP3 kinases. Proteins that have been shown to act as MAP3 kinases in the p38 pathway include among others, TAK1 (Yamaguchi *et al.*, 1995, Shibuya *et al.*, 1996, Shirakabe *et al.*, 1997) and ASK1 (Ichijo *et al.*, 1997).

As well as NF κ B, most TLRs tested have been shown to be capable of activating p38 MAP kinase. For IL-1 signalling, the activation of both the IKK complex and MAP kinases is dependent on TRAF6. Downstream of TRAF6 the MAP3 kinase, TAK1 is essential (Wang *et al.*, 2001, Ninomiya *et al.*, 1999). TAK1 is linked to TRAF6 via the adaptor TAB2 (Takaesu *et al.*, 2000). The signalling mechanism requires phosphorylation of IRAK, upstream of TRAF6. Phosphorylated IRAK recruits TRAF6 to the receptor complex and allows TRAF6 to interact with a preformed complex consisting of TAK1, TAB1 and TAB2 at the cell membrane

5.2 A52R Drives p38 MAP Kinase Activation

I previously showed that A52R acts as a potent inhibitor of NF κ B activation induced by IL-1 and various TLRs, and that it was capable of interacting with both TRAF6 and IRAK2 (Chapter 3, Harte *et al.*, 2003). Further, I demonstrated that A52R had no inhibitory effect on TLR3- or TRIF- mediated IRF3 activation (Fig. 4.1c, 4.3b) nor on MyD88 or TRAF6 mediated IRF5 or IRF7 activation (Fig. 4.9, 4.10). However, the effect of A52R on MAP kinase activation was not determined.

Therefore, the effect of A52R on p38 MAP kinase activation was examined. For this, the Stratagene PathdetectTM System, which is based on the ability of p38 MAP kinase to phosphorylate and activate the transcription factor CHOP, was used. This is assayed by an increase in the ability of a Gal4-CHOP fusion protein, which has been phosphorylated by p38 MAP kinase to transactivate the pFR luciferase reporter. The pFR reporter contains Gal4 binding sites in its promoter. Fig. 5.1 shows that treatment of cells with IL-1, expression of CD4-TLR4 or ectopic expression of TLR3 together with poly(I:C) stimulation, led to activation of p38 MAP kinase. Surprisingly, ectopic expression of increasing amounts of a plasmid encoding A52R enhanced IL-1-, TLR4- and TLR3- mediated p38 MAP kinase activation (Fig. 5.1). This was in contrast to the inhibitory effect of A52R on IL-1/TLR-induced NF κ B activation (Fig. 3.2, 3.4, 3.5). In fact, expression of A52R alone in unstimulated cells led to activation of p38 MAP kinase (Fig. 5.2a) and JNK (Fig. 5.2b), whereas A52R alone had no effect on basal levels of NF κ B activity (Fig. 3.4). Thus, A52R has opposite effects on IL1/TLR-induced NF κ B and MAP kinase activation, and can in fact activate MAP kinases in the absence of any other stimulus.

5.3 Activation of p38 by A52R Requires Interaction with TRAF6

Given the fact that Δ A52R only interacted with IRAK2 (Fig. 3.17) and not TRAF6 (Fig. 3.16) the ability of Δ A52R to induce MAP kinase activation was tested. Fig. 5.3 shows that in contrast to A52R, Δ A52R failed to drive p38 and JNK activation. Thus, the failure of Δ A52R to activate p38 and JNK correlated with its inability to interact with TRAF6, suggesting a role for TRAF6 in the stimulatory effect of A52R on MAP kinases. Interestingly, Δ N, which encodes amino acids 59-190 of A52R, was also capable of activating p38 MAP kinase in a reporter gene assay system, although the level of activation was not as dramatic as the effect of the full length A52R. In contrast, Δ C, like Δ A52R was no longer capable of driving p38 activation (Fig. 5.4).

5.4 A52R Activates p38 via the TRAF Domain of TRAF6

I next sought to examine more closely how the interaction between A52R and TRAF6 was responsible for the p38 MAP kinase activation. Fig. 5.5 shows that consistent with previous work (Harte *et al.*, 2003), A52R interacted with the TRAF domain of TRAF6 (amino acids 289-522). The TRAF domain of TRAF6, here termed Δ TRAF6, has been reported to act as a dominant negative, inhibiting IL-1-induced p38 and JNK activation (Baud *et al.*, 1999). I therefore hypothesised that if the A52R-TRAF domain interaction was important for A52R induced p38 activation, overexpression of Δ TRAF6 would sequester A52R and prevent p38 MAP kinase activation. To test this I examined p38 activation by Western blot analysis in HEK293 cells using a phospho specific p38 antibody. Fig. 5.6 shows that transfection of cells with Δ TRAF6 alone had no effect on levels of phospho-p38 (upper panel, lane 2). Consistent with the reporter gene based assay (Fig. 5.1,

5.2), overexpression of A52R led to an increase in phospho-p38 (Fig. 5.6 upper panel, compare lane 3 to lane 1). Co-expression of Δ TRAF6 with A52R completely inhibited the A52R-induced increase in p38 phosphorylation (Fig. 5.6 upper panel, lane 4), presumably by sequestering A52R and preventing its interaction with endogenous TRAF6. Thus A52R activates p38 by engaging the TRAF domain of TRAF6. As a control, A52R was shown to have no effect on I κ B α protein levels (Fig. 5.6 third panel). Cells were also blotted for expression of A52R (Fig. 5.7 sixth panel), however, there is more A52R present in lane 4. Nevertheless, overexpression of Δ TRAF6 in combination with this increased amount of A52R was able to prevent A52R from activating p38. I next sought to confirm that the TRAF6 interaction was critical the p38 activation observed. Using TRAF6^{-/-} and TRAF6^{+/+} MEFs, the ability of ectopic expression of TRAF6 and A52R to induce p38 phosphorylation was examined (Fig. 5.7). Overexpression of TRAF6 activated p38 in both the TRAF6^{-/-} and TRAF6^{+/+} cells as expected (Fig. 5.7 top panel lanes 3 and 7). However, overexpression of A52R also drove p38 activation in both cell lines (Fig. 5.7 top panel lanes 2 and 6). Thus, A52R may have other ways of activating p38 MAP kinase as well as through TRAF6. As a control cells were blotted for total p38 (Fig. 5.7 middle panel) and TRAF6 expression (Fig. 5.7 bottom panel).

5.5 A52R Enhances TLR-Induced IL-10 Induction While Inhibiting NF κ B-Dependent Genes

Previous studies have shown that LPS-induced IL-10 production is p38-dependent (Foey *et al.*, 1998, Ma *et al.*, 2001). Therefore, I wondered whether A52R would have an effect on IL-10 induction. Firstly, the effect of A52R on the IL-10

promoter using a reporter gene assay was examined. Interestingly, A52R was capable of driving the IL-10 promoter in a dose-dependent manner in HEK293 cells (Fig. 5.8). In order to examine whether the induction of IL-10 was TRAF6-dependent, I next assessed the ability of A52R to activate this reporter in TRAF6^{-/-} MEFs. In the absence of TRAF6, the ability of A52R to drive the IL-10 promoter was abolished compared to a 3-fold induction in normal MEFs (Fig. 5.9), thus implicating TRAF6 in the activation of the IL-10 promoter by A52R. In fact A52R was capable of driving the IL-10 promoter in a dose-dependent manner in TRAF6^{+/+} MEFs (Fig. 5.9b). I next used the murine macrophage cell line RAW 264.7 to analyse the effect of A52R on IL-10 protein production. The p38 inhibitor SB203580 has previously been shown to inhibit LPS induced IL-10 production in human PBMCs (Foey *et al.*, 1998) and in a human monocyte cell line THP-1 (Ma *et al.*, 2001). Consistent with this, here SB203580 inhibited LPS/TLR4-induced IL-10 production (Fig. 5.10b). In addition, the JNK inhibitor SP600125 also inhibited LPS-induced IL-10 production (Fig. 5.10b). Expression of A52R alone did not lead to IL-10 production in unstimulated cells (Fig. 5.11). However, expression of A52R strongly enhanced LPS-induced IL-10 production (Fig. 5.11). This is suggestive of a potent effect on TLR-induced IL-10 production, given that only a small fraction of the RAW 264.7 cells stimulated by LPS to release IL-10 would be expected to be expressing A52R in this transient transfection system. The fact that A52R failed to drive the IL-10 promoter in TRAF6^{-/-} MEFs suggested that the TRAF6-A52R interaction was critical for the effect of A52R on IL-10 production. This conclusion prompted us to examine the role of TRAF6 on LPS induced IL-10 production. Consistent with the effect of A52R on LPS induced IL-10, ectopic expression of TRAF6 enhanced (Fig. 5.12a), while Δ TRAF6 inhibited (Fig. 5.12b)

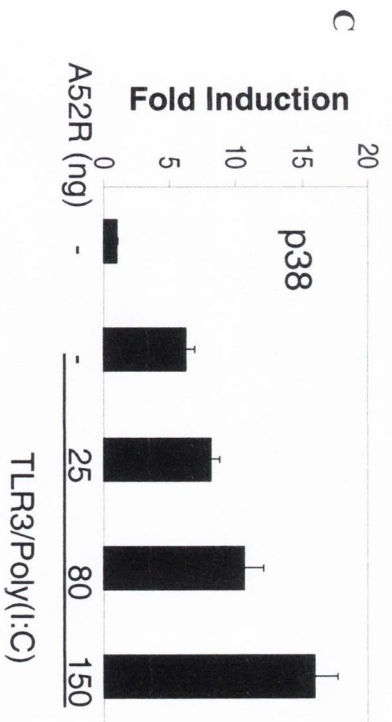
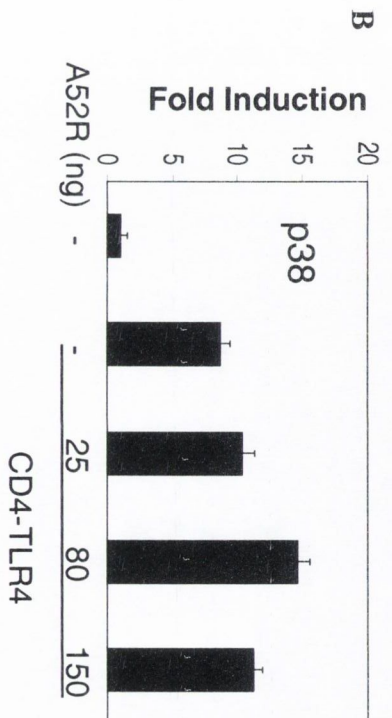
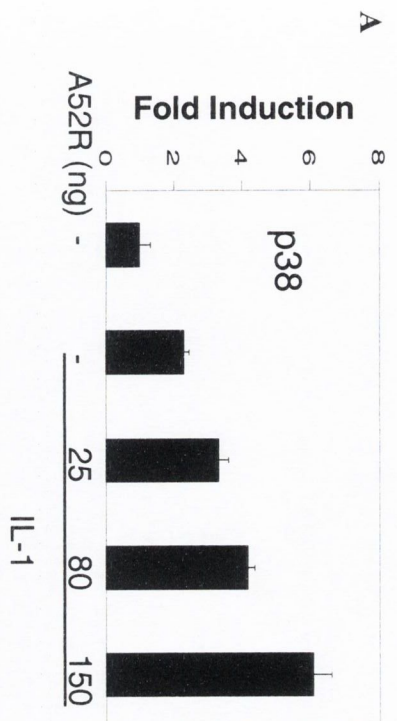
LPS induced IL-10 production. Next I sought to compare the effect of A52R on IL-10 production to its effects on two NF κ B dependent genes, the chemokines IL-8 (Jung *et al.*, 2002) and RANTES (Genin *et al.*, 2000). Stimulation of HEK-TLR4 cells with LPS led to IL-8 production (Fig 5.13a), while stimulation of HEK-TLR3 cells with poly (I:C) led to RANTES production (Fig. 5.13b). In both cases transient transfection with A52R inhibited chemokine production in a dose-dependent manner (Fig. 5.13). Thus A52R differentially modulates TLR-induced gene expression, by potentiating the p38-dependent gene IL-10, while inhibiting the NF κ B-dependent genes IL-8 and RANTES.

5.6 The Effect of A46R and N1L on IL-10 Induction

The ability of two other VV TLR antagonist, A46R (Bowie *et al.*, 2001, Stack *et al.*, 2005) and N1L (DiPerna *et al.*, 2004) to modulate IL-10 was compared to A52R. First, the ability of the viral proteins to drive the IL-10 promoter in HEK293 cells was then investigated. In contrast to A52R, ectopic expression of either A46R or N1L failed to drive the IL-10 promoter (Fig. 5.14). Furthermore, overexpression of A46R or N1L resulted in no significant increase or decrease in LPS induced IL-10 production (Fig. 5.15). A46R and N1L are known TLR antagonists and can inhibit NF κ B activation by multiple TLRs (Bowie *et al.*, 2001, Stack *et al.*, 2005, DiPerna *et al.*, 2004). Therefore, as a control, the ability of these proteins to inhibit an NF κ B-dependent gene, the chemokine IL-8 was tested. Consistent with published work both A46R and N1L inhibited LPS-induced IL-8 production in a dose-dependent manner (Fig. 5.16).

Fig. 5.1: A52R enhances IL-R/TLR induced p38 MAP kinase activation

HEK293 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected with increasing amounts of a plasmid encoding A52R as indicated, 0.25 ng CHOP Gal4 fusion vector and pFR luciferase reporter plasmids and Renilla luciferase as internal control. HEK293 cells were also transfected with 50 ng of plasmid encoding CD4-TLR4 (B). Six hours prior to harvesting cells were stimulated with 100 ng/ml IL-1 (A) or 25 μ g/ml poly(I:C) (C) and luciferase gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.



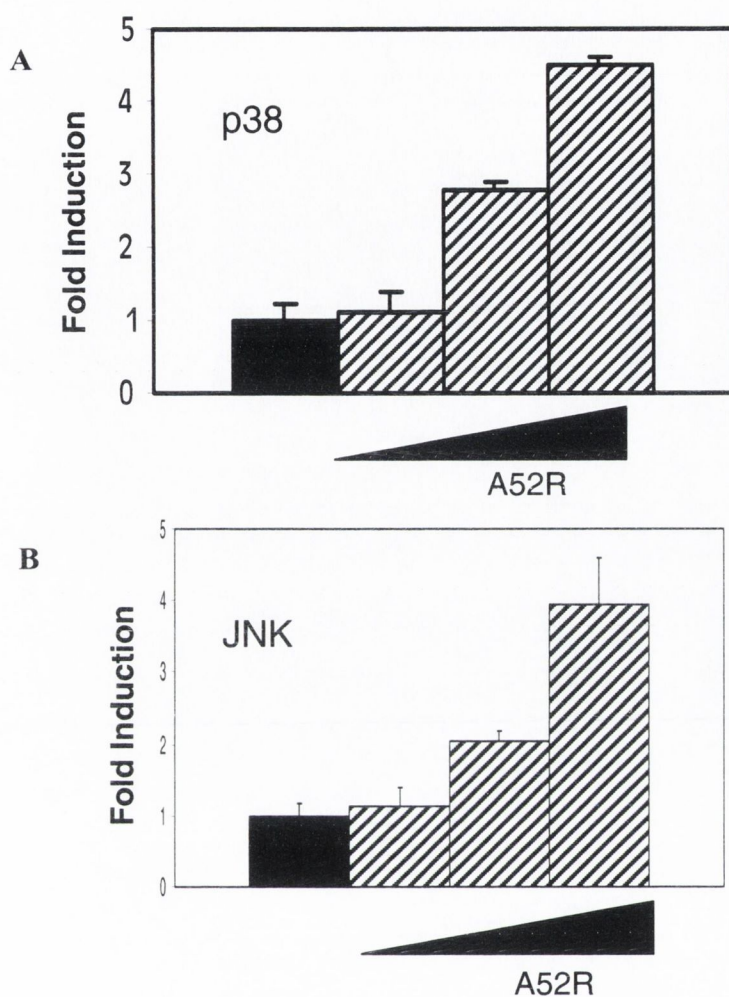


Fig. 5.2: A52R drives MAP Kinase Activation

HEK293 cells were (2×10^4 cells per ml) were seeded into 96-well plates transfected with increasing amounts (10, 30, 100 ng) of a plasmid encoding A52R, together with pFR luciferase, Renilla luciferase and 0.25 ng CHOP-Gal4 fusion vector (A) or 0.25 ng c-Jun-Gal4 fusion vector (B). After 24 hours the cells were harvested and the reporter gene activity was measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

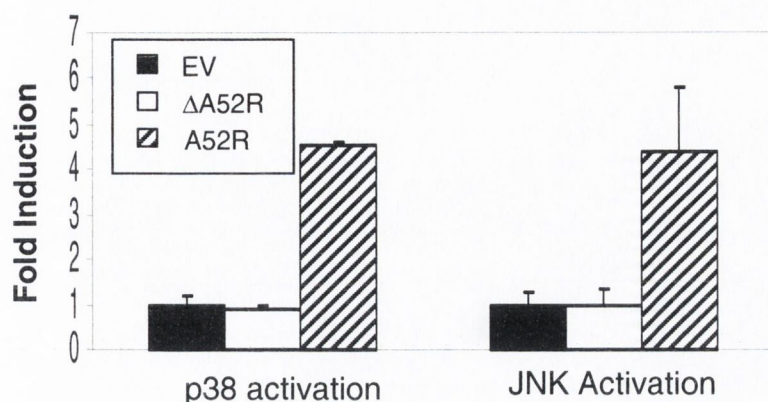


Fig. 5.3: ΔA52R dose not activate p38 and JNK

HEK293 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected with 100 ng of plasmid encoding A52R or ΔA52R, 0.25 ng CHOP Gal4 fusion vector or 0.25 ng c-Jun Gal4 fusion vector, pFR luciferase reporter plasmid and Renilla luciferase as internal control. After 24 hours the cells were harvested and the reporter gene activity measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

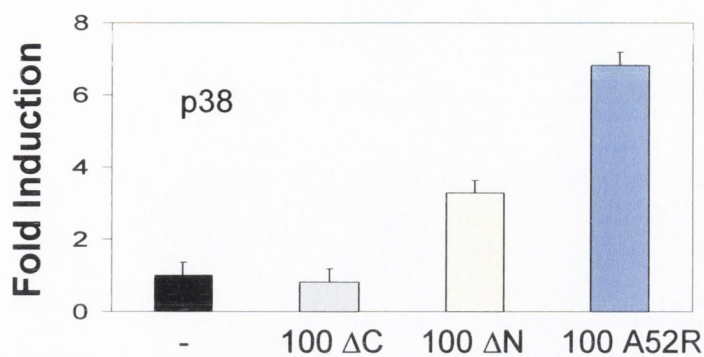


Fig. 5.4: ΔN Activates p38 MAP kinase

HEK293 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected with 100 ng of plasmid encoding ΔC, ΔN or A52R, 0.25 ng CHOP Gal4 fusion vector or and pFR luciferase reporter plasmid and Renilla luciferase as internal control. After 24 hours the cells were harvested and the reporter gene activity measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

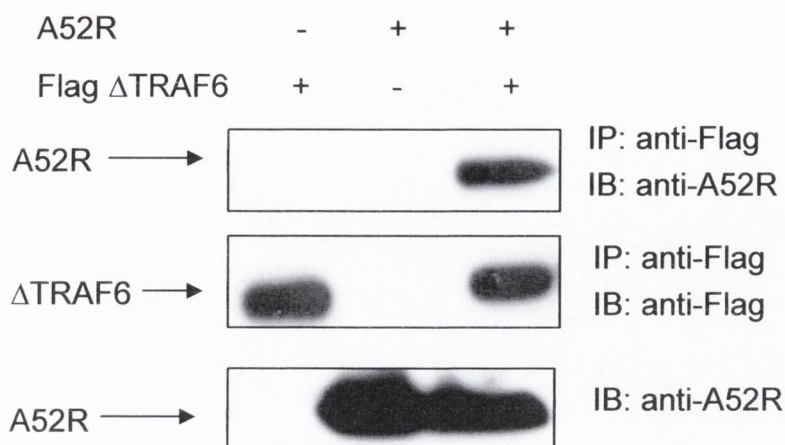


Fig. 5.5: A52R interacts with the TRAF domain of TRAF6

HEK293T cells (1.5×10^6) were seeded into 100 mm dishes 24 hours prior to transfection. Four μg of each construct was transfected where indicated (+). The total amount of DNA (8 μg) was kept constant by supplementation with pcDNA 3.1. Cells were harvested 24 hours post-transfection and immunoprecipitation experiments carried out as described (Section 2.7.5). The top panel corresponds to lysates immunoprecipitated with anti-flag and blotted for the presence of A52R. The appearance of a band in lane 3 that is not detected in lanes 1 and 2 is indicative of an interaction. The second panel corresponds to lysates immunoprecipitated with anti-flag and blotted for the same antibody. The lower panel shows lysates probed for A52R. Data is a representative of one experiment from a minimum of three separate experiments.

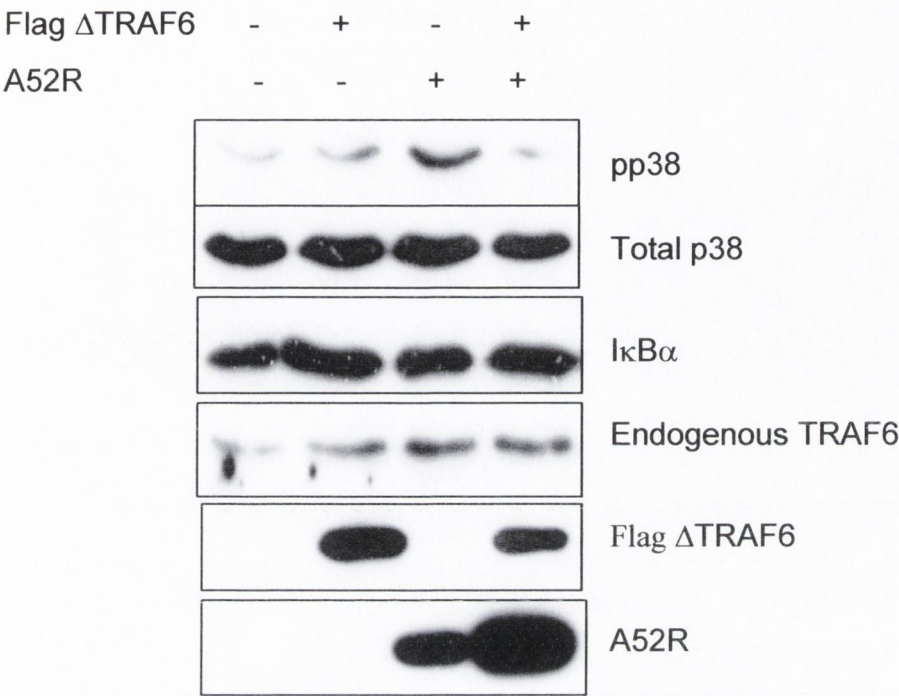


Fig. 5. 6: A52R activates p38 via the TRAF6 TRAF domain

HEK293 cells were seeded at 1×10^5 cells per ml in 6 well plates 24 hours prior to transfection. Cells were transfected with 1.15 μ g of expression plasmids Δ TRAF6 and/or A52R as indicated. Cells were harvested 24 hours later and lysates were subjected to SDS/PAGE and immunoblotting with antibodies to the indicated protein. Data is a representative of one experiment from a minimum of two separate experiments.

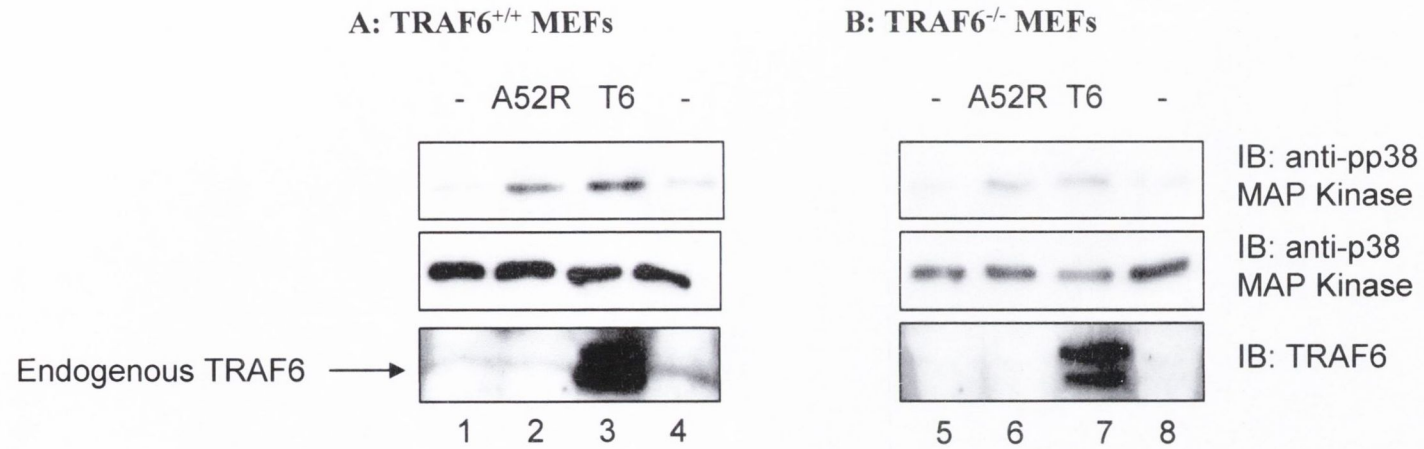


Fig. 5.7: A52R activates p38 in TRAF6^{+/+} and TRAF6^{-/-} MEFs

TRAF6^{+/+} and TRAF6^{-/-} MEFs were seeded at 1×10^5 cells per ml in 6 well plates 24 hours prior to transfection. Cells were transfected with 2.3 μ g of expression plasmids EV, A52R or TRAF6 as indicated. Cells were harvested 24 hours later and lysates were subjected to SDS/PAGE and immunoblotting with specific antibodies as indicated. Data is a representative of one experiment from a minimum of two separate experiments.

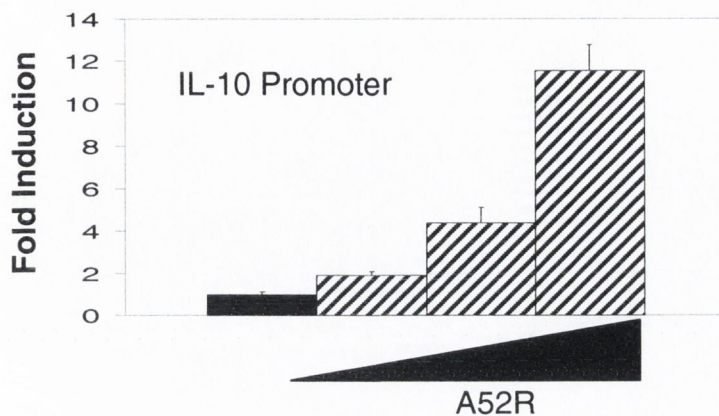


Fig. 5.8: A52R drives the IL-10 Promoter in HEK 293 cells

HEK293 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected 24 hours later with expression plasmids, IL-10 promoter luciferase reporter plasmid, Renilla luciferase as internal control and increasing amounts of A52R (10, 30 100 ng). After 24 hours the cells were harvested and the reporter gene activity measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

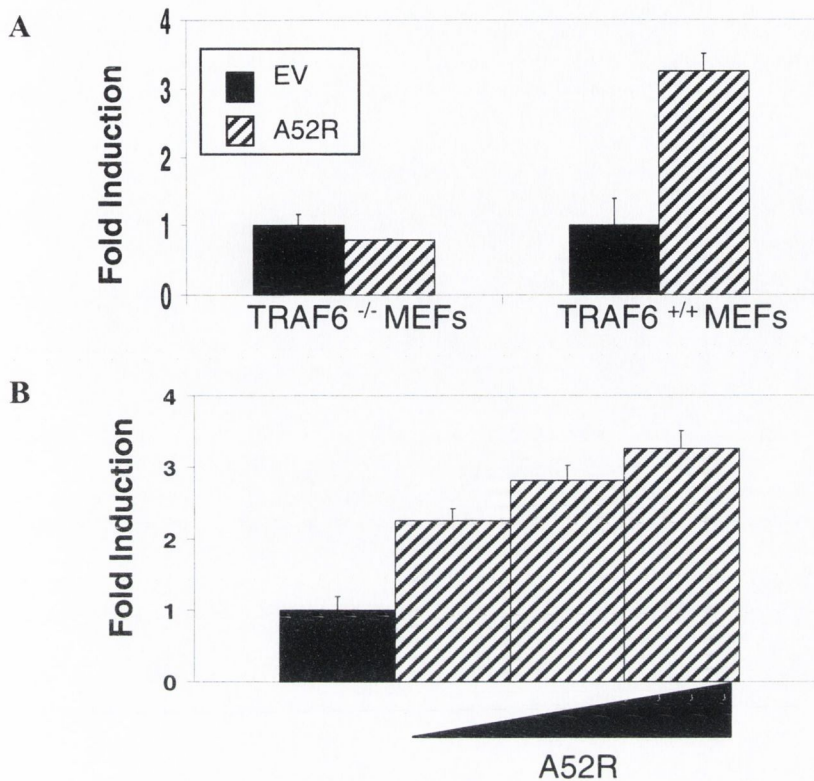


Fig. 5.9: A52R drives the IL-10 promoter in a TRAF6 dependent manner

TRAF6^{-/-} MEFs or TRAF6^{+/+} MEFs (2×10^4 cells per ml) were seeded into 96-well plates and transfected 24 hours later with expression plasmids, IL-10 promoter luciferase reporter plasmid, Renilla luciferase as internal control and 100 ng of a plasmid encoding A52R (A) or increasing amounts of A52R (10, 30 100 ng) (B). After 24 hours the cells were harvested and the reporter gene activity measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

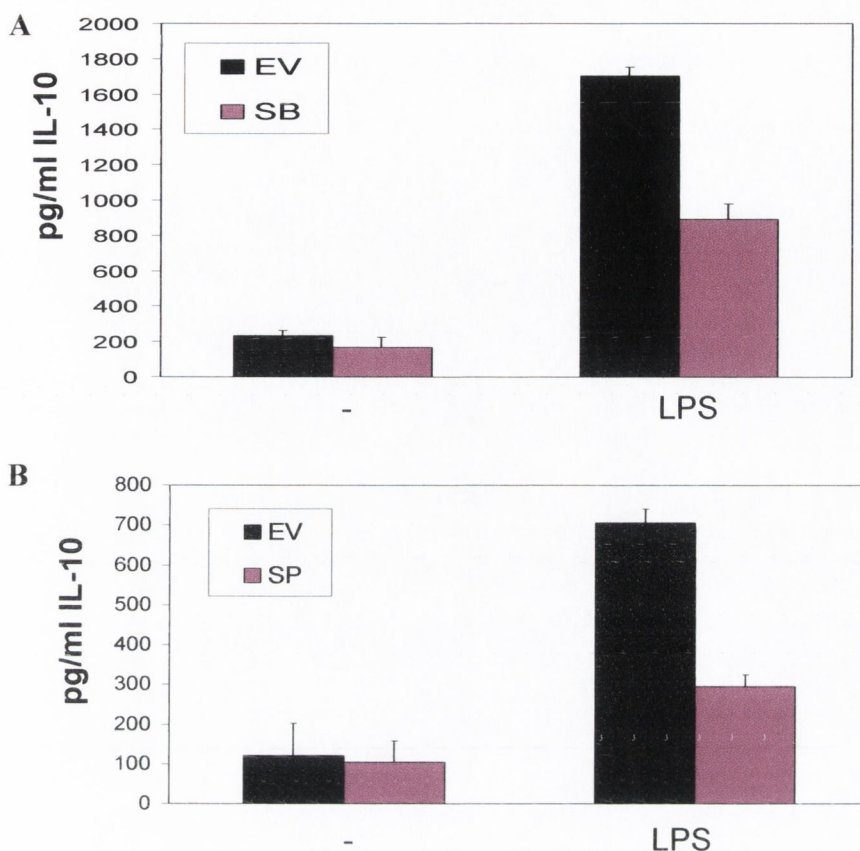


Fig. 5.10: LPS induced IL-10 Production is p38- and JNK-dependent

Murine Macrophage RAW 264.7 cells (2×10^4 cells per ml) were seeded into 96-well plates and treated 24 hours later with MAP Kinase inhibitors SB203580 (A) SP600125 (B) for two hours prior to stimulation with $1 \mu\text{g/ml}$ LPS. 24 hours after stimulation supernatants were harvested and assayed for IL-10 production by ELISA. Data is expressed as pg/ml $\pm$ s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

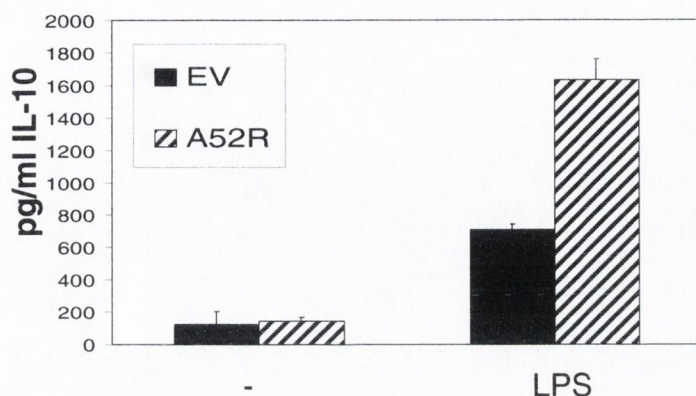


Fig. 5.11: The effect of A52R on LPS induced IL-10 production

Murine Macrophage RAW 264.7 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected with 120 ng of a plasmid encoding A52R 24 hours prior to stimulation with 1 μ g/ml LPS. 24 hours after stimulation supernatants were harvested and assayed for IL-10 production by ELISA. Data is expressed as pg/ml $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

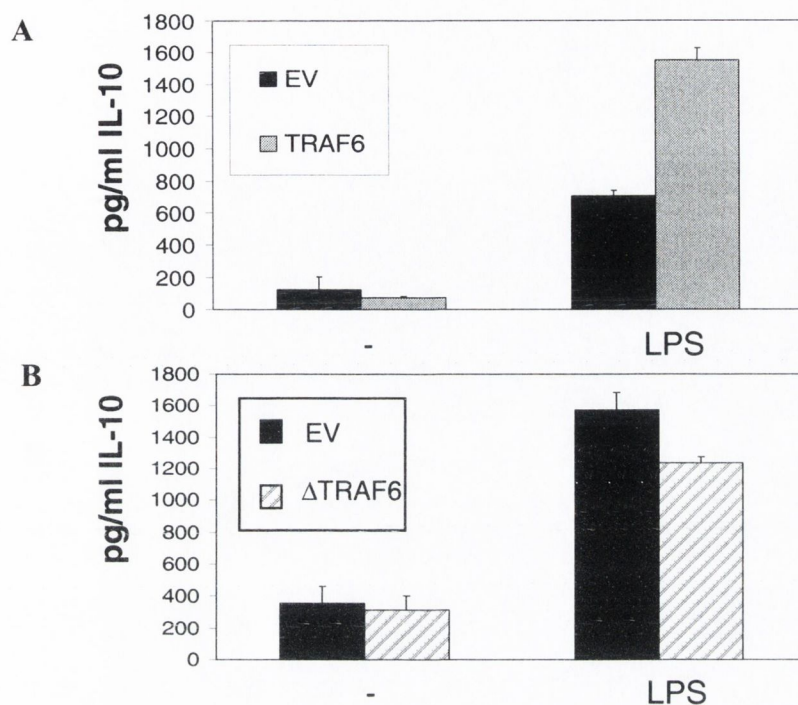


Fig. 5.12: The effect of TRAF6 and Δ TRAF6 on IL-10 production

Murine Macrophage RAW 264.7 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected with the indicated (ng) amounts of plasmids encoding TRAF6 (A) or Δ TRAF6 (B) 24 hours prior to stimulation with 1 μ g/ml LPS. 24 hours after stimulation supernatants were harvested and assayed for IL-10 production by ELISA. Data is expressed as pg/ml $\pm$ s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

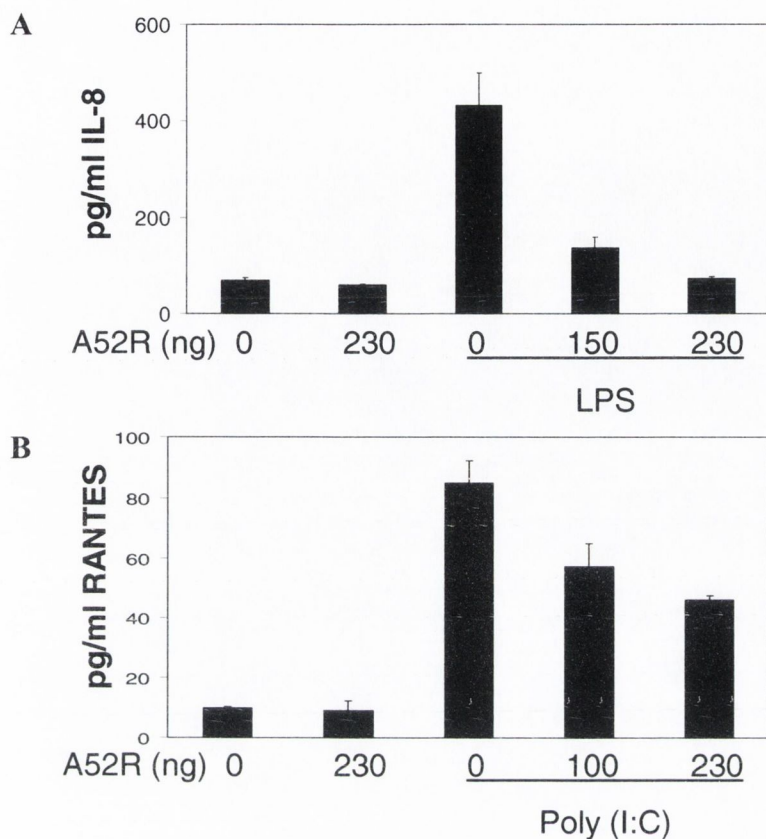


Fig. 5.13: The effect of A52R on IL-8 and RANTES production

HEK-TLR4 (A) or HEK-TLR3 (B) (2×10^4 cells per ml) were seeded into 96-well plates and transfected with the indicated (ng) amounts of A52R 24 hours prior to stimulation with 1 μ g/ml LPS (A) or 25 μ g/ml Poly (I:C) (B). 24 hours after stimulation supernatants were harvested and assayed for IL-8 production (A) or RANTES production (B) by ELISA. Data is expressed as pg/ml $\pm$ s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

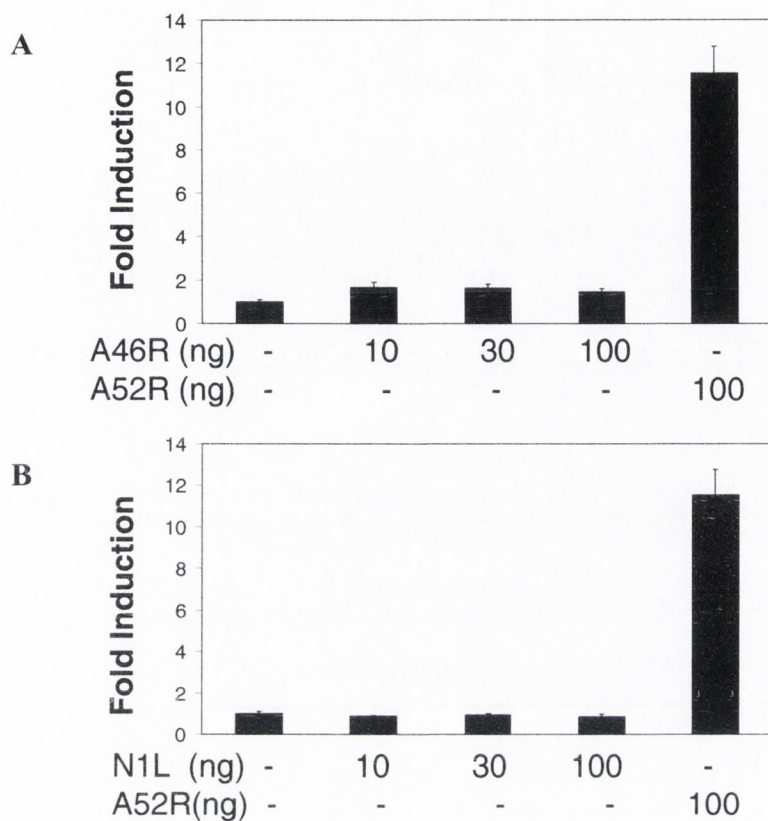


Fig. 5.14: A46R and N1L do not drive the IL-10 Promoter in HEK 293 cells

HEK293 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected 24 hours later with expression plasmids, IL-10 promoter luciferase reporter plasmid, Renilla luciferase internal control and increasing amounts of A46R (10, 30 100 ng) (A) or NIL (10, 30, 100 ng) (B) and 100 ng of a plasmid encoding A52R. After 24 hours the cells were harvested and the reporter gene activity measured. Data is expressed as mean fold induction $\pm$ s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

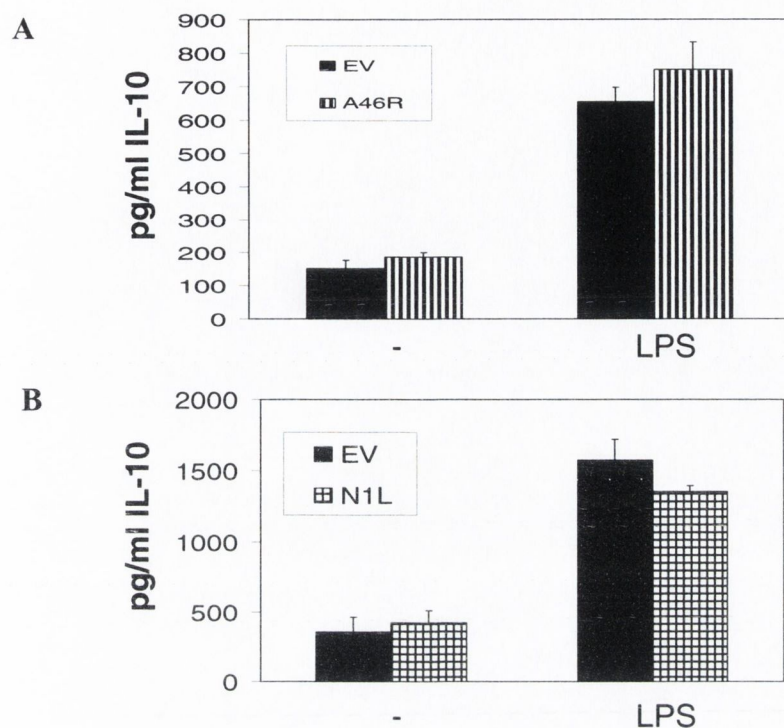


Fig 5.15: The effect of A46R and N1L on LPS induced IL-10 production

Murine Macrophage RAW 264.7 cells (2×10^4 cells per ml) were seeded into 96-well plates and transfected with 120 ng of a plasmid encoding A46R (A) or N1L (B) 24 hours prior to stimulation with 1 μ g/ml LPS. 24 hours after stimulation supernatants were harvested and assayed for IL-10 production by ELISA. Data is expressed as pg/ml $\pm$ s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

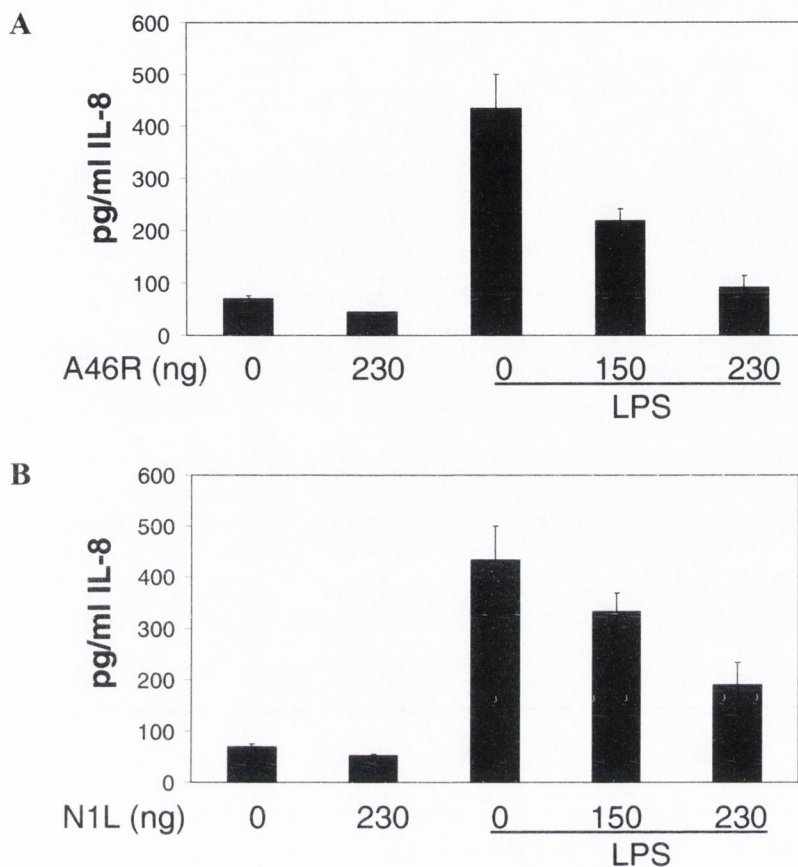


Fig. 5.16: The effect of A46R and N1L on LPS induced IL-8 production

HEK-TLR4 (2×10^4 cells per ml) were seeded into 96-well plates and transfected with the indicated (ng) amounts of A46R (A) or N1L (B) 24 hours prior to stimulation with $1 \mu\text{g/ml}$ LPS. 24 hours after stimulation supernatants were harvested and assayed for IL-8 production (A) or RANTES production (B) by ELISA. Data is expressed as pg/ml $\pm$ s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

5.7 Discussion

In this chapter the effect of the VV protein A52R on MAP kinase activation and LPS-induced IL-10 production was investigated. It was shown that A52R expression could activate both p38 and JNK MAP kinases. Further, A52R was shown to dramatically enhance TLR4-induced IL-10 production, which is known to be p38-dependent. In contrast inhibition of the TLR-induced NF κ B-dependent chemokines IL-8 and RANTES by A52R was observed. The fact that A52R differentially modulates TLR signalling reveals a further layer of complexity in the viral subversion of TLR signalling.

The ability of A52R to activate p38 was dependent on TRAF6. The fact that Δ A52R, which could not form a complex with TRAF6 (Fig. 3.16), and failed to drive p38 and JNK (Fig. 5.3) suggests that TRAF6 is crucial for mediating p38 and JNK activation by A52R (Fig. 5.2a, b). Further, expression of the TRAF domain of TRAF6, which A52R interacts with (Fig. 5.5), blocked the ability of A52R to activate p38 (Fig. 5.6). The observation that A52R activated p38 in the TRAF6^{-/-} MEFs (Fig. 5.7b) might reflect compensation by other TRAF family members and this possibility is currently under investigation in the laboratory. Given the fact that A52R interacted with the TRAF domain of TRAF6 and this domain is conserved among all TRAF family members, A52R may be interacting with other TRAF family members. A52R did not interact with TRAF2, the TRAF family member involved in TNF signalling (Fig. 3.9). Interestingly, densitometry analysis of the TRAF6 MEFs Western blots (using the ImageJ analysis) revealed a 6-fold increase in band intensity in lane 3 (i.e. the A52R containing lane) in the TRAF6^{+/+} MEFs compared with only a 2.5-fold increase in lane 6 in the TRAF6^{-/-} MEFs (Fig. 5.7).

The TRAF domain of TRAF6 is responsible for mediating oligomerisation of TRAF6 (Baud *et al.*, 1999). This facilitates the interaction of TRAF6 with TRAF6 regulated I κ B kinase activator 1 (TRIKA1), a dimeric ubiquitin conjugated enzyme complex composed of Ubc13 and Uvel1A (Wang *et al.*, 2001). This interaction causes a novel form of polyubiquitination involving Lys-63 of ubiquitin to occur. TRAF6 is ubiquitinated and TAK1 is activated which in turn phosphorylates MKK6 in the p38 kinase pathway (Wang *et al.*, 2001). In the absence of any upstream activation, enforced oligomerisation of TRAF6 leads to MAP kinase activation (Baud *et al.*, 1999). Thus, the fact that A52R targets the TRAF domain of TRAF6 (Fig. 5.5) and drives MAP kinase activation (Fig. 5.2) may suggest that A52R can cause oligomerisation of TRAF6 in the absence of upstream activation. Oligomerisation of TRAF6 should also lead to NF κ B activation. However, A52R can inhibit NF κ B activation (Fig. 3.4, 3.5, Bowie *et al.*, 2001, Harte *et al.*, 2003) but this is likely to be due to its ability to interact with IRAK2 (Fig. 3.7, Harte *et al.*, 2003) since Δ A52R which only interacted with IRAK2 and not TRAF6 (Fig. 3.16, 3.17) was still capable of inhibiting TLR-induced NF κ B activation (Fig. 3.18).

The fact that Δ A52R could not interact with TRAF6 (Fig. 3.16) suggests that the interaction site between A52R and TRAF6 is located between amino acids 145-190 of A52R. Although A52R does not have a typical TRAF6 bind motif (Pro-X-Glu-X-X-aromatic/acidic residue), a possible TRAF6 binding motif may be located at amino acids 149-154 of A52R. Instead of a proline that is usually found in a TRAF6 binding motif, an arginine is present in A52R. The glutamic acid and the aromatic/acidic residue that were found to be critical for TRAF6 binding (Ye *et al.*,

2002) are also found in A52R (Fig. 5.17). Interestingly, the unique biological function of TRAF6 is largely determined by its TRAF domain, and human CD40 and TRACE-R (both of which contain a TRAF6 binding motif) were found to interact with the TRAF domain of TRAF6 in crystallisation studies (Ye *et al.*, 2002). Indeed, like these two proteins, A52R also interacted with the TRAF domain of TRAF6 (Fig. 5.5). Furthermore, Δ N which contains amino acids 59-190 of A52R was also capable of driving p38 MAP kinase activation (Fig. 5.4) suggesting that Δ N might interact with TRAF6 since it contains the proposed TRAF6 binding motif. Further investigations into mapping the sites of interaction between A52R and TRAF6 are being carried out in the laboratory.

The effect of A52R on the activation of the MAP kinases p38 and JNK is consistent with the emerging theme that viral proteins engage host TRAF molecules to modulate signalling pathways. For example, the non-structural 5A (NS5A) protein of hepatitis C virus interacts with the TRAF domain of TRAF2 and can potentiate TNF α -induced JNK activation (Park *et al.*, 2003). Another viral protein, VP4 from rotaviruses, also interacts with TRAF2. However, this protein has opposite effects to A52R in that it activates NF κ B and inhibits JNK activation (LaMonica *et al.*, 2001). In addition, similar to A52R, the oncogenic latent membrane protein 1 (LMP1) from Epstein-Barr Virus (EBV) has been shown to induce p38 MAP kinase through a TRAF6-dependent mechanism (Schultheiss *et al.*, 2001). Similar to our findings (Fig. 5.10) overexpression of a dominant negative form of TRAF6 entirely blocked p38 MAP kinase induction by LMP1 (Schultheiss *et al.*, 2001).

**MDIKIDISISGDKFTVTTRRENEERKKYLPLQKE
KTTDVIKPDYLEYDDLDRDEMSTILEEYFMYR
GLLGLRIKYGRLFNEIKKFDNDAEEQFGTIEELK
QKLRLNSEEGADNFIDYIKVQKQDIVKLTVYDCI
SMIGLCACVVDVWRNEKLFSRWKYCLRAIKLFI
NDHMLDKIKSILQNRLVYVEMS**

Fig. 5.17 Amino acid sequence of A52R

Full length A52R is 190 amino acids long. A possible TRAF6 binding motif is underlined. It is located between amino acids 149-154. A typical TRAF6 binding motif contains the consensus: P-X-E-X-X-aromatic/acidic residue. Instead of a proline, A52R has an arginine. However, the glutamic acid and the aromatic/acidic residue (phenylalanine in A52R) that are found in a typical TRAF6 binding motif are also present in A52R.

Several viruses can induce activation of MAP kinases in infected cells, for example human immunodeficiency virus type 1 (Kumar *et al.*, 1998), herpes simplex virus type 1 (Zachos *et al.*, 1999) and human cytomegalovirus (Rodems *et al.*, 1998). It has also been shown that the mitogenic Ras/MEK/MAPK pathway contributes to the replication of herpes simplex virus type 2 (Smith *et al.*, 2000) and influenza virus (Pleschka *et al.*, 2001). In addition, other studies have shown that the VV stimulated MAP kinase pathway is required for virus multiplication (de Magelhaes *et al.*, 2001, Andrade *et al.*, 2004). The fact that the VV protein A52R activates p38 in a TRAF6-dependent manner may be important to its role in virulence. Interestingly, we have shown that VV replication is impaired in TRAF6^{-/-} MEFs, or where p38 activation is blocked (I. Haga personal communication). Similarly, encephalomyocarditis (EMC) virus has been shown to induce p38 MAP kinase activation and suppression of this activation by the specific p38 MAP kinase inhibitor, SB203580, resulted in inhibition of EMC virus replication (Hirasawa *et al.*, 2003). Thus, indicating that p38 MAP kinase plays a key role in the replication of EMC virus. Furthermore, it has been demonstrated that replication of varicella-zoster virus (VZV) is influenced by the levels of JNK and p38 MAP kinase activation whereby inhibition of JNK resulted in a 2-fold increase in virus replication whereas a strong decrease in virus replication was observed after inhibition of p38 MAP kinase (Rahaus *et al.*, 2004).

The ability of A52R to activate p38, together with its known inhibitory effect on NFκB activation were shown to translate into effects on gene induction by TLRs. LPS-induced IL-10 (which is known to be p38 dependent) was enhanced in the presence of A52R (Fig. 5.11), while TLR-induced NFκB-dependent chemokine

production was inhibited (Fig. 5.13). The effect of A52R on LPS-induced IL-10 was likely to be at the level of the promoter, since A52R expression led to activation of an IL-10 promoter (Fig. 5.8). However, A52R expression did not lead to IL-10 protein in the absence of LPS (Fig. 5.11), suggesting that induction of IL-10 promoter by A52R was not sufficient to lead to protein expression. Rather, A52R may synergise with LPS at the level of the IL-10 promoter (presumably in a p38-dependent fashion), leading to enhanced IL-10 production when the TLR4 pathway is fully activated. TRAF6 was clearly shown to have a role in the synergistic effect, since A52R could not induce the IL-10 promoter in TRAF6^{-/-} MEFs (Fig. 5.9), and ectopic expression of TRAF6 led to a similar effect on IL-10 protein in the presence of LPS (Fig. 5.12a) as seen for A52R. Interestingly, IRAK1 has also recently been shown to be critically involved in LPS induced IL-10 production (Huang *et al.*, 2004b). Further, LMP1 from EBV, which as mentioned can interact with TRAF6 and potentiate p38, can also induce IL-10 production (Schultheiss *et al.*, 2001, Vockerodt *et al.*, 2001). The enhancement of IL-10 production by A52R (and indeed TRAF6) may also have a post-translational component since IL-10 mRNA contains destabilising AU -rich elements (ARE) in its 3' untranslated region (Powell *et al.*, 2000). It is known that p38 activation leads to stabilisation of cytokine mRNA containing these repeats (Dean *et al.*, 2004). p38 MAP kinase activation has been shown to have a post-translational role in enhancing mRNA stability for example, the mRNA encoding IL-8 is stabilised following p38 MAP kinase activation (Holtmann *et al.*, 1999). The precise mechanism of how p38 regulates mRNA stability remains unknown. However, it is clear that it involves MAP kinase activated protein kinase 2, an inflammatory mRNA ARE and deadenylation (Dean *et al.*, 2004). Interestingly, IL-1 and TLR4

but not TLR3 can lead to stabilisation of unstable chemokine mRNAs. The ability of IL-1 and TLR4 to induce mRNA stabilisation depends on their differential use of TIR adaptors; MyD88 and Mal efficiently enhance mRNA stability whereas TRIF and TRAM are relatively inactive (Datta *et al.*, 2005).

IL-10 is a pleiotropic cytokine that inhibits inflammatory and cell mediated immune responses (Moore *et al.*, 2001). TLR induced IL-10 has been shown to have a role in the generation of T regulatory cells and to lead to the inhibition of Th-1 responses (Netea *et al.*, 2004a, Netea *et al.*, 2004b, Higgins *et al.*, 2003). TLR-mediated anti-inflammatory signals are beneficial after the elimination of pathogens; however, they can induce dangerous immunosuppressive mechanism if activated too early during a severe infection. Recently it has become clear that TLR-induced IL-10 represents an important target of immune subversion for some pathogens. For example, the *Yersinia* virulence factor LcrV interacts with TLR2 leading to immunosuppression by induction of IL-10 (Sing *et al.*, 2002). TLR2^{-/-} mice (Sing *et al.*, 2002a) and IL-10^{-/-} mice (Sing *et al.*, 2002b) are less susceptible to oral *Yersinia enterocolitica* infection than wild type controls. The fungus *Candida albicans* has been shown to trigger immunosuppression through TLR2 induced IL-10 and subsequent survival of T regulatory cells (Netea *et al.*, 2004b). In addition, immune escape of mouse mammary tumour virus which leads to persistent infection has been shown to be dependent on TLR4 triggered production of IL-10 (Jung *et al.*, 2002). Therefore the enhancement of TLR4-induced IL-10 production by A52R may represent a vaccinia immune subversion mechanism. Another study showed that VV infection induced IL-10 in human monocytes (Slezak *et al.*, 2000). The ability of A52R to enhance TLR-induced IL-10

production may be contributing to the IL-10 production observed in infected human monocytes. Unlike other viruses such as EBV (Moore *et al.*, 1990) and poxviruses Orf (Fleming *et al.*, 1997) VV does not encode an IL-10 homologue. Evidence that elevated IL-10 levels would favour the virus have been confirmed in that, VV replication was impaired in IL-10^{-/-} mice (van Den Broek *et al.*, 2000).

To date TLR2, 3, 4, 7, 8 and 9 have been shown to be activated by viral proteins and nucleic acids (Section 1.). However no TLR has yet been shown to recognise VV. Nevertheless VV encodes other proteins that have been shown to specifically target the IL-1R/TLR superfamily; these are A46R and N1L (Bowie *et al.*, 2001, DiPerna *et al.*, 2004). A46R is a viral TIR domain containing protein and interacts with TIR adaptor proteins leading to inhibition of multiple IL-1R/TLR signalling (Bowie *et al.*, 2001, Stack *et al.*, 2005), while N1L has been shown to suppress NFκB activation by IL-1R-TLRs by targeting the IKK complex (DiPerna *et al.*, 2004). However, the ability to enhance TLR-induced IL-10 is unique to A52R, since overexpression of neither A46R nor N1L resulted in an increase (or decrease) in TLR stimulated IL-10 production (Fig. 5.15). Further, A52R, A46R and N1L are not functionally redundant since a single deletion of each gene affected virus virulence (Harte *et al.*, 2003, Stack *et al.*, 2005, Bartlett *et al.*, 2002).

This study sheds further light on how VV interacts with the TLR system, and reveals that A52R is a multi-functional VV protein that can not only inhibit TLR-mediated NFκB activation (Bowie *et al.*, 2001, Harte *et al.*, 2003) and NFκB dependent genes, but can also, via TRAF6, activate p38 MAP kinase and subsequently potentiate TLR-induced IL-10. Both of these activities of A52R are likely to contribute to its role in virulence (Harte *et al.*, 2003).

Chapter 6

Final Discussion

6.1 Final Discussion

The co-evolution of viruses and their host has had a significant impact on how each has evolved. The consequences of this ancient and dynamic battle are evident in both host and virus genomes. Viruses and other invading pathogens have exerted unrelenting pressure on the mammalian host, necessitating the development of a complex and host immune system. Therefore, the successful replication of viruses within the host requires strategies to evade the host attack. Many viruses, including vaccinia, have an extensive inventory of gene products that defend these viruses against host immune attack.

The immune response to poxvirus infection is complex (Reviewed in Smith and Kotwal, 2002). Early in infection the innate immune response attempts to control the infection using complement, IFN, natural killer cells and an inflammatory response, while in a matter of days antibodies directed against the virus are produced. To circumvent the host immune attack, the VV genome contains numerous genes encoding proteins involved in immunomodulation and immunoevasion. For example, VV secretes decoy receptors and binding proteins for IL-1, IL-18, TNF, chemokines, IFN- α/β and IFN- γ and anti-apoptotic proteins (Reviewed in Alcamí and Koszinowski, 2000). However, it is still unclear as to how the host response against VV is initiated.

Given the importance of the TLR superfamily in initiating an appropriate innate immune response to infectious agents and in directing the later adaptive response, the question arose as to whether VV would have strategies to subvert the function of this family of receptors. The discovery that the VV protein A46R contained a

putative TIR domain and the subsequent identification of A52R during a BLAST search with A46R raised the possibility that these two proteins could be involved in some aspect of immunomodulation of the TLR superfamily (Bowie *et al.*, 2000).

A52R was subsequently implicated in the evasion of the host TLR response since a role for A52R in VV virulence was clearly established (Harte *et al.*, 2003). In this study, A52R was shown to block every IL-1/TLR pathway to NF κ B tested and to interact with two key signalling molecules, TRAF6 and IRAK2 and. Here, the interaction with IRAK2 was shown to be sufficient for inhibition of IL-1/TLR-induced NF κ B activation. A52R was shown to activate p38 MAP kinase in a TRAF6-dependent manner and subsequently potentiate LPS-induced IL-10. These activities of A52R are likely to contribute to its role in virulence. It was also demonstrated that A52R had no effect on TLR-mediated IRF activation.

In addition to A52R, VV encodes other proteins that target the IL-1/TLR pathway. These are A46R and N1L (Bowie *et al.*, 2000, Stack *et al.*, 2005, DiPerna *et al.*, 2004). A46R has recently been shown to act to target multiple TIR adaptors, thereby interfering with downstream TLR signalling pathways (Stack *et al.*, 2005). A46R was also shown to contribute to VV virulence (Stack *et al.*, 2005). Unlike A52R, A46R is conserved amongst VV strains and it is found in variola virus, the causative agent of smallpox. However, as shown in this study A46R and A52R are not functionally redundant. Whereas A52R is a potent TLR3- induced NF κ B inhibitor (Fig. 3.4) it had no effect on TLR3- or TRIF- mediated IRF3 activation (Fig. 4.1b, 4.5a) nor on MyD88 or TRAF6 mediated IRF5 or IRF7 activation (Fig. 4.8, 4.9). In contrast, A46R blocked all IRF-mediated signalling (Fig. 4.8, 4.9) and

had little effect on TLR3-mediated NF κ B activation (Fig. 4.2c). Thus A52R inhibits all TLR-NF κ B pathways, while A46R seems to block all TLR-IRF pathways.

N1L is another intracellular VV protein that contributes to virulence (Bartlett *et al.*, 2003), which has also been shown to function by antagonising TLR signalling, but at the level of I κ B kinases and related kinases involved in IRF3 activation (DiPerna *et al.*, 2004). Interestingly, overexpression of either A46R or N1L did not activate the IL-10 promoter in HEK293 cells. Furthermore, the ability of A52R to enhance TLR-induced IL-10 is unique to A52R, since overexpression of neither A46R nor N1L resulted in an increase (or decrease) in LPS stimulated IL-10 production. Like A52R, N1L can also inhibit TLR-NF κ B pathways, however, the fact that A52R potentiates TLR-mediated p38 MAP kinase leading to enhanced IL-10 production could be its unique contribution to VV virulence. Thus, the importance of modulating TLR signalling is demonstrated by the retention by VV of at least three distinct mechanism of disrupting these pathways. No TLR has yet been shown to recognise VV. However, the attenuated phenotypes seen in the absence of A52R, A46R and N1L provide evidence for a role for TLRs in containing VV infections. Possible VV PAMPs detected by TLRs could be proteins on the surface of the intracellular mature virion or extracellular enveloped proteins particles (potentially detected by TLR2 or TLR4 (Vidya and Cheng, 2003, Rassa and Ross, 2003), intracellular dsRNA produced during VV replication (potentially detected by TLR3 (Alexopoulou *et al.*, 2001), or the dsDNA genome itself (potentially detected by TLR9 which responds to the dsDNA genome from herpes simplex virus (Krug *et al.*, 2004, Lund *et al.*, 2004).

A further VV protein, K1L, has also been shown to inhibit NF κ B activation through a mechanism of inhibiting I κ B α degradation. However, this study did not investigate if the activation of NF κ B was TLR-dependent. Nevertheless, the fact that VV encodes four immunomodulatory proteins that specifically target NF κ B suggests that this is an important strategy to evade immune detection.

Given the fact that A52R interacted with IRAK2 (Fig. 3.8) and that this interaction was shown to be required for inhibition of IL-1/TLR induced NF κ B activation, I propose a role for IRAK2 in IL-1/TLR induced NF κ B activation. Consistent with this, a role for IRAK2 in TLR2&1, TLR2&6, TLR3, TLR4 and TLR5-mediated NF κ B activation was suggested by using a dominant negative version of IRAK2 (Fig. 3.14). Evidence is beginning to emerge that IRAK1 is not essential for IL-1/TLR-mediated NF κ B activation. It has been reported that TLR3-mediated NF κ B activation is through an IRAK1-independent pathway (Jiang *et al.*, 2003b). Another study also demonstrated that inflammatory cytokine production in response to TLR7 and TLR9 ligands was normal in IRAK1 deficient cells (Uemastu *et al.*, 2005), implying an IRAK1-independent mechanism of action.

A52R was also shown to have no effect on TLR3-induced IRF3 or MyD88 or TRAF6 induced IRF5 and IRF7 activation. Therefore, it is possible that IRAK2 may have no role in TLR-mediated IRF activation. Recently, it has been reported that IRAK1 is essential for TLR7 and TLR9-induced IRF7 activation (Uematsu *et al.*, 2005).

The results presented in this thesis highlight the fact that the specific role of IRAK2 in IL-1/TLR signalling needs to be addressed in more detail. Biochemical analysis and transfection based experiments have revealed much about the mechanism of action of IRAK1 and IRAK4. However, very little is known about the exact function IRAK2 plays in TLR signalling. Hence, analysis of the interactions of IRAK2 other IL-1/TLR signalling components should reveal critical information as to the precise mechanism of action of IRAK2. This information will also shed light on which IL-1/TLR signalling pathways use IRAK2. Furthermore, the generation of an IRAK2 knockout mouse will be required to clarify the exact role of IRAK2 in TLR signalling.

The interaction of A52R with TRAF6 was required for p38 MAP kinase activation and was also accountable for the enhanced TLR-stimulated IL-10 production observed (Fig. 5.11). Consistent with this, LPS-induced IL-10 production is known to be p38-dependent (Foey *et al.*, 1998, Ma *et al.*, 2000). The fact that A52R still drove some p38 activation in TRAF6 knockout cells suggests that A52R might use other mechanisms to achieve p38 activation (Fig. 5.7). Indeed, A52R may be interacting with other TRAF family members, for example, TRAF7. TRAF7 has been reported to specifically interact with MEKK3 and potentiated MEKK3-mediated AP-1 and CHOP activation (Xu *et al.*, 2004). A52R also drove activation of JNK in a reporter gene assay (Fig. 5.2b) and Δ A52R, which no longer interacted with TRAF6, failed to activate JNK (Fig. 5.3) suggesting a role for TRAF6 in the stimulatory effect of A52R on JNK activation. A52R-induced JNK activation may also have a role in enhancement of TLR-induced IL-10 production by A52R. This hypothesis needs to be addressed in greater detail. Although VV does not encode

an IL-10 homologue, it has been reported the VV replication is impaired in IL-10^{-/-} mice (Van Den Broek *et al.*, 2000). In addition, another study showed that VV infection induced IL-10 in human monocytes, with a more significant increase in IL-10 expression observed following LPS treatment (Slezak *et al.*, 2000). Therefore, the ability of A52R to enhance TLR-induced IL-10 production may be contributing to the IL-10 production observed in infected human monocytes.

Advances in our understanding of how TLRs signal may allow a range of challenges in immunology and medicine to be tackled, for example autoimmunity and sepsis (Beutler 2004). Many of the current therapeutic strategies employed to combat autoimmune diseases, such as rheumatoid arthritis, involve blocking cytokines such as TNF. These cytokines are now known to be induced in response to upstream TLR signalling. Therefore, targeting TLR signalling directly is ultimately likely to be an even more effective approach to blocking inflammation. Thus, the development of inhibitors of TLR signalling may give rise to powerful new therapies with use in a range of contexts, including sepsis, transplantation and autoimmunity. The role of TLRs has begun to be investigated in all the major aspects of organ transplantation including ischemia-reperfusion injury, acute and chronic rejection and post-transplant infection (Andrade *et al.*, 2005). For example, during reperfusion, ischemic organs are infiltrated with activated CD4⁺ T cells and monocytes/macrophages, which can secrete cytokines, chemoattractants and other inflammatory mediators. Using a quantitative real-time PCR technique, Andrade *et al.*, examined mRNA expression levels of TLRs in lung tissue collected during ischemia and reperfusion in human lung transplantation. mRNA levels of most TLRs correlated with the mRNA levels of IL-1 β , IL-6, IL-8, IL-10 and IFN γ

(Andrade *et al.*, 2005). This observation suggests that the TLR expression levels and activation may contribute to the regulation of cytokine gene expression.

Viral proteins such as A52R may represent useful strategies for immunosuppression, since such proteins have usually been optimised and modified during evolution to be maximally effective against their targets. The identification of an A52R peptide that inhibited cytokine secretion in response to TLR activation, but not TNF α or PMA activation (McCoy *et al.*, 2005), reveals the possible therapeutic potential of this viral inhibitor.

6.2 Future Work

- 1 Direct interactions between A52R and TRAF6 and IRAK2 remain to be demonstrated. A yeast-2-hybrid approach and mass spectroscopy analysis are currently being carried out in an attempt to detect a direct interaction. In addition, interactions of A52R with endogenous TRAF6 and IRAK2 in VV-infected cells should be assessed. Using the antibody raised against A52R, attempts to co-immunoprecipitate A52R with specific host proteins can be undertaken. Further truncations of A52R can be generated, while co-immunoprecipitates will determine if these truncations retain the ability to form a complex with TRAF6 or IRAK2. In addition, the proposed role for IRAK2 in TLR-induced NF κ B activation is currently under investigation.
- 2 Further experiments are necessary to ratify the hypothesis that A52R activates p38 MAP kinase through its interaction with TRAF6, given the observation that A52R activated p38 MAP kinase in TRAF6^{-/-} MEFs. This

experiment suggests that other TRAF family members may be compensating for the lack of TRAF6, thus facilitating p38 activation. Therefore, future studies to characterise if A52R interacts with other TRAF family members e.g. TRAF3, 4, 5 and 7 should be considered. The exact mechanism of how A52R enhances LPS-induced IL-10 and the effects on mRNA stability should also be scrutinised. This should reveal critical functional information as to how A52R exerts its effects. The importance of IL-10 enhancement to VV virulence should be addressed in greater detail. Experiments using VV and VV lacking A52R-infected cells could be analysed for differences in IL-10 production.

- 3 Further analysis of the effects of A52R on gene induction should be determined. This could be investigated using cells stably expressing A52R. A gene chip analysis would reveal the genes that are blocked and/or enhanced by A52R in response to TLR stimulation. In addition, this analysis will determine a role for TRAF6 and IRAK2 in gene induction. However, the vast amount of data generated from a gene chip analysis may mean that the most relevant results may not be immediately obvious. Therefore, a more effective approach would involve a comparative analysis of the effects of A52R and A46R on specific IRF regulated genes given the fact that A46R but not A52R had an inhibitory effect on TLR stimulated IRF activation. This type of analysis is fundamental to fully comprehend the exact functional role of each of these proteins.

- 4 McCoy *et al.*, (2005) identified an A52R peptide that blocked TLR-mediated NF κ B activation. Therefore, further investigation into the therapeutic potential of A52R will be carried out. A52R peptides will be generated and tested for the ability to specifically inhibit TLR signalling and/or enhance TLR-induced IL-10 production. This could be done using reporter gene assays and further clarified by testing the effect of the peptides on TLR-mediated gene induction (e.g. RANTES, IL-8 or IL-10 which can be measured by ELISA). Finally, any bioactive peptide will be tested *in vivo*, in a mouse model system. For example, a mouse model of septic shock could be used, whereby intravenous injection of LPS induces high levels of serum IL-12 and IFN γ , which are symptoms of shock (McGuirk *et al.*, 2000). Mice could be pre-injected with either bioactive or control peptide 1 hour prior to challenge with LPS, and the effects on IL-12 and IFN γ levels assessed. Any peptides that demonstrated activity in this model should then be tested in a more detailed *in vivo* study.

Chapter 7

References

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Appendices

APPENDIX I: Addresses of Suppliers

A.L.C. Interantional s.r.l.	Via Carlo Porta, 3 1-20093 Cologno Monzese Milan Italy
Argus Fine Chemicals	Sussex Innovation Centre Science Park Square Brighton United Kingdom
Axxora (UK) Ltd. (Formerly Alexis Corporation)	PO Box 6757 Bingham Nottingham NG13 8LS United Kingdom
Amersham Biosciences	SE-751 84 Uppsala Sweden
Babco (Berkely Antibody Company) Covance Research products, Inc.	PO Box 7200 Denver PA 17517 USA
Biosynth AG	Rietlistr. 4 Postfach 125 9422 Staad Switzerland
Cell Signalling Technology	166B Cummings Centre Beverly MA 01915 USA

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Corning B.V. Life Sciences	European Office and Distribution Centre Koolhovenlaan 12 1119 NE Schiphol-Rijk The Netherlands
Dunnes Stores	Outlets Nationwide
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Greiner GmbH	Maybachstrasse P.O. Box 1162 D-7443 Frickenhausen Germany
Invitrogen	PO Box 2312, 9704 CH Groningen, The Netherlands
LabTech	1 Acorn House The Broyle Ringmer East Sussex BN8 5NN United Kingdom

Molecular Devices Ltd.	135 Wharfdale Road Winnersh Triangle Winnersh Wokingham RG41 5RB United Kingdom
New England Biolabs	32 Tozer Road, Beverly, MA 01915-5599, USA
Novagen	Merck Biosciences Ltd. Padge Road Beeston/Nottingham NG9 2JR United Kingdom
Pierce	3747 N. Meridian Road P.O. Box 117 Rockford IL 61105 USA
Promega Corporation	2800 Woods Hollow Road Madison WI 53711-5399 USA
R&D Systems Europe	19 Barton Lane Abingdon Science Park Abingdon, Oxon OX14 3NB United Kingdom
Santa Cruz Biotechnology, Inc.	2161 Delaware Avenue Santa Cruz CA 95060 USA

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Dorset BH17 7BR
United Kingdom

Stratagene Cloning Systems

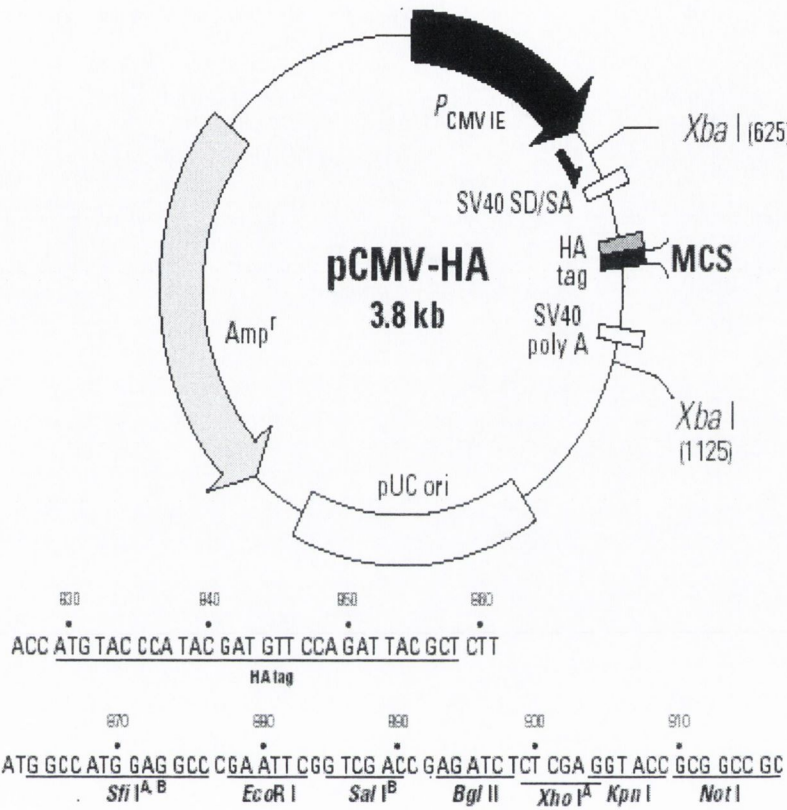
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USA

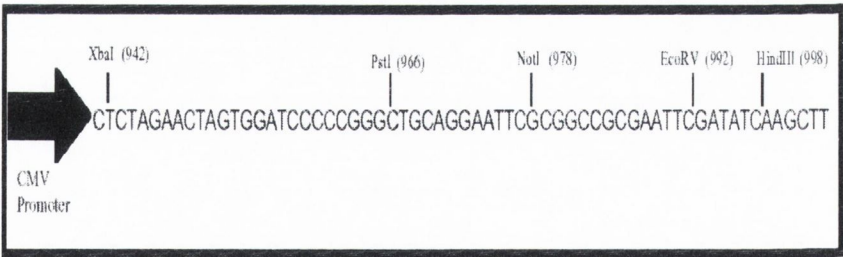
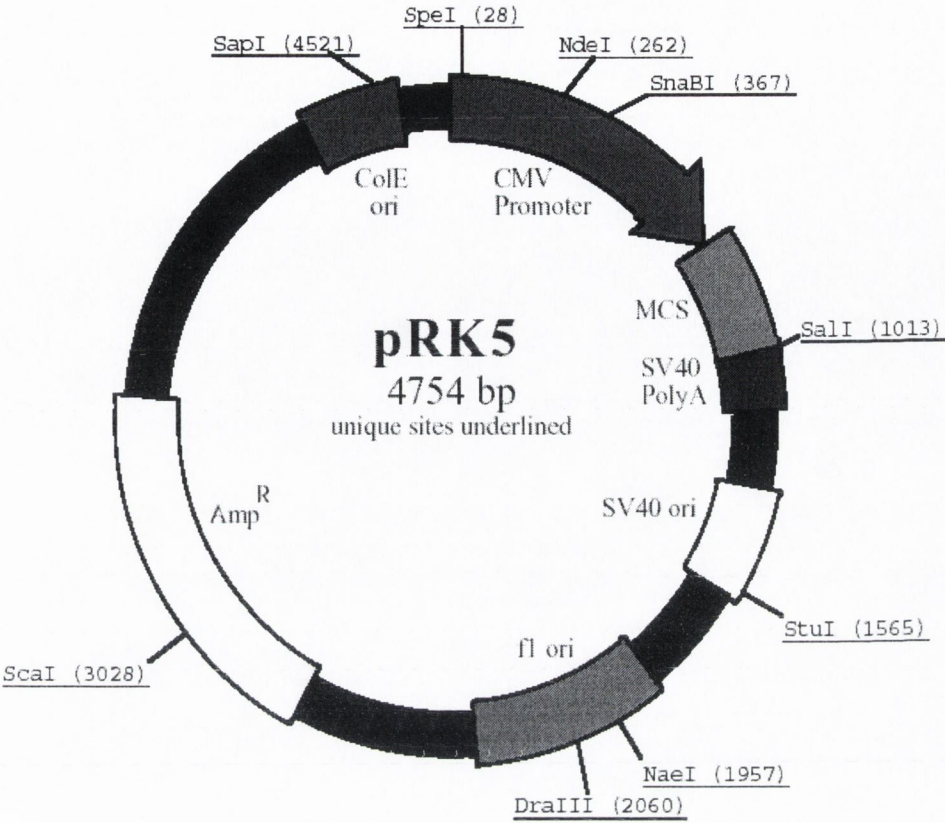
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Publications

The Poxvirus Protein A52R Targets Toll-like Receptor Signaling Complexes to Suppress Host Defense

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Abstract

Toll-like receptors (TLRs) are crucial in the innate immune response to pathogens, in that they recognize and respond to pathogen associated molecular patterns, which leads to activation of intracellular signaling pathways and altered gene expression. Vaccinia virus (VV), the poxvirus used to vaccinate against smallpox, encodes proteins that antagonize important components of host antiviral defense. Here we show that the VV protein A52R blocks the activation of the transcription factor nuclear factor κ B (NF- κ B) by multiple TLRs, including TLR3, a recently identified receptor for viral RNA. A52R associates with both interleukin 1 receptor-associated kinase 2 (IRAK2) and tumor necrosis factor receptor-associated factor 6 (TRAF6), two key proteins important in TLR signal transduction. Further, A52R could disrupt signaling complexes containing these proteins. A virus deletion mutant lacking the A52R gene was attenuated compared with wild-type and revertant controls in a murine intranasal model of infection. This study reveals a novel mechanism used by VV to suppress the host immunity. We demonstrate viral disabling of TLRs, providing further evidence for an important role for this family of receptors in the antiviral response.

Key words: nuclear factor κ B • signal transduction • vaccinia virus • immunomodulation • Toll-like receptor

Introduction

Members of the IL-1 receptor (IL-1R)/Toll-like receptor (TLR)* superfamily are key mediators in innate and adaptive immunity (1). The superfamily is defined by the presence of a cytosolic motif termed the Toll/IL-1 receptor (TIR) domain (1). The family includes receptors for the proinflammatory cytokines IL-1 and IL-18 as well as the TLR members, which participate in the recognition of pathogens by responding to pathogen-associated molecular

patterns and activating signaling pathways leading to altered gene expression (1, 2). 10 mammalian TLRs have been identified to date. TLR4, TLR5, and TLR9 are essential in the respective recognition of LPS, bacterial flagellin, and unmethylated CpG motifs that are present in bacterial DNA (3–6). TLR2 recognizes bacterial lipoproteins and other Gram-positive molecular patterns, but only when present as a heterodimer in combination with either TLR1 or TLR6 (7–12).

TLRs have also been implicated in sensing viral infections (13–17). Most notably, TLR3 is activated in response to poly(I:C), a synthetic double-stranded RNA (dsRNA) mimic of viral dsRNA, which is a molecular pattern produced by most viruses (15). Poly(I:C) activation of cells via TLR3 led to the activation of the transcription factor nuclear factor (NF)- κ B and the production of type I interferons, which are important in antiviral innate immunity (15).

A. Bowie and L. O'Neill contributed equally to the work.

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*Abbreviations used in this paper: dsRNA, double-stranded RNA; IP, immunoprecipitation; IRAK, IL-1 receptor-associated kinase; Mal, MyD88 adaptor-like; NF, nuclear factor; TAB, TAK1 binding protein; TAK1, TGF- β -activated kinase 1; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRAF, TNF receptor associated factor; VV, vaccinia virus.

Stimulation of IL-1R/TLR family members with the appropriate ligands leads to activation of NF- κ B and also the mitogen-activated protein kinases p38, JNK, and p42/44. The activation of NF- κ B by IL-1, IL-18, TLR2, TLR7, and TLR9 is absolutely dependent on the cytoplasmic TIR domain-containing protein MyD88 (16, 18–20), which is recruited to receptor TIR domains (21–23). However, both TLR4 and TLR3 are able to activate NF- κ B, by both a MyD88-dependent and MyD88-independent pathway (15, 24). For TLR4, the MyD88-independent pathway may be mediated by MyD88 adaptor-like (Mal, also known as TRAP), as it has been demonstrated that Mal is recruited to TLR4, and is involved in NF- κ B activation (25, 26). Activation of NF- κ B by the MyD88-dependent pathway can proceed via recruitment of IL-1 receptor-associated kinase (IRAK) and/or IRAK2, while Mal can recruit IRAK2 (25). IRAK or IRAK2 activation in turn leads to recruitment of TNF receptor-associated factor 6 (TRAF6). TRAF6 is required for the ubiquitination and activation of TGF- β -activated kinase 1 (TAK1), which, in complex with TAK1 binding protein 1 (TAB1), phosphorylates I κ B kinase (IKK) leading to NF- κ B activation (27).

Vaccinia virus (VV), the vaccine used to eradicate smallpox, encodes many immunomodulatory proteins to evade the host immune response (28). Previously we demonstrated that VV protein A52R down-regulated the activation of NF- κ B by the IL-1R/TLR superfamily members IL-1R, IL-18R, and TLR4 (29). Here we show that A52R inhibits multiple TLR pathways to NF- κ B, including TLR3, and provide evidence to suggest the mechanism of action. Further we show that A52R contributes to VV virulence in vivo. Thus, we demonstrate viral targeting of TLR signaling molecules, and reveal a novel mechanism used by VV to suppress the host immune response.

Materials and Methods

Expression Plasmids. Chimeric TLR receptors CD4-TLR1, CD4-TLR2, CD4-TLR4, CD4-TLR5, and CD4-TLR6 composed of the extracellular domain of CD4 fused to the transmembrane domain and cytosolic tail of the TLR were a gift from R. Medzhitov (Yale University, New Haven, CT). TLR3 was a provided by K. Fitzgerald and D. Golenbock (University of Massachusetts Medical School, Worcester, MA). AU1-MyD88, Myc-IRAK2, and Myc-IRAK2 expression vectors were a gift from M. Muzio, Mario Negri Institute, Milano, Italy (23). IRAK, Flag-TRAF6, Flag-TRAF6 domain (amino acids 289–522), and Flag-TRAF2 expression plasmids were provided by Tularik Inc. (San Francisco, CA). Flag-TAK-1 and HA-TAB-1 expression plasmids were a gift from H. Sakurai (Tanabe Seiyaku Co., Osaka, Japan). Construction of A52R, Flag-A52R, and HA-Mal expression plasmids was described previously (25, 29).

Antibodies. Polyclonal antibodies were raised against a purified, bacterially expressed glutathione S-transferase (GST) fusion of A52R, encoded by a plasmid synthesized by inserting full-length A52R downstream of GST in the bacterial expression vector GEX4T2. Other antibodies used were Anti-flag M2 monoclonal antibody, anti-flag M2 conjugated agarose, anti-myc

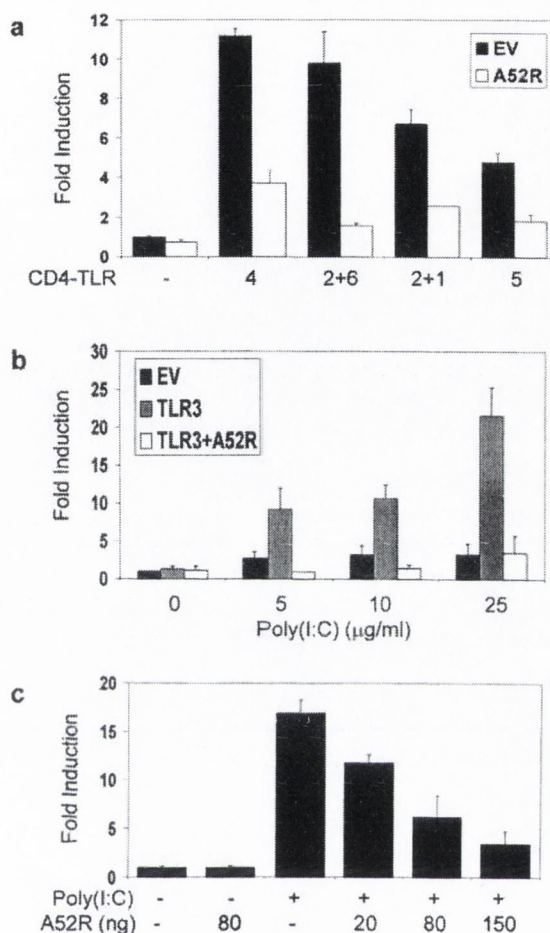


Figure 1. A52R inhibits NF- κ B activation by multiple TLRs. (A) 293 cells were transfected with constitutively active CD4-TLRs (50 ng TLR4 or TLR5, or 25 ng each of TLR2 and TLR6 or TLR2 and TLR1) in the presence of 80 ng empty vector (EV) or plasmid encoding A52R, together with NF- κ B reporter plasmid. (B) Cells were transfected with empty vector (EV), 0.5 ng TLR3, or 0.5 ng TLR3 plus 150 ng A52R. 6 h before harvesting cells were stimulated with 0–25 μ g/ml poly(I:C). (C) Cells were transfected with 0.5 ng TLR3 and stimulated with 25 μ g/ml poly(I:C) where indicated (+), in the presence of increasing amounts of A52R (20–150 ng). In all cases, luciferase activity was measured after 24 h.

monoclonal antibody clone 9E10 (all from Sigma-Aldrich), anti-AU1 monoclonal antibody (BabCO), anti-HA polyclonal antibody (Y-11), and anti-TRAF6 (H-274; both from Santa Cruz Biotechnology, Inc.). Anti-IRAK antibody was a gift from K. Ray (GlaxoSmithKline).

NF- κ B Reporter Gene Assay. HEK 293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κ B-luciferase reporter gene, and *Renilla*-luciferase internal control as described previously (25). The total amount of DNA per transfection was kept constant at 220 ng by addition of pcDNA3.1 (Stratagene). After 24 h, reporter gene activity was measured (25). Data are expressed as mean fold induction $\pm$ SD relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Immunoprecipitation and Immunoblotting. HEK 293T cells were seeded into 100-mm dishes (1.5×10^6) 24 h before transfection.

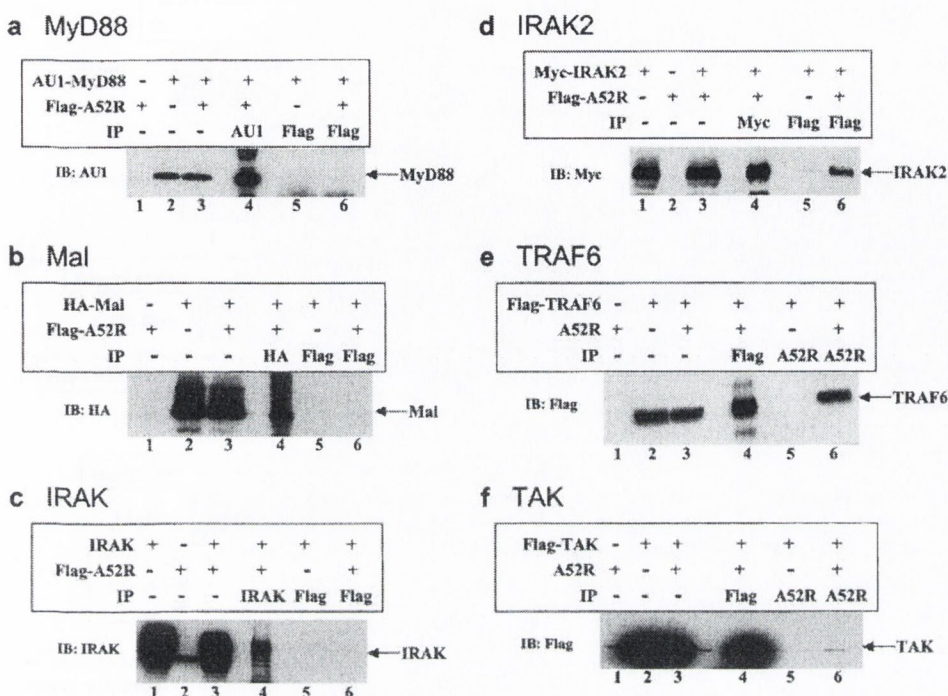


Figure 2. A52R immunoprecipitates with both IRAK2 and TRAF6. 293T cells were transfected with 4 μ g A52R (either flag-tagged or untagged, as indicated) plus 4 μ g of AU1-MyD88 (A), HA-Mal (B), IRAK (C), Myc-IRAK2 (D), Flag-TRAF6 (E), or Flag-TAK-1 (F). After 24 h lysates were subject to immunoprecipitation, and subsequent SDS-PAGE and immunoblotting with the antibodies indicated. Lysates were analyzed to ensure appropriate and equivalent expression of the proteins (lanes 1–3). To assess the efficiency of immunoprecipitation the immune complex (1/7th total lysate) was blotted with the same antibody used for immunoprecipitation (lane 4). Single transfection of each construct was also performed to act as a specificity control for the immunoprecipitating antibody (lane 5). The remainder of the immune complex was probed for the presence of associated protein (lane 6). The appearance of a band in lane 6 indicates an interaction. Representative of two (F) or three (A–E) independent experiments.

Transfections were performed using FuGENE 6 (Roche) according to manufacturer's instructions. For coimmunoprecipitations, 4 μ g of each construct was transfected. Where only one construct was expressed the total amount of DNA (8 μ g) was kept constant by supplementation with vector DNA. For competition experiments where the effect of increasing A52R expression on complex formation between two signaling molecules was examined, 2 μ g of each signaling molecule expression plasmid was transfected in the presence of increasing amounts of A52R expression plasmid. The total amount of DNA was kept constant in each sample using empty vector. In all cases, cells were harvested 24 h after transfection in 750 μ l of lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 containing 1 mM PMSF and protease inhibitor cocktail [Sigma-Aldrich], and 1 mM sodium orthovanadate). For immunoprecipitation, the indicated antibodies were precoupled to either protein A sepharose or protein G sepharose for 1 h at 4°C, washed, and then incubated with the cell lysates for 2 h at 4°C. The immune complexes were washed twice with lysis buffer and once with lysis buffer without NP-40 and glycerol. Associated proteins were eluted from the beads by boiling in 35 μ l sample buffer and fractionated by SDS PAGE. 30 μ l of the immune complex was immunoblotted for co-precipitating protein and the remaining 5 μ l was blotted directly for the protein directly recognized by the immunoprecipitating antibody. For immunoblotting, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence (Amersham Biosciences).

Recombinant VV Viruses. A VV mutant lacking 95.2% of the A52R gene (v Δ A52R) was constructed by transient dominant selection (30). A plaque purified wild-type virus (vA52R-wt) and a revertant virus (vA52R-rev) in which the A52R gene was reinserted at its natural locus were also isolated. The virulence of the viruses was investigated in a mouse intranasal model. Female, 6-wk-old Balb/c mice were anaesthetized and inoculated with 10⁴ PFU of VV in 20 μ l of phosphate-buffered saline. A control group

was mock infected with PBS. Each day the weights of the animals and signs of illness were measured as described previously (31).

Results

A52R Inhibits NF- κ B Activation by Multiple TLRs. We have previously shown that A52R blocks the activation of NF- κ B by IL-1, IL-18, and TLR4, with little inhibitory effect on the activation of NF- κ B by TNF (29). Given the emerging role of TLRs in antiviral responses, we tested the effect of A52R on NF- κ B activation by other TLRs. Chimeric versions of the TLRs, comprising the murine CD4 extracellular domain fused to the cytoplasmic domain of a human TLR render TLRs constitutively active, and have proved useful in probing TLR signaling pathways (5, 11, 32). Some TLR cytoplasmic domains can induce gene expression as homodimers (TLR4 and TLR5), while others signal as heterodimers (TLR1, TLR2, and TLR6; references 5 and 11). Using these chimeras TLR signaling can be examined in the absence of exogenous activator. Induction of an NF- κ B-dependent reporter gene by overexpression of either CD4-TLR4 or CD4-TLR5, or a combination of either CD4-TLR2 and 6, or CD4-TLR2 and 1 in HEK293 cells was inhibited in all cases by coexpression of A52R (Fig. 1 A).

DsRNA is a molecular pattern associated with viral infection, and TLR3 has been shown to sensitize cells to activation by polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue (15). Therefore, we also tested the effect of A52R on TLR3-dependent NF- κ B activation induced by poly(I:C). Transfection of HEK293 cells with TLR3 led to strong dose-dependent activation of NF- κ B by poly(I:C), which was not seen in the absence of TLR3 (Fig. 1 B). This

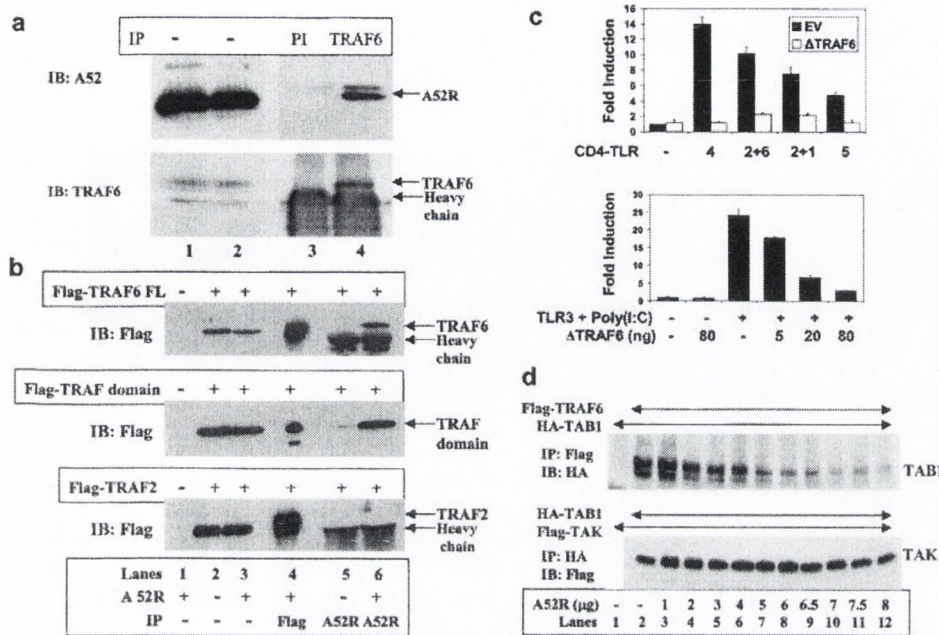


Figure 3. Analysis of the interaction of A52R with TRAF6. (A) A52R immunoprecipitates with endogenous TRAF6. Lysates from 293T cells transfected with A52R were immunoprecipitated with either preimmune serum (PI, lane 3) or anti-TRAF6 serum (lane 4), followed by immunoblotting with either A52R (top panel) or TRAF6 (bottom panel) antibody. Direct immunoblot of cell lysates confirmed equal expression of A52R and TRAF6 (lanes 1 and 2). (B) A52R immunoprecipitates with the TRAF6 TRAF domain, but not with TRAF2. 293T cells were transfected with A52R plus TRAF6 (top panel), Δ TRAF6 (residues 289–522 consisting of the TRAF domain of TRAF6, middle panel), or TRAF2 (bottom panel). Lysates were prepared and immunoprecipitated with A52R or Flag antibody as indicated, before immunoblot with Flag antibody. (C) Inhibition

of TLR-induced NF- κ B activation by Δ TRAF6. 293 cells were transfected with constitutively active CD4-TLRs as in Fig. 1, in the presence of 80 ng empty vector (EV) or plasmid encoding Δ TRAF6, together with NF- κ B reporter plasmid (top panel). In the bottom panel, 293 cells were transfected with 0.5 ng TLR3 and stimulated with 25 μ g/ml poly(I:C) where indicated (+). Luciferase activity was measured after 24 h. (D) A52R causes the dissociation of a TRAF6-TAB1-containing signaling complex but has no effect on a TAB1-TAK complex. A52R was cotransfected with either Flag-TRAF6 and HA-TAB1 (top panel) or HA-TAB1 and Flag-TAK1 (bottom r panel). Lysates were prepared and immunoprecipitated and immunoblotted as indicated. A52R can be seen to interfere with the TRAF6/TAB2 complex.

TLR3-dependent induction of NF- κ B was completely blocked by A52R (Fig. 1 B) in a dose-dependent manner (Fig. 1 C). Thus, A52R is a global inhibitor of signaling by the TLR family, with TLR3 being particularly sensitive.

A52R Interacts with TRAF6 and IRAK2. The activation of NF- κ B by different TLRs is mediated by a common set of signaling molecules (1). The ability of A52R to inhibit NF- κ B activation by multiple TLRs suggested that its effects might be due to its interaction with a molecule whose function is critical to signaling by all TLRs. Thus, the ability of A52R to associate with characterized mediators of signaling of the TLR family was examined in coimmunoprecipitation (coIP) experiments. No complex formation was detected when A52R was coexpressed with MyD88 (Fig. 2 A), Mal (Fig. 2 B), or IRAK (Fig. 2 C), as indicated by the lack of a band in lane 6 in each case. Under the same conditions complexes were detected between MyD88 and IRAK2, Mal and IRAK2, and IRAK and TRAF6 (unpublished data) as has been demonstrated previously (23, 25, 33). However, upon expression of A52R with IRAK2 a clear complex of A52R with IRAK2 was immunoprecipitated, using antibody to either A52R (Fig. 2 D, lane 6), or IRAK2 (unpublished data). The next signaling mediator downstream of the IRAK family is TRAF6 (33). Coexpression of A52R with TRAF6 resulted in the formation of a complex with high stoichiometry, as detected by IP with an antibody to either A52R (Fig. 2 E, lane 6), or TRAF6 (unpublished data). TRAF6 is responsible for activating TAK1 that

forms a complex with its two coactivators TAB1 and TAB2 (27). A52R was coexpressed with either TAK1 or TAB1 to determine if it associates with either of these downstream mediators of TRAF6 signaling. A weak but reproducible interaction was detected between A52R and TAK1 (Fig. 2 F, lane 6). No interaction was detectable between A52R and TAB1 (unpublished data). These results indicate that A52R is capable of interacting with both IRAK2 and TRAF6. The interaction with TAK1 is likely to occur via endogenous TRAF6.

A52R Associates with the TRAF6 TRAF Domain. We next examined the specificity and functional consequences of the interaction of A52R with TRAF6-containing complexes. Fig. 3 A shows that A52R could be immunoprecipitated with endogenous TRAF6 (Fig. 3 A, top panel, compare lanes 3 and 4). To determine the regions in TRAF6 responsible for interacting with A52R, truncated versions of TRAF6 were coexpressed with A52R and tested for their ability to interact by IP. A truncated version of TRAF6 composed of just the TRAF domain was able to interact with A52R to the same extent as the full length TRAF6 (Fig. 3 B, lanes 5 and 6, compare top and middle panels). TRAF2 is a TRAF family member with a role in TNF signaling (34). A52R has only minimal inhibitory effect on the activation of NF- κ B by TNFR (29) and consistent with this, no interaction between A52R and TRAF2 was detected (Fig. 3 B, bottom panel). A role for TRAF6 in the TLR pathways inhibited by A52R was confirmed by testing the effect of

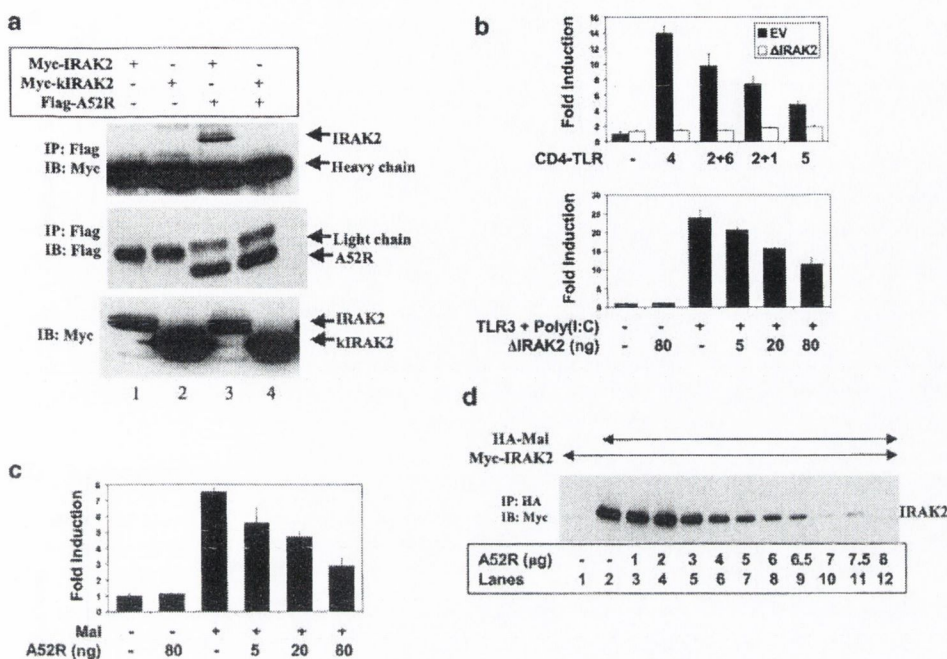


Figure 4. Role of IRAK2 in inhibition of TLR-induced NF- κ B activation by A52R. (A) A52R requires the IRAK2 death domain for association. 293T cells were cotransfected with A52R and either IRAK2, or kIRAK2 (lacking the death domain and containing residues 97–590). Lysates were immunoprecipitated and immunoblotted as indicated. The band in lane 3, top panel, indicates interaction with full-length IRAK2. (B) Inhibition of TLR-induced NF- κ B activation by kIRAK2. 293 cells were transfected with constitutively active CD4-TLRs, as in Fig. 1, in the presence of 80 ng empty vector (EV) or plasmid encoding kIRAK2 (Δ IRAK2), together with NF- κ B reporter plasmid (top panel). In the bottom panel, 293 cells were transfected with 0.5 ng TLR3 and stimulated with 25 μ g/ml poly(I:C) where indicated (+). Luciferase activity was measured after 24 h. (C)

A52R inhibits the activation of NF- κ B by Mal. 293 cells were transfected with 10 ng Mal where indicated (+), A52R, and NF- κ B reporter plasmid. Luciferase activity was measured after 24 h. (D) A52R causes the dissociation of a Mal-IRAK2-containing complex. A52R was cotransfected with IRAK2 and Mal. The amount of IRAK2-Mal complex formed was assessed by immunoprecipitation using anti-HA antibody, followed by immunoblotting with anti-myc antibody. A52R can be seen to interfere with the Mal/IRAK2 complex.

dominant negative TRAF6 (containing only the TRAF domain): Fig. 3 C shows that each CD4-TLR combination was inhibited (top panel), while 80 ng of plasmid encoding for dominant negative TRAF6 abolished the stimulatory effect of poly(I:C) (bottom panel). This is consistent with previous published work showing inhibition of TLR3-dependent NF- κ B activation by dominant negative TRAF6 (15).

A52R Disrupts a TRAF6-TAB1-containing Complex. To test whether the ability of A52R to target TRAF6 could explain inhibition of TLR-induced NF- κ B activation, the effect of A52R on the ability of TRAF6 to form active signaling complexes necessary for the activation of NF- κ B was assessed. As an example of a TRAF6 complex important for NF- κ B activation, we looked at the effect of A52R on a complex containing TRAF6 and TAB1 (27). We detected a TRAF6-TAB1-containing complex by IP (Fig. 3 D, top panel), and as the expression of A52R increased, the amount of TAB1 capable of coIP with TRAF6 decreased steadily (Fig. 3 D, top panel). This effect was specific to TRAF6 because the expression of increasing levels of A52R had no effect on the formation of a TAB1-TAK1 complex (Fig. 3 D, bottom panel).

A52R Disrupts IRAK2-dependent Signaling. Fig. 2 showed that A52R could also interact with IRAK2-containing complexes. We therefore examined the specificity and functional consequences of this interaction. The death domain of IRAK2 was required for association with A52R, as a truncated version of IRAK2 termed kIRAK2, which lacks the death domain, was unable to interact with A52R (Fig. 4 A, top panel, compare lanes 3 and 4). Similar to

TRAF6, there was a correlation between inhibition of TLR-induced NF- κ B activation by A52R, and a role for IRAK2 in these pathways: Fig. 4 B shows that each CD4-TLR induced signal that was sensitive to A52R was also blocked by dominant negative IRAK2 (top panel). Further, we obtained the first evidence that IRAK2 has a role in TLR3-dependent poly(I:C)-induced NF- κ B activation, as dominant negative IRAK2 dose dependently inhibited this signal (Fig. 4 B, bottom panel). Thus, IRAK2 has a wide-ranging role in many TLR pathways to NF- κ B activation, providing a further rationale for the inhibitory effect of A52R on TLR signaling.

The activation of NF- κ B by Mal requires IRAK2 (25). Given that IRAK2 is a target for A52R, the effect of A52R on the ability of Mal to activate NF- κ B was examined. Overexpression of Mal activated NF- κ B, and this activation was inhibited by the coexpression of A52R in a dose-dependent manner (Fig. 4 C). As Mal interacts with IRAK2 (25), we hypothesized that A52R may inhibit the activation of NF- κ B by Mal by preventing the formation of an active Mal-IRAK2 signaling complex. Therefore, the effect of A52R expression on the ability of Mal to interact with IRAK2 was tested. As the expression of A52R increased, the amount of IRAK2 capable of coimmunoprecipitation with Mal decreased steadily (Fig. 4 D). This decrease in complex formation was not due to a decrease in the expression of either IRAK2 or Mal since direct immunoblot showed equal expression of both signaling molecules as the expression of A52R increased (unpublished data). Thus, increasing expression of A52R is able to inhibit the acti-

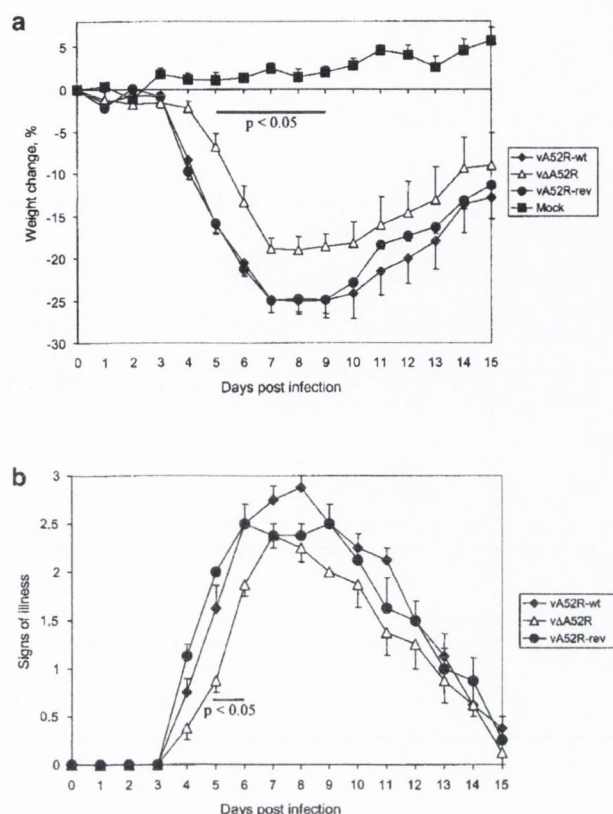


Figure 5. Gene A52R contributes to virus virulence. Groups of four female, Balb/c mice, 6-wk-old were infected intranasally with 10^4 PFU of vA52R-wt, vΔA52R, or vA52R-rev. Each day animals were weighed and the signs of illness scored. Data are presented as (A) the mean weight of each group of animals compared with the mean weight of the same group on day 0, and (B) the mean signs of illness score. Error bars = SEM. Horizontal bars represent days on which there was a statistically significant difference ($P < 0.05$, Student's *t* test) between the vΔA52R and both control groups.

vation of NF- κ B by Mal, and this correlates with dissociation of an active Mal-IRAK2 signaling complex.

A52R Contributes to VV Virulence. Finally, in order to assess the importance of A52R and TLR inhibition to VV virulence, the role of A52R in the VV life cycle was investigated by the construction of a deletion mutant lacking the A52R gene and by the comparison with wild-type and revertant controls. The loss of the A52R gene did not affect the replication of the virus in cell culture or the plaque size (unpublished data). However, in a murine intranasal model the deletion mutant caused reduced weight loss (Fig. 5 A) and milder signs of illness (Fig. 5 B) compared with controls. This result was confirmed in a repeat experiment. Thus, the A52R protein contributes to virus virulence and this is likely to be due to the inhibition of TLR signaling.

Taken together, these results suggest that A52R from VV is able to inhibit TLR-induced NF- κ B activation by associating with key signaling molecules and thus disrupting the formation of active signaling complexes, which contributes to virus virulence.

Discussion

In this report we identify the VV protein A52R as a broad intracellular inhibitor of signaling by TLRs and show it contributes to virus virulence in vivo. In addition to inhibition of the IL-1R, the IL-18R, and TLR4 (29), A52R was shown here to inhibit NF- κ B induction by multiple TLRs, including TLR3, which has been implicated in antiviral innate immunity.

There is intense interest in the IL-1R/TLR family, given its emerging central importance in the innate immune response to diverse pathogens (1). During the course of viral infection the body mounts several lines of host defense involving constituents of the IL-1R/TLR superfamily. The cytokines IL-1 and IL-18 are key regulators of the innate and adaptive immune response to viral infection. In particular IL-1 is responsible for inducing a fever response during viral infection, which is antagonized by the production of a soluble IL-1 β binding protein (B15R) by VV (31). IL-18 is a potent inducer of IFN- γ , and administration of IL-18 has been shown to elicit antiviral effects in VV-infected mice (35), while deletion of the VV IL-18 binding protein caused virus attenuation in vivo (36). As for the TLR arm of the family, recent work has suggested that they are crucial mediators of an innate immune response to viral infection. TLR3 has been shown to activate NF- κ B and the production of IFN- α and IFN- β in response to poly(I:C), a synthetic analogue of dsRNA (15), which is produced by most viruses including VV at some stage in their life cycle. In addition to its role as the receptor that responds to LPS, TLR4 has been shown to mediate the cytokine-stimulating ability of the fusion protein of respiratory syncytial virus (RSV), while RSV replicated to a higher titre and persisted longer in TLR4-deficient mice than in control mice (13). TLR4 is also required by murine retroviruses for B cell activation (14). TLR2 signaling has recently been shown to be activated by the hemagglutinin protein from measles virus (17), while activation of TLR7 by certain imidazoquinoline compounds has been shown to account for the antiviral effects of these compounds (16). It is possible that other TLRs will also have a role in responding to viral infection. If the TLR family is truly important in antiviral host defense, one would assume that viral mechanisms to antagonize this family exist. Therefore, the discovery of VV A52R as an intracellular global inhibitor of TLR signaling lends strong support to the emerging role of TLRs in the host response to viral infection.

A52R could be coimmunoprecipitated with either IRAK2 or TRAF6. The strength of the interaction with each partner suggest that each interaction is direct, rather than A52R interacting with one protein via a 'bridge' with the other. This was theoretically possible since IRAK2 and TRAF6 interact (23). Importantly, the interaction of A52R with IRAK2 was able to disrupt IRAK2/Mal interactions, while the interaction with TRAF6 disrupted TRAF6/TAK1 interactions. An important control was a lack of effect on TAB1/TAK1 complexes, showing that the effect of A52R on TRAF6/TAB1 was via TRAF6 and not TAB1.

The work here does not exclude the possibility that A52R might disrupt other important TRAF6- (and IRAK2-) containing signaling complexes, and thus increase the range of downstream signals blocked by vaccinia. Disruption of signaling complexes by A52R provides a mechanistic explanation for its ability to inhibit TIR-dependent signaling. Some viral proteins, such as the hepatitis C virus nonstructural 5A protein (37) and the rotavirus capsid protein VP4 (38) are known to affect intracellular signaling by targeting TRAF2. However, this is the first demonstration of a viral protein targeting TRAF6. The observation that the TRAF domain of TRAF6 is targeted is of interest. The TRAF domain is responsible for mediating the oligomerization of TRAF6, which is required for its ubiquitination and activation of IKK via TAK (27, 39). A dominant negative version of TRAF6 composed of only the TRAF domain is thought to function by inhibiting oligomerization (33), and it is possible that the binding of A52R functions in a similar manner.

A52R is also the first viral protein identified to target IRAK2. This, together with our demonstration of a role for IRAK2 in all the TLR pathways tested, including TLR3, suggests an important role for IRAK2 in antiviral immunity and TLRs in general. The death domain of IRAK2 was required for the association. Functionally there is a clear difference between the death domain of IRAK and IRAK2. Based on results here, IRAK2 might be predicted to be more important for TLRs involved in the antiviral response than IRAK. Determination of the exact role of IRAK2 in antiviral immunity and TLR signaling awaits the generation of an IRAK2 knockout mouse.

The apparent redundant targeting of two signaling molecules present on common pathways may indicate the critical importance to the virus of inhibiting NF- κ B activated by TLRs. However, from recent work using knockout mouse studies it has become clear that although the IL-1R/TLR family share a common pool of downstream signaling molecules, specific molecules are used in different contexts leading to the range of different signals that are generated by TLRs. In response to LPS, TLR4 activates cytokine release from dendritic cells by a MyD88-dependent pathway, whereas NF- κ B activation and expression of costimulatory molecules can occur in the absence of MyD88 (40). We recently identified Mal, a novel TIR containing adaptor protein, which interacts with IRAK2 and can activate NF- κ B in response to TLR4 stimulation (25). Consistent with the targeting of IRAK2 by A52R, signaling triggered by Mal was sensitive to inhibition by A52R. Significantly, the MyD88-independent pathway, which may involve this Mal-IRAK2 axis (41), leads to the activation of the antiviral transcription factor IFN-regulatory factor 3 (IRF3) and induction of IRF3-responsive genes, and has been shown to be independent of TRAF6 (42). Thus, there would appear to be at least two types of signaling pathways activated by the IL-1R/TLR superfamily, those dependent on MyD88, IRAK/IRAK2, and TRAF6, and those activated by a Mal/IRAK2-dependent and TRAF6-independent pathway. Thus, the ability of A52R

to target both IRAK2 and TRAF6 would significantly increase the range of TIR domain-activated signaling pathways that VV is able to inhibit.

The importance of A52R, and by extrapolation TLRs, to virus virulence was demonstrated by deletion of A52R from VV, which reduced virulence. The attenuation seen by deletion of A52R is similar to that resulting from loss of IL-18 binding protein (36) or the type I IFN binding protein (43).

In conclusion, we have identified the VV protein A52R as an inhibitor of TLR signaling, which acts by a novel mechanism of targeting the two key signaling molecules IRAK2 and TRAF6. The targeting of these two molecules involved in a diverse range of TIR domain-dependent signaling pathways is likely to confer VV with a significant selective advantage in the presence of the host immune system. This study further highlights the emerging importance of TLRs in antiviral innate immunity.

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Vaccinia virus protein A46R targets multiple Toll-like–interleukin-1 receptor adaptors and contributes to virulence

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Viral immune evasion strategies target key aspects of the host antiviral response. Recently, it has been recognized that Toll-like receptors (TLRs) have a role in innate defense against viruses. Here, we define the function of the vaccinia virus (VV) protein A46R and show it inhibits intracellular signalling by a range of TLRs. TLR signalling is triggered by homotypic interactions between the Toll-like–interleukin-1 resistance (TIR) domains of the receptors and adaptor molecules. A46R contains a TIR domain and is the only viral TIR domain-containing protein identified to date. We demonstrate that A46R targets the host TIR adaptors myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like, TIR domain-containing adaptor inducing IFN- β (TRIF), and the TRIF-related adaptor molecule and thereby interferes with downstream activation of mitogen-activated protein kinases and nuclear factor κ B. TRIF mediates activation of interferon (IFN) regulatory factor 3 (IRF3) and induction of IFN- β by TLR3 and TLR4 and suppresses VV replication in macrophages. Here, A46R disrupted TRIF-induced IRF3 activation and induction of the TRIF-dependent gene regulated on activation, normal T cell expressed and secreted. Furthermore, we show that A46R is functionally distinct from another described VV TLR inhibitor, A52R. Importantly, VV lacking the A46R gene was attenuated in a murine intranasal model, demonstrating the importance of A46R for VV virulence.

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Abbreviations used: ERK, extra-cellular signal-regulated kinase; IRAK, IL-1 receptor-associated kinase; IRF3, interferon regulatory factor 3; JNK, c-Jun NH₂-terminal protein kinase; Mal, MyD88 adaptor-like; MOI, multiplicity of infection; MyD88, myeloid differentiation factor 88; PAM, pathogen-associated molecule; RANTES, regulated on activation, normal T cell expressed and secreted; SARM, sterile α and HEAT/Armadillo motifs-containing protein; TIR, Toll-IL-1 resistance; TLR, Toll-like receptors; TRAF, TNF receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor inducing IFN- β ; VV, vaccinia virus.

The discovery of the Toll-like receptor (TLR) family of proteins has revolutionized the understanding of how the innate immune system recognizes pathogens and initiates an effective and appropriate response (1, 2). TLRs are part of the larger IL-1R/TLR superfamily, which includes IL-1Rs, IL-18Rs, and a group of orphan receptors (3). The family is defined by the presence of a cytoplasmic Toll-like–IL-1 resistance (TIR) domain, which is responsible for mediating downstream signaling. So far, 13 TLRs have been identified; TLRs 1–9 are common to mouse and human, whereas TLR10 is only functional in humans, and TLRs 11, 12, and 13 have been found only in

mice (2–5). Many but not all of these receptors have been assigned a role in the initial detection of, and response to, specific pathogen-associated molecules (PAMs). In macrophages and neutrophils, this drives innate immune responses, such as inflammation and induction of microbicidal activity, whereas activation of TLRs expressed on dendritic cells leads to the initiation of adaptive immunity through induction of IL-12 and costimulatory molecules (6, 7).

Although interest focused on the role of TLRs in responding to bacteria initially, there are now three strong lines of evidence that TLRs are also involved in detecting viruses and initiating antiviral responses. First, some TLRs are required for cellular and whole animal responses to certain viruses, viral proteins, and nucleic acids (8, 9). Second, TLRs trigger antiviral signaling pathways leading to the induction of IFN responses (8, 9). Third, viral immune strategies used against TLRs have been identified (8, 9).

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Cell surface TLR2 and TLR4 may recognize viral glycoproteins on virions (8, 9). For example, measles virus hemagglutinin activates murine and human cells via TLR2, leading to induction of proinflammatory cytokines and up-regulation of surface expression of CD150, a receptor for measles virus (10). In terms of viral nucleic acids, TLR3 is activated in response to poly(I:C), a synthetic analogue of viral dsRNA (11), whereas TLR7 and TLR8 recognize ssRNA (from influenza, HIV, and vesicular stomatitis virus; references 12–14) and TLR9 responds to dsDNA from herpes simplex virus (15, 16).

That TLR signaling can induce an antiviral state was shown clearly by Doyle et al. (17) in that pretreatment of cells with poly(I:C) or lipid A (the moiety of LPS that is recognized by TLR4) inhibited the replication of a murine herpesvirus in macrophages. Even before the discovery of TLRs, it was known that viral replication and viral pathogenesis often involves NF- κ B activation (18). TLRs, like IL-1, mediate downstream signaling mainly through their cytoplasmic TIR domain. This domain mediates homotypic interactions between TLRs, and also recruitment of TIR-containing adaptor proteins, of which myeloid differentiation factor 88 (MyD88) is a prototypical example. Recruitment of MyD88 to TLR complexes leads to activation of IL-1 receptor-associated kinases (IRAKs), which engage with TNF receptor-associated factor (TRAF) 6, leading ultimately to the activation of mitogen-activated protein (MAP) kinases and the transcription factor NF- κ B (19, 20). MyD88 is involved in NF- κ B activation by every TLR tested thus far, except for TLR3 (21, 22). MyD88 adaptor-like (Mal; reference 23), another TIR adaptor protein, is required specifically for TLR2 and TLR4 signaling (19, 20).

An important MyD88-independent signaling pathway is the activation of the antiviral transcription factor IFN regulatory factor 3 (IRF3) by TLR3 and TLR4. IRF3 activation, together with NF- κ B activation, leads to IFN- β induction, which initiates the IFN-based antiviral response. TIR domain-containing adaptor inducing IFN- β (TRIF) is essential for MyD88-independent TLR3 and TLR4 signaling (21, 22, 24). TRIF activates IRF3 via TANK-binding kinase-1 (TBK1), leading to IFN- β expression (25, 26). TRIF probably associates directly with TLR3, but indirectly with TLR4, via a bridging interaction with a fourth adaptor that is unique to the TLR4 signaling pathway, TRIF-related adaptor molecule (TRAM, 27, 28).

Further evidence for the role of TLRs in responding to viruses came from the discovery of viral immune strategies used against TLRs. Vaccinia virus (VV), the poxvirus used to vaccinate against smallpox, encodes proteins that antagonize important components of host antiviral defense. Previously, we showed that VV protein A52R, which has no obvious similarity to host proteins, can block the activation of NF- κ B by multiple TLRs, in particular TLR3 (29, 30). A52R associates with both IRAK2 and TRAF6, and disrupts signaling complexes containing these proteins (30). Furthermore, deletion of the *A52R* gene from VV reduced virus virulence (30).

In contrast with A52R, the VV protein investigated in this work, A46R, has a TIR domain, and as such is the only viral member of the IL-1R/TLR family identified to date (29). Initial studies revealed that A46R could inhibit IL-1, but not TNF-induced NF- κ B activation (29). The effect on IL-1, together with the presence of a TIR domain in the protein suggested that A46R may have a role in immune evasion. However, the mechanism of action of A46R, its effect on TLR signaling pathways, and its potential role in virulence were not determined. Here, we show that A46R inhibits TLR-induced signaling, by associating with TIR-domain containing adaptor molecules. This is the first demonstration of direct viral targeting of TIR adaptors. We also demonstrate a role for A46R in VV virulence, and show that A46R and A52R are functionally distinct.

RESULTS

A46R inhibits multiple IL-1-induced signals

Previously, we showed that A46R was capable of suppressing NF- κ B activation induced by IL-1, whereas TNF-induced NF- κ B was unaffected (29). To further characterize the effects of A46R on cell signaling, first we determined the effect of A46R on other IL-1-dependent signals. The effect of A46R on IL-1-, but not TNF-induced NF- κ B-dependent reporter gene activation was confirmed (Fig. 1 a). To measure IL-1-induced transactivation of NF- κ B specifically, an assay was performed using an expression plasmid encoding the transactivation domain of the p65 subunit of NF- κ B fused to the DNA-binding domain of Gal4, together with a reporter plasmid under the control of a Gal4 upstream activation sequence (31). Reporter gene expression from this plasmid requires p65 transactivation through phosphorylation. Ectopic expression of A46R inhibited IL-1-induced p65 transactivation, but had no effect on basal levels of activity (Fig. 1 b). The effect of A46R on p65 transactivation provided a rationale for the inhibition of the NF- κ B-dependent reporter gene. In a similar assay (23), A46R blocked both c-Jun NH₂-terminal protein kinase (JNK; Fig. 1 c) and extracellular signal-regulated kinase (ERK; Fig. 1 d) activation induced by IL-1. Thus, A46R inhibited multiple distinct signals emanating from the IL-1 receptor.

A46R interacts with MyD88 and antagonizes MyD88-dependent signaling

The results from Fig. 1 suggest that A46R was acting close to the IL-1 receptor complex. This was also likely given that A46R has a TIR domain (29), as illustrated in Fig. 2 a, which shows an alignment of VV and variola virus A46R with several human TIR-containing proteins. Given that all IL-1 signals tested to date are dependent on the TIR domain-containing adaptor MyD88 (32, 33), including p65 transactivation (31) and JNK activation (34), we reasoned that A46R may target MyD88 via a TIR domain interaction. To test this hypothesis, coimmunoprecipitation studies were performed with A46R and MyD88 expressed ectopically. Fig. 2 b shows a clear interaction between A46R and

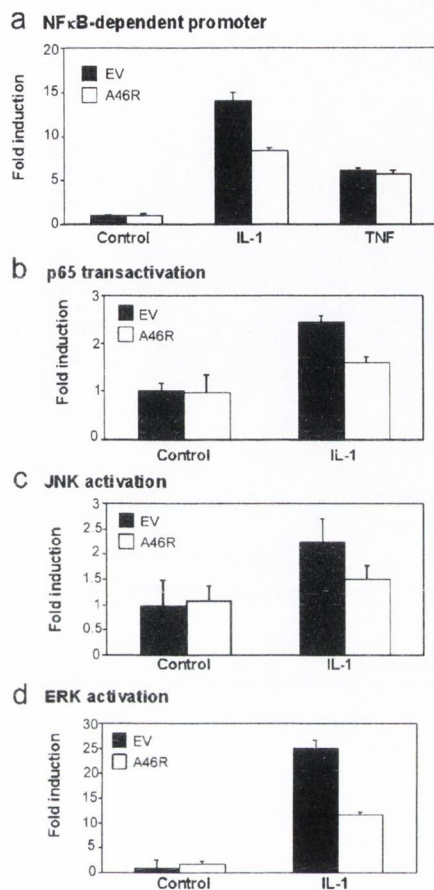


Figure 1. A46R inhibits multiple IL-1-dependent signals. HEK 293 cells were transfected with 100 ng A46R or pcDNA3.1 (EV) and the NF- κ B (a), p65 (b), JNK (c), or ERK (d) reporter plasmids as described in Materials and methods. 6 h before harvesting, the cells were stimulated with either 100 ng/ml IL-1 or 100 ng/ml TNF as indicated, and luciferase reporter gene activity was measured.

MyD88 (left). As a control, the ability of A46R to interact with TRAF2, an adaptor used by TNF but not IL-1 (35), was tested in parallel. In this case, no interaction was detected (Fig. 2 a, right), consistent with the lack of effect of A46R on TNF signaling (Fig. 1 a). These results were confirmed by GST-pulldown experiments, whereby purified GST-A46R interacted with ectopically expressed MyD88 in a cell lysate, but not with TRAF2 (Fig. 2 c). A46R also failed to interact with TAB1, a signaling molecule downstream of MyD88 (unpublished data). The interaction of A46R with MyD88 was also demonstrated in cells infected by VV and transfected to express MyD88, to use the A46R protein at its physiological concentration (Fig. 2 d). As a control for specificity, this was compared with a VV deletion mutant lacking the A46R gene (Δ A46R), whereupon no band for A46R was detected (Fig. 2 d). A46R also interacted with MyD88 in a yeast two-hybrid pairwise assay (unpublished data), thus demonstrating that the association was direct.

Apart from its role in IL-1R signaling, MyD88 is also used by murine TLRs 1, 2, 4, 5, 6, 7, and 9 (2). Therefore, we tested the effect of A46R on signaling via these TLRs in RAW264.7 cells, a murine macrophage cell line that expresses most TLRs (36). Stimulation of RAW264.7 cells with MALP-2 (TLR2 and 6), Pam₃Cys (TLR2 and 1), LPS (TLR4), flagellin (TLR5), R848 (TLR7), or CpG DNA (TLR9) led to induction of the NF- κ B-dependent reporter gene (Fig. 2 e). In each case, the presence of A46R caused strong inhibition of induction, whereas it had no suppressive effect on control levels (Fig. 2 e). Thus, A46R inhibited MyD88-dependent signaling by both IL-1R and TLRs, presumably by interacting with MyD88 via its TIR domain.

A46R targets the TLR4 receptor complex

The effect of A46R on TLR4 signaling was examined in greater detail. A chimeric form of TLR4, comprising the murine CD4 extracellular domain fused to the cytoplasmic domain of human TLR4, which renders TLR4 constitutively active (37), was used. Overexpression of CD4-TLR4 induced NF- κ B, and this was inhibited by coexpression of A46R in a dose-dependent manner (Fig. 3 a, left). In fact, the highest concentration of A46R-expressing plasmid almost completely prevented TLR4-induced NF- κ B activation, whereas it did not suppress basal levels of reporter gene activity (Fig. 3 a, left, white bars). TLR4-induced activation of the MAP kinases p38 and ERK was also inhibited by A46R expression (Fig. 3, middle and right, respectively).

Given that TLR4-induced NF- κ B, p38, and ERK are only partially MyD88 dependent (38), it was difficult to account for the potent effects of A46R on these signals by an interaction with MyD88 alone. Therefore, we assessed the ability of A46R to target other TIR domain-containing proteins involved in the TLR4 receptor complex. Fig. 3 b shows that A46R could be immunoprecipitated with TLR4 itself, suggesting that A46R can also interact with the TLR4 TIR domain. Next, we tested the ability of A46R to target two important TLR4 TIR adaptors, Mal and TRAM. Mal and TRAM are thought to interact directly with TLR4, and subsequently recruit MyD88 and TRIF, respectively (23, 27, 39). Both Mal and TRAM are essential for robust TLR4-dependent NF- κ B activation (19, 23, 27, 28). Consistent with this, A46R immunoprecipitated with both Mal (Fig. 3 c) and TRAM (Fig. 3 d). The interactions with TLR4, Mal, and TRAM were confirmed by GST-pulldown experiments (Fig. 3 e). Furthermore, Mal was shown to coimmunoprecipitate with A46R in infected cells, associate directly with A46R in the yeast two-hybrid pairwise assay, and rMal interacted with GST-A46R in vitro (unpublished data).

Next, we demonstrated that the inhibitory effects of A46R on TLR4 signaling pathways also resulted in a suppression of gene induction. Fig. 3 f shows that LPS stimulation of HEK293 cells expressing TLR4 led to the release of IL-8, which is NF- κ B and p38 dependent (not depicted). Transient transfection of these cells with A46R suppressed IL-8 release by ~50%. Thus, the viral TIR domain of A46R can target

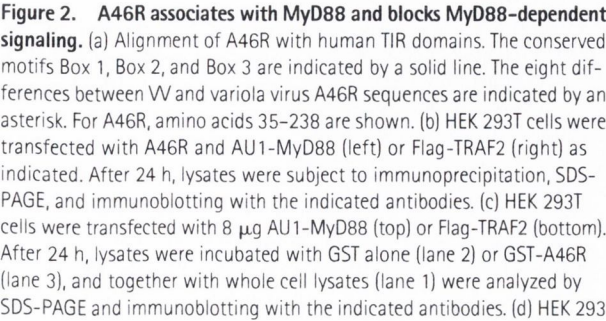


Figure 2. A46R associates with MyD88 and blocks MyD88-dependent signaling. (a) Alignment of A46R with human TIR domains. The conserved motifs Box 1, Box 2, and Box 3 are indicated by a solid line. The eight differences between VV and variola virus A46R sequences are indicated by an asterisk. For A46R, amino acids 35–238 are shown. (b) HEK 293T cells were transfected with A46R and AU1-MyD88 (left) or Flag-TRAF2 (right) as indicated. After 24 h, lysates were subject to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies. (c) HEK 293T cells were transfected with 8 μ g AU1-MyD88 (top) or Flag-TRAF2 (bottom). After 24 h, lysates were incubated with GST alone (lane 2) or GST-A46R (lane 3), and together with whole cell lysates (lane 1) were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. (d) HEK 293T

cells were transfected with 4 μg of myc-MyD88. After 24 h, cells were infected with viruses either containing (vWT-A46R) or not (v Δ A46R) the A46R gene (MOI = 1) and harvested 24 h after infection. Lysates were subjected to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies. Whole cell lysates were analyzed for expression of A46R. (e) Murine macrophage RAW 264.7 cells were transfected with the pRL-TK reporter gene and the NF- κ B luciferase construct as described in Materials and methods, together with pcDNA3.1 or 100 ng A46R. Cells were stimulated for 6 h with 10 nM MALP-2 (MALP), 5 $\mu\text{g}/\text{ml}$ Pam3Cys (Pam), 1 $\mu\text{g}/\text{ml}$ LPS, 250 ng/ml Flagellin (Flag), 1 μM R-848, or 5 $\mu\text{g}/\text{ml}$ CpG DNA. Cells were harvested 24 h after transfection and the reporter gene activity was measured.

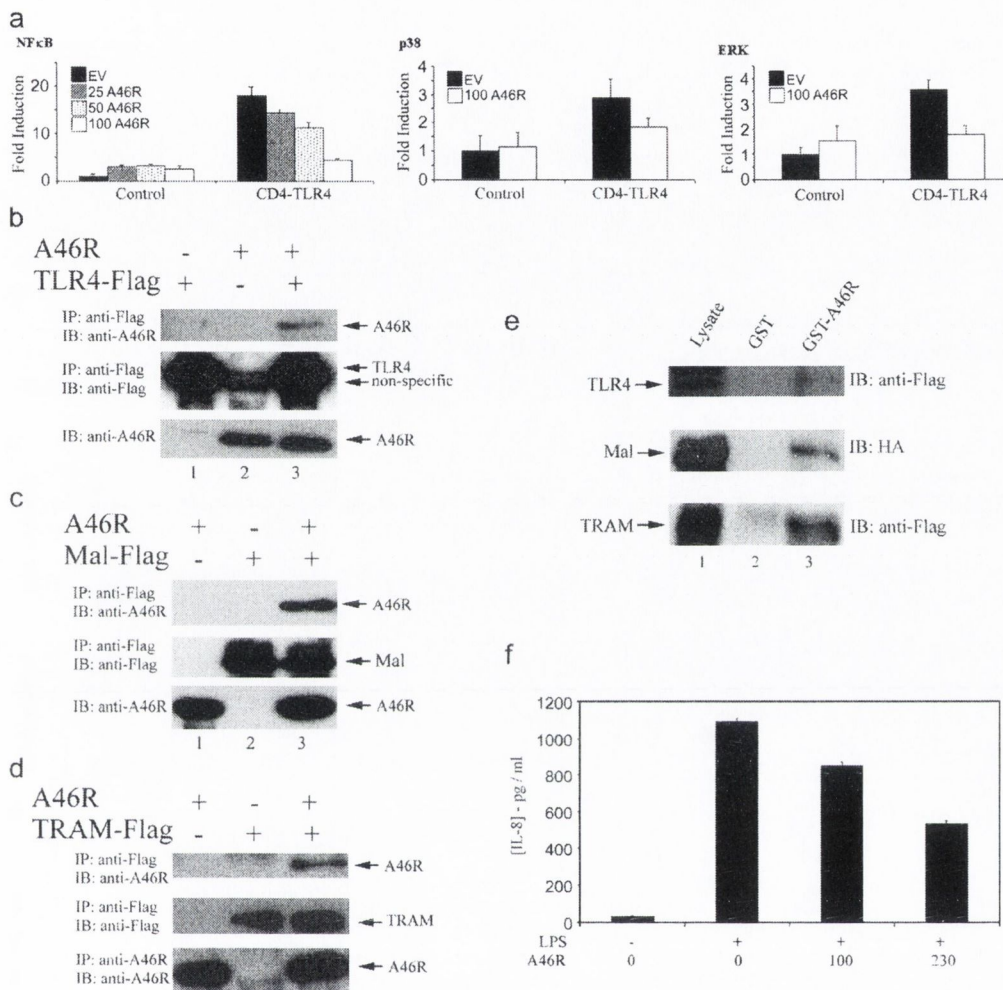


Figure 3. A46R inhibits TLR4 signaling and interacts with TLR4, Mal, and TRAM. (a) HEK 293 cells were transfected with 50 ng CD4-TLR4, 25–100 ng A46R, or pcDNA3.1 (EV) and the NF- κ B (left), p38 (middle), or ERK (right) reporter plasmids as indicated. Cells were harvested 24 h after transfection, and luciferase reporter gene activity was measured. (b–d) HEK 293T cells were transfected with A46R and Flag-TLR4 (b), Flag-Mal (c), or Flag-TRAM (d) as indicated. After 24 h, lysates were subject to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies.

(e) HEK 293T cells were transfected with 8 μ g of Flag-TLR4 (top), Flag-Mal (middle), or Flag-TRAM (bottom). After 24 h, lysates were incubated with GST-A46R (lane 3) or GST alone (lane 2), and together with whole cell lysate (lane 1), were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. (f) HEK-TLR4 cells were transfected with the indicated amounts (ng) of A46R 24 h before stimulation with 1 μ g/ml LPS, and 24 h after stimulation, supernatants were harvested and assayed for IL-8 by ELISA.

multiple human TIR domain-containing proteins important for TLR4 signaling, leading to antagonism of signaling pathways and subsequent suppression of gene induction.

A46R antagonizes TRIF-dependent pathways

TRIF was identified as a TIR adaptor molecule capable of directing both TLR4- and TLR3-induced IRF3 activation, leading to IFN- β induction, which is independent of MyD88 (21, 22, 24). The MyD88-independent late activation of NF- κ B by TLR4 was also explained by the discovery of TRIF (21). Given the central role of TRIF in the activation of the antiviral transcription factor IRF3, we wondered

whether A46R would also target this TIR adaptor directly. Co-expression of A46R and TRIF, with subsequent coimmunoprecipitation, demonstrated that these two proteins could indeed form a complex (Fig. 4 a). An interaction between A46R and TRIF was confirmed by a GST-pull-down experiment (Fig. 4 b) and also by a yeast two-hybrid pairwise assay (not depicted).

To determine the functional relevance of this interaction, the effect of A46R on TRIF-dependent signals was examined. For this, IRF3 activation by TLR4 and TLR3 was assessed by using a Gal4-IRF3 fusion protein together with a Gal4-dependent reporter plasmid, which requires IRF3

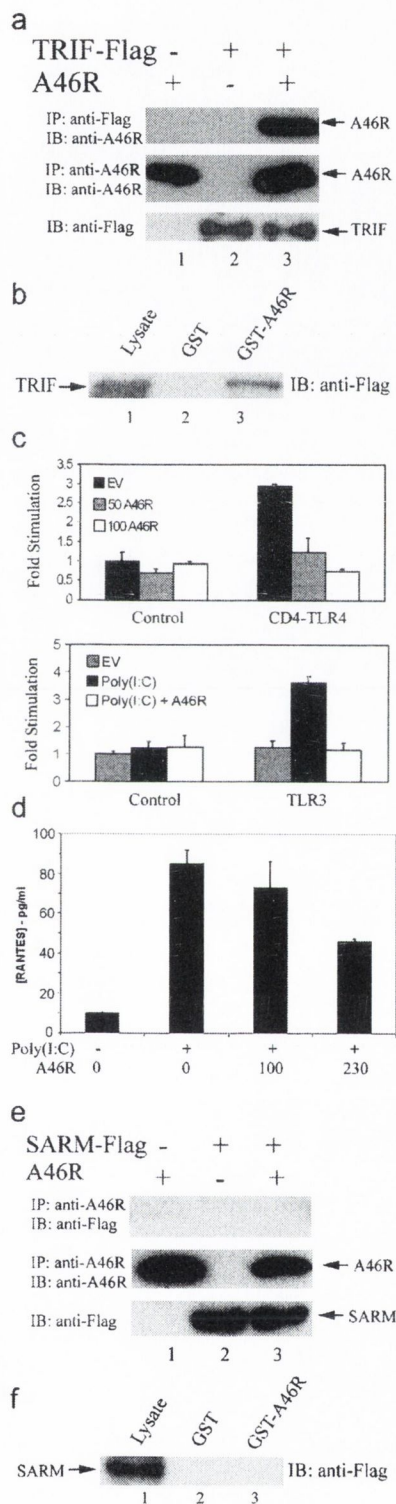


Figure 4. A46R associates with TRIF and inhibits TRIF-dependent signaling and gene induction. (a) HEK 293T cells were transfected with A46R and Flag-TRIF as indicated. After 24 h, lysates were subject to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies.

transactivation to express the reporter gene (27). Ectopic expression of CD4-TLR4 resulted in a threefold induction of this IRF3-dependent reporter, and this was completely blocked by coexpression of A46R (Fig. 4 c, top). Although the interaction of A46R with TLR4 and TRAM probably contributes to this inhibitory effect, induction of an IFN- β promoter reporter gene by ectopic expression of TRIF was also blocked by A46R (unpublished data), suggesting a direct effect on TRIF, which is downstream of TLR4 and TRAM. Further TLR3-dependent activation of IRF3 by poly(I:C), which is entirely TRIF dependent, was also completely blocked by A46R (Fig. 4 c, bottom). A46R also inhibited LPS and poly(I:C)-mediated induction of an ISRE-dependent reporter (unpublished data). In addition, no interaction between A46R and TLR3 was detected (unpublished data). Furthermore, poly(I:C)-induced regulated on activation, normal T cell expressed and secreted (RANTES) release from HEK293 cells stably expressing TLR3 was inhibited by A46R (Fig. 4 d). This represents inhibition of gene induction that is entirely independent of MyD88 (27). Therefore, A46R blocks TLR-induced IRF3 activation and subsequent gene induction by directly targeting TRIF.

Thus, A46R was capable of interacting with four TIR adaptors known to have a key role in IL-1R and TLR-induced signaling. In contrast, A46R did not interact with the fifth human intracellular TIR domain-containing protein, sterile α and HEAT/Armadillo motifs-containing protein (SARM) in a coimmunoprecipitation (Fig. 4 e) or GST-pulldown (Fig. 2 f) assay. This is consistent with the fact that SARM does not lead to NF- κ B or IRF3 activation (40), and provided an important specificity control for A46R interactions.

A46R is expressed early during infection and contributes to VV virulence

To determine when A46R was expressed during the virus life cycle, cells were infected for different lengths of time in the presence or absence of cytosine β -D-arabinofuranoside (AraC) (an inhibitor of virus DNA replication and, therefore, of intermediate and late genes) and analyzed by immunoblotting. Fig. 5 a (top) shows that A46R expression was detected in the presence of AraC, whereas a late protein

(b) HEK 293T cells were transfected with 8 μ g Flag-TRIF. After 24 h, lysates were incubated with GST-A46R (lane 3) or GST alone (lane 2), and together with whole cell lysates (lane 1), were analyzed by SDS-PAGE and immunoblotting with anti-Flag Ab. (c) HEK 293 cells were transfected with the IRF3 reporter plasmids (as described in Materials and methods) with either 50 ng CD4-TLR4 (top) or 0.5 ng TLR3 (bottom) and 50–150 ng A46R or pcDNA3.1 (EV) as indicated. (bottom) Cells were stimulated with 25 μ g/ml poly(I:C) 6 h before harvesting. Luciferase activity was measured after 24 h. (d) HEK-TLR3 cells were transfected with the indicated amounts (ng) of A46R 24 h before stimulation with 25 μ g/ml poly(I:C), and 24 h after stimulation supernatants were harvested and assayed for RANTES by ELISA. (e and f) As in a and b, except Flag-SARM was transfected instead of Flag-TRIF.

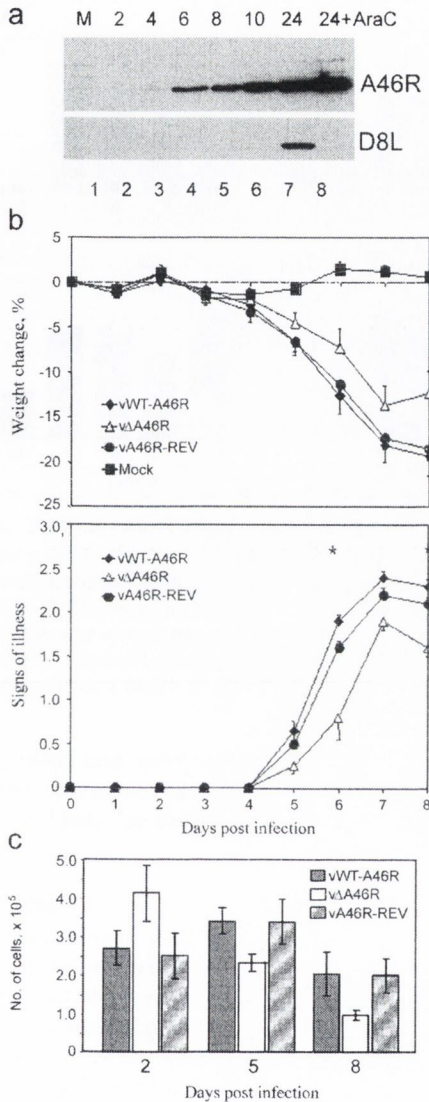


Figure 5. A46R is expressed early during virus infection and contributes to virus virulence. (a) BSC-1 cells were mock infected (M) or infected with VV Western reserve (MOI = 5) in the absence or presence of 40 μ g/ml cytosine β -D-arabino-furanoside (AraC). Cells were harvested at the indicated times (h) after infection, and lysates were subjected to SDS-PAGE and immunoblotting, using either anti-A46R (top) or anti-D8L (bottom) Ab. (b) A46R contributes to virus virulence. Groups of 15 female, 6-wk-old Balb/c mice were infected intranasally with 5×10^3 PFU of vWT-A46R, v Δ A46R, or vA46R-REV. Each day, animals were weighed and the signs of illness were scored. Data are presented as the mean weight of each group of animals compared with the mean weight of the same group on day 0 (top graph), and the mean signs of illness score (bottom graph). Error bars are SEM. Asterisks represent days on which there was a statistically significant difference ($P < 0.05$; Student's t test) between the vWT-A46R and both control groups. (c) Number of cells recruited to the lungs of VV-infected mice. On days 2, 5, and 8 after infection, mice were killed, lungs were harvested, and cell suspensions were prepared. The total number of viable lung cells per animal was determined by Trypan blue exclusion. Data are means $\pm$ SEM.

(D8L) was not (Fig. 5 a, bottom), showing early expression. A46R remained in infected cells up to 24 h after infection.

To explore the role of A46R in VV virulence, a VV deletion mutant lacking the A46R gene was constructed (v Δ A46R). As a control, a revertant virus was also constructed in which the A46R gene was reinserted into the v Δ A46R mutant (vA46R-REV). Western blot analysis confirmed expression of A46R in wild-type virus (vWT-A46R) and vA46R-REV, but not in v Δ A46R (Fig. 2 d and not depicted). Mice were infected intranasally with v Δ A46R, vA46R-REV, or vWT-A46R and were weighed and assessed for signs of illness daily. Fig. 5 b shows that v Δ A46R was attenuated relative to both vWT-A46R and vA46R-REV in terms of reduced weight loss (Fig. 5 b, top graph) and milder signs of illness (bottom graph). Assessment of the total number of cells in lungs after infection revealed a difference in the kinetics of the host response to v Δ A46R compared with vA46R-REV or vWT-A46R, in that when the virus lacked A46R, the number of cells present on day two was increased, whereas on days 5 and 8, it was reduced. The difference in cell recruitment between the vWT-A46R and v Δ A46R on day 5 was statistically significant ($P < 0.05$). The p-value for the difference between vWT-A46R and v Δ A46R on day 8 was 0.09, whereas the value for the difference between vA46R-REV and v Δ A46R was 0.04.

A46R and A52R are not functionally redundant

Previously, we showed that the poxviral protein A52R could also inhibit TLR signaling and contribute to virulence (30). A52R does not resemble any host proteins, but does have some similarity to A46R (29, 30). A52R blocked TLR-induced NF- κ B activation by targeting TRAF6 and IRAK2, which act downstream of TIR adaptors (30). Therefore, we looked in more detail at the effects of both proteins on signaling by a single TLR, to ascertain whether or not they were functionally redundant. We chose TLR3, given its proposed role in the antiviral response, and also because it signals via a single TIR adaptor, TRIF, thus making the interpretation of the results more definitive. Analysis of NF- κ B and IRF3 activation by TLR3 demonstrated a clear difference between A46R and A52R. Fig. 6 a shows that A52R was a potent inhibitor of poly(I:C)/TLR3-induced NF- κ B activation, whereas A46R had little effect. In contrast, Fig. 6 b shows that IRF3 activation induced by poly(I:C)/TLR3 was sensitive to A46R, but not A52R. Furthermore, A52R was not capable of inhibiting IL-1 or TLR4-induced MAP kinase activation (not depicted), which were clearly blocked by A46R (Fig. 1, c and d, and Fig. 3, a and b). Hence, A46R and A52R are not functionally redundant in that both are required to effectively shut down TLR3 signaling, whereas only A46R is capable of inhibiting TLR-induced MAP kinase activation.

The results demonstrate that A46R has a viral TIR domain with which it targets TIR adaptor molecules, resulting in inhibition of both MyD88-dependent and TRIF-depen-

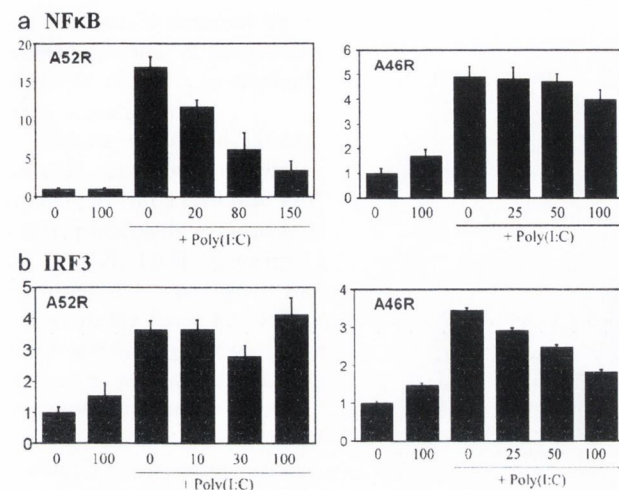


Figure 6. A46R and A52R target different TLR3-mediated signaling pathways. (a and b) HEK 293 cells were transfected with 0.5 ng TLR3 and the indicated amounts (ng) of either A52R or A46R, together with the NF- κ B (a) or IRF3 (b) reporter plasmids as described in Materials and methods. Cells were stimulated with 25 μ g/ml poly(I:C) 6 h before harvesting where indicated. Luciferase activity was measured after 24 h. Relative stimulation is shown on the y axis.

dent signaling and gene induction. Furthermore, A46R is functionally distinct from A52R and contributes to VV virulence, most likely due to its inhibitory effects on TIR-dependent signaling.

DISCUSSION

The identification of viral immune evasive strategies and the analysis of the molecular aspects of host-pathogen interactions are crucial to enhancing understanding of microbial pathogenesis and immunity to infection. Given the emerging importance of the TLR system in the antiviral response, understanding how viruses target this receptor family is of particular interest. During a database search for novel TIR domain-containing proteins, A46R from VV was identified (29). This was potentially interesting because many poxviral immunomodulatory proteins, such as cytokine-binding proteins, bear sequence similarity to host factors (41). To date, A46R is the only identified viral TIR domain-containing protein. In this paper, we show that A46R is an intracellular inhibitor of multiple TLR-dependent signaling pathways, define host signaling molecules that it targets, and demonstrate that the protein contributes to VV virulence in vivo.

The observation that A46R blocked all IL-1 signals tested (NF- κ B, JNK, and ERK activation) suggested that it was acting close to the IL-1R complex. Furthermore, the presence of a TIR domain within A46R, the knowledge that TIR domains participate in homotypic interactions, and the fact that all the signals blocked by A46R were MyD88 dependent (31–34), suggested that MyD88 may be sequestered by A46R. Coimmunoprecipitation and GST-pull-

down experiments demonstrated an interaction between A46R and MyD88. This also suggested that A46R would antagonize TLR signaling, given the central role of MyD88 in many of these pathways. In fact, A46R inhibited every murine TLR pathway to NF- κ B activation known to involve MyD88 in a mouse macrophage cell line, together with TLR4-mediated NF- κ B, p38, and ERK activation, and IL-8 induction in human 293 cells.

Inhibition of TLR4-dependent NF- κ B signaling in human cells was particularly potent, which led us to test the effect of A46R on other TIR domain-containing proteins with a role in this pathway. Altogether, five such proteins are known to be involved in TLR4-mediated NF- κ B activation, namely the receptor itself, MyD88, Mal, TRAM, and TRIF (19–23, 28). A46R was found to be capable of associating with all five of these proteins, and hence it probably prevents the formation of the TLR4 receptor complex, thus accounting for its potent inhibition of NF- κ B activation. Although these results were based on overexpression, several lines of evidence suggest that the interactions detected are specific, direct, and likely to occur in vivo. First, normally VV-expressed A46R interacted with MyD88 and Mal. Second, the interaction of A46R with MyD88, Mal, and TRIF were confirmed in yeast two-hybrid. Third, rMal interacted directly with GST-A46R in vitro. Finally, A46R displayed specificity for certain TIR domain-containing proteins and did not interact with TLR3 or SARM.

A46R is the first viral protein identified that can target host TIR domain-containing proteins. Given its ability to interact with different and diverse TIR domains (TRIF and TRAM are quite distinct in sequence from MyD88 and Mal; Fig. 1 a and reference 20), elucidation of the crystal structure of A46R should provide important information of general relevance as to how TIR domains interact. Because Box 2 of the TIR domain is particularly important in signaling (42), the extra amino acids surrounding the A46R Box 2 (Fig. 1 a) may represent inhibitory loops that account for the ability of A46R to prevent TIR-dependent signaling. This hypothesis is being explored using mutagenesis studies.

The fact that A46R associated with all four TLR4 adaptors may suggest that TLR4 is a particularly important target for VV immune evasion. Indeed, TLR4 has been proposed to have a role in responding to fusion (F) protein of respiratory syncytial virus (43), although the functional significance of this remains to be clarified (44). TLR4 is also activated by envelope proteins from both mouse mammary tumor virus and Moloney murine leukemia virus, which could also be coimmunoprecipitated with TLR4 (45). VV might interact with other or multiple TLRs that also use these adaptors. Possible VV PAMs detected by TLRs could be proteins on the surface of the intracellular mature virus or extracellular enveloped virus particles (potentially detected by TLR2 or TLR4; references 8, 9), intracellular dsRNA produced from the bidirectional transcription of the VV genome (potentially detected by TLR3; reference 11), or the dsDNA genome it-

self (potentially detected by TLR9, which responds to the dsDNA genome of herpes simplex virus; reference 15, 16). The role of TLRs in responding to VV PAMs is currently being investigated.

Although Mal and TRAM are yet to be directly implicated in responding to viruses, MyD88 (for TLR7; references 8, 9) and TRIF (for TLR3) have been shown to have a role, particularly in relation to type I IFN production (15, 16, 21, 22). TRIF is essential for both TLR4- and TLR3-mediated IRF3 activation and IFN- β production (21, 22) and probably has a key role in the initiation of the IFN-based antiviral response, leading to the inhibition of viral replication and spread. Although the relevance of TLR3 in responding to viruses *in vivo* has recently been questioned (46, 47), there is evidence that TRIF is important in controlling VV replication. Hoebe et al. (22) showed that macrophages from mice in which *trif* was disrupted supported the replication of VV to a higher titre than did macrophages from normal mice. Thus, the ability of A46R to inhibit TRIF-mediated IRF3 activation may be of primary importance to VV infection.

Consistent with its proposed role in antagonizing early innate TLR responses, A46R was expressed early during infection. Furthermore, deletion of A46R from VV caused attenuation in a murine intranasal model, and also led to enhanced levels of cells on day 2 in lungs of infected animals. The use of a revertant virus in which the A46R gene was reinserted into the deletion virus confirmed that the attenuation observed in the deletion virus, and the difference in lung cell numbers, were due solely to the absence of A46R. The ability of A46R to target intracellular TIR-dependent signaling most likely accounts for its role in virulence, probably by blocking the induction of immune response genes downstream of TLRs, as has been shown here for the chemokines IL-8 and RANTES. Possibly, inhibition of chemokine induction by A46R might account for the early enhanced levels of cells in the absence of A46R expression.

Previously, we identified another VV TLR antagonist, A52R, which could inhibit TLR-dependent NF- κ B activation. There are several lines of evidence that A46R and A52R are not functionally redundant. First, they target distinct TLR signaling molecules (30). Second, although they do have some overlapping effects (such as the inhibition of MyD88-dependent NF- κ B activation), their overall effects on TLR signaling are quite distinct. Although A52R is a good NF- κ B inhibitor, it has no inhibitory effect on MAP kinase activation (not depicted), nor on TLR3-mediated IRF3 activation (Fig. 6 b). In contrast, A46R blocks both MAP kinase activation and, importantly, TLR3- and TRIF-mediated IRF3 activation. Furthermore, A46R has little effect on TLR3-mediated NF- κ B activation, which A52R blocks potently (Fig. 6 a). This was surprising because A46R interacts with TRIF. But, presumably, the interaction of A46R with TRIF has a greater effect on downstream IRF3 activation compared with NF- κ B because these two path-

ways bifurcating from TRIF have been shown to be quite distinct (48, 49). Finally and crucially, the deletion of either A46R or A52R from VV causes attenuation (Fig. 5 b and reference 30) and, thus, both contribute to virulence and are nonredundant.

N1L is another intracellular VV protein that contributes to virulence (50), which has also been shown recently to function by antagonizing TLR signaling, but at the level of I κ B kinases and related kinases involved in IRF3 activation (51). Thus, the importance of blocking TLR signaling is demonstrated by the retention by VV of at least three distinct mechanisms of disrupting these pathways. Furthermore, the attenuated phenotypes seen in the absence of A46R, A52R, or N1L provide evidence for a role for TLRs in containing VV infections. The action of these intracellular TLR inhibitory proteins would be expected to be restricted to infected cells, whereas immunomodulatory proteins secreted from VV-infected cells, such as cytokine, chemokine, and IFN-binding proteins, can act on ligands produced from both infected and uninfected cells.

Finally, A46R is also found in variola virus, the causative agent of smallpox. Concern about the threat of the use of variola as a bioweapon has led to a renewed desire to understand this human pathogen. However, little is known about the role of human TLRs in sensing variola virus. Given that VV A46R targets human adaptors, the knowledge that A52R is truncated in variola virus, together with the fact that the VV and variola virus A46R amino acid sequences differ by only eight residues (Fig. 2 a), it is likely that variola virus A46R would have an important role in interactions with the human TLR system.

MATERIALS AND METHODS

Expression plasmids. Sources of expression plasmids were as follows: AU1-MyD88 and Flag-TLR4 (M. Muzio, Mario Negri Institute, Milan, Italy; references 52, 53), flag-TRAF2 (Tularik Inc.), chimeric receptor CD4-TLR4 (R. Medzhitov, Yale University, New Haven, CT), TLR3 (D. Golenbock, University of Massachusetts Medical School, Worcester, MA), Flag-TRIF (S. Akira, Osaka University, Osaka, Japan), and Myc-MyD88 (L. O'Neill, Trinity College, Dublin, Ireland). Construction of A46R, Flag-A46R, HA-Mal, Flag-TRAM, and Flag-SARM were described previously (23, 27, 29, 40). The glutathione S-transferase (GST) fusion of A46R was synthesized by inserting full-length A46R in the bacterial expression vector GEX4T2.

Antibodies and reagents. Anti-A46R polyclonal Ab was raised against a purified, bacterial-expressed A46R-GST fusion protein. Other antibodies used were anti-Flag M2 mAb, anti-Flag M2-conjugated agarose, anti-myc mAb clone 9E10 (all obtained from Sigma-Aldrich), anti-AU1 mAb (BabCO), anti-HA polyclonal Ab (Y-11), anti-TRAF2 Ab (both obtained from Santa Cruz Biotechnology, Inc.), and anti-D8L mAb (54).

Human rIL-1 α was a gift from the National Cancer Institute and human rTNF- α was a gift from S. Foster (Zeneca Pharmaceuticals, Macclesfield, England). TLR agonists used were poly(I:C) (Amersham Biosciences), LPS (Sigma-Aldrich), R-848 (a gift from D. Golenbock, University of Massachusetts Medical School, Worcester, MA), flagellin (a gift from A. Gewirtz, Emory University, Atlanta, GA), phosphothioate CpG DNA (Sigma-Aldrich), synthetic tripalmitoyl lipopeptide Pam₃Cys-Ser-(Lys)₄ (Pam3Cys; Invivo-gen), and macrophage-activating lipopeptide 2 kD (MALP-2; Qbiogene).

Reporter gene assays. HEK 293 cells (2×10^4 cells per well) or RAW264.7 cells (4×10^4 cells per well) were seeded into 96-well plates and transfected 24 h later with expression vectors and luciferase reporter genes using GeneJuice (Novagen). In all cases, 20 ng/well of pRL-TK reporter gene (Promega) was cotransfected to normalize data for transfection efficiency. The total amount of DNA per transfection was kept constant at 220 ng (HEK293) or 200 ng (RAW264.7) by addition of pcDNA3.1 (Stratagene). After 24 h, reporter gene activity was measured (30). Data are expressed as the mean fold induction $\pm$ SD relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

For NF- κ B assays, 60 ng of a κ B-luciferase reporter gene was used (30). For MAP kinase reporter assays, the Pathdetect System (Stratagene) was used, whereby 0.25 ng c-jun-, 2 ng Elk1-, or 0.25 ng CHOP-Gal4 fusion vectors were used in combination with 60 ng pFR-luciferase reporter to measure JNK, ERK1/2, and p38 activation, respectively. For the p65 transactivation assay, 1 ng of a p65 Gal4 fusion vector was used in combination with 60 ng pFR-luciferase reporter (31). For the IRF3 assay, an IRF3-Gal4 fusion vector (3 ng) was used in combination with 60 ng pFR luciferase reporter (27).

Immunoprecipitation and immunoblotting. HEK293 cells were seeded into 10-cm dishes (1.5×10^6 cells) 24 h before transfection with GeneJuice. For coimmunoprecipitations, 4 μ g of each construct was transfected. Cells were harvested after 24 h in 850 μ l of lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 containing 0.01% aprotinin, 1 mM sodium orthovanadate, and 1 mM PMSF). For assessment of the A46R-TLR4 interaction, the cells were harvested after 48 h and lysed for 1 h in a different lysis buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.05% CHAPS, 0.5% Triton X-100, 250 mM NaCl containing 0.01% aprotinin, 1 mM sodium orthovanadate, and 1 mM PMSF). For assessment of interactions involving VV-expressed A46R, cells were infected (multiplicity of infection [MOI] = 1) with VV Western Reserve 24 h after transfection for 90 min at 37°C. The virus inoculum was aspirated and cell monolayers were overlaid with 2.5% FBS DMEM and harvested 24 h after infection in lysis buffer. For all immunoprecipitations, the appropriate antibodies were precoupled to either protein A or protein G-Sepharose for 1 h at 4°C before incubation with the cell lysates overnight at 4°C. The immune complexes were precipitated, washed, and analyzed by SDS-PAGE and immunoblotting (30).

For analysis of the kinetics of A46R expression, confluent monolayers of BSC-1 cells were infected (MOI = 5) for 90 min at 37°C. After removal of inoculum, cell monolayers were overlaid with 2.5% FBS DMEM in the presence or absence of 40 μ g/ml cytosine β -D-arabinofuranoside (Sigma-Aldrich). Cell lysates were analyzed by immunoblotting using anti-A46R or anti-D8L Ab.

GST pulldown assays. Plasmid GEX.4T2-A46R or GEX.4T2 was transformed into *Escherichia coli* BL21 (DE3) and grown in Terrific Broth. Protein expression was induced with 0.7 mM IPTG. Cells were lysed in NETN and proteins were purified from the insoluble fraction by glutathione sepharose 4B affinity chromatography (Amersham Biosciences).

For GST pulldown experiments, HEK 293T cells were transfected and harvested as described for coimmunoprecipitation. 800 μ l of cell lysate was added to purified GST-fusion protein coupled to glutathione-sepharose and incubated for 2 h at 4°C. The immune complexes were precipitated, subjected to SDS-PAGE, and were analyzed by immunoblotting.

Determination of cytokine concentrations. HEK293 clonal cell lines expressing either TLR3 (HEK-TLR3) or TLR4 and MD-2 (HEK-TLR4; reference 27) and were used for determination of cytokine production. Cells (2×10^4 cells per well) transfected with the A46R expression plasmid for 24 h were stimulated with 1 μ g/ml LPS or 25 μ g/ml poly(I:C) 24 h later. Supernatants were harvested 24 h later and IL-8 and RANTES concentrations were determined by ELISA (R&D Systems). Experiments were performed four times in triplicate and data are expressed as the mean $\pm$ SD from one representative experiment.

Recombinant VV viruses. A VV mutant (strain WR) lacking 93.5% of the A46R gene (v Δ A46R) was constructed by transient dominant selection (55). A plaque-purified wild-type virus (vWT-A46R) and a revertant virus (vA46R-REV) in which the A46R gene was reinserted at its natural locus were also isolated. The virulence of the viruses was investigated in a mouse intranasal model. Female, 6-wk-old BALB/c mice were anesthetized and inoculated with 5×10^3 plaque-forming units of VV in 20 μ l of phosphate-buffered saline. A control group was mock-infected with phosphate-buffered saline. Each day, the weights of the animals and signs of illness were measured as described previously (56). On days 2, 5, and 8, single cell suspensions of lung cells were prepared by sieving lungs through a 100- μ m nylon mesh followed by hypotonic lysis of erythrocytes. Cell viability was assessed using trypan blue exclusion. Statistical significance was assessed using Student's *t* test. The animal experiments were conducted under the appropriate licence and regulations stipulated by the Animals (Scientific Procedures) Act 1986, UK government.

Alignment of TIR domains. TIR domains from human proteins with assigned functions were aligned with VV and variola virus A46R using Clustal W. The alignment was viewed and adjusted using GeneDoc (57).

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