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Studies Into The Effects Of The Vaccinia Virus Protein A46R On Interleukin-1/Toll-Like Receptor Signalling Pathways

Thesis Submitted To The University Of Dublin
For The Degree Of Doctor Of Philosophy

By

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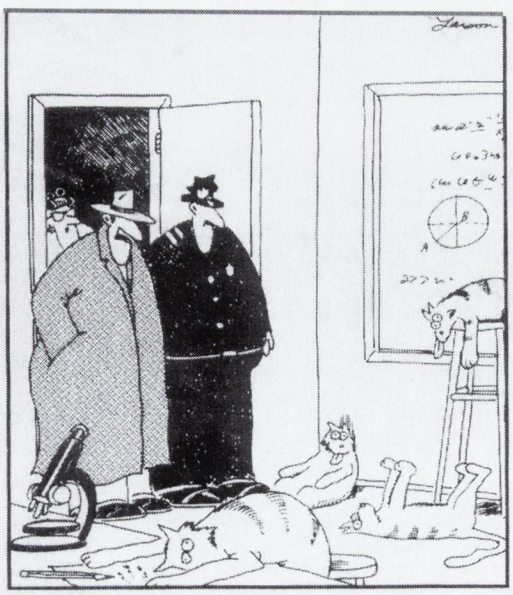

"Notice all the computations, theoretical scribblings, and lab equipment, Norm. . . . Yes, curiosity killed these cats."

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Abstract

Poxviruses circumvent the host immune response by encoding proteins that can antagonise host defence mechanisms. The vaccinia virus (VV) protein, A46R, was identified based on sequence similarity to the Toll/Interleukin-1/Resistance homology (TIR) domain, the cytosolic signalling domain of the interleukin-1 receptor (IL-1R) and Toll-like receptors (TLRs). TLRs play a crucial role in host defence against invading micro-organisms by mediating pro-inflammatory signalling cascades, mainly through their cytoplasmic TIR domains. A46R is the only viral protein so far identified to have a TIR domain. Initial studies revealed that A46R could inhibit IL-1, but not tumour necrosis factor (TNF) α-induced NFκB activation (Bowie *et al.,* 2000), suggesting a role for the protein in immune evasion. This study shows that A46R also blocked multiple signals emanating from TLR4, suggesting that it was acting on a molecule close to the receptor complex that was also required for IL-1 signalling. Immunoprecipitation and GST-pulldown experiments revealed that A46R targeted MyD88, a TIR adaptor molecule used by both IL-1 and TLR4, while further studies showed A46R blocked MyD88-dependent pathways in murine macrophages. This is the first example of a viral protein targeting MyD88.

Examination of other TIR-containing proteins showed that A46R could target Mal, an adaptor exclusively used by TLR2 and TLR4, while it did not associate with non-TIR containing adaptors such as TRAF2. A46R was found to inhibit TLR-4 dependent signalling pathways and to interact with other members of the TLR4 receptor complex, including TRIF and TRAM. It did not interact with SARM, a TIR adapter molecule which does not have a positive role in TLR signalling. Both MyD88- and TRIF-

dependent gene induction were also blocked by A46R. TLR3 and TLR4 induce TRIF-dependent anti-viral signalling pathways leading to IRF3 activation and type I interferon induction; these were found to be sensitive to A46R. TLR7 and TLR9 induce MyD88-dependent type I interferon induction. These results highlight the ability of A46R to block all arms of TLR-induced type I interferon induction.

Other VV proteins have also been found to inhibit TLR-induced activation of NFκB. A52R inhibits TLR3-induced NFκB (Harte *et al.,* 2003), but not IRF3 activation. In contrast, A46R inhibits TLR3-induced IRF3 activation, but not NFκB. Therefore, the two poxvirus proteins are not redundant in their function, but target different signalling pathways emanating from TRIF. N1L has been shown to inhibit many pathways leading to the activation of NFκB, including TLR signals. However, N1L acts at a point further downstream than A46R, at the level of IκB kinases and related kinases involved in NFκB and IRF3 activation (DiPerna *et al.*, 2004). Thus the importance to VV of blocking TLR signalling is demonstrated the retention of the virus of at least three distinct mechanisms of disrupting these pathways. Deletion of *A46R* has been shown to attenuate VV in a murine intranasal model, again demonstrating that A46R is not redundant in function with either A52R or N1L. The inhibitory effects of A46R on TLR-mediated NFκB and IRF3/7 activation and TLR-induced gene expression may be important to its role in virulence.

Chapter One

Introduction

1.1 The Immune System

A main goal of the immune system is to protect us against invading pathogens, such as bacteria, viruses and parasites. Mechanical protection afforded by skin and epithelia can keep out many intruders, but pathogens often possess specialised mechanisms to breach epithelial barriers. Rapid deployment of cells and molecules of the innate immune system is the next layer of defence. In many cases, lasting protection is afforded through acquired immunity. The trait of immunological memory allows re-exposure to many pathogens to be experienced without significant morbidity. Both the innate and adaptive arms of the immune system work cooperatively to protect the host from microbial infections.

1.1.1 Adaptive Immunity

Adaptive immunity detects non-self through recognition of pepetide 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 triggers clonal expansion of the lymphocyte and further production of antigen-specific antibodies (reviewed by Iwasaki *et al.*, 2004). 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 recognise fragments of antigens bound to the major histocompatibility complex (MHC) on the surface of an APC. The two types of peptide-binding proteins – MHC class I and MHC class II –then stimulate cytotoxic T lymphocytes (CTLs) and helper T cells (T_H). Naïve T_H cells, when stimulated with

cognate antigens by APCs, differentiate into two cell subsets: T_H1 and T_H2. T_H1 cells secrete interferon (IFN)-γ and promote mainly cellular immunity, whereas T_H2 cells produce IL-4, IL-5, IL-10 and IL-13 and primarily promote humoral immunity (reviewed by Abbas *et al.*, 1996).

This mechanism accounts for the generation of immunological memory, which provides a significant adaptive fitness to vertebrate animals. However, the adaptive immune system has two main limitations. First, randomly generated antigen receptors are unable to determine the source and the biological context of the antigen for which they are specific. Second, 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, typically for 4-7 days, which is too much of a delay to combat quickly replicating microbial invaders. However, the adaptive immune system does not function independently. Indeed, almost every aspect of the adaptive immune response is controlled by a combination of permissive and instructive signals, which are provided by the universal and evolutionarily ancient innate immune system (reviewed by Medzhitov, 2001).

1.1.2 Innate Immunity

The innate immune system detects the presence and nature of infection, rapidly provides the first line of host defence, and controls the initiation and determination of the effector class of the adaptive immune response. Its strategy is based on the early detection of conserved microbial structures that are relatively invariant within a given class of microorganisms. These structures are called pathogen-associated molecular

patterns (PAMPs), and the receptors of the innate immune system that directly recognize PAMPs are called pattern-recognition receptors (PRRs). PAMPs have several characteristic features important for their immunogenic activities. First, they are produced by microorganisms but not by host cells, which allows the host organism to discriminate between self molecules and microbial-associated non-self. Second, they are usually essential for the survival or pathogenicity of the microorganism, and have therefore been conserved over the course of evolution of the pathogen, even though their recognition by the host immune system creates a negative selective pressure. Finally, PAMPs often, but not always, represent a 'molecular signature' of a pathogen class. Therefore recognition of PAMPs by the innate immune system not only signals the presence of infection, but also provides valuable information regarding the type of infecting pathogen. Pattern-recognition receptors (PRRs), the receptors of the innate immune system (TABLE 1.1) that recognise PAMPs, are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. Their principal functions include opsonisation, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signalling pathways, induction of interferon and initiation of apoptosis (reviewed by Medzhitov, 2001).

TABLE 1.1: Pattern Recognition Receptors (Adapted from Medzhitov (2001) and Dziarski (2004))

Abbreviations Used:

CARD, caspase-recruitment domain; CRP, C-reactive protein; dsRNA, double stranded RNA; LBP, LPS-binding protein; LDL, low density lipoprotein; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MARCO, macrophage receptor with collagenous structure; MBL, mannan-binding lectin; NFκB, nuclear factor-κB; NOD, nucleotide-binding oligomerization domain; PGN, peptidoglycan; PGRP, PGN recognition protein; PKR, dsRNA activated protein kinase; RIG-I, retinoic acid inducible gene-I; SAP, serum amyloid protein; TIR, toll/interleukin/resistance TLR, toll-like receptor.

TABLE 1.1: Pattern Recognition Receptors

PRR	Protein/Domains	Ligands	Function
Secreted			
MBL	C-type lectin	Terminal mannose residues	Activation of the lectin pathway of complement
CRP, SAP	Pentraxins	Phosphorylcholine on microbial membranes	Opsonisation, activation of classical complement pathway
LBP	Lipid-transfer protein family	LPS	LPS recognition
Cell-surface			
CD14	Leucine-rich repeats	LPS, PGN	Co-receptor for TLRs
Macrophage mannose receptor	C-type lectin	Terminal mannose residues	Phagocytosis
Macrophage scavenger receptor	Scavenger receptor cysteine-rich domain	LPS, dsRNA, oxidised LDL, anionic polymers	Phagocytosis, LPS clearance, and lipid homeostasis
MARCO	Scavenger receptor cysteine-rich domain	Bacterial cell walls	Phagocytosis
TLRs	Leucine-rich repeats TIR domain	See TABLE 1.2	Activates NFκB and MAPKs; induction of type I interferon
Intracellular			
PKR	dsRNA binding domain Protein kinase domain	dsRNA	Activates NFκB and MAPKs; inhibition of translation and induction of apoptosis in virally infected and stressed cells
NODs	Leucine-rich repeats Nucleotide-binding oligomerisation domain CARD domain	Ligands for many NOD proteins are unknown. NOD1 and NOD2 were shown to recognise PGN	Activates NFκB and MAPKs; some family members may be involved in the induction of apoptosis
TLRs	Leucine-rich repeats TIR domain	See TABLE 1.2	Activates NFκB and MAPKs; induction of type I interferon
PGRP	PGN-binding cleft	PGN	Activation of prophenoloxidase cascade, amidase activity, activation of TLR, activation of *Imd* pathway, induction of phagocytosis, antibacterial activity
RIG-I	CARD domain RAN helicase domain	dsRNA	Induction of type I interferon

1.2 The IL-1R/TIR Superfamily

The Toll-like receptors (TLRs) are PRRs that have an essential function in immunity. Their discovery originated in the observation that a receptor in *Drosophila melanogaster* termed Toll, a maternal-effect gene that functions in a pathway that controls dorsoventral axis formation in fruitfly embryos (Hashimoto *et al.*, 1988), displayed remarkable sequence similarity in its intracellular domain to that of a mammalian receptor for the cytokine interleukin-1 (IL-1), termed the type I IL-1 receptor, IL-1R1 (Gay and Keith, 1991). However, the IL-1RI and Toll had very distinct ectodomains, comprised of immunoglobulin folds and leucine-rich repeats respectively. The conserved intracellular domain is now called the Toll/IL-1R/Resistance (TIR) domain. It was subsequently shown that, like IL-1RI, Toll had a role in host defence in that it mediated the induction of the antifungal peptide drosomycin in *Drosophila* (Lemaitre *et al.*, 1996). Further, the intracellular signalling pathways of IL-1R1 and Toll were shown to use some common signalling intermediates, and both receptors led to activation of the Rel family of transcription factors, typified by nuclear factor κB (NFκB) in mammals and Dif in flies (reviewed by Imler and Hoffmann, 2001).

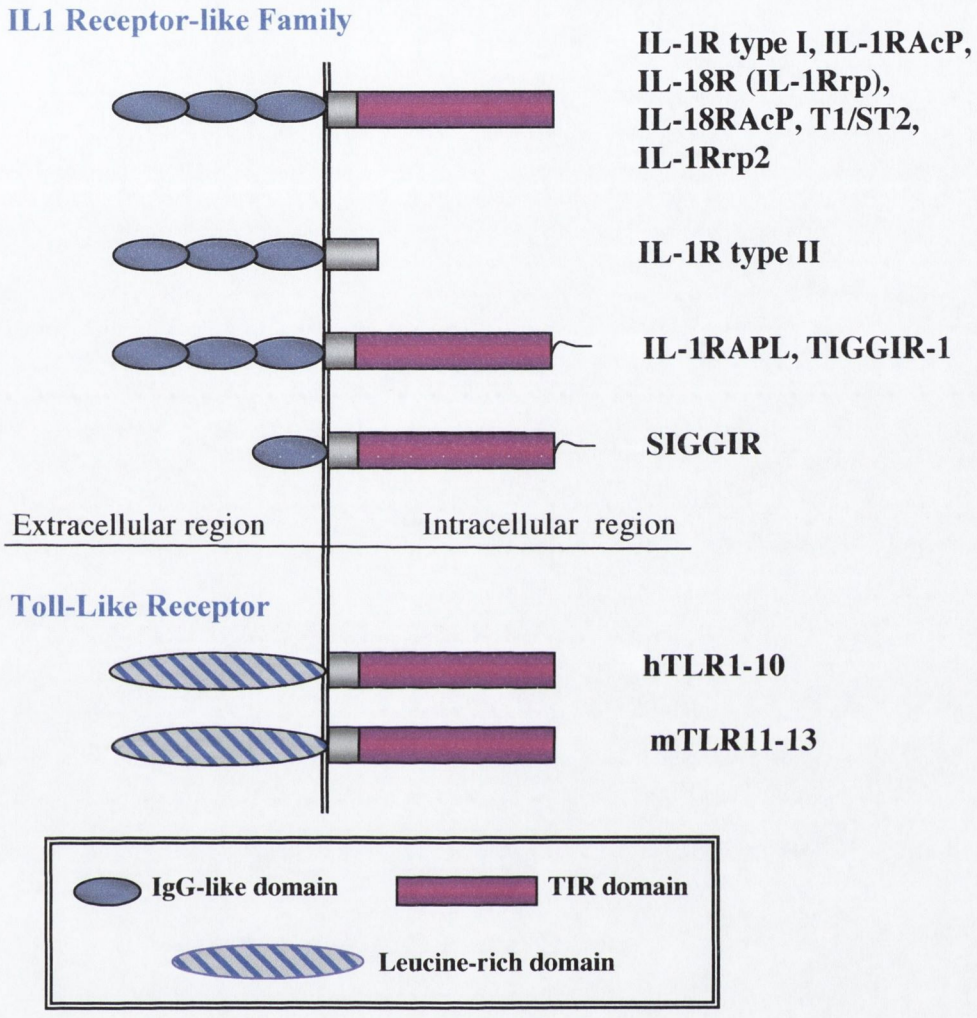
Subsequently, mammalian receptors began to be identified that were more Toll-like than IL-1R-like in that, as well as the intracellular TIR domain, they also had extracellular leucine-rich repeats. The first mammalian receptor in this class to be identified was termed human Toll, hToll (Medzhitov *et al.*, 1997). It was shown that a constitutively active chimera of this receptor with the extracellular portion of murine CD4 could induce expression of the pro-inflammatory cytokines IL-1, IL-6 and IL-8 and also of the costimulatory molecule CD80 (which is required for the activation of

naïve T cells) when expressed in THP-1 human monocytic cells. Further, hToll was shown to activate NFκB. More mammalian Toll-like receptors were identified and cloned, and hToll was renamed TLR4 (Medzhitov *et al.*, 1997).

Today, the TIR superfamily includes plant disease resistance proteins such as N protein and RPP1, and eight Toll receptors in *Drosophila* (dToll 1-8). The family splits broadly into those molecules that are IL-1R1-like, in that they have extracellular immunoglobulin domains, and those that are Toll-like, in that they have extracellular leucine-rich repeats. Mammalian members comprise TLRs 1-13, the IL-1R family members (Figure 1.1) and the intracellular TIR adapters (Figure 1.2), and elucidation of their function is an intensive area of research at present. Consistent with their function as PRRs, TLRs are expressed mainly in the cell types that are involved in the first line of defence, such as dendritic cells, macrophages, neutrophils, mucosal epithelial cells and dermal endothelial cells, as well as on T and B lymphocytes (Applequist *et al.*, 2002).

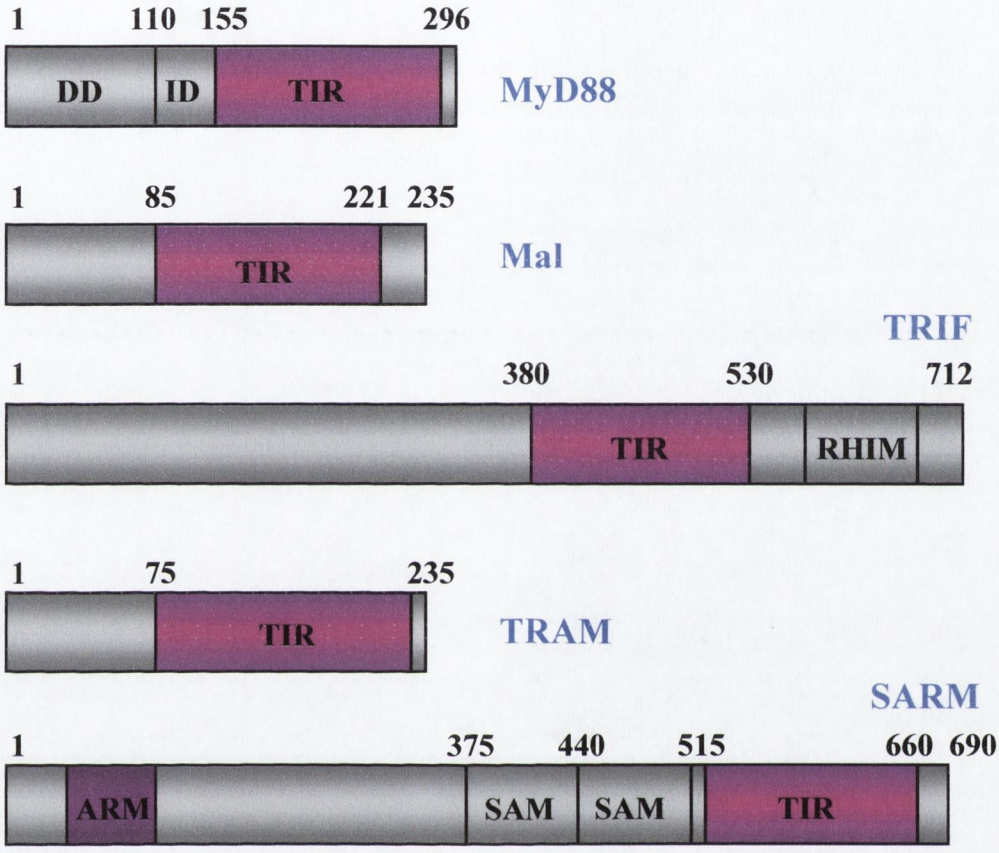
The crystal structures of the TIR domains for TLR1 and TLR2 have been obtained and analysed; they contain a central five-stranded β-sheet, which is surrounded by five α-helices on each side (Xu *et al.*, 2000). These two secondary structural elements are connected by loops: for example, the BB loop connects the strand β-B and the helix α-B. The conserved boxes 1 and 2 and the BB loop are adjacent and display most of their side-chains for interaction with adapter molecules (Xu *et al.*, 2000). The three-layer αβα sandwich conformation adopted by the TIR domains of TLR1 and TLR2 is similar to that of the bacterial protein CheY (Figure 1.14.A) (Xu *et al.*, 2000). Amino acid

Figure 1.1 Mammalian members of the IL-1R/TLR superfamily



The IL1R/TLR superfamily share a region of homology in their intracellular domain termed the Toll/IL-1 Receptor (TIR) domain. The IL-1R subgroup are typified by having multiple IgG-like domains in their extracellular domain whereas the TLR subgroup have a leucine-rich repeat domain in its place. Abbreviations used: IL-1R, IL-1 receptor; IL-1RAcP, IL-1R accessory protein; IL-1RAPL, IL-1R accessory protein like; IL-1Rrp, IL-1R related protein; SIGGIR, single immunoglobulin IL-1 receptor related molecule; TIGGIR-1, three immunoglobulin domain-containing IL-1R-related; TIR, TLR/IL-1R/Resistance; TLR, Toll-like receptor.

Figure 1.2 Schematic diagram of the TIR adapters, MyD88, Mal, TRIF, TRAM and SARM



Schematic diagram showing the structures of the TIR adapter molecules MyD88, Mal, TRIF, TRAM and SARM. Abbreviations used: ARM, armadillo repeat motif; DD, death domain; ID, intermediate domain; Mal, MyD88 adapter-like; MyD88, myeloid differentiation factor 88; RHIM, RIP homotypic interaction motif; SAM, sterile-α motif; SARM, SAM and ARM protein; TIR, Toll/IL-1R/Resistance; TRAM, TRIF-related adapter molecule; TRIF, TIR-domain-containing adapter inducing interferon-β.

sequence conservation among the TIR domains is generally around 20–30% (reviewed by Akira and Takeda, 2004).

Activation of the transcription factor NF-κB in response to IL-1 and several of the TLR ligands has been extensively documented and the pathway characterising its release from the cytoplasm well characterised (reviewed in Baeuerle and Henkel, 1994). A separate pathway regulating the transactivating potential of NFκB *via* phosphorylation of its p65 subunit has also been described, adding another level of complexity to the regulation of NFκB dependant gene expression by pro-inflammatory stimuli such as IL-1 and TNF (reviewed in Vermeulen *et al.*, 2002). In addition to NFκB activation, IL-1, LPS, peptidoglycan and other TLR ligands activate the three mitogen activated protein kinases (MAPK) (p42/p44, p38, and JNK MAPK). These are key pathways involved in regulating gene expression in response to diverse stimuli such as mitogens, growth factors, LPS, ultraviolet light and cytokines. Also, the abilities of TLRs 3, 4 7 and 9 to induced the expression of type I interferon *via* the activation of interferon regulatory factors (IRFs) have recently been demonstrated.

1.3 IL-1 Receptor-Like members of TIR Superfamily

IL-1 is a key inflammatory cytokine that mediates profound effects in virtually every organ system of the body. It activates the rapidly acting innate immune response, is a potent stimulator of both haematopoiesis and the adaptive immune system, and places the organism in a state of readiness to deal with injury or infection. 'IL-1' actually refers to two molecules, IL-1α and IL-1β. The expression of these molecules is regulated independently, but they bind to the same receptor and exert identical biological effects. In addition to the IL-1 family members (IL-1α, IL-1β and IL-1ra)

and IL-18, the past few years have seen the description of six novel proteins that belong to the IL-1 family. All of the genes encoding members of the IL-1 family, with the exception of the IL-18 gene which maps to chromosome 11q, map to a cluster on chromosome 2. These novel IL-1-like cytokines have been designated IL-1F5 - IL-1F10 (Sims *et al.*, 2001).

1.3.1 Type I IL-1 Receptor

The type I IL-1 Receptor (IL-1R1) was the first mammalian example of a TIR domain-containing protein, and is the receptor to which IL-1α and IL-1β bind. Its extracellular, ligand-binding portion comprises three immunoglobulin-like domains (Ig domains). Crystallography studies have shown that the two terminal Ig domains of the receptor are held in a rigid orientation by an inter-domain disulphide bond, whereas the membrane-proximal Ig domain is connected to the other two by a flexible tether. The receptor appears to wrap around the IL-1 molecule, such that IL-1β interacts with the receptor in two places: a larger area of contact in the groove between the first and second domains, and a smaller area on the side of the third domain (Vigers *et al.*, 1997). A homologue of the type-I IL-1R, known as IL-1R accessory protein (IL-1RAcP), is also required for IL-1 signalling. It has no affinity on its own for either IL-1α or IL-1β, but once IL-1 has bound the receptor, IL-1RAcP is recruited to the ligand-receptor pair to form the high affinity receptor complex that is necessary and sufficient for signal transduction (reviewed by Sims, 2002).

There are two natural regulators of IL-1. One is a dummy form of IL-1, called the 'IL-1R antagonist' (IL-1ra) (Eisenberg *et al.*, 1990), which resembles IL-1α and IL-1β (Nicklin *et al.*, 1994). IL-1ra binds to the type I receptor with an affinity that is slightly

higher than that of IL-1α and IL-1β (Greenfeder *et al.*, 1995), thus preventing IL-1α and IL-1β from doing so. The IL-1 binding protein, called the type II IL-1 receptor, IL-1RII (McMahan *et al.*, 1991) resembles the IL-1RI in its extracellular portion and has a high affinity for IL-1β (Lang *et al.*, 1998). However, it has a very short cytoplasmic domain and cannot mediate any biological signals. The importance of type II IL-1R in regulating IL-1 action is demonstrated by pox viruses, which have acquired and retained a soluble version of this molecule, deletion of which affects the virulence of the virus (Alcami and Smith, 1992; Spriggs *et al.*, 1992).

1.3.2 IL-18 Receptor

IL-18 is a potent inducer of interferon-γ (IFN-γ), upregulates the cytolytic activity of cytotoxic lymphocytes and natural killer cells, and promotes the development and differentiation of T cells, usually to the T-helper type-1 phenotype. It may also play a role in chronic inflammatory diseases (reviewed by Akira, 2000). The IL-18 receptor (IL-18R) complex is remarkably similar to the IL-1R complex in that IL-18R binds IL-18 with a relatively low affinity, but can bind with high affinity by recruiting an IL-1RAcP homologue, the IL-18R accessory protein, IL-18RAcP. As is the case for the IL-1R complex, both molecules are required for IL-18 signal transduction (reviewed by Sims, 2002). A small protein called IL-18-binding-protein (IL-18bp), which is not obviously related to the IL-18R, can bind to IL-18 and prevent its interaction with IL-18R, thereby regulating IL-18 activity (Novick *et al.*, 1999).

1.3.3 Other IL-1R Homologues

In addition to the two subunits in each of the IL-1R and IL-18R complexes, and the type II IL-1R, five other members of the IL-1R family are known, all of which are

presumed to be capable of mediating biological responses. IL-1RAPL/TIGIRR-2 (IL-1R accessory protein like/three immunoglobulin domain-containing IL-1R-related) (Born *et al.,* 2000; Carrie *et al.,* 1999) and TIGIRR-1/IL-1RAPL2/IL-1R9 (Born *et al.,* 2000; Ferrante *et al.,* 2001; Jin *et al.,* 2000; Sana *et al.,* 2000) are both orphan receptors with no known ligands. IL-1Rrp2 (IL-1R related protein 2; Lovenberg *et al.,* 1996) is reported to be responsive to the novel IL-1 homologues, IL-1F9 and IL-1F5 (Debets *et al.,* 2001).

Single Ig-domain containing IL-1 receptor related (SIGIRR) (Thomassen *et al.,* 1999) is also a family member although it only possesses one extracellular Ig-domain, similar to IL-18 binding protein (Aizawa *et al.,* 1999). A salient feature of IL-1RAPL, TIGIRR-1 and SIGIRR is the addition of a carboxy-terminal tail of roughly 100 amino acids. The tails of APL and TIGIRR are similar in sequence, but have no homology to the SIGIRR tail (reviewed by Sims, 2002). SIGIRR has been identified as a negative regulator of TLR and IL-1R signalling and interacts with TLRs 4, 5 and 9, the IL-1R, TRAF6 and IRAK-1 (Wald *et al.*, 2003).

T1/ST2 (Klemenz *et al.,* 1989; Tominaga, 1989; Werenskiold *et al.,* 1989) was the first identified member of the TIR family unable to activate NFκB, although it can activate MAPKs (Brint *et al.*, 2002). T1/ST2 negatively regulates IL-1R1 and TLR4. TLR4 recognises LPS (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999) and, in contrast to wild-type mice, ST2-deficient mice failed to develop endotoxin tolerance (Brint *et al.*, 2004).

1.4 Toll-Like Members of TIR Superfamily

So far, thirteen TLRs have been identified; TLRs 1-9 are common to mouse and human, TLR10 is only functional in humans, and TLRs 11-13 have been found only in mice (Takeda *et al.*, 2003; Dunne and O'Neill, 2003; Zhang *et al.*, 2004). The extracellular domain of TLRs contains 19–25 copies of the LRR motif. LRRs are involved in ligand recognition and signal transduction in a diverse set of proteins (Kobe & Deisenhofer, 1995). Each repeat consists of 24–29 amino acids and contains the leucine-rich sequence XLXXLXLXX, and another conserved sequence $X\Phi XX\Phi X_4FXXLX$ (Bell *et al.*, 2003), where X denotes any amino acid and Φ a hydrophobic amino acid. The repeats comprise a β-strand and an α-helix connected by loops. The LRR region in the TLRs is separated from the transmembrane region by a so-called 'LRR carboxy-terminal domain', which is characterised by the consensus motif $CXC(X_{23})C(X_{17})C$. The LRR domains of TLRs form a horseshoe structure, and it is thought that the concave surface of the LRR domains is involved directly in the recognition of various pathogens (Akira and Takeda, 2004). Combinations of overexpression studies, *ex vivo* studies and knockout mice have been widely used to identify the main ligands recognised by different TLRs (TABLE 1.2) Remarkably, despite the conservation among LRR domains, different TLRs can recognise several structurally unrelated ligands (Akira and Takeda, 2004). Heterodimerisation of TLRs may partly account for the wide variety of ligands recognised by TLR2, and this is discussed further in section 1.4.1.

The subcellular localisation of different TLRs correlates to some extent with the molecular patterns of their ligands. TLR1, TLR2, TLR4 and TLR6 are all located on the cell surface and are recruited to phagosomes after activation by their respective

ligands (Ozinsky *et al.*, 2000; Underhill *et al.*, 1999). Depending on the cell type, TLR3 is expressed either on the cell surface or on intracellular surfaces (Matsumoto *et al.*, 2003). In contrast, TLR7 and TLR9, which are involved on the recognition of nucleic-acid-like structures, are not expressed on the cell surface (Ahmad-Nejad *et al.*, 2002; Heil *et al.*, 2003). In this context, pathogen-derived RNA and DNA motifs are generated primarily during intracellular infection and are thus recognised intracellularly. For example, TLR9 has recently been shown to be expressed in the endoplasmic reticulum, and it is recruited to endosomal/lysosomal compartments after stimulation with CpG-containing DNA (Latz *et al.*, 2004). Similar events occur in Loxoribene- and imidazoquinoline-driven signalling *via* TLR7 (Heil *et al.*, 2003). TLR3, which recognises dsRNA may be expressed either on the cell surface or on intracellular surfaces depending on the cell type (Matsumoto *et al.*, 2003), because pathogen-derived nucleic acids may also be present in the extracellular environment, possibly due to lysis of infected cells.

It is unclear how TLRs actually recognise their ligands. How a single receptor such as TLR4 can recognise so many divergent ligands is still an unanswered question. However, in plants it has been demonstrated that recombination events between the leucine-rich repeats (LRRs) of related R genes result in the reversal of ligand specificity, indicating that this domain is responsible for binding the virulence factor expressed by the pathogen (Holt *et al.*, 2003). Since the extracellular portion of all TLR molecules contains LRR domains, there may also be a large region on the TLR proteins capable of interacting with viral envelope proteins, for example. Direct binding of ligands by TLRs 2, 5 and 9 has been demonstrated, and will be discussed in sections 1.4.1, 1.4.4 and 1.4.6 respectively.

TABLE 1.2: Ligands recognised by TLRs (adapted from Akira and Takeda, 2004)

Receptor	Ligands	Origin of Ligands
TLR1/TLR2	Soluble factor	*Neisseria meningitides*
	Lipoproteins (tri-acylated)	Bacteria
	JBT3002	Chemical
TLR2	Lipotechoic Acid	Gram positive bacteria
	Peptidoglycan	Gram positive bacteria
	Lipoproteins/Lipopeptides	Various pathogens
	Glycolipids	Spirochetes
	Lipoarabinomannan	Mycobacteria
	Atypical Lipopolysaccharide	*Porphyromonas gingivalis*
	Atypical Lipopolysaccharide	*Leptospira interrogans*
	Zymosan	Fungi
	GPI anchor	*Trypanosoma cruzi*
	Outer membrane protein A	*Klebsiella*
	Porin	*Neisseria*
	HSP60, HSP70, HSPgp96	Host
	Phenol Soluble Modulin	*Staphylococcus epidermidis*
TLR3	dsRNA	Virus
	dsRNA	*Schistosoma mansoni*
	poly(I:C)	Synthetic dsRNA
	mRNA	Host
TLR4	Lipopolysaccharide	Gram negative bacteria
	HSP60	*Chlamydia pneumoniae*
	Flavolipin	Flavobacteria
	Fusion protein	Respiratory syncytial virus
	Envelope proteins	Murine retroviruses
	Taxol	Plant
	Heat Shock Proteins 60, 70	Host
	Fibronectin 7EDA domain	Host
	Oligosaccharides of Hyaluronan	Host
	Fibrinogen	Host
TLR5	Flagellin	Flagellated bacteria
TLR2/TLR6	Lipoprotein (di-acylated)	*Mycoplasma*
	Lipotechoic acid	Gram positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinoline	Synthetic compounds
	Loxoribine	Synthetic compounds
	Bropirimine	Synthetic compounds
	ssRNA	Viruses
TLR8	Imidazoquinoline	Synthetic compounds
	ssRNA	Viruses
TLR9	CpG-containing DNA	Bacteria and Viruses
	Chromatin-IgG complexes	Host
	Hemozoin	Malaria
TLR11	N.D.	Uropathogenic bacteria

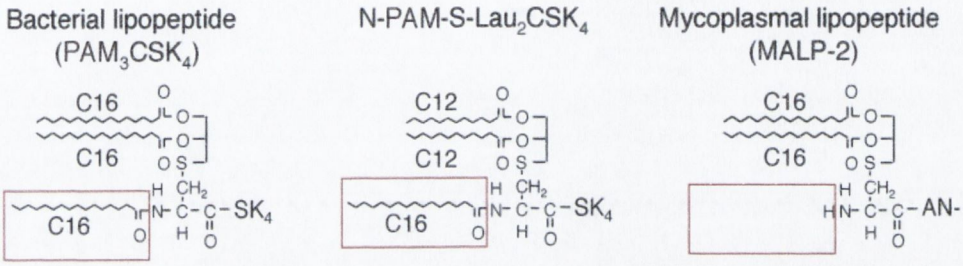
1.4.1 TLR2

TLR2 has been shown to be essential in recognising a multitude of PAMPs (Figure 1.3) including bacterial lipoproteins (Underhill *et al.*, 1999; Brightbill *et al.*, 1999), Gram positive bacterial cell wall components such as peptidoglycan (Takeuchi *et al.*, 1999) and lipotechoic acid (Schwandner *et al.*, 1999), yeast zymosan (Underhill *et al.*, 1999; Ozinsky *et al.*, 2000), measles hemagglutinin protein (Bieback *et al.*, 2002) and GPI anchors from *Trypanosoma cruzi* (Campos *et al.*, 2001). This unusually broad range of ligands recognised by TLR2 is explained, in part, by cooperation between TLR2 and at least two other TLRs: TLR1 and TLR6; i.e. the formation of heterodimers between TLR2 and either TLR1 or TLR6 dictates the specificity of ligand recognition (Figure 1.3). For example, TLR2 dimerises with TLR 6 to form a functionally active complex (Ozinsky *et al.*, 2000), which is essential in recognising the diacylated mycoplasmal lipopeptide MALP-2 (Takeuchi *et al.*, 2001). TLR2 can also dimerise with TLR1 to recognise a triacylated bacterial lipopeptide (Takeuchi *et al.*, 2002). The fact that the TLR10 amino acid sequence is most related to TLR1 and TLR6 suggests that it too may be a partner receptor for TLR2. The ligand for TLR10 is as yet unknown.

Vasselon and colleagues (2004) showed that the extracellular domain of TLR2 interacted directly with synthetic bacterial lipopeptide (sBLP), a potent analogue of bacterial lipoproteins. Specific binding of fluorescent sBLP to purified soluble TLR2 (sTLR2) required sCD14. However, sCD14 was not part of the complex formed by sTLR2 and sBLP.

It was once thought that TLR2 was the receptor involved in LPS signalling but data from TLR4 deficient mice (Hoshino *et al.*, 1999) and TLR2 knockout mice (Takeuchi

Figure 1.3 Lipopeptides recognised by TLR2 heterodimers (taken from Akira and Hemmi, 2003)



All membrane-anchored lipoproteins contain a lipoylated amino-terminal residue. The immunostimulatory activity of lipoproteins is attributed to the lipid portion. The lipoylation of the amino-terminal cysteinyl residue by a diacylglyceryl moiety *via* a thioether bond is a feature common to all known bacterial membrane lipoproteins but some lipoproteins undergo further acylation at the cysteinyl residue *via* an amide bond. PAM_3CSK_4 and N-PAM-S-Lau_2CSK_4 contain a triacylated cysteinyl residue at their N-terminus, whereas MALP-2 is only diacylated. MALP2 is recognized by a heterodimer between TLR2 and TLR6 whereas PAM_3CSK_4 and N-PAM-S-Lau_2CSK_4 are preferentially recognized by a heterodimer between TLR2 and TLR1.

et al., 1999) proved that this was not the case. However, some types of LPS (from *Leptospira interrogans* and *Porphyromonas gingivalis*) that are structurally different from those from enterobacteria have been shown to activate through TLR2 (Werts *et al.*, 2001; Hirschfield *et al.*, 2001).

1.4.2 TLR3

dsRNA is a molecular pattern produced by most viruses at some point in their life cycle. It has long been known to have immunostimulatory activity, partly because of its ability to activate the dsRNA-dependent protein kinase R (PKR). PKR has a central role in the antiviral response by inactivating the eukaryotic translation initiation factor 2 (eIF2) in response to dsRNA. Not only does this block viral and cellular protein synthesis, but it also causes NFκB and the MAPK activation, leading to the induction of type I interferons and apoptosis of infected cells, thus preventing viral spread (Williams, 1999). However, cells derived from PKR knockout mice still responded to the dsRNA mimic, polyinosine-polycytidilic acid, poly(I:C), suggesting the existence of another receptor which recognised dsRNA (Maggi *et al.*, 2000).

Thus Alexopoulou *et al.* (2001) tested whether a TLR could recognise poly(I:C), a synthetic analogue of dsRNA. When cells that were unresponsive to poly(I:C) were transfected with various TLRs, those expressing TLR3 showed marked responsiveness to poly(I:C), in terms of NFκB activation. Poly(I:C) activation of cells *via* TLR3 also led to the induction of type I interferons, which was abrogated in cells from TLR3 knockout mice. Further, TLR3 knockout mice were significantly resistant to poly(I:C)-induced shock compared to wild-type mice (Alexopoulou *et al.*, 2001). These results suggested a role for TLR3 in the host anti-viral response. The role of TLR3 in innate

immunity has recently been expanded to include non-viral pathogens with the discovery that dsRNA from the eggs of the Helminth parasite *Schistosoma mansoni* activates TLR3 in dendritic cells (Aksoy *et al.*, 2005).

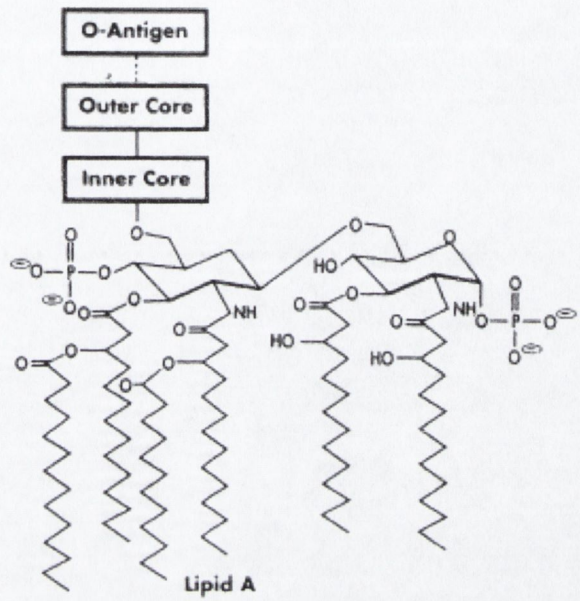
Pathogens continuously mutate and evolve to escape immune surveillance, including detection by TLRs. It is thought that activation of TLRs by host-derived ligands released during injury and infection may be very important in alleviating the sole need of pathogen for activation of the immune system. The endogenous ligand for TLR3 has been identified as single-stranded mRNA which activates TLR3-dependent NFκB. This probably occurs *via* double-stranded sequences such as hairpin loops within the mRNA secondary structure (Kariko *et al.*, 2004).

Interestingly, unlike all other TLRs, TLR3 contains an alanine residue instead of the usual conserved proline residue in the position equivalent to proline-712 of murine TLR4. This could be a reflection of the fact that the TLR3 signalling mechanism differs from that of other TLRs, with TRIF being the only TIR adapter molecule involved (Yamamoto *et al.*, 2002b).

1.4.3 TLR4

Lipopolysaccharide (LPS) constitutes the outer leaflet of the outer membrane of Gram-negative bacteria and consists of three covalently linked regions; lipid A, core oligosaccharide, and an O side chain. The innermost layer, lipid A, which is responsible for the toxicity of LPS, consists of six fatty acyl chains linked to two glucosamine residues (Figure 1.4). LPS is a potent activator of macrophages as a causative agent of endotoxic shock. Stimulation of macrophages with LPS results in

Figure 1.4 Structure of LPS, the TLR4 activator (taken from Guha and Mackman, 2000)



LPS is the principal component of the outer membrane Gram-negative bacteria. It consists of three covalently linked regions; lipid A, core oligosaccharide, and an O side chain. The innermost layer, lipid A, which is responsible for the toxicity of LPS, consists of six fatty acyl chains linked to two glucosamine residues

the production of various cytokines such as TNF-α, IL-1, IL-6, IL-10, macrophage inflammatory protein-1α/β (MIP-1α/β) and inflammatory effector substances such as prostanoids, leukotrienes, and nitric oxide (reviewed in Guha and Mackman, 2000)

Lipopolysaccharide-binding protein (LBP), a 60 kDa serum glycoprotein that binds the lipid A moiety of LPS (Tobias *et al.*, 1995), is a lipid transferase catalyzing LPS transfer from the outer membrane to CD14, another LPS binding molecule (Schumann *et al.*, 1990). CD14 is an approximately 50 kDa glycoprotein, which is expressed on myleomonocytic cells as a glycophosphatidyl inositol (GPI) anchored molecule (membrane CD14, mCD14) or as a soluble molecule in circulation (sCD14) (Wright *et al.*, 1990). In CD14-negative cells such as endothelial cells and fibroblasts, the soluble form of CD14 present in serum can functionally replace membrane-bound CD14. Occupation of the LPS/LBP/CD14 ternary complex on the cell surface triggers activation of several members of the MAP kinase family and NFκB. Although LPS is bound primarily by LBP and CD14, the fact that CD14 lacks a transmembrane domain suggested the existence of an additional co-receptor to initiate signal transduction. Indeed, LPS-mediated cellular activation still occurs in CD14-deficient mice (Haziot *et al.*, 1996).

This putative co-receptor was identified through analysis of a strain of mice, C3H/HeJ, that is hyporesponsive to LPS. Positional cloning of the *lps* locus demonstrated that C3H/HeJ mice harbour a missense point mutation within the cytoplasmic portion of TLR4 that results in the substitution of histidine for a proline that is highly conserved among TLR family members (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). Mice deficient in the *Tlr4* gene were generated by gene disruption techniques. Macrophages

and B cells from these TLR4 knockout mice were hyporesponsive to LPS to a similar extent to C3H/HeJ mice (Hoshino *et al.*, 1999).

To respond efficiently to LPS, TLR4 requires an accessory protein, MD2. MD2 is a 20- to 30-kDa glycoprotein, which binds to the extracellular domain of TLR4 (Shimazu *et al.*, 1999). Photoaffinity labelling studies have recently shown that LPS binds directly to the TLR4-MD2 complex and that both molecules are in close proximity to the bound LPS (da Silva Correia *et al.*, 2001). Transfection with either TLR4 or MD-2 alone did not confer responsiveness to LPS, but co-transfection with both TLR4 and MD-2 did. MD-2 is physically associated with TLR4 on the cell surface (Shimazu *et al.*, 1999). LPS has to interact with TLR4/MD-2 complexes to trigger the LPS signal. However, the molecular mechanisms underlying LPS transfer from CD14 to TLR4/MD-2 remain to be clarified. TLR4 is in the Golgi apparatus as well as on the plasma membrane, and rapid recycling of TLR4/CD14/MD-2 complexes between the plasma membrane and the Golgi apparatus is a prominent phenomenon. However, LPS signalling commences on the plasma membrane and is independent of trafficking to the Golgi (Latz *et al.*, 2002)

TLR4/MD-2 has a variety of ligand other than LPS. Murine, but not human, TLR4/MD-2 also senses Taxol, a natural plant toxin that triggers microtubule polymerisation (Kawasaki *et al.*, 2000). Interestingly, TLR4/MD-2 is activated by endogenous mediators in the innate immune system, which include a member of the collectin family of preimmune opsonins surfactant protein-A (SP-A) (Guillot *et al.*, 2002), an antimicrobial peptide murine β-defensin (Biragyn *et al.*, 2002) and molecular chaperones with immunogenic activities such as heat-shock proteins (HSPs) (Vabulas

et al., 2002; Ohashi *et al.*, 2000). It is hard to imagine that such a variety of ligands independently and specifically interact with TLR4/MD-2 and it is very important that the molecular mechanisms for the recognition of these ligands are revealed. TLR4 does not heterodimerise with other TLRs, but instead functions as a homodimer (Zhang *et al.*, 2002).

1.4.4 TLR5

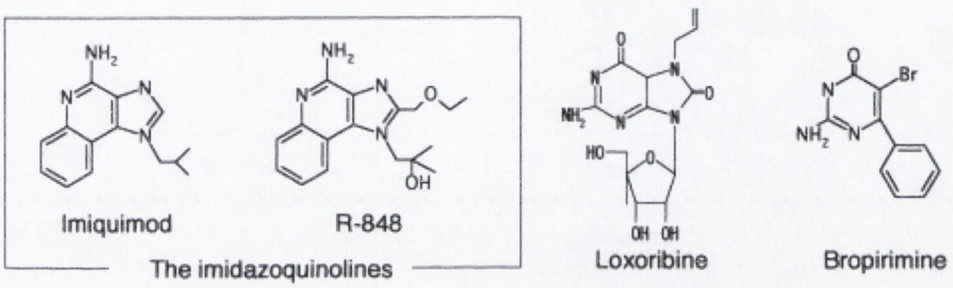
Flagellin is a 55-kDa monomer obtained from bacterial flagella, a polymeric rod-like appendage from the outer membrane of Gram-negative bacteria that propels the organism through its aqueous environment. Flagellin is also a potent proinflammatory factor. Flagellated bacteria, purified flagellin, and medium conditioned by flagellated bacteria all induce NFκB activation and inducible nitric oxide synthase (iNOS) expression in transformed human epithelial cells and murine macrophages. These actions of flagellin have been shown to be mediated through TLR5 (Hayashi *et al.*, 2001). An unusual aspect of this TLR ligand is that, unlike most other PAMPs, flagellin is a protein, and it does not undergo any post-translational modification that would distinguish it from host proteins. However, the amino- and carboxy-termini of flagellin are extremely conserved, presumably because they form a hydrophobic core of the flagella and have significant structural constraint on variability (Samatey *et al.*, 2001). This extreme structural conservation and the vitally important function of flagellin for bacterial mobility explain why it was selected as a target for recognition by TLR5. Flagellin has been shown to bind directly to TLR5. Also, a sequence in the extracellular domain of hTLR5 has been identified that is required for the binding of flagellin to occur (Mizel *et al.*, 2003), providing for the first time a molecular basis for the agonist specificity of a TLR.

Interestingly, TLR5 is expressed on the basolateral, but not apical, surface of intestinal epithelium, where it can sense flagella from pathogenic bacteria such as *Salmonella* (Gewirtz *et al.*, 2001). Such polarised expression of TLR5 (and presumably other TLRs) on surface epithelia might provide an important mechanism of discrimination between commensal and pathogenic bacteria, as pathogenic, but not commensal, microbes can cross the epithelial barriers.

1.4.5 TLR7 & TLR8

Imiquimod (R-837) and resiquimod (R-848) are low-molecular weight compounds of the imidazoquinoline family (Figure 1.5) that have potent anti-viral and anti-tumour properties in animal models (Miller *et al.*, 1999). Hemmi *et al.* (2002) found that the response to these imidazoquinolines is completely abolished in MyD88 knockout mice and that human TLR7 confers responsiveness to R-848. Jurk *et al.* (2002) showed that R-848 induced NFκB activation in a dose-dependent manner when HEK293 cells were transfected with human TLR8. In contrast, HEK293 cells transiently transfected with murine TLR8 did not activate NFκB after stimulation with R-848, which suggests that TLR8 is non-functional in mice. This mute phenotype is in accordance with the observation that TLR7-deficient mice do not respond to R-848, even though TLR8 is present. Another low-molecular-weight immune modifier, loxoribine (7-allyl-7,8-dihydro-8-oxo-guanosine), activates NFκB *via* TLR7 in a MyD88-dependent manner (Heil *et al.*, 2003). The imidazoquinolines and the guanosine analogue, loxoribine, are structurally similar to nucleic acids. Recently, the natural ligand for TLR7 was discovered to be a single-stranded RNA (ssRNA) derived from viruses (Lund *et al.*, 2004; Heil *et al.*, 2004; Diebold *et al.*, 2004). Further studies are required to disclose the structure-activity relationship of TLR7 and its ligands.

Figure 1.5 Structures of TLR7 activators; the imidiazoquinolines, loxoribine and bropirimine (taken from Hemmi and Akira, 2003)



TLR7 recognizes small synthetic immune modifiers including imiquimod (R-837), resiquimod (R-848), loxoribine, and bropirimine, all of which are already applied or promising for clinical use against viral infections and cancers.

1.4.6 TLR9

Unmethylated CpG motifs are prevalent in bacterial but not vertebrate genomic DNA. Oligodeoxynucleotides (ODN) containing CpG motifs activate host defense mechanisms leading to innate and acquired immune responses. The recognition of CpG motifs requires TLR9 (Hemmi *et al.*, 2000) and TLR9 binds directly (Latz *et al.*, 2004) and sequence specifically (Bauer *et al.*, 2001) with CpG. The optimal CpG motif for human TLR9 was GTCGTT, whereas the optimal murine sequence was GACGTT. However, consistent with the fact that CpG stimulation and the resultant signalling *via* TLR9 causes maturation and/or acidification of endosomes (Ahmad-Nejad *et al.*, 2002; Hacker *et al.*, 1998), TLR9 only binds with CpG at acidic pH (Rutz *et al.*, 2004). Bauer *et al.* (2001) showed that transfection of either human or murine TLR9 conferred responsiveness in HEK293 cells in a CD14- and MD2-independent manner, yet required species-specific CpG-DNA motifs for initiation of the Toll/IL-1R signal pathway *via* MyD88. Recently, TLR9 has been implicated in type I interferon production in response to infection with herpes simplex virus type-1 (HSV-1) (Hochrein *et al.*, 2004), probably through recognition of immunostimulatory viral CpG motifs.

The malaria pigment, hemozoin (HZ), is a novel non-DNA ligand for TLR9 (Coban *et al.*, 2005). The physiological role of HZ-induced, TLR9-mediated innate immune activation in malaria infection and the host response against it is currently under investigation, as is the molecular mechanism by which TLR9 discriminates between CpG DNA and HZ.

Similar to other TLRs, TLR9 also responds to host-derived molecules. It is well established that, in the absence of infection, damaged host tissues provoke inflammation and that this might be mediated by TLRs that sense products released by damaged cells. TLR9 senses host DNA bound to histones or anti-histone antibodies (Leadbetter *et al.*, 2002). .

In humans, the expression of TLR9 is restricted to B cells and plasmacytoid dendritic cells (pDCs; Kadowaki *et al.*, 2001). The PDCs, which also express large amounts of TLR7, are characterized by their ability to rapidly synthesise large amounts of type I interferon (IFNα and IFNβ) in response to viral infection. In contrast to other dendritic cell subsets which express a broad profile of TLRs, the TLR profile in pDCs is restricted to TLR7 and TLR9 and this is the reason why these cells are often used to study the immunostimulatory effects of CpG DNA and the associated role of TLR9 (Rothenfusser *et al.*, 2002).

1.4.7 TLR11

Using TLR4 as a guide, Zhang and colleagues (2004) searched for similar gene sequences in the databases of the National Centre for Biotechnology Information. They discovered TLR11 in a mouse liver expressed sequence tag database. Further analysis revealed that TLR11 is particularly abundant in the kidney and the bladder. When infected with so-called uropathogenic bacteria, mice deficient in TLR11 harboured 10,000 times as many bacteria in their kidneys as normal mice. Murine TLR11 senses bacteria that infect the kidney and provokes an immune response that leads to bacterial clearance. Humans have a truncated form of TLR11 that is probably inactive. It is speculated that this could be the reason why humans are susceptible to urinary tract

infections. The similarity between TLR11 and TLR5 suggests that a flagellin-like protein, such as the one found in the pili of uropathogenic bacteria, might bind to TLR11.

1.5 TIR Adapter Molecules

Activation of the various TLRs leads to different patterns of gene expression (Figure 1.8). The molecular mechanisms underlying these differences can be explained, at least in part, by the existence of several TIR adapter molecules, which are used by different TLRs (Figure 1.2).

1.5.1 MyD88

MyD88, myeloid differentiation factor 88, 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 (Lord *et al.*, 1990). As both the medium and IL-6 induced growth arrest and terminal differentiation along the macrophage lineage in M1 cells, the 12 cloned cDNAs were referred to as myeloid differentiation (MyD) primary response genes. Four years later in 1994, the C-terminal portion of MyD88 was found to have a TIR domain (Hultmark *et al.*, 1994). The N-terminal part of MyD88 encodes a so-called death domain (DD), a protein-protein interaction motif of ~ 90 amino acids that was originally identified in apoptosis promoting proteins (Feinstein *et al.*, 1995). The DD and TIR domain of MyD88 are linked by an intermediate domain (ID; residues 110-157) (Figure 1.2) which is involved in the recruitment of IRAK-4. The MyD88 short protein, MyD88s, lacks this ID and acts as an inhibitor of IL-1 and LPS-induced NFκB activation (Burns *et al.*, 2003).

MyD88 knockout mice have been generated and it was found that MyD88 deficient cells did not respond to IL-1, implying that MyD88 is the only adapter involved in IL-1 responses (Kawai *et al.*, 1999). MALP2, a ligand for the TLR2-TLR6 receptor pair, did not activate NFκB in MyD88 deficient macrophages (Kawai *et al.*, 1999). Further, MyD88 is an absolute requirement for TLR5, TLR7 and TLR9 signalling, as MyD88-null mice did not respond to flagellin (Hayashi *et al.*, 2001), R-848 (Hemmi *et al.*, 2002) or CpG stimulation (Hemmi *et al.*, 2000). However, LPS-induced activation of NFκB and JNK, although delayed, still occurred (Kawai *et al.*, 1999), as did LPS-induced DC maturation (Kaisho *et al.*, 2001), implying that an additional adapter was required for a subset of LPS-inducible, TLR4 dependent signals. TLR3 signalling to NFκB in MyD88-deficient mice was identical in terms of kinetics to wild-type mice (Kawai *et al.*, 1999), again implying the presence of an additional adapter.

1.5.2 Mal/TIRAP

The results from MyD88 knockout mice indicated that other adapters similar to MyD88 must exist; the first of these to be described was MyD88 adapter-like (Mal) or TIR domain-containing adapter protein (TIRAP; Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). Mal is a 235 amino acid protein, and its N-terminus shows no similarity to any known protein motifs. Its C-terminus TIR domain is similar to that of MyD88, although it does not contain a conserved box 3. Mal both homodimerises, and forms heterodimers with MyD88 and TLR4, and activates NFκB, JNK and ERK1/2 when overexpressed. Dominant negative forms of Mal inhibit NFκB, which is activated by TLR4 or LPS, but not NFκB activated by IL-1R or IL-18R (Fitzgerald *et al.*, 2001).

When Mal knockout mice were generated they were found to have delayed kinetics in the activation of NFκB and MAPKs in response to LPS, similar to that observed in MyD88 deficient mice. Mal knockout mice responded normally to the TLR3, TLR5, TLR7 and TLR9 ligands, as well as to IL-1 and IL-18, but were severely impaired in their responses to ligands for TLR2. These data showed that Mal is not specific to TLR4 signalling and does not participate in the MyD88-independent pathway. Instead, Mal has a crucial role in the MyD88-dependent pathway shared by TLR2 and TLR4 (Horng *et al.*, 2002).

Interferon regulatory factor (IRF) 3 was activated in a Mal- and MyD88 independent manner in response to LPS in that IRF3 activation and homodimer formation occurred as normal in wild-type, Mal-deficient and MyD88 deficient macrophages (Yamamoto *et al.*, 2002a; Kawai *et al.*, 1999). LPS induction of IFN-inducible genes encoding IP-10 (inducible protein 10), GARG-16 (glucocorticoid attenuated response gene 16) and IRG-1 (immune-responsive gene 1) was comparable in wild type, Mal-deficient and MyD88-deficient macrophages (Yamamoto *et al.*, 2002a).

Results from the Mal knockout and MyD88 knockout mice did not preclude the possibility that MyD88 might compensate for the Mal-deficiency and *vice versa* during the LPS response. Mal-MyD88 double knockout mice were therefore generated. Genes encoding IP-10, IRG-1 and GARG-16 were induced normally in response to LPS in double knockout macrophages. LPS-induced expression of co-stimulatory molecules was observed normally in double knockout dendritic cells (Yamamoto *et al.*, 2002a), consistent with LPS-induced DC maturation being MyD88-independent (Kawai *et al.*, 1999). These studies suggested that there must be another adapter, which

would function in TLR4 signalling on the MyD88-Mal-independent pathway. In addition, because activation of IRF3 by TLR3 did not require either MyD88 or Mal, another adapter was suggested to exist for TLR3 signalling also (Yamamoto *et al.*, 2002a).

1.5.3 TRIF

Almost simultaneously, two groups reported the existence of a third TIR domain-containing adapter, called TRIF (TIR-domain containing adapter Inducing IFN-β) or TICAM-1 (TIR-containing adapter molecule-1) (Yamamoto *et al.*, 2002b; Oshiumi *et al.*, 2003a). The carboxy-terminal TIR domain of TRIF shows little similarity to those of Mal and MyD88. The conserved sequences in other TIR domain-containing proteins, (F/Y)D in box 1, RD in box 2 and FW in box 3 are missing, but the proline residue that is highly conserved among TIR domains is present. Overexpression of TRIF activated the NFκB dependent promoter in a similar fashion to MyD88 and Mal. However, TRIF, but neither Mal nor MyD88, activated the IFN-β promoter (Yamamoto *et al.*, 2002b). TRIF also activated both IRF3 and IRF7 (Fitzgerald *et al.*, 2003a). TRIF associated with TLR3 and IRF3 and a dominant negative version of TRIF comprising its TIR domain alone inhibited TLR3-mediated activation of both the NFκB and IFN-β promoters. (Yamamoto *et al.*, 2002b). These data indicated that TRIF is involved in MyD88-independent activation of TLR3 signalling.

Yamamoto and co-workers (2002b) showed that distinct domains of TRIF are responsible for activation of IFN-β and NFκB: the N-terminal portion of TRIF is essential for activation of IFN-β promoter activity, whereas both N-terminal and C-terminal portions of TRIF are involved in the activation of NFκB. Two separate groups

expanded on these data and showed that TRIF interacted with TRAF6 (TNF receptor-associated factor 6) through TRAF6-binding motifs found in the N-terminal portion of TRIF and the TRAF domain of TRAF6 (Sato *et al.*, 2003, Jiang *et al.*, 2004). Disruption of TRAF6 binding motifs of TRIF disabled it from binding TRAF6, and resulted in a reduction in the TRIF-induced activation of the NFκB but not the IFN-β promoter (Sato *et al.*, 2003; Jiang *et al.*, 2004). TRIF contains a receptor inhibitor protein (RIP) homotypic interaction motif (RHIM) at its C-terminus that is essential for binding of RIP1 and RIP3, two serine-threonine kinases linked to TNF-mediated NFκB activation. A mutant TRIF, in which four amino acids (QLGL) in the RHIM domain were replaced by four alanine residues, could no longer activate NFκB (Meylan *et al.*, 2004), which was surprising considering TRIF can activate NFκB by interacting with TRAF6 at its N-terminus (Sato *et al.*, 2003). However, RIP1 is probably more important for TLR3-mediated NFκB activation *via* TRIF because TLR3-induced NFκB activation was normal in TRAF6-deficient mice (Gohda *et al.*, 2004).

TBK1 (TANK-binding kinase 1), an essential transcription factor for IFN-β expression (Fitzgerald *et al.*, 2003b; Sharma *et al.*, 2003), associates with the N-terminal portion of TRIF to activate IRF3. Overexpression of TRIF fails to induce the expression of IFN-β, RANTES (regulated on activation normal T cell expressed and secreted) or IP-10 reporter genes in *Tbk1*-deficient cells (McWhirter *et al.*, 2004). Co-expression of TBK1 with TRIF induced phosphorylation of TRIF, as is the case of the endogenous TRIF modification in response to LPS, and this modification was dependent on the kinase activity of TBK1. Furthermore, TBK1-associated TRIF is a phosphorylated form. Collectively, TRIF, TBK1 and IRF3 form a complex, which was dependent on TBK1 kinase activity (Sato *et al.*, 2003). The fact that TBK1 associates with the N-

terminal of TRIF is consistent with the previous report in which the N-terminal, but not the C-terminal, truncated TRIF failed to activate the IFN-β promoter (Yamamoto *et al.*, 2002b; Sharma *et al.,* 2003; Fitzgerald *et al.,* 2003a). It is also consistent with the fact that the TRIF C-terminal RHIM domain alone can activate the NFκB, but not the IFN-β promoter or the ISRE reporter gene (Meylan *et al.,* 2004). Although TRAF6 and TBK1 might not associate with the same residues of TRIF, it seems that TRAF6 physically blocks the association between TRIF and TBK1 because the increased expression of the TRAF domain of TRAF6 repressed the association of TRIF-TBK1. Moreover, not only the TRAF domain but also full-length TRAF6 inhibited TRIF-induced IFN-β promoter activation (Sato *et al.*, 2003).

TRIF knockout mice were defective in both TLR3- and TLR4-mediated IFN-β expression and IRF3 activation, suggesting that TRIF is essential for MyD88-independent activation of IRF3 in response to TLR3 and TLR4 signalling. Poly(I:C)-induced NFκB activation was severely impaired in TRIF deficient cells, indicating that TRIF plays a major role in TLR3-mediated NFκB activation (Yamamoto *et al.*, 2003a; Hoebe *et al.*, 2003). However, TRIF knockout mice displayed normal LPS-induced MyD88-dependent activation of IRAK-1, NFκB, and MAP kinases demonstrating that TRIF is not involved in the LPS-induced MyD88-dependent signalling. However, the mice were impaired in inflammatory cytokine production, which needs activation of MyD88-dependent signalling and were resistant to LPS-induced septic shock (Yamamoto *et al.*, 2003a). These data suggest that cooperation between MyD88-dependent and -independent signalling pathways are required for TLR4 mediated production of inflammatory cytokines. Mice deficient in both MyD88 and TRIF showed complete loss of NFκB activation in response to TLR4 stimulation (Yamamoto

et al., 2003a; Hoebe *et al.*, 2003) and macrophages lacking both TRIF and MyD88 did not upregulate any genes in response to LPS (Hirotani *et al.*, 2005). The anti-viral role of TRIF was highlighted in mice with the so-called *Lps2* mutation. The response of macrophage cultures to infection by VV was demonstrably impaired when the *Trif* gene was disrupted, in that macrophage monolayers from *Lps2* homozygotes supported the replication of *Vaccinia* to a higher titre than did macrophages from normal mice (Hoebe *et al.*, 2003).

When overexpressed, TRIF has been observed to potently induce apoptosis. TRIF-induced apoptosis was mediated by its C-terminal domain, which interacts with the death-domain containing protein kinase, RIP1. TRIF-induced apoptosis was blocked by the dominant negative mutants of FADD (Fas-associated protein with death domain) and caspase-8 and by the caspase inhibitors ZAD-fm and CrmA, but not by a p53 mutant and Bcl-2. These observations suggest that TRIF induces RIP/FADD/caspase-8 dependent, mitochondrion-independent apoptosis (Han *et al.*, 2004).

1.5.4 TRAM

A fourth TIR adapter termed TRIF related adapter molecule (TRAM) or TIR-domain containing protein (TIRP) or TIR-domain containing adapter molecule-2 (TICAM-2) (Fitzgerald *et al.*, 2003a; Bin *et al.*, 2003; Oshiumi *et al.*, 2003b) was subsequently identified. The gene encoding TRAM is located on human chromosome 5 and is most similar in sequence to TRIF, particularly in Box 1 of its TIR domain (Bin *et al.*, 2003). TRAM is a small TIR domain containing protein with just 235 amino acids, which like TRIF induces NFκB, IFN-β and IRF3 activation (Fitzgerald *et al.*, 2003a). Early investigations into TIRP by Bin and colleagues (2003) suggested that it had a role in

IL-1R signalling. They found that TIRP interacted with IL-1R and IL-1RAcP, but not TLR2 and TLR4. They also showed that a TIRP dominant negative inhibited IL-1 signalling and not that of TNF-α (Bin et al., 2003, JBC). However, dominant negative constructs, when highly expressed, have the potential to bind and interfere with proteins that might otherwise not be related to a specific signal transduction pathway (Fitzgerald *et al.*, 2003a). The TRAM knockout mouse confirmed that TRAM does not play a role in IL-1R signalling (Yamamoto *et al.,* 2003b), but rather has a specific role in TLR4 signalling. TRAM is the only TIR adapter shown to be specific to one TLR.

TRAM-deficient mice showed normal responses to ligands for TLR2, TLR3, TLR7, TLR9 and IL-1β, but defective macrophage responses to TLR4 ligand (LPS) with respect to cytokine production and B cell activation. LPS-induced IRF3 activation and expression of interferon-inducible genes was impaired in TRAM-deficient cells, but poly(I:C) responses were normal *i.e.* TLR4-mediated, but not TLR3-mediated, activation of the MyD88-independent signalling pathway was impaired in TRAM deficient mice. TRAM was also found to be responsible for the late-phase activation of NFκB observed in both MyD88- and Mal-deficient and double knockout mice (Yamamoto *et al.,* 2003b). TRAM has been found to act as a physical bridge between TLR4 and TRIF and functionally transmits LPS-TLR4 signalling to TRIF, which in turn activates IRF3 (Oshiumi *et al.*, 2003b).

Most TIR domain BB loop sequences have a conserved proline, which when mutated to histidine renders the protein unable to signal (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001; Poltorak *et al.*, 1998). Unlike the other TIR adapter molecules, TRAM contains a cysteine residue at this position (113) which when mutated to a histidine specifically

inhibited LPS-induced NFκB (Fitzgerald *et al.,* 2003a), confirming TRAM's TLR4-specific role. Consistent with this result, TRAM interacts with TLR4, but TRAM C113H does not. TRAM P112H had no inhibitory activity on any TLR pathway to NFκB, including the TLR4 pathway, confirming the importance of the C113 for this response. However, neither TRAM C113H nor TRAM P112H mutants induced NFκB activation.

1.5.5 SARM

SARM (SAM and ARM protein) was described in 2001 as containing sterile-α (SAM) and HEAT-Armadillo (ARM) motifs and was subsequently annotated as having a TIR domain (O'Neill *et al.*, 2003). SARM is a 690 amino acid protein with a surprisingly high degree of sequence similarity to proteins in *Drosophila melanogaster* and *Caenorhabditis elegans*. As well as a TIR domain, the protein contains two sterile-α motif (SAM) domains and an Armadillo repeat motif (ARM). The SAM domain is widespread in signalling and nuclear proteins and is thought to have a role in development. SAM proteins can homo- and hetero-oligomerise, and mediate protein-protein interactions. The Armadillo repeat is a 40 amino acid tandem repeat that mediates the interaction of β-catenin with its ligands. This domain can form structural complexes with other proteins, such as the small GTPase Ras. In terms of its TIR domain, Box 1 appears to be particularly conserved (O'Neill *et al.*, 2003).

C. elegans encodes two proteins that contain TIR domains. One of these, TIR-1, which is an orthologue of SARM, is involved in the cellular response to infection; the other, TOL-1, is not (Couillault *et al.*, 2004). These results suggest that TIR-1 is not coupled

to an upstream TIR domain protein. By extension, Couillault and colleagues propose that the orthologues of TIR-1 in other species, including SARM for which no function is known, may not be coupled to receptors. Preliminary results suggest that this is the case. Because of its TIR domain, it had been inferred that SARM would function like the other four TIR adapter proteins to activate NFκB- or IRF3-dependent reporter gene expression (O'Neill *et al.*, 2003). However, overexpression of human SARM did not activate the NFκB or ISRE reporter genes (Liberati *et al.*, 2004). These data do not preclude the possibility that SARM functions in the mammalian immune response pathway. SARM may not function in the HEK 293 cells used in these experiments because of tissue or cell-type specificity or it may require activation by an upstream signalling component before it can signal effectively. It is also possible that SARM may function in an NFκB or IRF3 independent TLR signalling pathway or that SARM may mediate TLR-independent immune responses.

1.6 NFκB

Activation of NFκB and MAPKs by TLRs leads to the induction of various genes that function in host defence, including inflammatory cytokines, chemokines, major histocompatibility complex (MHC) and co-stimulatory molecules. Mammalian TLRs also induce multiple effector molecules such as inducible nitric oxide synthase and antimicrobial peptides, which can directly destroy microbial pathogens (Thoma-Uszynski *et al.*, 2001).

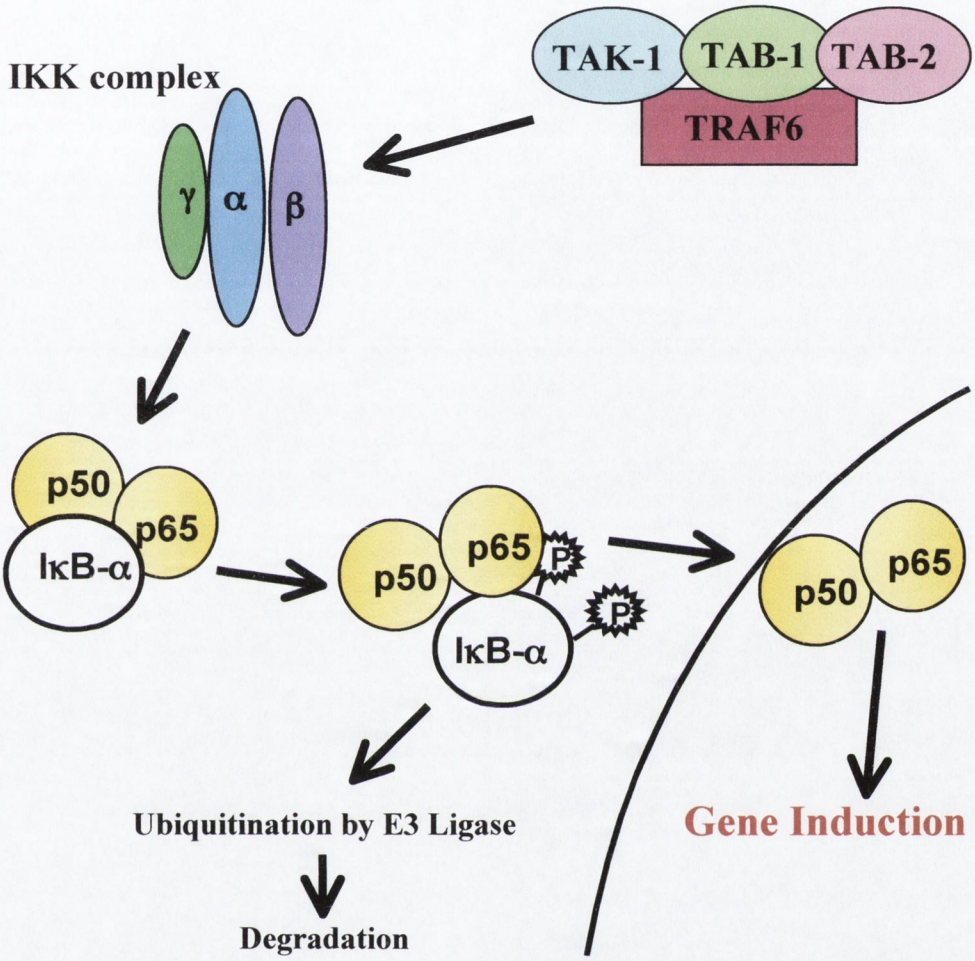
NFκB is a transcription factor originally identified by its ability to bind the promoter of the kappa light chain gene in B cells (Sen and Baltimore, 1986) but has since been shown to be present and functional in many other cell types. At present, five

mammalian members of the NFκB family have been identified; p65, c-Rel, RelB, p50/p105 and p52/p100. Each member of this family contains a highly conserved 300 amino acid long N-terminal Rel-homology domain (RHD), which acts as the dimerisation and DNA binding domain, and the nuclear localisation signal. The RHD is also the binding site for inhibitory molecules of NFκB (IκB; reviewed by May and Ghosh, 1998). Members of the NFκB family are able to form both hetero- and homodimers to produce the NFκB complex; however the term NFκB is generally used to describe p50/p65 homodimers, the predominant form of NFκB (Thanos and Maniatis, 1995). Mice lacking p50 and p65 were extremely sensitive to the toxic effects of LPS, thus highlighting the important anti-bacterial function of NFκB activation (Gadjeva et al., 2004). Most NFκB dimers are transcriptionally active; however some, like p50 and p52 homodimers, are thought to be transcriptionally inactive or repressive as they lack the transactivation domain required for transcriptional activity (Baeuerle and Henkel, 1994; Plaksin *et al.,* 1993).

1.6.1 Regulation of NFκB by IκB

NFκB is retained in an inactive state in the cytosol *via* interaction with a family of inhibitory molecules called IκBs (Figure 1.6). Each member of this family has a multiple ankyrin repeat domain that mediates interaction of the RHD of NFκB molecules, thus masking the nuclear localisation sequence of NFκB. The best characterised of the IκBs is IκB-α, which is responsible for the transitory activation of NFκB in response to cytokines (Sun *et al.,* 1993). IκB-α becomes phosphorylated on Ser32 and Ser36 upon activation of specific upstream kinases (Brown *et al.,* 1995), now known as the IκB kinases (IKKs). Phosphorylated IκB-α is then ubiquitinated at Lys21 and Lys22 which targets it for degradation by the 26S proteasome, thereby

Figure 1.6 NFκB activation *via* IKK complex and TRAF6



Upon stimulation of cells with a variety of signals such as the pro-inflammatory cytokine IL-1, the IKK complex is activated by the upstream TAK1/TAB1/TAB2 complex. As a result of this activation, the IKK complex is able to phosphorylate the inhibitory protein IκB-α, causing the release of NFκB from IκB-α. IκB-α is subsequently ubiquitinated and targeted for degradation. NFκB translocates to the nucleus where it causes induction of genes involved in the inflammatory and immune response. NFκB is shown as a p50/p65 heterodimer in this diagram.

releasing NFκB dimers from the cytoplasmic NFκB-IκB complex and allowing them to translocate to the nucleus (Figure 1.6) (Chen *et al.,* 1995). IκB-α is quickly resynthesised to allow inhibition of the pathway (Krappmann and Scheidereit, 1997). IκB-β is regulated in a similar manner although its resynthesis is delayed, leading to persistent NFκB activation in response to its degradation. Recent studies, however, have indicated that another level of NFκB regulation may occur by balancing continuous movement of NFκB complexes between nuclear and cytosolic compartments. Structural and biochemical analyses have shown that only one of the two nuclear localisation signals in an NFκB dimer is masked by IκB-α in an NFκB-IκB complex, which allows the complex to shuttle to the nucleus. At the same time, the nuclear export signal (NES) that is located at the amino-terminal of IκB-α functions to expel the NFκB-IκB complex from the nucleus (reviewed by Li and Verma, 2002).

Not all members of the IκB family are inhibitory for NFκB signalling. IκB-ξ is highly homologous to the IκB family member Bcl-3 (Kitamura *et al.*, 2000) and its transcription is rapidly induced by stimulation with TLR ligands and IL-1. IκB-ξ-deficient cells show severe impairment of IL-6 and other cytokine production in response to a variety of TLR ligands as well as IL-1, but not in response to TNF-α. Endogenous IκB-ξ specifically associates with the p50 subunit of NFκB, and is recruited to the NFκB binding site of the IL-6 promoter on stimulation (Yamamoto *et al.*, 2004).

1.6.2 The IκB Kinases

The active IκB kinase (IKK) has been identified as a complex consisting of the initial formation of IKK-α (IKK1) and IKK-β (IKK2) as a heterodimer, followed by the

addition of either a dimer or a trimer of IKK-γ (also known as NEMO, NFκB essential modulator) (Karin and Ben-Neriah, 2000). This complex is frequently referred to as the "signalosome". The different functions of IKK-α and IKK-β have been revealed by the analysis of knockout mice. Selective ablation of IKK-β prevents NFκB activation by known proinflammatory stimuli including TNF-α, IL-1 and LPS (Li *et al.*, 1999a, Tanaka *et al.*, 1999), while IKK-α knockouts show no defects in these signalling pathways (Li *et al.*, 1999b, Hu *et al.*, 1999). These data suggest that despite their close proximity within the IKK signalling complex, both kinases have very different functions.

IKK-γ acts as a regulatory subunit, stabilising the interaction between the kinase and the helix-loop-helix domains of the IKKs. This interaction is essential for kinase activity and phosphorylation of IκB resulting in activation of NFκB. No IKK or NFκB activity can be elicited in IKK-γ deficient cells that are treated with TNF-α, IL-1, LPS, or dsRNA. IKK-γ has no catalytic domain so its only known function to date is that of a scaffold protein for the other IKKs (reviewed by Karin, 1999).

Recently two homologues of IKK-α and IKK-β have been described, IKK-i (Shimada *et al.*, 1999), also called IKK-ε (Peters *et al.*, 2000), and TBK-1 (TANK-binding kinase-1), also called NAK (NFκB activating kinase; Tojima *et al.*, 2000) or T2K (TRAF2-associated kinase; Bonnard *et al.*, 2000). Both of these kinases participate in activation of NFκB-dependent transcription, but they do not appear to act as IκB kinases, but rather function further upstream in the signalling pathway (reviewed by Peters and Maniatis, 2001). Recently both of these kinases have been identified as

essential components of the IRF-3 signalling pathway and are involved in mediating the host anti-viral response to infection (Fitzgerald *et al.*, 2003b; Sharma *et al.*, 2003).

1.6.3 Activation of the IKKs

Activation of the IKK complex involves the phosphorylation of two serine residues located in the activation loop within the kinase domain of IKK-α (S176 and S180) or IKK-β (S177 and S181). The kinase responsible for the activation of IKK is a continuing matter of debate. Certain MAPK kinase kinases (MAP3K), including MEKK1 (MAPK/ERK Kinase Kinase 1), MEKK2, MEKK3 and NIK (NFκB inducing kinase) are capable of phosphorylating these serine residues *in vitro* and of activating NFκB when overexpressed (Karin and Ben-Neriah, 2000). Furthermore, dominant negative mutants of MEKK1 or NIK can inhibit NFκB activation induced by certain stimuli. However, none of these kinases has been definitely proven to be an IKK kinase *in vivo*.

Another candidate for the IKK kinase is TAK1 (TGF-β activated kinase-1), a kinase that was originally identified as a mediator of transforming growth factor-β (TGF-β) signalling and is a member of the MAPK kinase kinase (MAP3K) family of proteins (Yamaguchi *et al.*, 1995). TAK1 has been shown to be activated in an IL-1 dependant manner, causing the association of the complex with TRAF6 and activation of TAK1 kinase activity (Ninomiya-Tsuji *et al.*, 1999). TRAF6 functions as part of a unique E3 complex with Ubc13 and Uev1A, and is the target of non-classical ubiquitination (Deng *et al.*, 2000). This ubiquitination plays a regulatory role and does not lead to proteasome-mediated degradation. Once activated, TAK1 can directly phosphorylate IKK-β and MKK6, leading to the activation of both the NFκB and JNK signalling

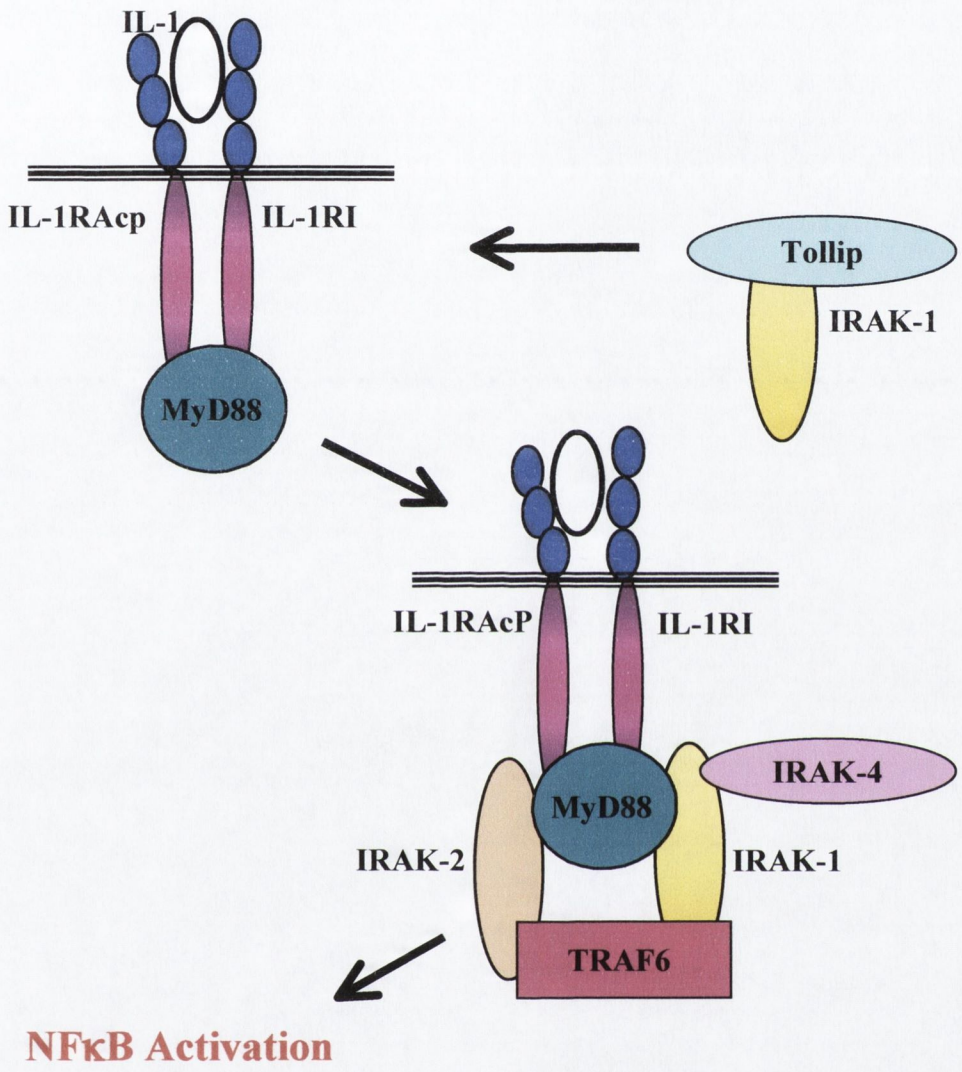
pathways (Wang *et al.*, 2001). TAK1 is also activated upon stimulation by TNF, LPS (Irie *et al.*, 2000; Lee *et al.*, 2000), poly(I:C) (Jiang *et al.*, 2003) and possibly other TLR ligands. Therefore, TAK1 is probably a common component shared by several different signalling pathways.

1.6.4 From IL-1 Receptor to IKK

Binding of IL-1 to its receptor allows recruitment of the IL-1 receptor accessory protein (IL-1RacP), which leads to the formation of a series of transitory multi-protein complexes intracellularly, resulting in the activation of IKKs and subsequently NFκB. The initial event in this process involves recruitment of the adaptor protein MyD88 to IL-1R/IL-1RAcP (Figure 1.7; Burns *et al.*, 1998; Muzio *et al.*, 1997; Wesche *et al.*, 1997) *via* TIR-TIR interactions (Burns *et al.*, 1998). The death domain (DD) of MyD88 has been shown to interact with under-phosphorylated IL-1 receptor associated kinase 1 (IRAK-1) (Wesche *et al.*, 1997).

The IRAK family of kinases are key mediators of IL-1 signalling. The first member of this family to be identified was IRAK-1, which contains a DD. IRAK-1 is recruited to the IL-1 receptor complex upon IL-1 stimulation (Cao *et al.*, 1996b). This process is thought to be mediated by a binding protein named Tollip. IRAK-1 is kept in a complex with Tollip prior to IL-1 stimulation, and recruitment of the Tollip-IRAK-1 complex to the receptor occurs through association of Tollip with IL-1RAcP (Burns *et al.*, 2000). Interestingly, Tollip has been shown to have an inhibitory role in TLR2 and TLR4 signalling (Zhang and Ghosh, 2002). MyD88 and IRAK-1 associate *via* their death domains, initiating the kinase activity of IRAK-1. The initial phosphorylation of IRAK-1 weakens its affinity for Tollip, making it more accessible for interaction with MyD88. Neither Tollip nor MyD88 are able to maintain interaction with hyper-

Figure 1.7 Recruitment of signalling molecules to the IL-1 receptor complex



IL-1 binds IL-1RI, causing recruitment of both IL-1RAcP and MyD88. MyD88 interacts with the IL-1R1/IL-1RAcP complex *via* the TIR domain. IL-1 also stimulates the release of IRAK-1 from Tollip. IRAK-1 and MyD88 interact via their death domains. IRAK-2 is also recruited to the complex. IRAK-4 causes phosphorylation and activation of IRAK-1 allowing IRAK-1 to bind TRAF6. This series of interactions ultimately results in activation of NFκB.

phosphorylated IRAK-1, thereby resulting in the release of IRAK-1 from the receptor complex. Truncated forms of IRAK-1 have been shown to inhibit IL-1R1 and IL-1RAcP-induced NFκB activation in reporter gene assays (Muzio *et al.,* 1997). Interestingly, the kinase activity of IRAK-1 has been shown not to be essential for NFκB activation, as kinase deficient mutants are still able to drive NFκB (Li, X., *et al.,* 1999). The requirement for IRAK-1 in IL-1 signalling has been demonstrated using IRAK-1 deficient mice (Swantek *et al.,* 2000), since embryonic fibroblasts from these mice fail to activate NFκB in response to treatment with IL-1 but not TNF. Also these mice show attenuated but not eradicated production of IL-6 and TNF in response to injection with IL-1β implying a level of redundancy among IRAK family members (Thomas *et al.,* 1999).

Other members of the IRAK family include IRAK-2 (Muzio *et al.,* 1997), IRAK-M (Wesche *et al.,* 1999) and the recently identified IRAK-4 (Li, S. *et al.,* 2002). IRAK-2 lacks kinase activity and there is evidence that IL-1R1 may be able to directly recruit IRAK-2 (Muzio *et al.,* 1997), while the IL-1RAcP can interact with IRAK-1. IRAK-M expression is primarily restricted to myeloid cells (Wesche *et al.,* 1999) and may be involved in negative regulation of signalling. Overexpression of IRAK-M in HEK 293 cells has been shown to prevent the formation of the IRAK-1/TRAF6 complex upon stimulation with IL-1β and TLR ligands (Kobayashi *et al.,* 2002).

The identification of IRAK-4 provided the link between the receptor complex and IRAK activation. The fact that IRAK-4 was able to phosphorylate IRAK-1 *in vitro* suggested that IRAK-4 might be the IRAK-1 kinase. However, it has recently been discovered by Qin and colleagues (2004) that the IRAK-4 kinase inactive mutant had

the same ability as wild-type IRAK-4 to restore IL-1-mediated signalling in human IRAK-4-deficient cells. They also found that the kinase activity of IRAK-4 was not necessary for IL-1 induced IRAK-1 phosphorylation, suggesting that IRAK-1 phosphorylation can probably be achieved either by auto-phosphorylation or by trans-phosphorylation through IRAK-4. In support of this, only the impairment of the kinase activity of both IRAK-4 and IRAK-1 efficiently abolished the IL-1 pathway (Qin *et al.*, 2004). However, consistent with the fact that IRAK-4 is a necessary component of the IL-1 pathway (Suzuki *et al.,* 2002; Picard *et al.*, 2003; Medvedev *et al.*, 2003), it was discovered that IRAK-4 was required for the efficient recruitment of IRAK-1 to the receptor complex (Qin *et al.*, 2004). To summarise, IRAK-4 interacts with IRAK-1 and TRAF6 in an IL-1 dependant manner and this interaction enables phosphorylation of IRAK-1 (Li, S. *et al.,* 2002).

The role of IRAK-1 is to recruit TNF receptor associated kinase 6 (TRAF6) (Figure 1.7; Cao *et al.*, 1996a). A dominant negative form of TRAF6 inhibited the stimulation of NFκB by IRAK, thus placing TRAF6 downstream of IRAK on the signalling pathway from IL-1 (Muzio *et al.*, 1998). The importance of TRAF6 in IL-1 signalling has been shown through the use of knockout cells, which are defective in NF-κB activation and JNK activity (Lomaga *et al.*, 1999). The structure of TRAF6 consists of an N-terminal ring finger and a zinc finger domain, followed by a coiled coil region and a well conserved C-terminal TRAF domain (Ishida *et al.*, 1996). The crystal structures of TRAF6 alone and in a complex with peptides from the TNF receptor family members CD40 and RANK, have allowed identification of a TRAF6 binding motif of sequence Pro-X-Glu-X-X- Ar/Ac (aromatic/acidic). This sequence is also present in IRAK-1, IRAK-2 and IRAK-M but not IRAK-4 (Ye *et al.*, 2002). The

interaction of TRAF6 with IRAK-1 through this motif results in the translocation of TRAF6 from the membrane to the cytosol where it then interacts with and activates the TAK1/TAB1/TAB2 complex (Qian *et al.*, 2001). TRAF6 must be ubiquitinated in order to activate the TAK1/TAB1 (TAK1 binding protein 1)/TAB2 complex (Wang *et al.*, 2001). TAK1 can then phosphorylate downstream targets such as the IKKs, finally culminating in NF-κB activation (Figure 1.6). Figure 1.7 shows formation of the IL-1R complex, leading to recruitment of MyD88, IRAKs and TRAF6.

1.7 Activation of MAP Kinases by IL-1

p42/p44 MAPK, also called extracellular-signal regulated kinase 1/2 (ERK1/2) is activated by diverse stimuli such as mitogens, growth factors and IL-1. Activation of p38 and c-Jun N-terminal kinase (JNK) occurs in response to stress stimuli such as pro-inflammatory cytokines IL-1 and TNF, UV-irradiation and osmotic shock. They are all activated by dual phosphorylation on the tripeptide Thr-X-Tyr motif by upstream kinases termed MAP kinase kinase (MKK). These kinases have very tightly defined specificities and their activation is highly regulated. The sequence surrounding the phosphorylation site of p42/p44, p38 or JNK defines which MKK will recognise and phosphorylate it (Cano and Mahadevan, 1995). MKK4 and 7 are upstream of JNK and target the TPY motif of JNK. MKK3 and 6 phosphorylate p38 MAP kinases at the TGY motif and MKK1 and 2 activate p42/p44 MAP kinase at the TEY motif (reviewed in (Dong *et al.*, 2002). MKKs are similarly regulated by upstream MAP3Ks, whose physiological functions and specificities remain elusive.

The proximal signalling molecules involved in TLR4-induced activation of ERK, p38 and JNK are not well defined. LPS is thought to activate ERK1/2 proteins *via* the

Ras/Raf-1/MEK pathway, JNK proteins *via* the MEKK1/MEK4 pathway and p38 proteins *via* activation of MEK3 (Guha *et al.*, 2001; Delgado *et al.*, 2000; Swantek *et al.*, 1997). The mechanisms of activation of MAPKs by other TLRS are unclear. However, it has been demonstrated that the TIR adapter molecules MyD88 and TRIF, *via* their associations with TRAF6, are involved in TLR-induced activation of the MAP kinases. TRAF6 induces activation of TAK1 and MKK6 leading, in turn, to the activation of NFκB, JNK and p38 (Wang *et al.*, 2001).

1.8 Type I Interferon Induction

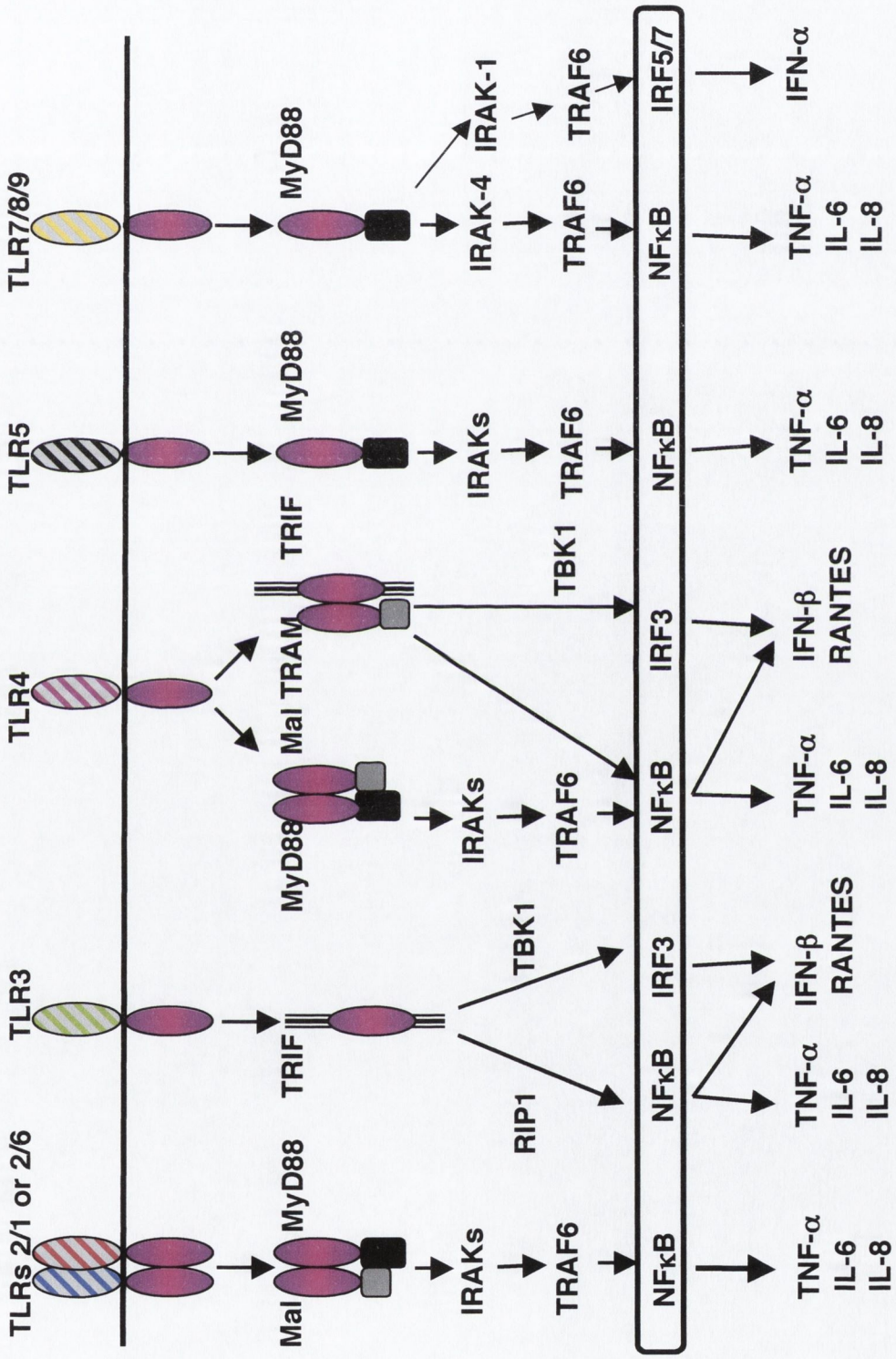
In unstimulated cells, IRF3 is present in the cytoplasm. Viral infection results in the phosphorylation of the C-terminus of IRF3, leading to its dimerisation and interaction with the co-activators CREB-binding protein (CBP) and p300. The IRF3 complex then translocates to the nucleus, where it activates promoters containing IRF3-binding sites. IRF3 transcriptionally activates the promoters for the IFN-β, interferon-stimulated genes 15 and 56 (ISG56 and ISG15), IP10 and RANTES genes (Grandvaux *et al.*, 2002).

Recombinant IKK-ε and TBK-1 directly phosphorylate IRF3 on C-terminal residues known to be required for its activation (McWhirter *et al.*, 2004). LPS-induced expression of IFN-β, but not proinflammatory cytokines, was markedly reduced in *TBK1*$^{-/-}$ cells. Similar defects were also observed in poly(I:C)-stimulated and virus-infected *TBK1*$^{-/-}$ cells but *IKK-ε*$^{-/-}$ cells showed normal IFN-β induction. However, LPS and poly(I:C)-induced upregulation of IFN-β and IFN-inducible genes were completely abolished in *TBK1*$^{-/-}$*IKK-ε*$^{-/-}$ cells, showing that IKK-ε is critically involved in these responses (Hemmi *et al.*, 2004).

Figure 1.8 Differences in TLR signalling pathways arise at the level of the TIR adapter molecules

The discovery of the MyD88-independent pathway led researchers to characterize the signalling pathways of the various TLRs, the activation of which lead to different patterns of gene expression. The molecular mechanisms underlying such differences can now be explained, at least in part, by the existence of several TIR adaptor molecules which are used by different TLRs. MyD88 mediates the TLR-signalling pathway that activates IRAKs and TRAF6 and leads to the activation of the IKK complex. This pathway is used by TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 and releases NFκB from its inhibitor so that it translocates to the nucleus and induces expression of inflammatory cytokines. Mal is involved in the MyD88-dependent signalling pathway through TLR2 and TLR4. By contrast, TLR3- and TLR4-mediated activation of IRF3 and the induction of type I interferon are observed in a MyD88-independent manner. TRIF is essential for this pathway. The non-typical IKKs, IKK-α and TBK1, mediate activation of IRF3 downstream of TRIF. TRAM is specific to the TLR4-mediated, MyD88-independent/TRIF-dependent pathway.

Figure 1.8 Differences in TLR signalling pathways arise at the level of the TIR adapter molecules



Apart from pDCs (Prakash *et al.*, 2005), IRF7 is not expressed constitutively in cells; rather expression is induced by type I interferon, LPS, and virus infection (Lin *et al.*, 2000; Sato *et al.*, 2000). As with IRF3, virus infection or stimulation with type I interferon appears to induce the phosphorylation of IRF7 at its carboxy terminus, a region that is highly homologous to the IRF3 C-terminal end. IRF7 localises to the cytoplasm in uninfected cells and translocates to the nucleus after phosphorylation (reviewed by Lin *et al.*, 2000). pDCs constitutively express IRF7 and rapidly produce IFN-α in response to viral infection, TLR7 and TLR8 ligands (ssRNA and imidazoquinolines) and the TLR9 ligand (CpG DNA) (Hemmi *et al.*, 2003; Kerkmann *et al.*, 2003). IFN-α induction by these TLR ligands was abolished in MyD88-deficient cells, demonstrating that TLR7, TLR8 and TLR9 were likely to have a unique mechanism that activates the genes encoding IFN-α in pDCs using MyD88. It was recently discovered that IRF7 and MyD88 co-localise in the cytoplasm and that IRF7 translocates from the cytoplasm to the nucleus in response to TLR9 ligand in a MyD88-dependent way (Kawai *et al.*, 2004; Honda *et al.*, 2004).

Previous studies have shown that both IRF3 and IRF7 are required for efficient type I interferon production (reviewed in (Servant *et al.*, 2002; Zhang and Pagano, 2002)). However, their roles are different in these processes. In the early phase of viral infection, pre-existing IRF3 is activated and induces expression of IFN-β and IFN-α4. These early produced interferons transcriptionally induce IRF7; and upon viral infection, the induced high level of IRF7 is activated by phosphorylation. Activated IRF7 then trans-activates multiple IFN genes, leading to robust production of type I interferons in response to viral infection (Servant *et al.*, 2002; Zhang and Pagano, 2002). Thus, IRF3 stimulates the expression of certain interferons early in infection

and IRF7 then amplifies the expression of other interferon genes not induced during the early stage.

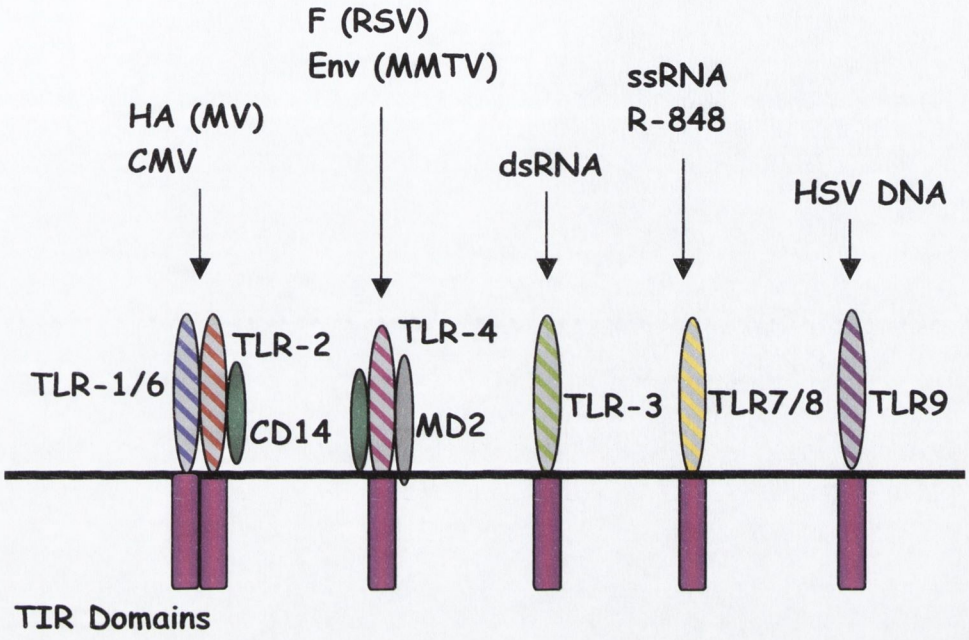
In addition to the spatial difference in their functions in virus-induced type I interferon production, IRF3 and IRF7 recognise different DNA binding sites or have distinct affinities for the same DNA site. As mentioned above, the active form of IRF3 (but not IRF7) is associated with some co-activators (Servant *et al.*, 2002). These different properties between IRF3 and IRF7 lead to differential gene activation by these two transcription factors.

1.9 TLRs and Viruses

TLRs play an important role in detecting viruses (Figure 1.9) and initiating anti-viral responses (Figure 1.10). Highlighting their importance in anti-viral immunity, a number of viral immune strategies employed against TLRs have been identified. VV encodes a number of proteins which interfere in TLR signalling and these will be discussed further in section 1.11.

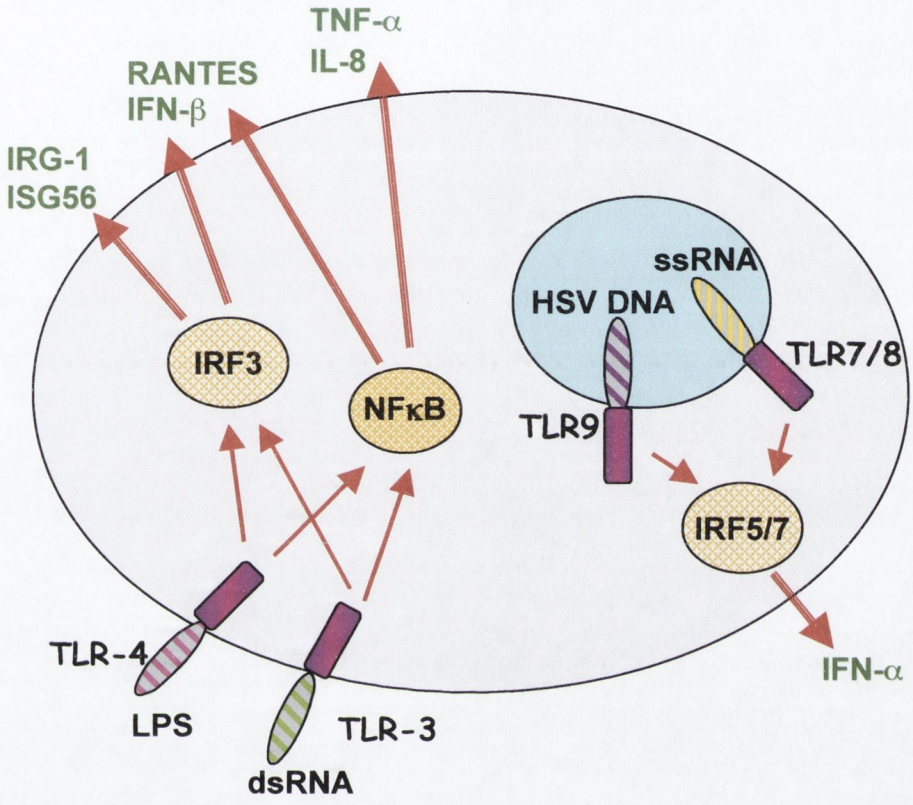
Accumulating evidence shows that TLRs can respond to viral proteins and nucleic acids. Cell-surface TLR2 and TLR4 may recognise viral glycoproteins on virions (Vaidya *et al.*, 2003; Rassa and Ross, 2003). For example, measles virus haemaglutinin activates murine and human cells *via* TLR2 (Bieback *et al.*, 2002). TLR4/MD-2 responds to viral products, including the fusion (F) protein of respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000) and the envelope proteins of the murine mouse mammary tumour virus (MMTV) and Moloney murine leukaemia virus (Rassa *et al.*, 2002). In terms of viral nucleic acids, TLR3 has been shown to be activated in

Figure 1.9 TLRs 'recognise' viral PAMPs



TLRs respond to specific viral PAMPs. TLR2 can dimerise with either TLR1 or 6 in order to respond to a variety of activators, including human cytomegalovirus (CMV) or the HA protein from measles virus (MV). TLR4 recognises fusion (F) protein from respiratory syncytial virus (RSV) and envelope protein from the retrovirus mouse mammary tumour virus (MMTV). dsRNA is a molecular pattern produced by most viruses at some point in their life cycle. TLR3 is activated by the dsRNA analogue, poly(I:C). TLR7 responds to ssRNA from a variety of sources, and also to small anti-viral compounds such as R-848. TLR9 is activated by viral DNA containing immunostimulatory CpG motifs.

Figure 1.10 TLRs trigger anti-viral signalling pathways



A number of TLRs have been associated with virus recognition, and subsequent induction of anti-viral immunity. Stimulation of TLRs 3, 4, 7 and 9 results in activation of NFκB and IRFs 3, 5 and 7, leading to induction of type I interferon and the production of interferon stimulated genes such as IRG-1, ISG56 and RANTES and proinflammatory cytokines such as TNF-α and IL-8.

response to poly(I:C), a synthetic analogue of viral dsRNA (Alexopoulou *et al.*, 2001), while TLR7 and TLR8 have been shown to recognise ssRNA (from influenza, HIV and vesicular stomatitis virus) (Lund *et al.*, 2004; Heil *et al.*, 2004; Diebold *et al.*, 2004) and TLR9 responds to dsDNA from herpes simplex virus (Lund *et al.*, 2003; Krug *et al.*, 2004).

That TLR signalling can induce an anti-viral state was clearly shown by Doyle *et al.* (2002). Pre-treatment of cells with poly(I:C) or lipid A, the moiety of LPS recognised by TLR4, inhibited the replication of a murine herpesvirus in macrophages. Even before the discovery of TLRs, it was well-known that viral replication and viral pathogenesis often involves NFκB activation (Histcott *et al.*, 2001). The anti-viral transcription factor, IRF3, is activated by TLR3 and TLR4 in a MyD88-independent fashion. Together with NFκB activation, IRF3 activation leads to IFN-β induction, which initiates the interferon-based anti-viral response.

In addition to production of inflammatory cytokines, ligand stimulation of TLR7, TLR8, and TLR9 can induce anti-viral IFN-α in pDCs (Hemmi *et al.*, 2000; Hemmi *et al.*, 2002; Hemmi *et al.*, 2003; Ito *et al.*, 2002), a subset of DCs is known for their ability to produce a large amount of type I interferons upon viral infection (Asselin-Paturel *et al.*, 2001; Nakano *et al.*, 2001; Martin *et al.*, 2002). Recent studies showed that TLR7- and TLR9-mediated IFN-α induction requires the formation of a complex consisting of MyD88, TRAF6, and IRF7 (Kawai *et al.*, 2004) and also IRAK-1 (Uematsu *et al.*, 2005).

TLRs cooperate in the induction of anti-viral immunity. Both the TLR9 and TLR3 signalling pathways are activated *in vivo* on inoculation with mouse cytomegalovirus and each pathway contributes to innate defence against systemic viral infection by leading to type I interferon production. However, neither pathway offers full protection against mouse cytomegalovirus infection in the absence of the other (Tabeta *et al.*, 2004).

TRIF-dependent signalling plays an important role in the anti-viral immune system and its presence is significant in containing viral replication. The response of macrophage cultures to infection by VV was demonstrably impaired when the *Trif* gene was disrupted, in that macrophage monolayers from *Lps2* homozygotes supported the replication of *Vaccinia* to a higher titre than did macrophages from normal mice (Hoebe *et al.*, 2003). Hoebe and colleagues (2003) also demonstrated that when infected with mCMV, robust production of type I interferon was observed in the serum of normal mice, but no type I interferon was detected in the serum of *Lps2* mutant homozygotes. Hence, the antiviral effect conferred by TRIF is very broad with regard to the virus species and is correlated with type I interferon production.

Highlighting the importance of TRIF in anti-viral immunity is the fact that it is a target of the hepatitis C virus (HCV) immunomodulatory protein, NS3/4A. The NS3/4A serine protease causes specific proteolysis of TRIF, reducing its abundance and inhibiting poly(I:C)-activated signalling through the TLR3 pathway before its bifurcation to IRF3 and NFκB. This represents a uniquely broad mechanism of viral immune evasion which potentially limits the expression of multiple host defence genes,

thereby promoting persistent infections with this medically important virus (Li *et al.*, 2005).

Although viral activation of TLRs can result in an anti-viral response, for some viruses this interaction creates a cellular environment more beneficial for virus replication. Activation of TLR2 by measles virus (MV) not only resulted in the expression of inflammatory cytokines, but also in increased expression of the MV entry receptor CD150, thereby allowing infection of target cells. These data suggest that viral activation of TLR2 could result in increased infection and viral spread (Murabayashi *et al.*, 2002). Also, herpes simplex virus 1 (HSV-1) induced a blunted cytokine response in $TLR2^{-/-}$ mice with reduced levels of proinflammatory cytokines in the brain and reduced mortality compared with wild-type mice being observed (Kurt-Jones *et al.*, 2004). These differences could not be accounted for on the basis of virus levels. Thus, these studies suggest that the TLR2-mediated cytokine response is detrimental for the host during infection with HSV-1 (Kurt-Jones *et al.*, 2004).

TLR3 has been implicated in the enhancement of inflammatory responses in the brain. When $TLR3^{-/-}$ mice were infected with West Nile virus (WNV), viral load, inflammatory responses and neuropathology in the brain were reduced compared to wild-type mice. Also, $TLR3^{-/-}$ mice were more resistant to lethal WNV infection. However, because $TLR3^{-/-}$ mice had impaired cytokine production and enhanced viral load in the periphery, a role for TLR3 in facilitating WNV entry into the brain, thus causing lethal encephalitis, has been proposed (Wang et al., 2004).

Similar to MV, mouse mammary tumour virus (MMTV) binding to DCs upregulates expression of its entry receptor, transferrin receptor I (Burzyn *et al.*, 2004). An observed link between resistance to MMTV-induced tumourigenesis and a mutant TLR4 allele led to the hypothesis that MMTV might utilise this receptor to activate its initial cellular targets and thereby achieve infection. MMTV can activate B cells and NFκB in C3H/NeN mice, but cannot do so in C3H/HeJ mice which have a defective TLR4 (Rassa *et al.*, 2002). Recently, Jude and co-workers (2003) observed that MMTV-mediated activation of the TLR4 pathway might also subvert the anti-viral adaptive immune response. In C3H/NeN mice, MMTV-mediated activation of B cells led to production of IL-10 (Jude *et al.*, 2003), a cytokine known to repress induction of TH1-type cellular immune responses (Moore *et al.*, 2001). In contrast, IL-10 induction did not occur in C3H/HeJ mice, suggesting that mice of this strain could produce a stronger TH1 response against MMTV (Jude *et al.*, 2003).

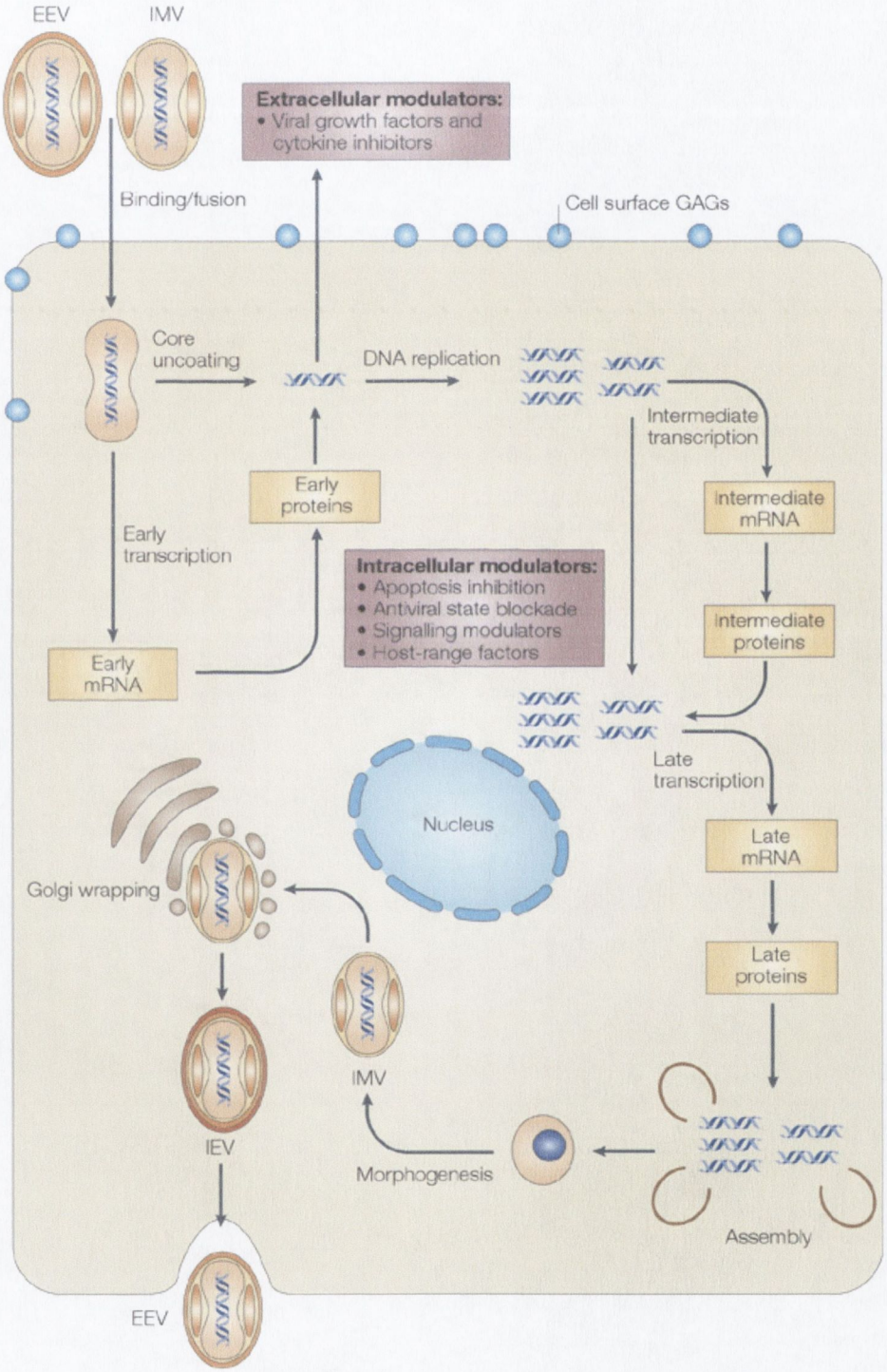
1.10 The *Poxviridae*

The *Poxviridae* comprise a large family of highly complex DNA viruses that replicate in the cytoplasm of both vertebrate cells (*Chordopoxvirinae*) and invertebrate cells (*Entomopoxvirinae*). An overview of the poxvirus replication cycle is shown in Figure 1.11. The replication cycle is divided into early, intermediate and late phases, with each class of gene being transcribed in a strictly regulated temporal cascade that is dependent upon prior expression of genes from the previous class. Early genes are expressed before DNA replication and encode immunomodulators and genes that are needed for DNA replication. In contrast, the intermediate and delayed classes of genes are expressed only after DNA replication has started. Some intermediate genes encode

Figure 1.11 An overview of the poxvirus replication cycle (taken from McFadden, 2005)

All poxviruses replicate in the cytoplasm of infected cells by a complex, but largely conserved, morphogenic pathway. Two distinct infectious virus particles — the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) — can initiate infection. The IMV and EEV virions differ in their surface glycoproteins and in the number of wrapping membranes. The binding of the virion is determined by several virion proteins and by glycosaminoglycans (GAGs) on the surface of the target cell or by components of the extracellular matrix. Fully permissive viral replication is characterized by three 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 (not shown in the figure) 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 (CEV; not shown), which is either extruded away from the cell by actin-tail polymerization (not shown) or is released to form free EEV. EEV might also form by direct budding of IMV, therefore bypassing the IEV form. Poxviruses also express a range of extracellular and intracellular modulators, some of which are defined as host-range factors that are required to complete the viral replication cycle. Poxviruses can be markedly diverse in their portfolio of specific modulators and host-range factors, which determine tropism and host range. Non-permissive poxvirus infections generally abort at a point downstream of the binding/fusion step.

Figure 1.11 An overview of the poxvirus replication cycle (taken from McFadden, 2005)



late transcription factors, and late genes encode other immunomodulators and proteins that form the new virus particles (reviewed by Moss, 1996).

The most notorious member of the *Chordopoxvirinae*, variola virus, is a member of the *Orthopoxvirus* genus and is the causative agent of smallpox, a disease which was very important historically (reviewed by (Esposito and Fenner, 2001). Smallpox was once the most serious disease of mankind, and caused vast epidemics in human populations. The history of the world has been changed by the early death of rulers from smallpox or from the appearance of smallpox in armies. Five reigning European monarchs died from smallpox during the 18th century and an invasion of Canada by American troops during the Revolutionary War failed when an epidemic of smallpox swept through the American troops (Hopkins, 1983).

Vaccinia virus (VV), also a member of the *Orthopoxvirus* genus, is the vaccine that was used to eradicate smallpox. In 1796 Edward Jenner used a virus taken from the hand of a milkmaid (probably cowpox virus as she had acquired the infection from a cow) to introduce vaccination to the world. In 1939 Alan Downie showed that the smallpox vaccines being used in the 20th century were distinct from cowpox virus and henceforth they became known as vaccinia virus (after vacca, Latin for cow). VV is the most intensively studied of the poxviruses. Despite this, its origin and natural host remain unknown (reviewed by (Esposito and Fenner, 2001)).

1.10.1 Classification of Poxviruses

The general properties of *Poxviridae* include a large complex virion containing enzymes that synthesise mRNA, a genome composed of a single linear double-stranded

DNA molecule of 130-300 kilobase pairs (kbp) with a hairpin loop at each end, and a cytoplasmic site of replication. As stated previously, the *Poxviridae* are divided into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae*, based on vertebrate and insect host range. The *Chordopoxvirinae* consist of eight genera (TABLE 1.3). Members of a genus are genetically and antigenically related and have a similar morphology and host range. The *Entomopoxvirinae* have been divided into three genera based on the insect host of isolation (Moss, 1996).

TABLE 1.3: Family Poxviridae (Moss, 1996)

Subfamilies	Genera	Member Viruses	Features
Chordopoxvirinae	*Orthopoxvirus*	Camelpox, cowpox, ectromelia, monkeypox, racoonpox, skunkpox, taterapox, Uasin Gishu, vaccina, variola, volepox	Brick-shaped virion, ~200 kbp, wide to narrow host range
	Parapoxvirus	Auzduk disease, chamois contagious ecthyma, orf, pseudocowpox, parapox of deer, seal pox	Ovoid virion, ~140 kbp, infects mainly ungulates
	Avipoxvirus	Canarypox, fowlpox, juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, peacockpox, penguinpox, sparrowpox,starlingpox, turkeypox	Brick-shaped virion, ~260 kbp, infects birds, arthropod transmission
	Capripoxvirus	Goatpox, lumpy skin disease, sheeppox	Brick-shaped virion, ~150 kbp, ungulates, arthropod transmission
	Leporipoxvirus	Hare fibroma, myxoma, rabbit fibroma, squirrel fibroma	Brick-shaped virion, ~160 kbp, infects leoprids and squirrels, arthropod transmission causes localised tumours,
	Suipoxvirus	Swine pox	Brick-shaped genome, ~170 kbp, narrow host range
	Molluscipoxvirus	*Molluscum contagiosum*	Brick-shaped virion, ~180 kbp, exclusively human host, causes localised tumours in epidermis, spread by contact
	Yatapoxvirus	Tanapox, Yaba monkey tumour	Brick-shaped virion, ~145 kbp, infects primates and rodents
Entomopoxvirinae	*Entomopoxvirus* A	*Melolontha melolontha*	Ovoid virions, ~260-370 kbp, infects Coeloptera
	Entomopoxvirus B	*Amsacta moorei*	Ovoid virions, ~225 kbp, infects Lepidoptera and Orthoptera
	Entomopoxvirus C	*Chirionimus luridus*	Brick-shaped virions, 250-380 kbp, infects Diptera

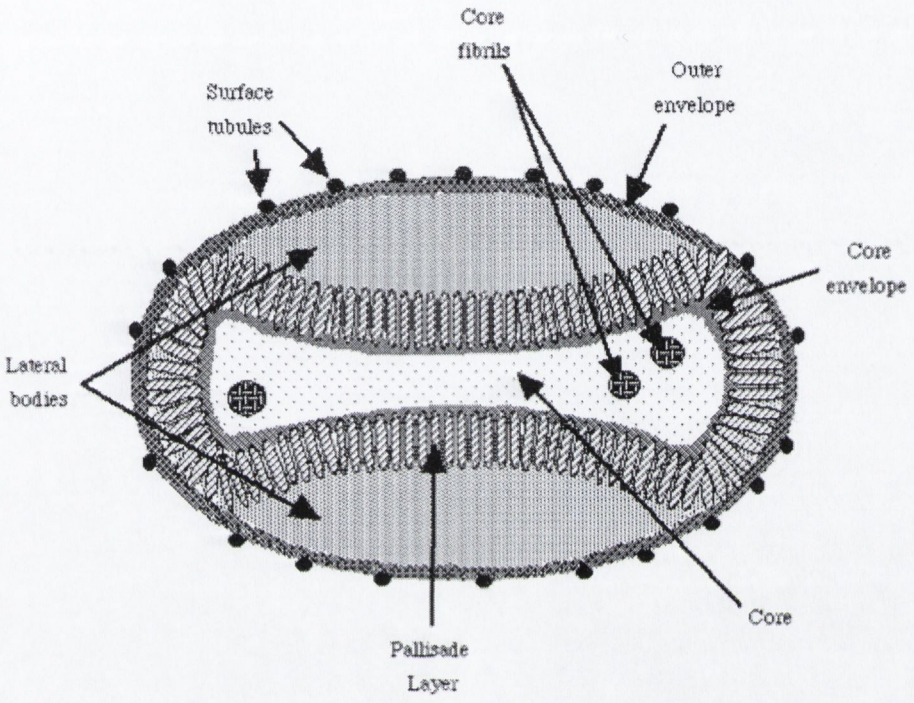
1.10.2 Structure of the Poxvirus Virion

Poxvirus virions are larger than those of other animal viruses and are just discernable by light microscopy. Vaccina virus, which serves as a model for the family, is approximately 350 by 270 nm. Two forms of infectious vaccinia virions are known, an intracellular form and an extracellular form (Figure 1.12). The intracellular form has a lipid-containing surface membrane and can be released by disruption of the infected cell. The extracellular form has a second, lipid-containing envelope around it, which contains glycoproteins that are not present in the intracellular form. Within the virion are a core, which in mammalian viruses appears dumbbell-shaped, and one or two flanking lateral bodies. The core contains the viral DNA complexed with protein. The lateral bodies are proteinaceous. Altogether, the virion contains more than 30 structural proteins (Strauss and Strauss, 2002).

1.10.3 Poxvirus Genome

Poxviruses have linear, double-stranded DNA genomes that vary from about 130kbp in parapoxviruses to about 300kbp in avipoxviruses. Inverted terminal repeats (ITRs), which are identical but oppositely oriented sequences at the two ends of the genome have been identified in all poxviruses thus far examined. The length of the ITRs is, however, variable even within a genus. The two strands of vaccinia DNA are connected by hairpin loops to form a covalently continuous polynucleotide chain. The loops are A+T rich, cannot form a completely base-paired structure, and exist in two forms that are inverted and complementary in sequence. Similar telomeric structures have been found in variola, cowpox and Shope fibroma viruses and are undoubtedly characteristic of the entire family (Moss, 1996).

Fig. 1.12 Structure of the Poxvirus Virion



Poxvirus virions are oval or "brick-shaped" particles, 200-400 nm long, which can be visualised by light microscopy. External forms contain two membranes, while intracellular particles only have one. The external surface is ridged in parallel rows of tubules, which are sometimes arranged helically. Thin sections in electron microscopy reveal that the outer surface is composed of lipid and protein which surrounds the core. The core, composed of tightly compressed nucleoprotein, is dumbbell-shaped and is surrounded by two "lateral bodies" whose function is unknown. (http//www-micro.msb.le.ac.uk/3035/poxviruses.html)

There is no obvious distribution of genes in the genome with respect to function. However, genes often seem to be arranged in a fashion that would minimise transcriptional interference. Specifically, an early transcriptional termination signal for a given gene may often be embedded in an adjacent late gene where it will be ignored, thus economising on genome usage. Also, genes seem to be arranged in a fashion that minimises divergent and convergent transcription. This minimises the generation of double-stranded RNA (dsRNA) from contiguous transcripts, since this is a PAMP recognised by PRRs, as discussed earlier.

One generalisation about poxviral genomes is that the central regions are 'essential' for growth in the reductionist infectivity model of cell culture. This is because this region generally codes for housekeeping genes and is highly similar between strains and families. The terminal regions, on the other hand, are nonessential for growth in cell culture and indeed, the virus can tolerate large deletions from the termini without any phenotype when grown in some cell lines. It is in these regions that ORFs with high identity to host sequences are located, which are unmistakeably acquired from the host organism.

1.10.4 Bioterrorism and Smallpox

As a concept, bioterrorism is not new. The alleged catapulting of plague-ridden corpses over enemy walls in the 1346 siege of Caffa (the modern Crimean port of Feodosia, Ukraine) and the dispatch of smallpox-impregnated blankets to Indians by British officers to Indians in the Seven Years War (1754-1763) have frequently been cited as examples of bioterrorism (Hopkins, 1983).

The last case of endemic smallpox occurred in Somalia on 26th October 1977, and eradication of the disease was declared in 1980, nearly two centuries after the introduction of prophylactic inoculations with cowpox and vaccinia virus. With no natural reservoir, variola virus has existed only in laboratories; indeed the last case of smallpox was due to infection acquired in a laboratory in the United Kingdom in 1978. By 1994, only the Centres for Disease Control and Prevention (CDC), in Atlanta, and the Vektor Institute, in Novosibirsk, Russia, retained variola virus isolates (reviewed in (Breman, 2002)). However, there is concern that variola virus resides outside these laboratories and could be used as a bioweapon by terrorists. Possible sources are virus in countries that claim they destroyed their stocks but did not, and virus obtained from laboratories in the former Soviet Union. Variola is considered as an ideal bioterrorist weapon because of its highly transmissible nature and the fact that the civilian populations of most countries contain a high number of susceptible persons, (reviewed by Mayr, 2003).

Before the 2001 anthrax attacks (Jernigan *et al.*, 2002), the US scientific community had for several years been bolstering its biodefence research capacity. The anthrax attacks greatly accelerated this expansion as part of a national defence plan, which includes efforts to provide a knowledge base for the development of effective countermeasures against agents of bioterror, such as diagnostics, therapeutics and vaccines, and to translate this knowledge into the production and delivery of such measures (Fauci *et al.*, 2003). Possible bioterror agents such as anthrax, smallpox, plague, tularaemia, viral haemorrhagic fevers and *Clostridium botulinum* toxin are given top priority because they are highly lethal and readily deployed as weapons.

1.11 Vaccinia Virus Infection and Host Response

The common host response mounted against poxvirus infection covers all arms of the immune system. Early in the immune response, the innate immune system is called into battle. Interferon, complement, natural killer cells and inflammatory cells act rapidly try to keep the poxvirus under control until a more powerful acquired immune response is mounted. Humoral and cell-mediated immunity is very important for virus clearance and the prevention of reinfection. Poxvirus specific antibodies play many roles, including virus neutralisation, opsonisation and antibody-dependent cell-mediated cytotoxicity. The cell mediated immune response, poxvirus specific CTLs, is thought to play the most important role in controlling poxvirus infection (reviewed by Smith and Kotwal, 2002).

The mouse model has been used extensively to examine VV infection and pathogenesis. Respiratory challenge of BALB/c mice with VV led to early virus replication in the lung and upper respiratory tract followed by dissemination of virus to other visceral organs and to the brain. Leukocytes, predominantly macrophages and T lymphocytes, and a number of chemokines and inflammatory mediators were detected in the lungs of infected mice. In contrast, intradermal inoculation was followed by highly localised viral replication and by an influx of neutrophils and T lymphocytes into the dermis. These findings indicated marked differences in disease pathogenesis and immunopathology following VV inoculation by different routes (Reading and Smith, 2003a).

VV confers long-term immunity to variola virus infection by cross-reacting antibodies and CTLs. VV is also used for the expression of foreign genes used to vaccinate

against other disease-causing agents, such as rabies (reviewed by Smith and Kotwal, 2002). Although most human infections, or immunisations, result in very mild, self-limiting local infection, occasionally dissemination occurrs, and the development of a very serious and sometimes fatal neurological side-effect ensues, known as post-vaccinial encephalitis (Goldstein *et al.*, 1975). In most cases however, VV infections remain localised to the infection site and usually ends in complete recovery (reviewed by Smith and Kotwal, 2002).

1.12 Vaccinia Virus Immunoevasion

To circumvent the host immune response, the VV genome contains numerous genes encoding proteins involved in immunomodulation and immunoevasion. In fact, a VV-encoded secreted polypeptide structurally related to complement control proteins was the first description of a soluble viral factor that influences the immune response (Kotwal and Moss, 1988). Other VV proteins involved in immunoevasion include anti-apoptotic proteins, secreted decoy receptors and binding proteins for IL-1, IL-18, TNF, chemokines, IFN-α/β and IFN-γ. VV immunomodulatory proteins can also inhibit serine proteases, intracellular IFN effector pathways and TLR signal transduction (reviewed by Alcami and Koszinowski, 2000). The presence of genes encoding these proteins in poxvirus genomes illustrates the co-evolution of viruses and their hosts; indeed the mechanisms used by viruses to interfere with the normal functioning of the host immune system can be informative about the function of our immune system

1.12.1 E3L and K3L

Protein kinase R (PKR) is a ribosome associated dsRNA-triggered protein kinase that has been attributed an important role in the intracellular inhibition of viral protein

synthesis as well as in the control of cell proliferation. The amino terminal region of PKR contains two dsRNA-binding domain and the kinase domain is located in the C-terminal region of the protein. PKR bound to dsRNA undergoes an autophosphorylation event and subsequently phosphorylates various substrates including the α subunit of eukaryotic translation initiation factor eIF-2. This leads to the interference of eIF-2B-mediated exchange of GTP for GDP necessary for the catalytic function of eIF-2. This ultimately leads to the inhibition of protein synthesis at the level of initiation (reviewed by Langland *et al.*, 2002).

The products of two VV genes, *K3L* and *E3L*, cause the IFN-resistant phenotype of VV by interference with the activity of PKR. The two inhibitors act in different ways, but E3L inhibits PKR activity approximately 50- to 100-fold more efficiently than K3L (Davies *et al.*, 1993). E3L is a dsRNA binding protein and interferes with the binding of PKR to dsRNA (Sharp *et al.*, 1998). E3L also blocks IRF3 activation and prevents upregulation of the host cell IFN-β gene (Smith *et al.*, 2001). K3L competes with eIF-2α for its interaction with PKR (Davies *et al.*, 1993), reducing the level of phosphorylated eIF-2α in VV infected cells (Beattie et al., 1995). Deletion of E3L and K3L in VV renders the virus sensitive to IFN and severely limits host range and disease progression (Beattie *et al.*, 1995). To date, E3L and K3L orthologues have been identified in myxoma virus, Yaba-like disease virus, variola virus, Shope fibroma virus, swinepox virus and orf virus. The fact that poxviruses not only reduce the amount of free interferon ligand using secreted and membrane bound interferon receptors, but furthermore have developed two independent ways to modulate the intracellular interferon signal transduction pathway underlines the importance of the type I interferons in anti-viral immunity (reviewed by Seet *et al.*, 2003). Because PKR functions as a tumour suppressor gene by induction of apoptosis, E3L and K3L can be

categorised as anti-apoptotic proteins. However, E3L has been found to more important for prevention of apoptosis during infection (reviewed by Seet *et al.*, 2003).

1.12.2 A52R

The VV protein, A52R, which has no obvious similarity to host proteins, can block the activation of NFκB by multiple TLRs, in particular TLR3 (Bowie *et al.*, 2000, Harte *et al.*, 2003). IL-1, but not TNF-α, mediated NFκB activation was inhibited by A52R (Bowie *et al.*, 2000). A52R associates with both TRAF6 and IRAK-2, and disrupts signalling complexes containing these proteins. A vaccinia virus deletion mutant lacking the *A52R* gene was attenuated compared with wild-type and revertant controls in a murine intranasal model of infection (Harte *et al.*, 2003). Maloney and co-workers have recently discovered that A52R does not inhibit TLR-induced p38 or JNK MAPK activation. Rather, A52R can drive activation of these kinases, with the A52R-TRAF6 interaction being critical for these effects (manuscript in preparation). Also, LPS-induced IL-10 production, known to be p38-dependent (Ma *et al.*, 2000), was potentiated by A52R. IL-10 is a cytokine known to repress induction of T_H1-type cellular immune responses (Moore *et al.*, 2001), and induction of IL-10 by VV could represent a strategy to subvert the anti-viral adaptive immune response.

Just recently, a peptide derived from A52R that effectively inhibits cytokine secretion in response to TLR activation was identified. The peptide had no effect on cytokine secretion resulting from cell activation that was initiated independent of TLR stimulation. Crucially, the peptide was capable of dramatically reducing a significant inflammatory response in the middle ears of BALB/c which was induced by the administration of heat-inactivated *Streptococcus pneumoniae*. Thus, this peptide may

have application in the treatment of chronic inflammation initiated by bacterial or viral infections (McCoy *et al.*, 2005).

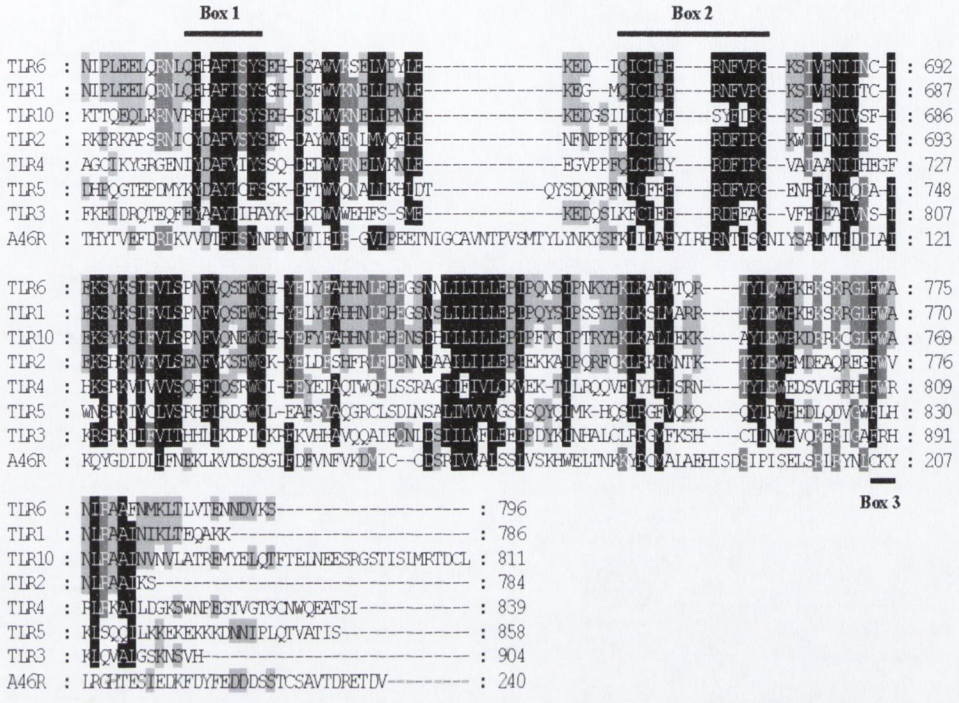
1.12.3 N1L

The published sequence of the VV WR *N1L* gene predicts a protein of 117 amino acids with a size of 13.8 kDa (Kotwal and Moss, 1988). The N1L protein is highly conserved in variola major virus (VAR) Bangledash-1975 (Massung *et al.*, 1994) and cowpox virus (CPV) GRI-90 (Shchelkunov *et al.*, 1998). While the *N1L* gene is not essential for replication in tissue culture, a mutant virus with the *N1L* gene disrupted was highly attenuated *in vivo* (Kotwal *et al.*, 1988). This work was confirmed using a revertant virus (Bartlett *et al.*, 2002). Because of N1L's sequence similarity to A52R, it was hypothesised by DiPerna *et al.* (2004) that N1L inhibited signalling to NFκB. N1L was found to inhibit signalling to NFκB by multiple signals including lymphotoxins, IL-1β, TNF-α, TLR2, TLR3 and TLR4. N1L also inhibits TRAF2-, TRAF6-, Mal-, MyD88-, TRAM- and TRIF-mediated NFκB activation, as is NFκB signalling by TBK-1, IKK-α, IKK-β and IKK-ε. N1L has been found to inhibit NFκB activation by interacting with components of the IKK complex, namely TBK-1, IKK-α, IKK-β and IKK-ε. N1L functions, at least in part, by associating with and inhibiting, the IKK complex. (DiPerna *et al.*, 2004).

1.13 Discovery of A46R

With the intention of finding previously unidentified members of the IL-1/TLR family, and using PROFILESEARCH, a VV ORF A46R that was related to the family at a statistically significant level was identified (Bowie *et al.*, 2000). Figure 1.13 shows an alignment of A46R with the TIR domain from a number of TLRs. TLR7, 8 and 9 are

Figure 1.13 Alignment of A46R from vaccinia virus with TLR TIR domains



Using PROFILESEARCH to search sequence databases for novel proteins containing TIR domains, the vaccinia virus protein A46R was identified as containing a putative TIR domain. Alignment studies show a high degree of similarity between A46R and the TIR domains of various TLRs. A solid bar indicates the three conserved regions of the TIR domain, boxes 1, 2 and 3, thought to be important in signalling. The region known as Box 1 is particularly strong in A46R, with the sequence DTFISY being as closely related to the Box 1 consensus as other proven family members.

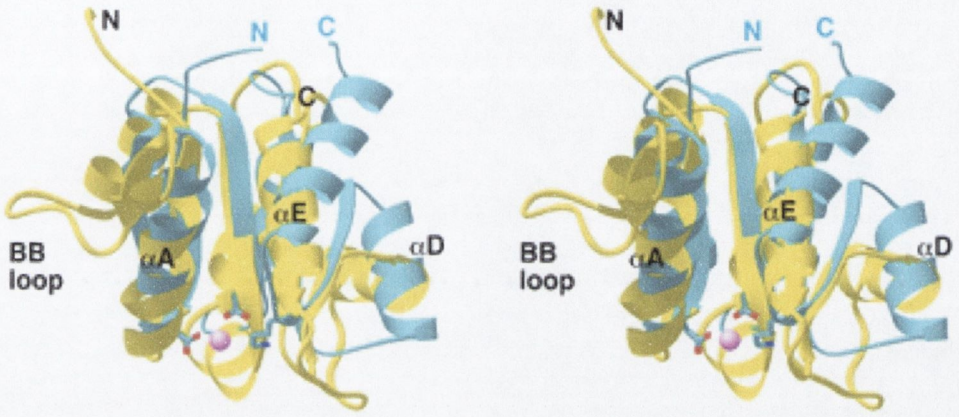
excluded from this alignment, as their TIR domains seem to be more distantly related to the TIR domain of A46R and the TLRs shown. Three regions of particular importance are termed boxes 1, 2 and 3 and are indicated with a heavy black line. Box 1 is a signature sequence of the family while boxes 2 and 3 contain amino acids shown to be important in signalling, based on mutational analysis of mainly IL1-R1 (Slack *et al.*, 2000). It is also known that a single proline to histidine mutation in box 2 of TLR4 renders mice insensitive to LPS (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). A46R displays sequence similarity with boxes 1 and 2 in particular, although the alignment predicts some areas of extra sequence in A46R, including within box 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.

As previously mentioned, the crystal structures of the TLR1 and TLR2 TIR domains have been solved, and the TIR domain has been shown to adopt a 'three-layer αβα sandwich' topology (Figure 1.14.A), like the bacterial chemotaxis regulator CheY (Xu *et al.*, 2000). Threading the A46R amino acid sequence through secondary structure prediction programmes revealed that A46R could also fold in such a manner (Figure 1.14.B). Therefore, the A46R protein does appear to contain a bona fide TIR domain.

Figure 1.15 shows that A46R displays a high degree of sequence conservation between different strains of VV, with Western Reserve (WR), Copenhagen, Ankara and Tian Tian all having versions, while many other orthopoxviruses also have a homologue of A46R, namely variola major, variola minor, camelpox, monkeypox, cowpox and ectromelia. This high degree of conservation could reflect an important role for A46R in pox viral pathogenesis.

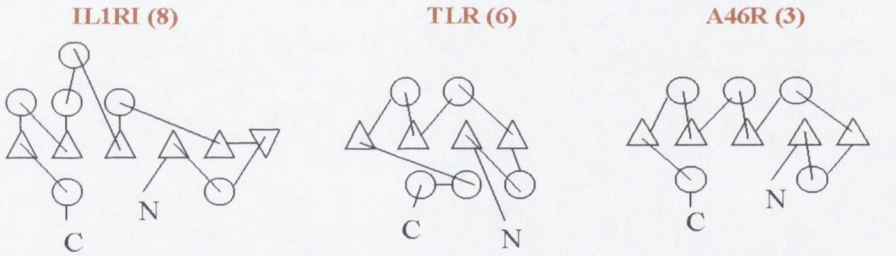
Figure 1.14 Secondary Structure prediction of A46R

A: Structural Comparison of TIR Domain and CheY (taken from Xu *et al.*, 2000)



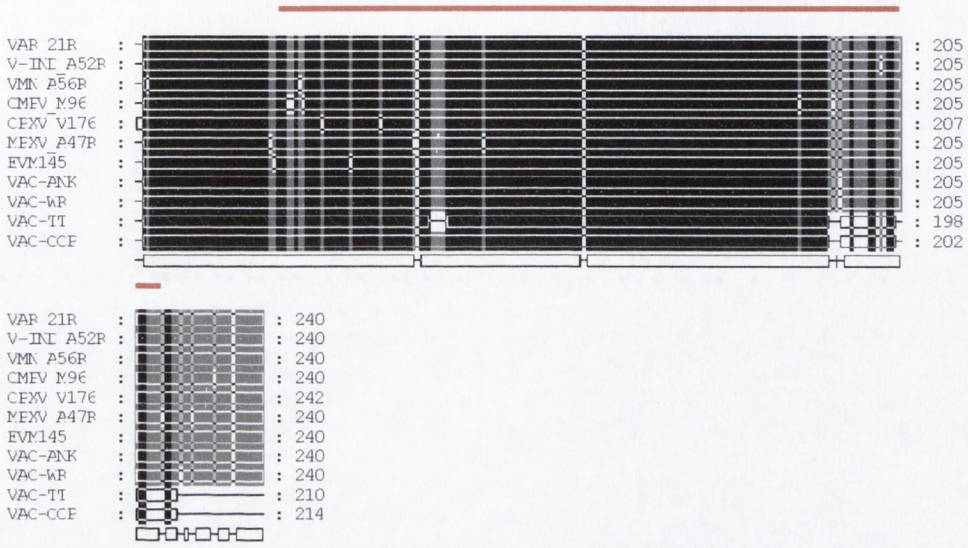
•CheY structure shown in cyan
• TIR domain of TLR2 in yellow

B: Topology Predictions for TIR domains



The crystal structures of the TIR domains for TLR1 and TLR2 have been determined and show that the domain adopts a three-layer αβα sandwich conformation, similar to that of CheY, a bacterial chemotaxis regulator. Threading the A46R amino acid sequence through secondary structure prediction programmes (B) revealed that A46R could also fold in such a manner, thus providing further evidence that A46R has a TIR domain.

Figure 1.15 A46R orthologues in different poxviruses



A summarising sequence alignment of A46R orthologues in different poxviruses. A black bar indicates residues conserved in 100% of the sequences listed, a dark-grey bar indicates conservation in 80 % and a light grey bar in 60 % of the sequences. A solid red line indicates the region of TIR domain similarity.

Key: VAR_21R, Variola major 21R protein; V-IND_A52R, variola (strain India) A52R; VMN_A56R, variola minor (strain Garcia) A56R; CMPV_M96, camelpox M-96; CPXV_V176, cowpox V176; MPXV_A47R, monkeypox A47R; EVM145, ectromelia EVM145; VAC-ANK, vaccinia (strain Ankara) 27.6k protein; VAC-WR, vaccinia (strain Western Reserve) SalF9R (A46R); VAC-TT, vaccinia (strain Tian Tan) TA57R, VAC-COP, vaccinia (strain Copenhagen) A46R.

A BLAST search with A46R identified A52R, another VV protein, as the highest scoring sequence producing significant sequence similarity. Initially it was thought that A52R was also a putative TIR-containing protein. However, a more careful analysis of the sequence, together with results from secondary structure prediction programmes, indicated that A52R was unlikely to contain a TIR domain. Interestingly, A52R and A46R have a region of strong sequence similarity spanning approximately 50 amino acids, in the region of A46R that seems most distantly related to other TIR domains. This region does not seem to be related to other known protein sequences and A52R is not conserved across different orthopoxviruses like A46R. For example, the gene encoding A52R in variola is disrupted.

The names A46R and A52R are based on the standard VV nomenclature of the Copenhagen strain (Goebel *et al.*, 1990). The convention adopted for naming OPV ORFs consists of using the *Hin*dIII restriction endonuclease DNA fragment letter, followed by the ORF number (from left to right) within the fragment, and L or R, depending on the direction of the ORF. For example, the B1R ORF is the leftmost one that starts in the *Hin*dIII B fragment and is read towards the right of the genome. An exception to this is the *Hin*dIII C fragment; the ORFs were numbered from right to left to avoid starting at the end of the genome which is highly variable (Goebel *et al*, 1990). A46R and A52R were cloned from the laboratory VV strain WR, where they were previously called SalF9R and SalF15R, into a mammalian expression vector.

Given the sequence similarity of A46R with the TIR domain, its apparent conservation within the orthopoxvirus family, the similarity between A46R and A52R and the fact

that the region in the VV genome from which these ORFs are expressed is rich in immunomodulatory genes, Bowie *et al.* (2000) hypothesised that A46R might represent a previously unidentified VV strategy directed against IL-1R/TLR signalling.

A46R was transfected into mammalian cells and tested for its ability to block activation of an NFκB-dependent reporter gene induced by IL-1R/TLR activators. Preliminary experiments found that when increasing amounts of the plasmid vector encoding A46R was transfected into mammalian cells, a dose-dependent pattern of expression was observed, a very useful phenomenon when planning future experiments. It was also found that ectopic expression of A46R could inhibit IL-1-induced NFκB activation, but did not have an inhibitory effect on TNF-mediated NFκB activation. These results were considered very promising and worthy of further investigation.

1.14 Aims and Objectives

The overall aim of this investigation was to characterise the effect of A46R on IL-1/TLR signalling. The specific aims of the project were as follows:

1. To confirm the preliminary data obtained by Bowie *et al.* (2000), and further characterise the effect of A46R on IL-1 signals, since previously only NFκB was tested.
2. To measure the effects of A46R on kinases, transcription factors and gene expression induced by TLR family members in order to more fully understand the subtleties and differences in signalling by different family members.
3. To identify the target of A46R in inhibition of IL-1 signalling and in TLR signalling also if such inhibition is observed.

4. To examine the interactions of A46R with the TLR system at the level of molecular associations and to map the sites of these associations.

These aims were carried out in parallel using two overall approaches. The first involved measuring the effect of A46R on IL-1 and TLR signalling pathways using a variety of reporter gene assays. This work led to the hypothesis that A46R might be exerting its effects at the level of the TIR adapter molecules. This theory was confirmed by means of the second approach which was exploring the protein-protein interactions of A46R TIR domain-containing proteins. Using these methods, a previously uncharacterised mechanism of poxviral immune evasion was revealed

Chapter Two

Materials and Methods

2.1 Materials

Human rIL-1α was a gift from the National Cancer Institute (Frederick, WA, USA), while human rTNF-α was a gift from S. Foster (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). The synthetic double-stranded RNA analogue, poly(I:C), was purchased from Amersham Biosciences. Lipopolysaccharide from *E. coli* (>99.9% pure with respect to protein and DNA) and macrophage-activating lipopeptide 2kDa (MALP2) were purchased from Alexis Corporation. Flagellin was a gift from A. Gerwirtz (Emery University, Atlanta, GA 30322, USA). Synthetic tripalmitoyl lipopeptide, Pam_3Cys-Ser-$(Lys)_4$, was purchased from Invivogen. R-848 was a gift from D.T. Golenbock (The University of Massachusetts Medical School, Worcester, MA 01605). Phorbyl 12-myristate 13-acetate (PMA) and phosphothioate CpG DNA were purchased from Sigma.

Polyclonal antibodies were previously raised against a purified, bacterially expressed Glutathione *S*-Transferase (GST) fusion of A46R, encoded by a plasmid synthesised by inserting full length A46R downstream of GST in the bacterial expression vector pGEX4T2. The phospho-ATF-2 (Thr71) antibody was purchased from Cell Signalling Technology as part of their p38 MAP Kinase assay kit. Anti-AU1 monoclonal antibody was purchased from Eurogentec. Anti-TRAF6 and anti-HA polyclonal antibodies were purchased from Santa Cruz Biotechnology. The antibody against the DNA binding domain of Gal4 was also purchased from Santa Cruz Biotechnology. An alternative monoclonal anti-HA antibody, was purchased from Babco. Anti-Mal polyclonal antibody was a kind gift from L.A. O'Neill (Trinity College, Dublin). Anti c-Myc (clone 9E10), anti-β-actin, anti-Flag M2, anti-mouse IgG (whole molecule)

peroxidase conjugate and anti-rabbit IgG (whole molecule) peroxidase conjugate antibodies were all purchased from Sigma.

The competent *E. coli* strain, NovaBlue, the UltraMobius™ 1000 plasmid purification kits and GeneJuice were all purchased from Novagen. Bacto™ Agar, Bacto™ Yeast Extract and Bacto™ Tryptone were purchased from Unitech. The human IL-8 and RANTES ELISA kits were purchased from R&D Systems.

All cell culture materials including DMEM, penicillin, streptomycin, gentamicin, trypsin and L-Glutamine were obtained from Sigma. Foetal calf serum was purchased from LabTech. Cell culture plastics were purchased from Greiner GmbH and Corning.

The human embryonic kidney (HEK) cell lines 293 (HEK 293) and 293T (HEK 293T), and HEK 293 cells stably transfected with IL1R1 (HEK 293R1) were gifts from Tularik Inc (San Francisco, CA 94080). The HEK 293 cells stably transfected with either pcDNA3.1 (HEK 293-pcDNA3.1) TLR3 (HEK 293-TLR3) or TLR8 (HEK 293-TLR8) were a gift from K.A. Fitzgerald (The University of Massachusetts Medical School, Worcester, MA 01605). The mouse leukaemic monocyte-macrophage cell line, RAW264.7, was a gift from S.R. Paludan (University of Aarhus, Denmark).

General laboratory chemicals were purchased from Sigma, with the exception of Glutathione Sepharose 4B (Amersham), Coelenterazine (Argus Fine Chemicals), passive lysis buffer (Promega), Luciferin (Biosynth), broad range prestained protein

marker (New England BioLabs Ltd.), Supersignal® West Pico chemiluminescent substrate (Pierce) and Re-Blot Plus (Chemicon).

2.2 Expression Vectors

The VV ORFs A46R and A52R were cloned by PCR amplification from Western Reserve (WR) DNA as described in Bowie *et al.* (2000). The empty vector pcDNA3.1 was purchased from Invitrogen. The NFκB reporter construct is described in Bowie *et al.* (2000). 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™ CHOP, Elk-1 and c-Jun *trans*-reporting system (pFA-CHOP, pFA-Elk-1, pFA-c-Jun, pFR-Luc, pFC-MEK3, pFC-MEKK and pFC-MEK1) and the ISRE-luciferase reporter gene construct were obtained from Stratagene. The plasmids encoding Gal4-IRF3 and Gal4-IRF7 were kind gifts from K.A. Fitzgerald (The University of Massachusetts Medical School, Worcester, MA 01605). The Gal4-p65 construct was a gift from L. Schmitz (German Cancer Research Centre, Heidelberg, Germany). The IL-8 promoter luciferase construct was a gift from E. Kiss-Toth (University of Sheffield, UK). The RANTES promoter luciferase construct was a gift from J. Hiscott (McGill University, Montreal, Canada).

The plasmids encoding AU1-tagged full-length MyD88, AU1-tagged MyD88 TIR domain, aa 152-296, (TIR-MyD88) and HA-tagged MyD88 death-domain, aa 1-151, (MyD88 DD) were gifts from M. Muzio (Mario Negri Institute, Milan, Italy). The plasmid encoding CD4-TLR4 was a kind gift from R. Medzhitov (Yale University, New Haven, CT, USA). The plasmid encoding Flag-hTLR3 was a gift from D.T.

Golenbock (The University of Massachusetts Medical School, Worcester, MA 01605). The plasmid encoding Flag-hTRIF was a gift from S. Sato (Research Institute for Infectious Diseases, Osaka University, Japan). Plasmids encoding Flag-tagged hTRAM, the TIR domain of TRAM, aa 63-235, (TIR-TRAM), the TIR domain of TRIF, aa 387-566, (TIR-TRIF), Mal, SARM and TBK1 were kind gifts from K.A. Fitzgerald (The University of Massachusetts Medical School, Worcester, MA 01605). The plasmid encoding the IL-1RAcP was a gift from W. Falk (Universität Regensburg, Germany). Plasmids encoding flag-tagged p38 MAP kinase, myc-tagged MyD88, AU1-tagged MyD88 Pro200H mutant (MyD88 P/H), HA-tagged full-length Mal, HA-tagged Mal Pro125His mutant (Mal P/H) and the Mal TIR domain, aa 74-235, (HA-tagged), were all gifts from L.A. O'Neill (Trinity College, Dublin). The plasmid encoding N1L was a gift from W.L. Marshall (The University of Massachusetts Medical School, Worcester, MA 01605). Plasmids encoding IKK-α, IKK-β, Flag-hTLR2 and Flag-TRAF2 were provided by Tularik Inc (San Francisco, CA 94080).

2.3 Cell Culture

2.3.1 Growth and maintenance of RAW 264.7 and HEK 293, 293T, 293R1, 293-TLR3 and 293-TLR8 cell lines

All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) foetal calf serum (FCS), 100 μg/ml gentamicin and 2 mM L-glutamine. The medium for the HEK 293-TLR3 and HEK 293-TLR8 cell lines also contained 1 mg/ml of the neomycin analogue geniticin (G-418) as a selection agent. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. For continuing cell culture, both of the cell lines cells were seeded at 1 x 10^5 cells/ml and sub-cultured

two or three times a week when cells were at 50-80% confluency. All HEK 293 cell lines were removed from the surface of the flask by incubation with 5 ml of trypsin-EDTA (0.05 mg/ml) for 1 minute. Complete medium (10 ml) was then added to the cells. The contents of the flask were then transferred to a 30 ml tube and they were centrifuged at 110 x g for 5 minutes in a PK121R desktop centrifuge (A.L.C.). 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 tube and centrifuged at 110 x g for 5 minutes. For all cell lines, the supernatants were removed and the pellet resuspended in 1 ml of complete medium. 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 a haemocytometer and a bright light microscope.

2.3.2 Cryo-Preservation of Cells

Cells were grown to 50-80% confluency, harvested and counted as previously described (Section 2.3.1). The cells were centrifuged at 110 x g for 5 minutes and the pellet was resuspended in the correct volume of FCS:dimethyl sulphoxide (DMSO) (9:1) to give a cell density of 2 x 10^6 cells. Aliquots (1 ml) of this suspension were placed in 1.5 ml cryotubes. These aliquots were placed at -20°C for 2 hours and then at -80°C for 3 hours or overnight before being stored in liquid nitrogen.

2.4 Plasmid Purification for Transient Transfection

2.4.1 Plasmid Transformation

The required number of tubes of the competent *E. coli* strain, NovaBlue (Novagen) were left to thaw on ice and mixed gently to ensure the cells were evenly suspended. 1 µl of the DNA solution was added to 25 µl of competent cells. The mixture was stirred gently and left on ice for 5 minutes. The tubes were then heat-shocked for exactly 30 seconds in a 42°C water bath and cooled on ice for 2 minutes. 125 µl of room temperature SOC medium were added to each tube. 50 µl aliquots were plated out onto Luria-Bertani (LB) agar, containing Ampicillin, and grown 16-18 hours at 37°C. Transformed cells were then single colony purified and used to purify plasmids for transfection.

2.4.2 Plasmid Purification Procedure

Plasmids were purified using Novagen UltraMobius™ 1000 plasmid purification system. 100 ml of LB broth (containing Ampicillin) was inoculated with a single colony from the single colony purification step above. Cells were grown to an optical density at 600 nm ($O.D._{600}$) of 3-5 at 37°C in a shaking incubator at 200 rpm (12-16 hours of growth). The cells were harvested by centrifugation at 5000 x g for 10 minutes using a GSA rotor in a Sorvall RC5C Plus centrifuge, and the supernatant decanted. Pellets were resuspended in 8 ml bacterial resuspension buffer. Cells were lysed for 5 minutes in 8 ml bacterial lysis buffer at room temperature and the reaction was stopped using 8 ml neutralisation buffer. The sample was then incubated on ice for 5 minutes. The neutralised lysate was centrifuged at 10,000 x g for 2 minutes to remove the bulk of the insoluble material. The cleared supernatant lysate was then decanted into a ClearSpin™ Filter unit and centrifuged in a clinical centrifuge at 2000 x

g for 3 minutes to filter the lysate. To minimise endotoxin levels, 2.4 ml of Detox Agent was added to the clarified and neutralised lysate, mixed gently and incubated on ice for 15 minutes. The lysate was transferred to an equilibrated Mobius 1000 Column and the entire volume allowed flow through by gravity. The column was washed with 20 ml Mobius Wash Buffer and the bound plasmid was eluted and using 5 ml Mobius Elution Buffer. The DNA was precipitated using 0.7 volumes of room temperature isopropanol and collected by centrifugation for 15,000 x g for 20 minutes. The pellet was then washed with 70% (v/v) ethanol and the DNA resuspended in a suitable volume of sterile water (0.25-0.5 ml). Plasmid DNA quality and concentration was determined using a UV spectrophotometer to measure the A_{260} and A_{280} values, and by running the samples on a 1% (w/v) agarose gel stained with ethidium bromide

2.5 Transient Transfection using GeneJuice

GeneJuice transfection reagent (Novagen), a proprietary nontoxic formulation of a cellular protein and a small amount of a novel polyamine, was used for transfection of RAW 264.7 cells and all HEK 293 cell lines. For 96 well plate transfections, cells were seeded at 2 x 10^4 cells per well (HEK 293) or 4 x 10^4 cells per well (RAW 264.7) and grown overnight. Cells were transfected in triplicate with 230 ng (HEK 293) or 200 ng (RAW 264.7) DNA per transfection. In all cases, the amount of DNA used per transfection was kept constant using the appropriate amount of relevant empty vector control, pcDNA3.1. The appropriate amount of GeneJuice (0.8 µl for HEK 293 cells or 0.5 µl for RAW 264.7 cells) was mixed with either 9.2 µl (HEK 293) or 9.5 µl (RAW 264.7) 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 transfections, the total DNA used was 1-2 μg, together with 8 μl GeneJuice and 92 μl serum-free DMEM. For transfection of 10 cm plates, 8-10 μg DNA was used in combination with 15 μl GeneJuice and 235 μl serum-free DMEM. All experiments were harvested at least 24 hours after transfection.

2.6 Luciferase Gene Reporter Assay

2.6.1 Preparation of Cellular Lysates

RAW 264.7 cells and all HEK 293 cell lines were transfected in 96 well plates as described in section 2.5. 6 hours post-stimulation or 24 hours post transfection (if no stimulation), medium was removed from HEK 293 cells and the cells were lysed for 15 minutes on a rocking platform at room temperature with 50 μl Passive Lysis Buffer (Promega). The RAW 264.7 cell line, however, were centrifuged at 1,000 x g for 5 minutes before removal of the medium and cells were then lysed as above. 20 μl of the supernatant was used to determine Firefly luciferase activity and an equivalent amount used for *Renilla* luciferase activity.

2.6.2 Reporter Gene Assays

The Stratagene PathDetect System™ was used for MAP kinase reporter assays. Each PathDetect *trans*-reporting system includes a unique fusion *trans*-activator plasmid that expresses a fusion protein. The fusion *trans*-activator protein consists of the activation domain of either the c-Jun, Elk1 or CHOP transcription activator fused with the DNA binding domain of the yeast Gal4 (residues 1-147). The transcription activators c-Jun, Elk1 and CHOP are phosphorylated and activated by c-Jun N-terminal kinase (JNK), ERK1/2 or p38 kinase, respectively, and their activity reflects the *in vivo* activation of

these kinases and the corresponding signal transduction pathways. The pFR-luciferase reporter plasmid contains a synthetic reporter with five tandem repeats of the yeast Gal4 binding sites that control expression of the *Photinis pyralis* (American firefly) luciferase gene. The DNA binding domain (DBD) of the fusion *trans*-activator protein binds to the reporter plasmid at the Gal4 binding sites. Phosphorylation of the transcription activation domain of the fusion *trans*-activator protein will activate transcription of the luciferase gene from the reporter plasmid. Expression (or activity) levels of luciferase reflect the activation status of the signalling events. When performing the MAP kinase assays, the CHOP (0.25 ng), c-jun (0.25 ng) or Elk1 (5 ng) Gal4 fusion vectors were used in combination with 60 ng pFR-luciferase reporter to measure p38, JNK and ERK1/2 activation respectively.

For the NFκB and ISRE reporter gene assays, 60 ng of either κB-luciferase or ISRE-luciferase reporter genes were used. For the p65, IRF3 and IRF7 transactivation assays, 1 ng p65-Gal4, 3 ng IRF3-Gal4 or 3 ng IRF7-Gal4 fusion vectors were used in combination with 60 ng pFR-luciferase reporter. The IL-8, IFN-β and RANTES assays were carried out using 60 ng of the IL-8, IFN-β or RANTES promoter luciferase reporter genes.

In all assays, 20 ng of *Renilla*-luciferase internal control (Promega) was used.

2.6.3 Measurement of Luciferase Activity

Firefly luciferase activity was assayed by the addition of 40 µl of luciferase assay mix (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM MgSO4, 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to the sample.

Coelenterazine was diluted in PBS to give a 2 μg/ml final concentration. *Renilla* luciferase was read by the addition of 40 μl of this solution to the sample. Luminescence was read using the Reporter microplate luminometer (Turner designs). Firefly luminescence readings were corrected for *Renilla* activity and expressed as fold stimulation over unstimulated empty vector control.

2.7 Western Blot Analysis

2.7.1 Preparation of Whole Cell Lysates using MAP Kinase Sample Buffer

Cells were seeded at a density of 1 x 10^5 cells/ml. After appropriate stimulation or transfection, cells were washed with phosphate buffered saline (PBS) and lysed in 100 μl MAPK 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 at 80% strength using a DAWE Soniprobe and boiled for 5 minutes.

2.7.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were resolved on a sodium dodecylsulphate (SDS) polyacrylamide gel using a constant voltage of 150 V. Samples were first run through a stacking gel (1 ml 30% bisacrylamide mix, 0.75 ml 1 M Tris pH 6.8, 60 μl 10% (w/v) ammonium persulphate and 6 μl TEMED made up to 6 ml with H_2O) to concentrate protein and then resolved according to size using 10-15% polyacrylamide gels (required volume of 30% bisacrylamide mix, 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 H_2O). Samples were 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 Immobilon-P polyvinylidene diflouride (PVDF) membrane (Sigma) 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% (v/v) methanol). The gel was placed on a layer of filter paper and sponge overlaid with polyvinylidene diflouride. A second piece of filter paper was placed on top followed by a second sponge. The entire assembly was placed in a cassette, the chamber filled with transfer buffer and a constant voltage of 100 V was applied for 1 hour.

2.7.4 Antibody Blotting

Membranes were blocked for non-specific binding by incubation in blocking buffer (5% (w/v) non-fat dried milk, Marvel™, (Dunnes Stores) in 1% (v/v) phosphate buffered saline (PBS)-Tween) at 4°C overnight or at room temperature for 1 hour. The membrane was washed for 5 minutes in PBS-Tween three times. The membrane was then incubated for 1 hour at room temperature or at 4°C overnight with the primary antibody of interest at 1:100 to 1:1000 dilutions depending on the antibody in question. Next, the membrane was washed five times and incubated with the appropriate secondary horseradish peroxidase linked enzyme for 1 hour at room temperature. The antibody diluent was 5% (w/v) Marvel™ 1% (v/v) PBS-Tween in all cases. Again the blots were washed five times. The blots were then washed in PBS without Tween for 5 minutes. Blots were developed by enhanced chemiluminescence (ECL) using Supersignal® according to manufacturers' instructions (Pierce). If required, blots were stripped by shaking in 10 ml Re-Blot Plus (Chemicon) for 15 minutes. The blots were then re-blocked and probed with the desired antibodies.

2.7.5 Coomassie Staining

Resolved gels were soaked in Coomassie blue stain (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 2.5 g/L Coomassie blue dye) for at least 1 hour and then washed several times with destain solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid) until bands appeared.

2.8 p38 MAPK Immunoprecipitation Kinase Assay

HEK 293R1 cells were transfected with Flag-tagged p38 and A46R and allowed to recover for 16 hours (details of plasmid concentrations used are described in Figure 3.6). Prior to harvesting, the cells were treated with either IL-1 (100 ng/ml) or TNF-α (100 ng/ml) for 15 minutes. Cells were lysed with 500 µl lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin). 50 µl of lysate was retained for blotting and Flag-p38 was immunoprecipitated with immobilised anti-Flag M2 antibody (Sigma). Beads were then washed three times with lysis buffer and then with kinase buffer (25 mM Tris pH 7.5, 5 mM β-Glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). Pellets were resuspended with 50 µl kinase buffer supplemented with 200 µM ATP and 2 µg ATF-2 fusion protein (Cell Signalling Technologies) and incubated for 30 minutes at 30°C. The reaction was terminated by addition of 25 µl 3X SDS sample buffer. Samples were boiled and then analysed by SDS-PAGE and Western blotted for phosphorylated ATF-2 or Flag-p38.

2.9 Immunoprecipitation and Immunoblotting

2.9.1 Antibody Precoupling

The relevant antibodies were precoupled to either protein A- (polyclonal antibodies and Flag monoclonal antibody) or protein G-sepharose (all other monoclonal antibodies) by incubation with 30 µl of 50% protein A/G slurry per immunoprecipitation sample on a roller, overnight at 4°C. Just before use, the beads were washed with 1 ml lysis buffer three times to remove uncoupled antibody.

2.9.2 Immunoprecipitation and Immunoblotting

HEK 293T cells were seeded into 10 cm dishes (1.5 x 10^6 cells) 24 hours prior to transfection. Transfections were carried out using GeneJuice™ transfection reagent (Novagen) as previously described. For co-immunoprecipitations, 4 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 empty vector (pcDNA3.1). For competition experiments where the effect of increasing A46R expression on complex formation between two signalling molecules was examined, 2 µg of each signalling molecule expression plasmid was transfected in the presence of increasing amounts of A46R plasmid. The total amount of DNA (10 µg) was kept constant in each sample using empty vector. In all cases the cells were harvested 24 hours post-transfection by scraping in 1 ml ice-cold PBS. The samples were centrifuged at 16,100 x g for 5 minutes and the supernatant discarded. The pellets were resuspended in 1 ml ice-cold PBS and centrifuged at 16,100 x g for 5 minutes. The supernatant was discarded and the cells were lysed in 850 µl of lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) NP-40 containing Aprotinin, 1

mM sodium orthovanadate, and 1 mM PMSF) for 20 minutes. PMSF (8.5 µl) was added to all samples, which were then centrifuged for 10 minutes at16,100 x g. 400 µl of lysate was added to the relevant precoupled antibodies and rolled overnight at 4°C. 12.5 µl 5X sample buffer was added to the remaining 50 µl of lysate, which was then boiled for 4 minutes and frozen immediately. The next day the immune complexes were washed twice with 1 ml lysis buffer and once with 1 ml lysis buffer without NP-40 and glycerol. All supernatant was removed and beads were resuspended in 30 µl 3X sample buffer. The samples were boiled for 4 minutes and either frozen immediately or analysed by western blot. 18 µl of the lysates were blotted for control purposes, 18 µl of the immune complex was immunoblotted for co-precipitating protein, and 10 µl was blotted for the protein directly recognised by the immunoprecipitating antibody.

2.10 Glutathione-*S*-Transferase (GST) Fusion Protein Interaction Assay

2.10.1 Preparation of GST-Fusion Proteins

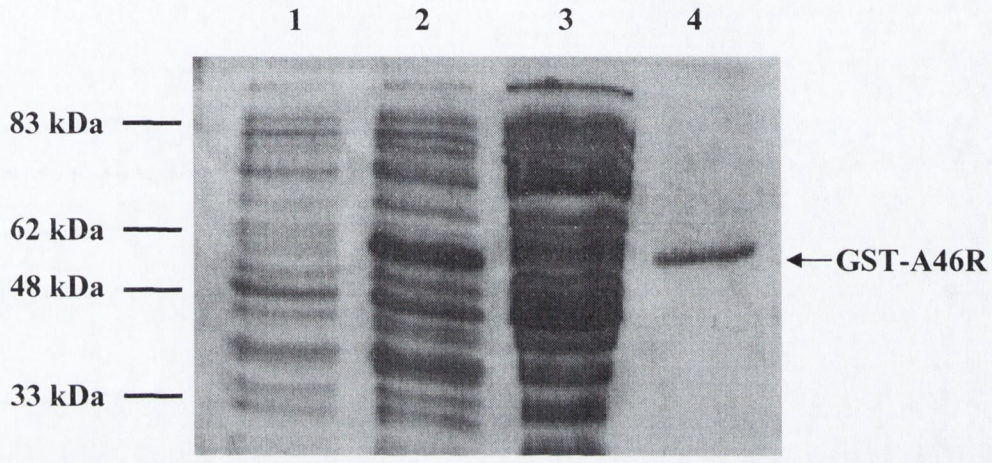
20 ml LB broth (containing Ampicillin) were inoculated with a single colony of *E. coli* BL21 cells transformed with pGEX-A46R or pGEX-Mal and incubated overnight at 37°C, 200 rpm.

The next day, the overnight culture was diluted 1:50 (i.e. 10 ml into 500 ml broth) in Terrific broth (TB), containing Ampicillin, (pGEX-A46R) or LB broth, containing Ampicillin, (pGEX-Mal). The culture was incubated at 30°C, 200 cycles/minute for approximately 3.5 hours or until $O.D._{600}$ of the culture is ≈ 0.5. IPTG was added to a final concentration of 70 µM (pGEX-A46R) or 50 µM (pGEX-Mal) and the culture was incubated for a further 4 hours. The culture was centrifuged using a GSA rotor in

a Sorvall RC5C Plus centrifuge at 7,750 x g for 10 minutes, 4°C in 250 ml tubes. The supernatant was decanted and the pellets frozen overnight at -70°C.

On the third day, the pellets were left to thaw on ice and then resuspended in 15 ml NETN (300 mM NaCl, 1 mM EDTA, 20 mM Trizma base pH 8.0, 0.5% NP-40 (v/v) plus 1 µl/ml leupeptin (stock @ 10 mg/ml), 5 µl/ml aprotinin, 10 µl/ml PMSF (100 mM stock) and 1 mg/ml lysozyme). The resuspended pellets were transferred to 30 ml centrifuge tubes and each pellet was sonicated on ice with 20 second pulses followed by 10 second pauses to prevent over heating of the preparation, 12 times. The suspensions were cleared of insoluble material by centrifugation using a SS-34 rotor at 14,500 x g for 30 minutes, 4°C, and the supernatants pooled in a 50 ml tube. 500µl glutathione sepharose (GSH) beads (50% slurry), that had previously been washed three times in 1 ml NETN plus inhibitors but without lysozyme, were added and the mixture was incubated for 2 hours at 4°C on a roller. The mixture was then centrifuged at 1000 x g for 10 minutes, 4°C and the supernatant discarded. The beads were washed with 25 ml ice-cold NETN (plus inhibitors), and rolled for 5 minutes at 4°C before being centrifuged at 1000 x g for 5 minutes. This washing step was repeated a further four times (five washes in total). After the final wash, the beads were resuspended in an equal volume of NETN (plus inhibitors) to make 50% slurry, transferred to an eppendorf and stored overnight at 4°C. The protein-containing slurry was then analysed by SDS-PAGE and Coomassie staining for the expression of the fusion protein (Figure 2.1).

Figure 2.1: GST-A46R Expression and Purification



Terrific broth (containing Ampicillin) was inoculated with an overnight culture of *E. coli* BL21 cells transformed with pGEX-A46R and incubated at 30°C, 200 rpm, until the O.D.600 of the culture is ≈ 0.5 (Lane 1). IPTG was added to a final concentration of 70 μM and the culture was incubated for a further 4 hours (Lane 2). The culture was pelleted by centrifugation and the supernatant removed. Cells were lysed in ice-cold NETN by sonication on ice. The suspension was cleared of insoluble material by centrifugation and the lysate was retained (Lane 3). The lysate was then incubated with GSH beads for 2 hours, after which the beads were stringently washed with NETN. After the final wash, the beads were resuspended in an equal volume of NETN to make 50% slurry, transferred to an eppendorf and stored at 4°C. The protein-containing slurry was then analysed by SDS-PAGE and Coomassie staining for the expression of the GST-A46R fusion protein (Lane 4).

2.10.2 Affinity Purification of Complexes using GST-Fusion Proteins

HEK 293T cells were seeded into 10 cm dishes (1.5 x 10^6 cells) 24 hours prior to transfection. Transfections were carried out using GeneJuice™ transfection reagent (Novagen) as previously described. For GST pulldown experiments, 8 μg of expression vector was transfected. The cells were harvested 24 hours post-transfection by scraping in 1 ml ice-cold PBS. The samples were centrifuged at 16,100 x g for 5 minutes and the supernatant discarded. The pellets were resuspended in 1 ml ice-cold PBS and centrifuged at 16,100 x g for 5 minutes. At this point, the supernatant was discarded and the cells were lysed in 850 μl of lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) NP-40 containing 10 μl/ml Aprotinin, 1 mM sodium orthovanadate, and 1 mM PMSF) for 20 minutes. PMSF (8.5 μl) was added to each sample and they were centrifuged for 10 minutes at 16,100 x g. 12.5 μl 5X sample buffer was added to 50 μl of lysate, which was then boiled for 4 minutes and frozen immediately. 800 μl of lysate was added to the appropriate amount of prewashed GST-fusion protein and incubated for 2 hours on a roller at 4°C. The samples were washed twice with 1 ml lysis buffer and once with 1 ml lysis buffer without NP-40 and glycerol. All supernatant was removed and beads were resuspended in 50 μl 3X sample buffer. The samples were boiled for 4 minutes and analysed by Western blot.

2.10.3 Production of recombinant Mal (rMal)

1 ml GST-Mal bound to GSH-agarose beads (50% slurry in lysis buffer) was washed four times with 5 ml PBS, discarding supernatant each time. Beads were rolled overnight in 5 ml PBS with 1 unit/ml Thrombin (Sigma) at 4°C. The next day, the

suspension was centrifuged at 1,500 x g for 5 minutes and supernatant was collected. Protein quantity and quality were analysed by SDS-PAGE and Coomassie staining (Figure 2.2).

2.11 Enzyme Linked Immunosorbent Assay (ELISA)

2.11.1 Preparation of samples – IL-8 ELISA

HEK 293R1 cells (2 x 10^4 cells per well) were seeded into 96-well plates and transfected with the A46R expression plasmid 24 h later. Cells were stimulated with 100 ng/ml IL-1 or 100 ng/ml TNF-α 24 h after transfection. Unstimulated cells transfected with empty vector were used as control. After 24h supernatants were harvested and stored at -20°C.

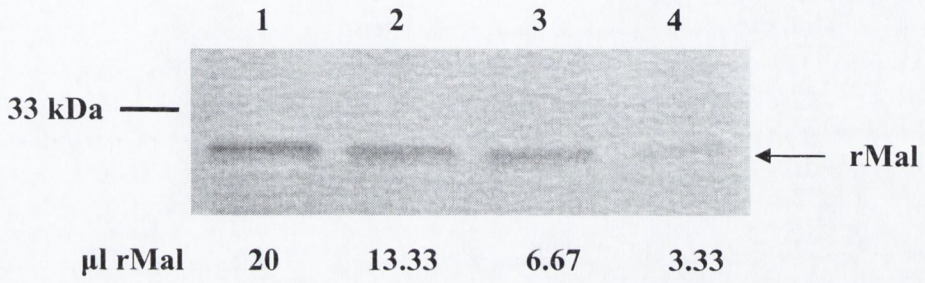
2.11.2 Preparation of samples – RANTES ELISA

HEK 293 cells stably expressing TLR 3 (2 x 10^4 cells per well) were seeded into 96-well plates and transfected with the A46R expression plasmid 24 h later where indicated. Cells were stimulated with 25 mg/ml poly(I:C) 24 h after transfection After 24 h, supernatants were harvested and stored at -20°C.

2.11.3 ELISA protocol

A 96 well plate was coated with 100 µl per well of either mouse anti-human IL-8 antibody diluted to 4 µg/ml in PBS or mouse anti-human RANTES antibody diluted to 1 µg/ml in PBS. The plate was covered and incubated overnight at room temperature and then washed three times with PBS/0.5% (v/v) Tween® 20. Plates were pounded dry on paper towels as a final step. The plate was blocked with 300 µl per well block-buffer (1% (w/v) BSA, 5% (w/v) Sucrose in PBS, with 0.05% (w/v) NaN_3) and

Figure 2.2: Purified Recombinant Mal (rMal)



1 ml GST-Mal bound to GSH-agarose beads were rolled overnight in 5 ml PBS with 1 unit/ml Thrombin at 4°C. When the supernatant was analysed by SDS-PAGE and Coomassie staining, pure recombinant Mal was observed at the expected molecular weight of just below 33 kDa.

incubated at room temperature for a minimum of 1 hour. The plate was washed and dried as before. Either IL-8 standards (in the range of 0.03-2 ng/ml) or RANTES standards (in the range of 0.015-1 ng/ml), and samples were added at 100 µl per well and incubated for 2 h at room temperature. Plates were then washed as before. 100 µl of either biotinylated goat anti-human IL-8 antibody (diluted to 20 ng/ml in reagent diluent (0.1% (w/v) BSA, 0.05% (v/v) Tween® 20 in Tris-buffered Saline) or biotinylated goat anti-human RANTES antibody (diluted to 10 ng/ml in reagent diluent) was added to each well. The plate was incubated at room temperature for 2 hours and then washed 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 (1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine)) was added to each well and incubated for 20 minutes in the dark at room temperature. Addition of 50 µl 2 N H_2SO_4 was added in order to stop this reaction. The optical density of each well was read at $O.D._{450}$ using a Versamax tunable microplate reader (Molecular Devices). Standard curves were constructed relating IL-8/RANTES concentrations to absorbance at 405nm and were used to determine IL-8/RANTES concentrations.

Chapter Three

A46R Inhibits IL-1 Signalling by Targeting MyD88

3.1 Introduction

Upon stimulation of cells with IL-1, a well characterised signalling pathway is activated, ultimately resulting in changes in gene expression. IL-1 binds IL-1RI, causing recruitment of both MyD88 and the IL-1RAcP. MyD88 interacts with the IL-1R1/IL-1RAcP complex via the TIR domain (Muzio *et al.*, 1997). IL-1 also stimulates the release of IRAK-1 from Tollip (Burns *et al.*, 2000). IRAK-1 and MyD88 interact via their death domains (Wesche *et al.*, 1997). IRAK-2 is also recruited to the complex (Muzio *et al.*, 1997). IRAK-4 causes phosphorylation and activation of IRAK-1 (Li *et al.*. 2002) allowing IRAK-1 to bind TRAF-6 (Cao *et al.*, 1996a). The IRAK-TRAF6 then forms a complex with another adapter protein, Pellino 1, and leaves the adapter to interact with TAK-1 (Jiang *et al.*, 2002). TAK1 is phosphorylated on the membrane, but activated in the cytosol (Jiang *et al.*, 2002). The activation of TAK-1 leads to the activation of IKK (Wang *et al.*, 2001), which in turn leads to the phosphorylation and degradation of IκB proteins (Brown *et al.*, 1995), and liberation of NFκB to activate transcription in the nucleus (Chen *et al.*, 1995).

TNF-α can activate the same transcription factors as IL-1, but it uses a distinct signalling pathway. The binding of TNF-α to its receptor results in the formation of homotrimeric clusters. This initiates the formation of an unusually large signalling complex (approximately 700 kDa). The TNF-α-TNF receptor complex recruits the DD-containing molecules FADD (Fas-associated death domain) and TRADD (TNF receptor associated death domain). TRADD then interacts with TRAF2 and the serine/threonine kinase RIP (receptor inhibitor protein). Originally the Ser/Thr protein kinase, NFκB-inducing kinase (NIK), was thought to activate the IKKs by associating

with TRAF2 (Regnier *et al.*, 1997), but more recently TAK1 has been implicated in TNF-α induced activation of the NFκB pathway (Ishitani *et al.*, 2003).

When A46R was first identified as a VV protein having a TIR domain, it was proposed that it may represent a potential viral antagonist of IL-1 and TLR signalling. In order to express A46R in mammalian cell cultures, the VV ORF A46R was subcloned into the mammalian expression vector pRK5 (Bowie *et al.*, 2000). Because TNF activates a signal transduction pathway that does not utilise TIR-domain containing proteins, the effect of A46R on this signalling pathway was used as a control. Initial studies revealed that A46R could inhibit IL-1, but not TNF-induced NFκB activation (Bowie *et al.*, 2000).

The aim of this investigation was to characterise the effect of A46R on IL-1 signalling. The initial investigation focused on confirming the preliminary data obtained by Bowie *et al.* (2000), and further characterising the effect of A46R on IL-1 signals, since previously only NFκB was tested. Furthermore, I wanted to identify the target of A46R in inhibition of IL-1 signalling, and to examine how A46R affects this molecule. Using luciferase assays and a p38 MAPK immunoprecipitation kinase assay, I found that A46R inhibits a variety of IL-1 signals including NFκB activation, p65 transactivation, and activation of the MAP kinases ERK, JNK and p38 without affecting TNF signalling. Using coimmunoprecipitation and GST-pulldown experiments, I discovered that A46R interacted with MyD88, a TIR domain-containing adapter molecule vital for IL-1 signalling, and that it inhibited all MyD88-dependent signals tested.

3.2 Results

3.2.1 Expression of A46R

Following transfection of increasing amounts of cDNA encoding A46R into HEK 293T cells (Figure 3.1), a band of the predicted molecular mass of 28.5 kDa for A46R was observed when the resultant immunoblot was probed with an antibody specific for A46R. A46R expression was dose-dependent. The non-specific band detected by the A46R antibody confirmed equal protein loading.

3.2.2 A46R inhibits IL-1, but not TNF-α, induced NFκB activation

Incubation of either HEK 293R1 (Figure 3.2) cells or RAW 264.7 cells (Figure 3.3) for 6 h with either 100 ng/ml IL-1 or 100 ng/ml TNF-α led to NFκB activation, albeit to different extents. A single dose of A46R inhibited IL-1-induced NFκB activation but did not affect basal levels of reporter gene expression. A46R did not have an inhibitory effect on TNF-α-induced NFκB activation. This experiment confirmed results previously obtained by Bowie *et al.* (2000). RAW 264.7 cells are a murine macrophage cell line, and this was the first time that A46R had been shown to function in a non-human system.

Next I began to examine other IL-1 signals. In order to specifically measure IL-1-induced transactivation of a promoter by the p65 subunit of NFκB, an assay was employed using an expression plasmid encoding the transactivation domain of the p65 subunit of NFκB fused to the DNA-binding domain of Gal4, together with a reporter plasmid with a Gal4 upstream activation sequence (Jefferies *et al.*, 2001). Reporter gene expression from this plasmid requires p65 transactivation through phosphorylation. A single dose of A46R inhibited IL-1-induced p65 transactivation,

but had no effect on TNF-α-induced p65 transactivation or on basal levels of activity (Figure 3.4). The effect of A46R on p65 transactivation provided a rationale for the inhibition of the NFκB-dependent reporter gene.

3.2.3 A46R inhibits IL-1, and not TNF-α, induced p38 MAPK activation

To assess the potential effects of A46R on p38 MAPK activation in downstream signalling from IL-1 and TNF, I used the Stratagene PathDetect™ System which is based on the ability of p38 MAPK to phosphorylate and activate the transcription factor CHOP. This is assayed by an increase in the ability of the Gal4-CHOP fusion protein to transactivate the pFR luciferase reporter, which contains Gal4 binding sites in its promoter. In a similar manner to the p65 transactivation system, incubation of HEK 293R1 cells for 6 h with either 100 ng/ml IL-1 or 100 ng/ml TNF-α led to p38 activation, albeit to different extents (Figure 3.5). A46R inhibited IL-1-induced p38 activation, but not the p38 signal induced by TNF-α. The specificity of this inhibition for IL-1 was further illustrated by the fact that the p38 signal generated by overexpression of MAPK ERK kinase 3 (MEK3), a known p38 MAPK kinase and positive control for this assay, is not inhibited by A46R. The basal level of activity was also uninhibited.

I subsequently attempted to confirm this effect using an alternative assay for p38, involving immunoprecipitation of a FLAG-tagged p38 construct from A46R transfected cells, the immunoprecipitate being assayed for its ability to phosphorylate ATF-2. Phosphorylation of ATF-2 was measured by immunoblotting samples using a phospho-ATF-2 antibody. Figure 3.6 shows that phosphorylation of ATF-2 was enhanced in anti-FLAG immunoprecipitates from cells stimulated with either TNF-α

(100 ng/ml) or IL-1 (100 ng/ml) for 15 minutes prior to harvesting when compared with anti-FLAG immunoprecipitates from unstimulated cells (compare lanes 2 and 4 to lane 1). However, phosphorylation of ATF-2 was inhibited when cells were stimulated with IL-1 and transfected with A46R (compare lanes 4 and 5). TNF-α induced phosphorylation of ATF-2 was not inhibited by A46R (compare lanes 2 and 3). The level of Flag-p38 in all samples is equivalent, as shown in the lower panel.

3.2.4 A46R inhibits IL-1 induced MAPK activation

To assess the potential effects of A46R on other MAP kinase signals induced by IL-1, I used the Stratagene PathDetect™ System which is based on the abilities of ERK and JNK to phosphorylate and activate the transcription factors Elk1 and cJun respectively, and serves as a readout for ERK and JNK activations. This is assayed by an increase in the abilities of the Gal4-Elk1 and Gal4-cJun fusion proteins to transactivate the pFR luciferase reporter, which contains Gal4 binding sites in its promoter. Incubation of HEK 293R1 cells for 6 h with IL-1 (100 ng/ml) led to activation of both JNK and ERK, albeit to different extents (Figure 3.7). A46R inhibited both IL-1-induced ERK and JNK activation, without affecting basal reporter gene activity.

3.2.5 A46R inhibits IL-8 gene induction

Having determined that A46R inhibits both NFκB and p38 MAPK activation induced by IL-1, I investigated whether or not A46R had an effect on IL-8 gene induction. Both NFκB (Baldwin A, 1996) and p38 (Parhar *et al.*, 2003) can effect the transcription of *IL-8*. Figure 3.8 shows that 6 h stimulation with IL-1 (100 ng/ml) causes a 50 fold increase in activity of an IL-8 promoter reporter gene construct, which was potently inhibited in a dose-dependent manner with increasing amounts of A46R.

It was not possible to compare this inhibition with an effect on TNF-α since TNF-α did not induce activation of the IL-8 promoter reporter gene. Therefore, the effect of A46R on the abilities of IL-1 and TNF-α to induce IL-8 production in HEK 293R1 cells was compared by ELISA (Figure 3.9). Although IL-1 and TNF-α induced IL-8 production to very different extents, it is still clear to see that IL-1-, and not TNF-α-, induced IL-8 production is inhibited by A46R. Therefore, the functional consequence of A46R inhibiting IL-1-induced NFκB and p38 MAPK activation in reporter gene assays is a suppression of cytokine induction.

3.2.6 A46R interacts with MyD88

Because A46R inhibits so many IL-1 signals, it was hypothesised that it must act close to the IL-1 receptor complex, before the signals bifurcate. Therefore, I investigated whether or not A46R targeted the TIR-adapter molecule, MyD88, which acts proximal to the IL-1 receptor complex. Coimmunoprecipitation studies were performed with ectopically expressed A46R and MyD88. Figure 3.10.A shows a clear interaction between A46R and MyD88. As a control, the ability of A46R to interact with TRAF2, an adaptor used by TNF but not IL-1 (Hsu *et al.*, 1996), was tested under the same conditions. In this case, no interaction was detected (Figure 3.11.A), consistent with the lack of effect of A46R on TNF signalling. These results were confirmed by GST-pulldown experiments, whereby purified GST-A46R was capable of interacting with ectopically expressed MyD88 in a cell lysate (Figure 3.10.B), but not with TRAF2 (Figure 3.11.B).

3.2.7 Exploring how A46R interacts with MyD88

I wished to determine how exactly A46R was interacting with MyD88. Using a number of MyD88 mutants, I discovered that A46R does not immunoprecipitate with the MyD88 Pro/His mutant (Figure 3.12.A), but does so with the TIR (Figure 3.13.A) and DD domains of MyD88 (Figure 3.14.A). These interactions, or lack of, were confirmed by GST-pulldown experiments, whereby purified GST-A46R was capable of interacting with ectopically expressed MyD88 TIR (Figure 3.13.B) and MyD88 DD (Figure 3.14.B) in a cell lysate, but not with MyD88 P200H (Figure 3.12.B).

3.2.8 Functional consequences of the A46R-MyD88 interaction

Having confirmed that A46R interacts with MyD88, I proceeded to investigate the functional consequences of this association. Overexpression of MyD88 induced activation of NFκB and other signals in reporter gene assays, and all these signals were inhibited by A46R. Transfection of increasing amounts of A46R cDNA potently inhibited MyD88-dependent NFkB activation (Figure 3.15) in a dose-dependent fashion. MyD88 P200H (MyD88 PH) also activated NFκB and, consistent with its inability to interact with this mutant, A46R did not affect this signal (Figure 3.15). The TIR domain of MyD88 (TIR MyD88) which acts as a dominant negative (Muzio *et al.* 1997), inhibited both MyD88- and MyD88 PH-mediated NFκB activation (Figure 3.15). A single dose of A46R cDNA was capable of blocking p65 transactivation, but had no effect on basal levels of activity (Figure 3.16.A). The effect of A46R on MyD88-dependent p65 transactivation was unsurprising considering the inhibition of the NFκB-dependent reporter gene.

MyD88 is also involved in IL-1-induced MAP kinase activation (Burns *et al.*, 1998). Overexpression of MyD88 led to the activation of the MAP kinases, ERK, JNK and p38 (Figure 3.16.B and 3.17), as measured by a *trans*-reporter system of either Elk1-Gal4, cJun-Gal4 or p38-Gal4 constructs and Gal luciferase (PathDetect® from Stratagene) which has been described earlier. A single dose of A46R was capable of inhibiting MyD88-mediated ERK, JNK and p38 activation (Figure 3.16.B and 3.17).

3.2.9 A46R does not affect CHOP-Gal4 fusion protein expression

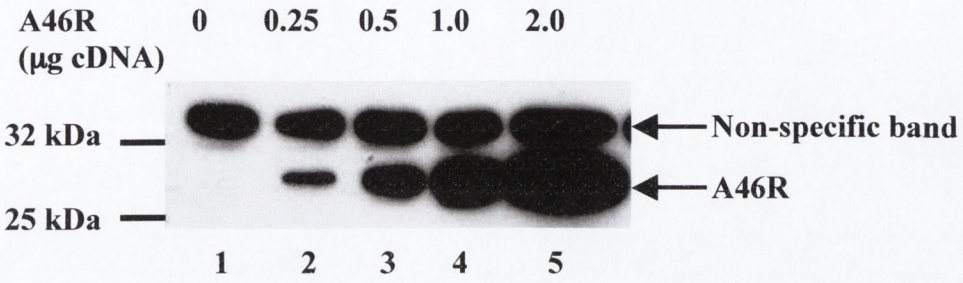
During the course of this investigation, the *trans*-reporter system consisting of either p65-Gal4, Elk1-Gal4, cJun-Gal4 or CHOP-Gal4 and Gal luciferase (PathDetect® from Stratagene), which serve as readouts for p65, ERK, JNK and p38 activations respectively, has been extensively used. A46R inhibited many of the signals in this system. To ensure that these inhibitory effects were not due to a suppression of the expression of the fusion proteins by A46R, I examined whether or not A46R affected the level of CHOP-Gal4 fusion protein expression. In this experiment, a p38 luciferase assay was performed as normal and remaining lysates were blotted with an antibody specific for the DNA-binding domain of Gal4 (Figure 3.18). As measured by luciferase assay, MyD88-induced p38 activation was, as expected, inhibited by A46R. The control values were unaffected (Figure 3.18.A). When the lysates were analysed by Western Blot using an anti-Gal4 DNA Binding Domain (DBD) antibody, I observed that the control levels of CHOP-Gal4 expression were unaffected by the presence of A46R. The presence of MyD88, however, appeared to enhance the levels of expression of CHOP-Gal4 and the reason for this is unknown. However, A46R did not affect the level of CHOP-Gal4 expression in this instance either (Figure 3.18.B). Overall, there was no correlation between CHOP-Gal4 protein levels and measured p38 activity.

Equal loading of samples was confirmed by stripping the blot and re-probing for β-actin (Figure 3.18.C). Identical results were obtained in an additional experiment.

3.2.10 Exploring how A46R inhibits MyD88-dependent signalling

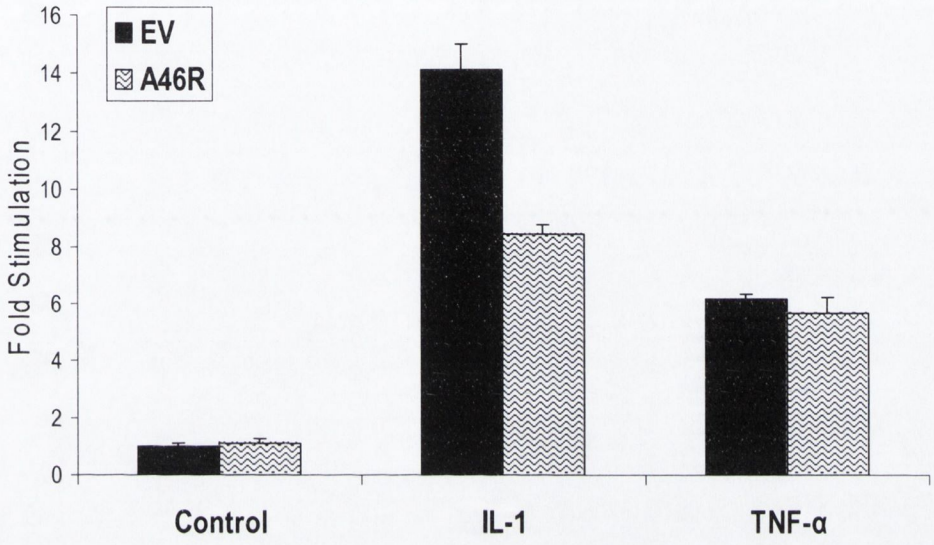
As A52R, a VV protein related to A46R, inhibits TLR signalling by disrupting the formation of signalling complexes (Harte *et al.*, 2003), it seemed possible that A46R might also function in this way. I therefore tested whether A46R would disrupt a MyD88-TRAF6 interaction. To investigate this possibility, MyD88 and TRAF6 were co-expressed, upon which an interaction was detected (Figure 3.19; top panel, lane 2). Whether or not this interaction involves just MyD88 and TRAF6, or is a multi-protein complex containing IRAK-1 for example has not been examined. The MyD88-TRAF6 complex was slightly affected by A46R concentrations in a dose-dependent manner (Figure 3.19; top panel, compare lanes 3–5 to lane 2).

Figure 3.1: Dose-dependent expression of A46R in mammalian cells



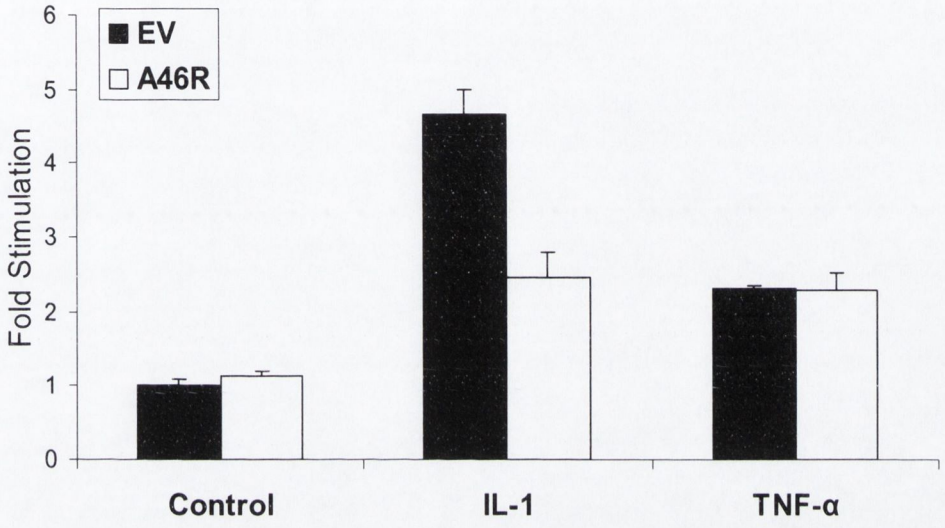
HEK 293T cells were seeded in 6 well plates (2 x 10^5 cells per well) 24 h prior to transfection. Increasing amounts of a plasmid vector encoding A46R (μg) was transfected. Cells were harvested 24 h post transfection and resolved by SDS-PAGE. The blot was probed with an antibody specific for A46R.

Figure 3.2: A46R inhibits IL-1, but not TNF-α, induced NFκB activation in HEK 293 cells



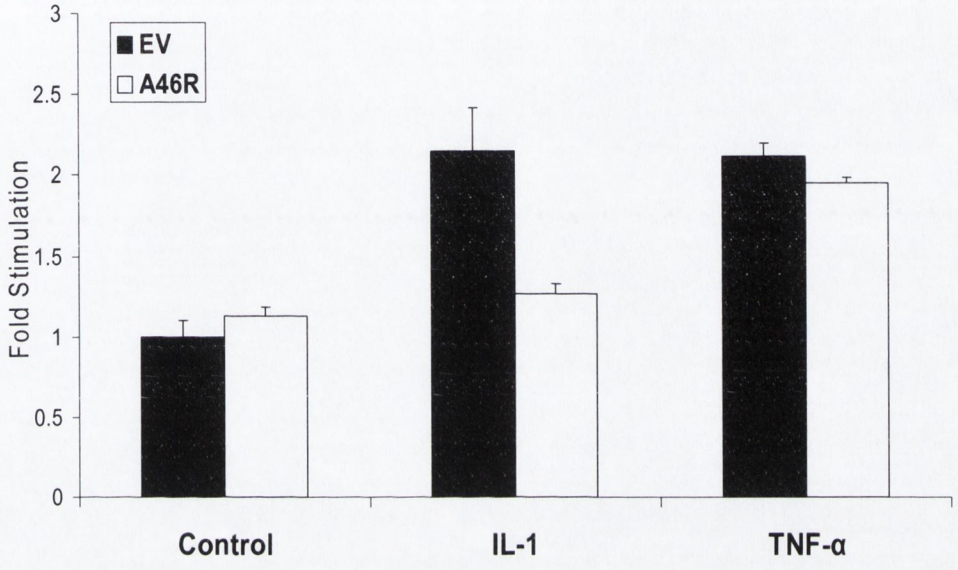
HEK 293R1 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and a κB-luciferase construct, along with either 150 ng empty vector (EV) or A46R expression vector. Six hours prior to harvesting, cells were stimulated with 100 ng/ml IL-1 or 100 ng/ml TNF-α. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.3: A46R inhibits IL-1, but not TNF-α, induced NFκB activation in RAW 264.7 cells



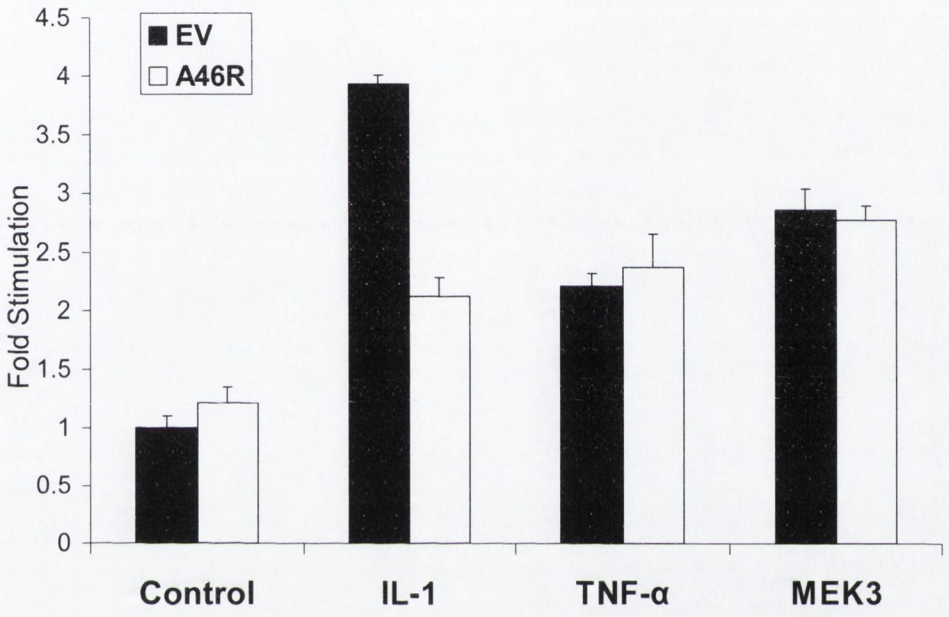
RAW 264.7 cells (4 x 10^4 cells per well) were transfected with an NFκB luciferase construct and a Renilla-luciferase internal control, together with 100 ng empty vector (EV) or cDNA encoding A46R. Six hours prior to harvesting, cells were stimulated with 100 ng/ml IL-1 or 100 ng/ml TNF-α. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.4: A46R inhibits IL-1, but not TNF-α, induced p65 transactivation in HEK 293 cells



HEK 293R1 cells (2 x 10^4 cells per well) were transfected with the pFR-luciferase reporter, a Renilla-luciferase internal control and a plasmid encoding the DNA-binding domain of the yeast protein Gal4 fused to p65, along with either 100 ng empty vector (EV) or A46R expression plasmid. Six hours prior to harvesting, cells were stimulated with 100 ng/ml IL-1 or 100 ng/ml TNF-α. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

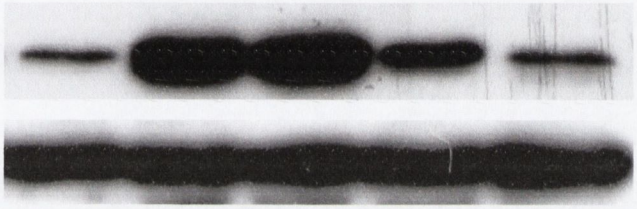
Figure 3.5: A46R inhibits IL-1, but not TNF-α, induced p38 MAP kinase activation in HEK 293 cells



HEK 293R1 cells (2 x 10^4 cells per well) were transfected with the pFR-luciferase reporter, a Renilla-luciferase internal control and a plasmid encoding the DNA-binding domain of the yeast protein Gal4 fused to CHOP, along with either 100 ng empty vector (EV) or A46R expression plasmid. 50 ng of MEK3, the positive control for this assay, was also transfected with and without A46R. Six hours prior to harvesting, cells were stimulated with 100 ng/ml IL-1 or 100 ng/ml TNF-α. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.6: A46R inhibits IL-1, and not TNF-α, induced p38 MAP kinase activation as measured by an ATF-2 kinase assay

Lanes	1	2	3	4	5
Flag-p38	+	+	+	+	+
ATF-2 Fusion	+	+	+	+	+
TNF-α	-	+	+	-	-
IL-1	-	-	-	+	+
A46R	-	-	+	-	+



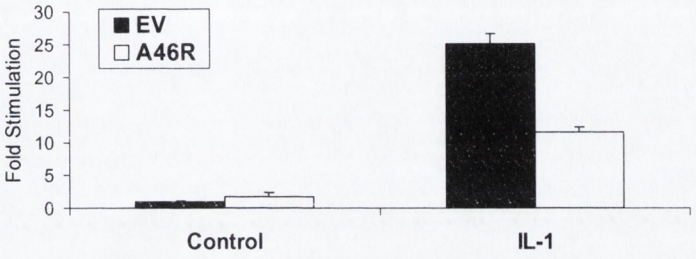
Anti-phospho ATF2

Anti-Flag

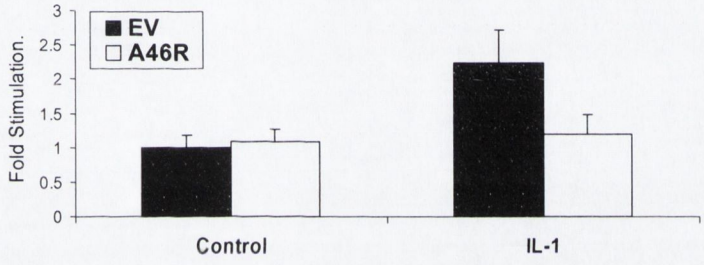
HEK 293R1 cells were seeded into 100 mm dishes (1.5 x 10^6 cells) and transfected with 4 μg A46R where indicated and 4 μg Flag-p38. The total amount of DNA (8 μg) was kept constant by supplementation with vector DNA. For the samples in lanes 2 to 5, the cells were treated with either TNF-α (100 ng/ml) or IL-1 (100 ng/ml) for 15 minutes prior to harvesting. Cells were harvested 24 h post transfection, 50 μl was removed for probing with anti-Flag to determine equal transfection and the kinase assay was performed on the remainder as described in section 2.8. Samples were assayed for phosphorylated ATF-2 or Flag-tagged p38 by immunoblotting. Identical results were obtained in an additional experiment.

Figure 3.7: A46R inhibits other IL-1-induced signals

A: ERK

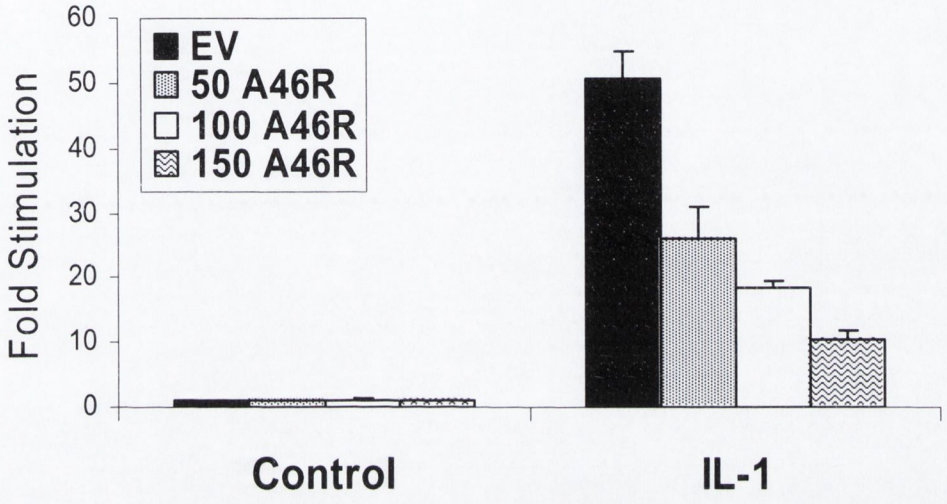


B: JNK



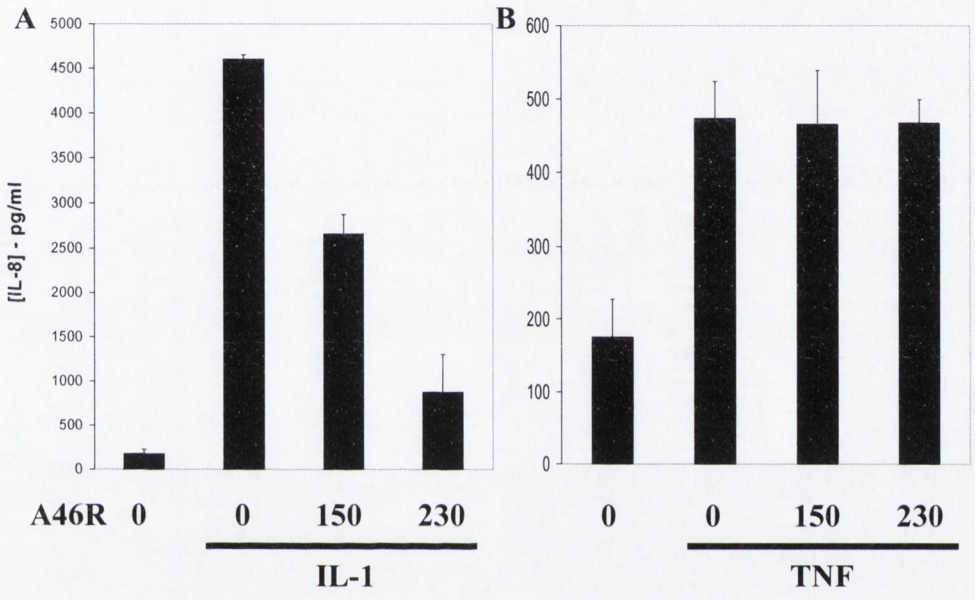
HEK 293R1 cells (2 x 10^4 cells per well) were transfected with the pFR-luciferase reporter, a Renilla-luciferase internal control and plasmids encoding either Gal4 fused to Elk1 (for ERK1/2) (A) or c-JUN (for JNK) (B), along with either 100 ng empty vector (EV) or A46R. Six hours prior to harvesting, cells were stimulated with 100 ng/ml IL-1. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.8: A46R inhibits IL-1-induced activation of the IL-8 promoter in a reporter gene assay



HEK 293R1 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and an IL-8-luciferase construct, along with empty vector (EV) and the indicated amount (ng) of A46R expression vector. Six hours prior to harvesting, cells were stimulated with 100 ng/ml IL-1. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.9: IL-1, and not TNF-α, induced IL-8 production is inhibited by A46R



HEK 293R1 cells (2 x 10^4 cells per well) were seeded into 96-well plates and transfected with either empty vector or the indicated amounts (ng) A46R expression plasmid 24 h later. 24 h after transfection, cells were stimulated with either 100 ng/ml IL-1 (A) or 100 ng/ml TNF-α (B). After 24h supernatants were harvested and the IL-8 concentration was determined by ELISA (R&D Biosystems). This experiment was performed once in triplicate and data is expressed as mean ± SD from one representative experiment.

Figure 3.10: A46R interacts with MyD88

A: Coimmunoprecipitation

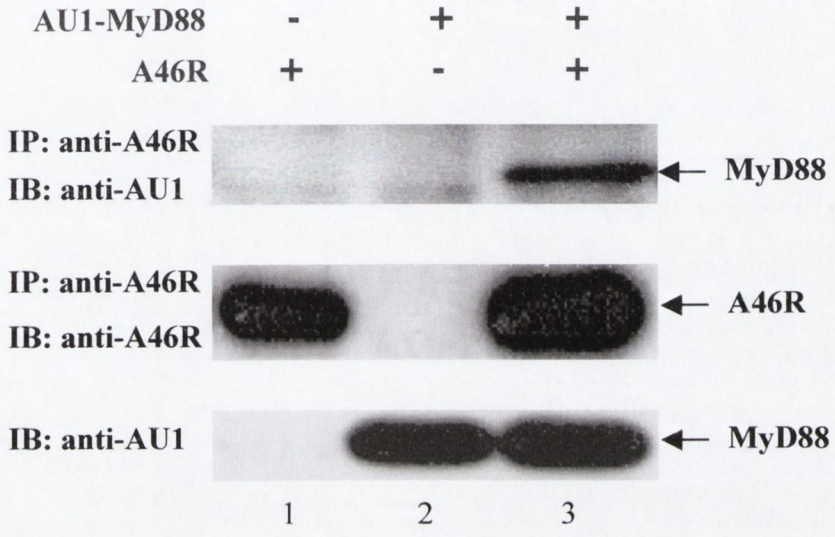
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection. 4 μg of each construct was transfected. Where only one construct was expressed the total amount of DNA (8 μg) was kept constant by supplementation with vector DNA. Cells were harvested 24 h post-transfection and the immunoprecipitation experiment carried out as described. The top panel corresponds to lysates immunoprecipitated with anti-A46R, and blotted with anti-AU1, to determine whether or not an interaction between two proteins has taken place. The band in lane 3 of the upper panel indicates an interaction between MyD88 and A46R. The second panel corresponds to lysates immunoprecipitated and blotted anti-A46R in order to show that equal levels of protein were immunoprecipitated. The lower panel corresponds to lysates directly blotted for overexpressed MyD88.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection. 8 μg of AU1-MyD88 was transfected. Cells were harvested 24 h post transfection and the GST-pulldown experiment carried out as described. Lane 1 corresponds to lysate directly blotted for expression of MyD88, lane 2 corresponds to lysate incubated with GST and lane 3 corresponds to lysate incubated with GST-A46R. The band in lane 3 indicates an interaction between A46R and MyD88.

Figure 3.10: A46R interacts with MyD88

A: Coimmunoprecipitation



B: GST Pulldown Assay

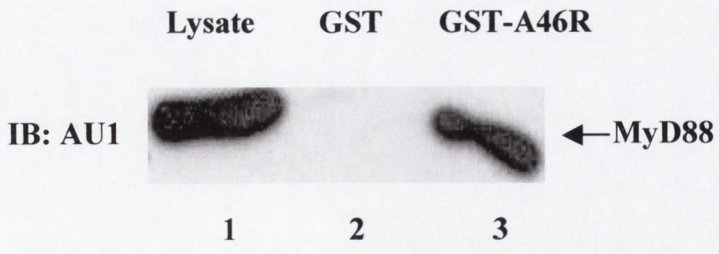


Figure 3.11: A46R does not interact with TRAF2

A: Coimmunoprecipitation

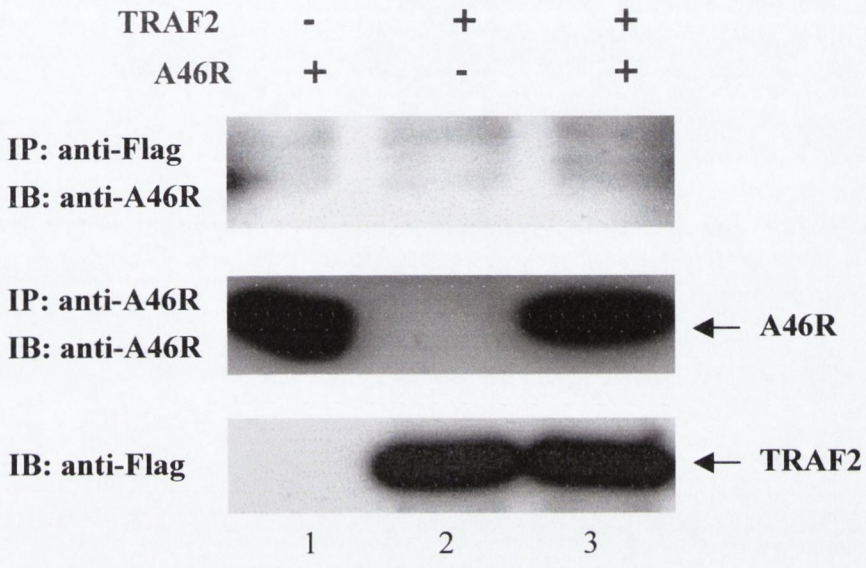
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The absence of a band in lane 3 of the upper panel indicates that no interaction occurred.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of Flag-TRAF2 expression vector and henceforth treated as described in Figure 3.10. The absence of a band in lane 3 indicates that no interaction occurred.

Figure 3.11: A46R does not interact with TRAF2

A: Coimmunoprecipitation



B: GST Pulldown Assay

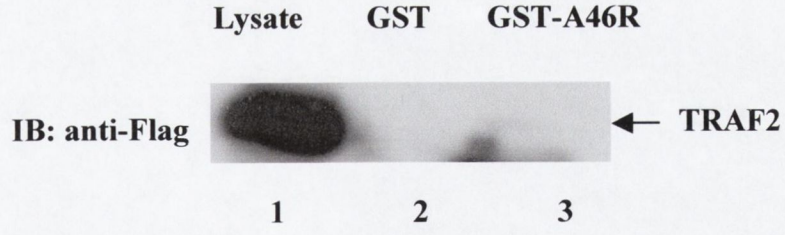


Figure 3.12: A46R does not interact with the MyD88 P200H mutant

A: Coimmunoprecipitation

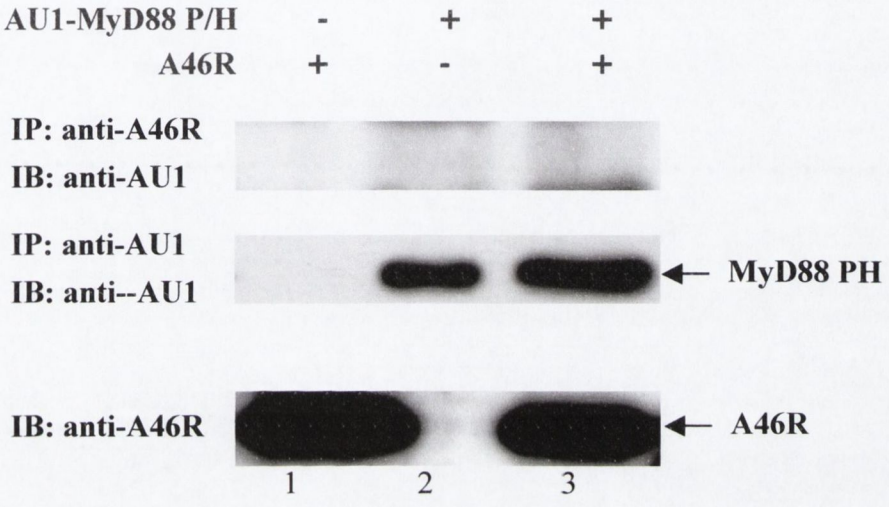
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The absence of a band in lane 3 of the upper panel indicates that no interaction occurred.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of AU1-MyD88 PH expression vector and henceforth treated as described in Figure 3.10. The absence of a band in lane 3 indicates that no interaction occurred.

Figure 3.12: A46R does not interact with the MyD88 P200H mutant

A: Coimmunoprecipitation



B: GST Pulldown Assay

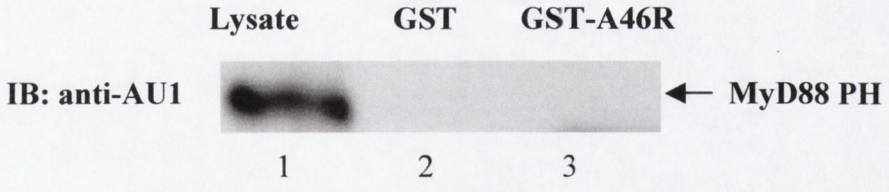


Figure 3.13: A46R interacts with the TIR domain of MyD88

A: Coimmunoprecipitation

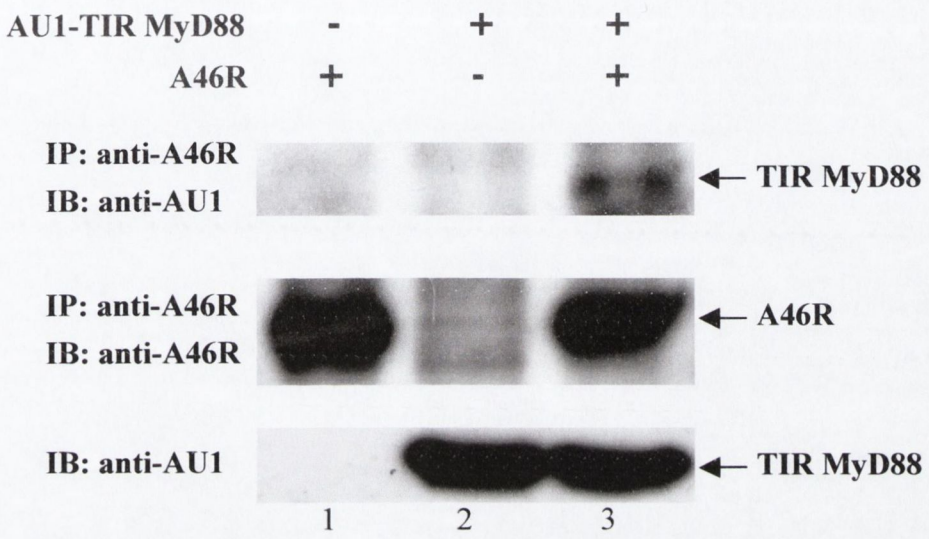
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The band in lane 3 of the upper panel indicates an interaction between the TIR domain of MyD88 and A46R.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding AU1-TIR MyD88 and henceforth treated as described in Figure 3.10. The band in lane 3 indicates an interaction between the TIR domain of MyD88 and A46R.

Figure 3.13: A46R interacts with the TIR domain of MyD88

A: Coimmunoprecipitation



B: GST Pulldown Assay

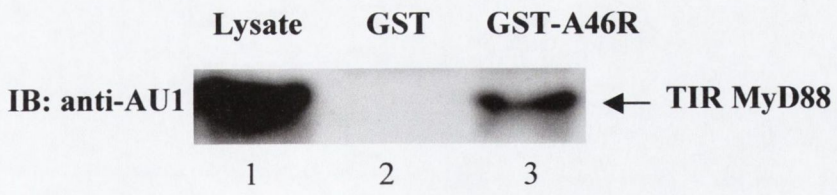


Figure 3.14: A46R interacts with the Death domain of MyD88

A: Coimmunoprecipitation

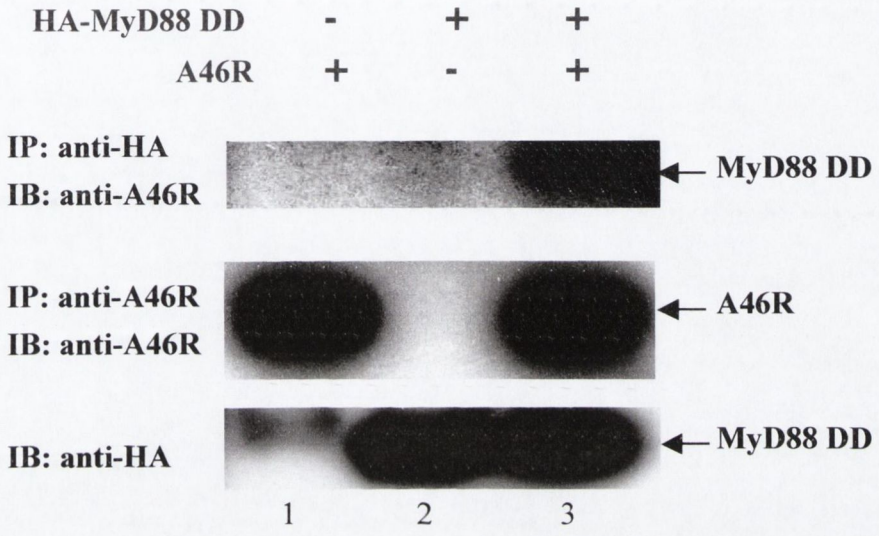
HEK 293T cells were seeded into 100 mm dishes (1.5 x 106 cells) 24 h prior to transfection and treated as described in Figure 3.10. The band in lane 3 of the upper panel indicates an interaction between the death domain of MyD88 and A46R.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 106 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding HA-MyD88 DD and henceforth treated as described in Figure 3.10. The band in lane 3 indicates an interaction between the death domain of MyD88 and A46R.

Figure 3.14: A46R interacts with the Death Domain of MyD88

A: Coimmunoprecipitation



B: GST Pulldown Assay

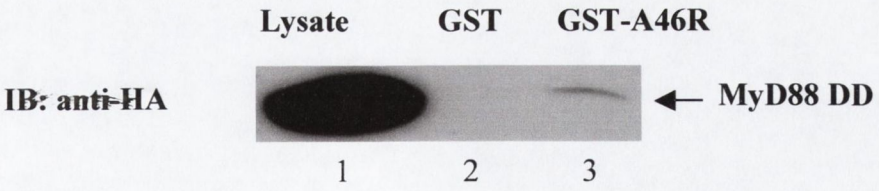
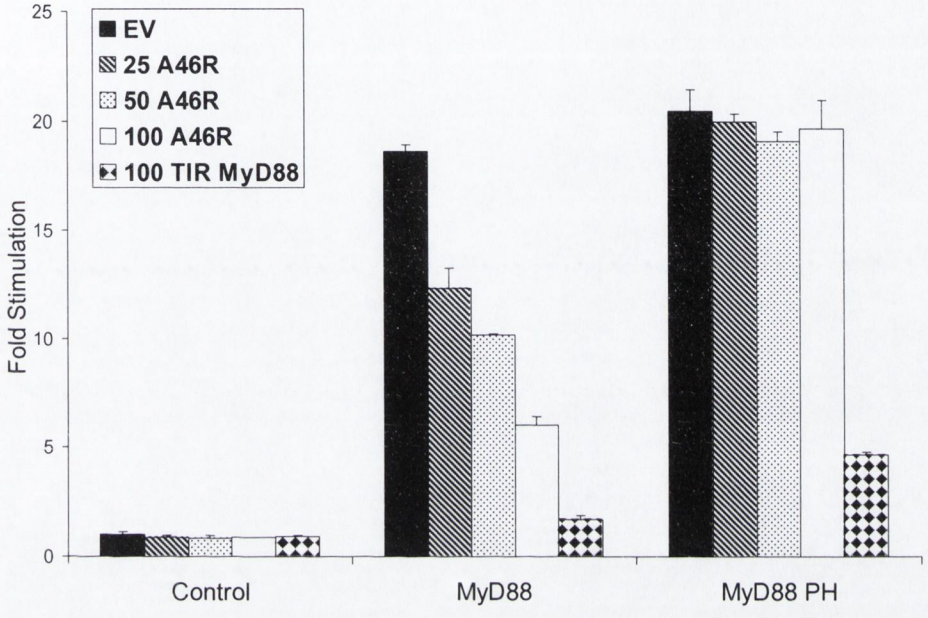


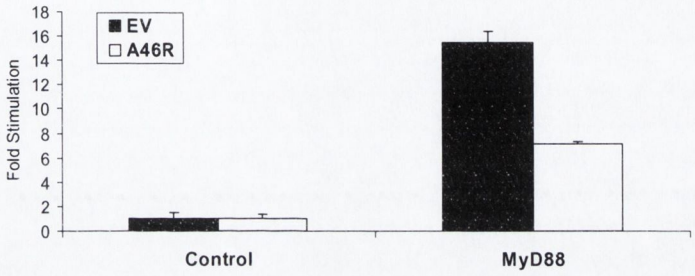
Figure 3.15: A46R inhibits MyD88-dependent NFkB activation



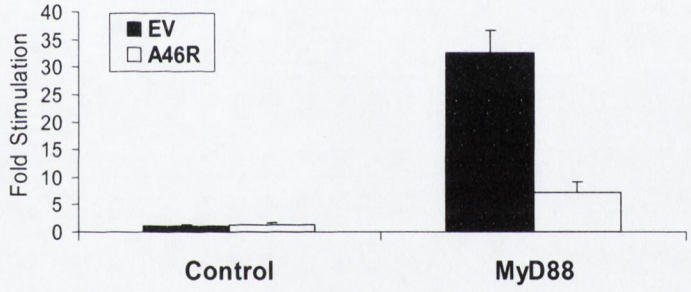
HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and a κB-luciferase construct together with 25 ng of either MyD88 or MyD88 PH, along with empty vector (EV), the indicated amount (ng) of A46R expression vector or 100 ng of plasmid encoding the TIR domain of MyD88 (TIR MyD88). Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.16: A46R inhibits MyD88-dependent signals

A: p65



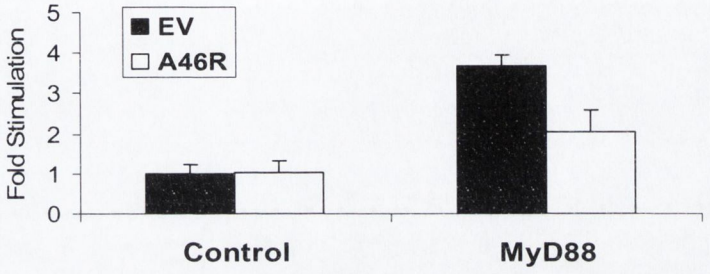
B: ERK



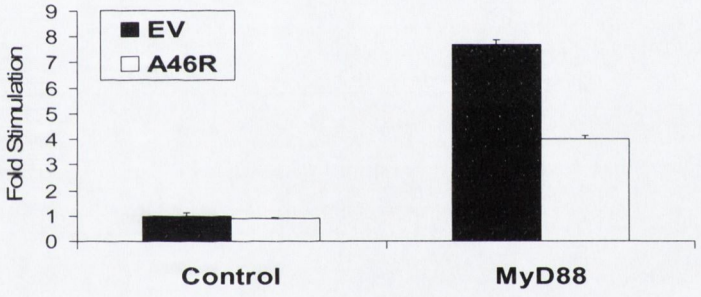
HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control, the pFR-luciferase reporter and 25 ng cDNA encoding MyD88, in the presence or absence of 100 ng empty vector (EV) or A46R expression plasmid, together with plasmids encoding Gal4 fused to p65 (A) or Elk1 (for ERK1/2) (B). Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.17: A46R inhibits MyD88-dependent signals

A: JNK

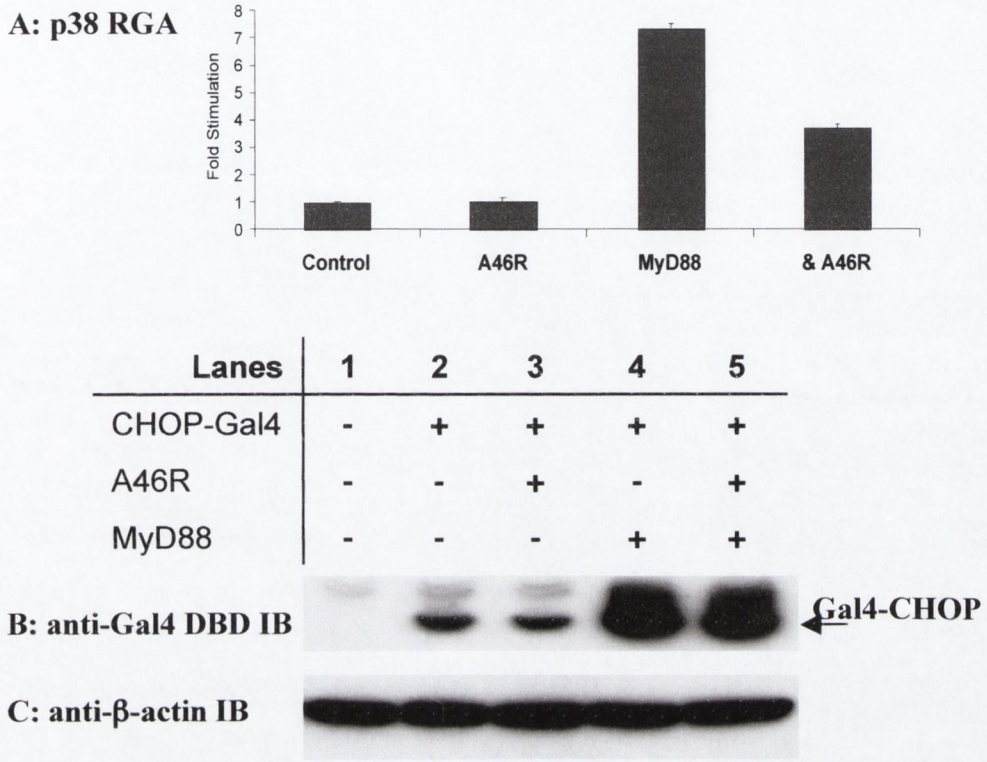


B: p38



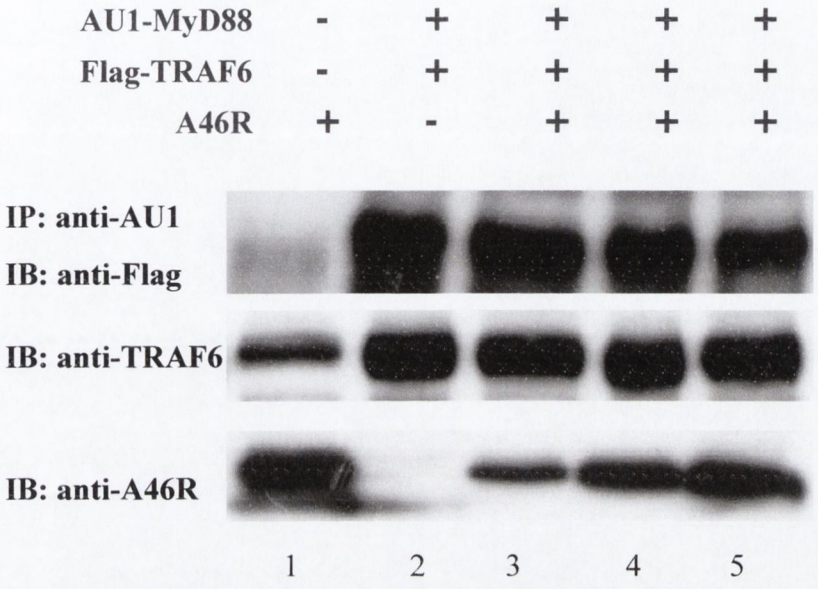
HEK 293 cells (2 x 104 cells per well) were transfected with a Renilla-luciferase internal control, the pFR-luciferase reporter and 25 ng cDNA encoding MyD88, in the presence or absence of 100 ng empty vector (EV) or A46R expression plasmid, together with plasmids encoding Gal4 fused to c-Jun (for JNK) (A) or CHOP (for p38) (B). Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 3.18: CHOP-Gal4 fusion protein expression control



HEK 293T cells (2 x 10^5 cells per well) were transfected with 600 ng pFR-luciferase reporter, 200 ng Renilla-luciferase internal control and 2.5 ng of plasmid encoding Gal4 fused to CHOP in the presence or absence of 250 ng MyD88, together with 1 μg A46R cDNA. Cells were harvested 24 hours after transfection, and analysed by both luciferase assay (A) and by western blot (B and C). Data are expressed as mean fold induction relative to control levels. Identical results were obtained in an additional experiment.

Figure 3.19: A46R slightly impairs formation of the MyD88-TRAF6 complex



HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection. 2 μg of both AU1-MyD88 and Flag-TRAF6 were transfected, in the presence of increasing amounts of A46R (2, 4 and 6 μg). Where only one construct was expressed the total amount of DNA (10 μg) was kept constant by supplementation with vector DNA. Cells were harvested 24 h post transfection and the immunoprecipitation experiment carried out as described. The top panel corresponds to lysates immunoprecipitated with anti-AU1 antibody, and blotted with anti-TRAF6 antibody, to determine whether the interaction between the two proteins has been affected by the presence of A46R. The lower two panels correspond to lysates directly blotted for TRAF6 and A46R.

3.3 Discussion

The identification of viral immunoevasive strategies and the analysis of the molecular aspects of host-pathogen interactions are crucial to enhancing our understanding of microbial pathogenesis and immunity to infection. Given the emerging importance of the TLR system in the anti-viral response, identifying and understanding how viruses target this receptor family is of particular interest. Both IL-1 as a potent pyrogen and IL-18 as an inducer of TNF, chemokines and IFN-γ, are important targets for viruses. The VV IL-1 receptor homologue, B15R, binds both human and murine IL-1β (Smith and Chan, 1991). The inactivation of B15R decreases virus virulence in mice after intercranial injection (Spriggs *et al.,* 1992), but enhances virulence when administered intranasally (Alcami and Smith, 1992). Many members of the poxvirus family, including the Molluscum contagiosum virus, vaccinia virus and cowpox virus encode functional IL-18 binding proteins, which have been shown to blunt the host response to viral infection (reviewed in Seet *et al.*, 2003). Specifically, the vaccinia virus IL-18-binding protein, C12L, promotes virulence by reducing IFNγ production and natural killer and T-cell activity (Reading and Smith, 2003b). During a database search for novel TIR domain containing proteins, A46R from VV was identified (Bowie *et al.*, 2000). This was potentially interesting, since many poxviral immunoevasive proteins, such as cytokine binding proteins, bear sequence similarity to host factors (Alcami and Koszinowski, 2000). To date, A46R is the only identified viral TIR domain-containing protein.

Given the sequence similarity of A46R with the TIR domain, Bowie *et al.* (2000) hypothesised that A46R might represent a previously unidentified VV strategy directed against IL-1R/TLR signalling. A46R was transfected into mammalian cells and tested

for its ability to block activation of an NFκB-dependent reporter gene induced by IL-1. Preliminary experiments found that ectopic expression of A46R could inhibit IL-1-induced NFκB activation, but did not have an inhibitory effect on TNF-α-induced NFκB activation. These results were considered very promising and worthy of further investigation. Although IL-1 and TNF-α activate similar signals, different adapter molecules are involved (no TIR molecules are involved in TNF signalling). In this study, I expanded on the initial results obtained by Bowie and colleagues and investigated the effect of A46R on a wide range of IL-1 signals, such as p65 transactivation, MAPK activation and IL-8 induction. A46R inhibited all IL-1 signals tested, but not TNF-α signals.

IL-1 stimulation of HEK 293R1 cells induces activation of NFκB and p38, both of which can effect the transcription of IL-8 (Baldwin A, 1996; Hoffman *et al.*, 2002). Considering A46R inhibits both IL-1 induced NFκB and p38 activation, it is unsurprising that A46R inhibited IL-1 induced IL-8 production. However, A46R did not inhibit production of IL-8 induced by TNF-α (Figure 3.9) nor PMA (C. Greene, personal communication). Transient transfection of HEK 293R1 cells with A46R suppressed IL-1-induced IL-8 release by approximately 80%. This is suggestive of a very potent effect on IL-8 induction given that not all of the cell population stimulated by IL-1 to release IL-8 would be expected to be expressing A46R in this transient transfection system.

Because other viral inhibitors of IL-1 signalling have been shown to be important for VV virulence, a VV deletion mutant lacking the A46R gene was constructed and demonstrated that the protein did in fact contribute to virus virulence *in vivo* (Stack *et*

al., 2005). Deletion of A46R from VV led to an attenuation of the virus in a murine intranasal infection model. The use of a revertant virus, in which the A46R gene was re-inserted into the deletion virus confirmed that the attenuation observed in the deletion virus was due solely to the absence of A46R (Stack *et al.*, 2005). The ability of A46R to target intracellular TIR-dependent signalling most likely accounts for its role in virulence, probably by blocking the induction of immune response genes downstream of IL-1 (Chapter 3) and TLRs (Chapter 6), as has been shown here for IL-1-dependent induction of the chemokine IL-8.

Because A46R inhibited so many IL-1 signals, it was hypothesised that it must act close to the IL-1 receptor complex before the signal bifurcates. Consistent with this theory, p38 activation induced by MEK3, a kinase which phosphorylates p38 and is therefore a downstream signalling molecule, is not inhibited by A46R (Figure 3.6). TNF activates a signal transduction pathway that does not require MyD88, but all IL-1 signals tested to date have been shown to be dependent on MyD88 (Adachi, *et al.*, 1998), including p65 transactivation (Jefferies *et al.*, 2001) and JNK activation (Burns *et al.*, 1998). Also, MyD88-deficient cells do not respond to IL-1 (Kawai, *et al.*, 1999). Therefore, I reasoned that A46R may target MyD88. This seemed likely given that A46R has a TIR domain (Bowie *et al.*, 2000), as illustrated in Figure 1.12 which shows an alignment of some human TIR-containing proteins with VV and variola virus A46R.

On further investigation, I found that A46R interacted with MyD88, and not with TRAF2, an adapter molecule involved in TNF-α signalling. This was consistent with the lack of effect of A46R on TNF-α signalling. Neither did A46R interact with TAB1, a signalling molecule downstream of MyD88 (data not shown). These interactions, or

lack of, were confirmed by GST-pulldown. I repeated the immunoprecipitation and GST-pulldown experiments using different conditions, including Flag-A46R, myc-MyD88 and an anti-MyD88 polyclonal antibody, and consistently observed A46R interacting with MyD88. The interaction of A46R with MyD88 has also been demonstrated in cells infected by VV and transfected to express MyD88, to use the A46R protein at its physiological level (Stack *et al.*, 2005). I tried to test whether or not A46R interacted with endogenous MyD88, but the anti-MyD88 antibody was not good enough. However, with increasingly sophisticated reagents and antibodies specifically for the study of TLR signalling becoming available all the time, it is highly probable that these experiments will be realised. A46R interacted with MyD88 in a yeast-two-hybrid pairwise assay (Stack *et al.*, 2005), thus demonstrating that the association was direct. To further demonstrate a direct interaction between A46R and MyD88, I attempted to generate recombinant MyD88, but was unable to cleave GST-MyD88. This is the first example of a viral protein targeting MyD88.

The functional consequence of the A46R-MyD88 interaction was that signals activated by ectopic expression of MyD88 were sensitive to A46R inhibition. A46R was capable of inhibiting MyD88-mediated activation of NFκB and p65 (Figure 3.15 and 3.16.A). MyD88-dependent MAP kinase activation was also inhibited (Figure 3.16.B and 3.17).

To elucidate how A46R inhibited MyD88-dependent signalling, I examined the MyD88-A46R interaction more closely. The S interface of TIR domains mediates the association between the receptor and adapter TIR domains, and the formation of this TIR domain complex is critical for receptor signalling (Xu *et al.*, 2000). Interactions at the S face are primarily mediated by a highly conserved region among the TIR

domains. Because a large number of TLRs signal through a common adapter molecule, MyD88, it is logical that the receptors must present a conserved surface area (i.e. the S interface) for coupling to the downstream adapter. The site of interaction between MyD88 and receptors of the TLR/IL-1R superfamily is purported to be an extended loop in box 2 of the TIR domain, the BB loop, which contains an RDx$\Phi_1\Phi_2$G motif (where x represents any amino acid and Φ represents a hydrophobic residue) with three highly conserved residues (Xu *et al.,* 2000). The Φ_2 proline residue is conserved in all TLRs, except for TLR3 (which does not use MyD88 to transmit its signals) in which it is replaced by alanine (Xu *et al.,* 2000). In the BB loop of A46R, the sequence is R-N-T-I-S-G. The arginine (R) and glycine (G) residues are conserved, the conserved aspartate (D) is replaced with the closely related asparagine (N) and Φ_1 is isoleucine, a hydrophobic residue. However, Φ_2 is not the conserved hydrophobic proline but a serine instead. Serine is a hydroxylated version of alanine, a hydrophobic residue. This hydroxyl group not only makes Serine slightly more hydrophilic than alanine, but also more reactive, which could be important in the function of A46R as an inhibitor. This hypothesis is being explored using mutagenesis studies; the inhibition and interaction profiles of all A46R mutants generated will be tested.

A single point mutation in the TIR domain of TLR 2 (P681H) disrupts signal transduction in response to stimulation by yeast and Gram-positive bacteria (Underhill *et al.*, 1999) without causing any significant structural differences to wild-type TLR 2 (Xu *et al.*, 2000). This same mutation does not affect the oligomerisation of the receptor, but renders TLR2 unable to immunoprecipitate with MyD88 (Xu *et al.*, 2000). Thus, it was hypothesised that the TLR4 Pro→His in the Φ_2 position of its TIR domain mutation might prevent interaction of TLR4 with MyD88, thus explaining the LPS non-

responder phenotype of CH3/HeJ mice (Buchanan and Gay, 1996). This theory was confirmed by Rhee and Hwang (2000). However, Dunne and colleagues (2003) observed MyD88 interacting with the TLR4 PH mutant and the necessity of the TLR4 conserved proline for recruitment of MyD88 remains controversial. Dunne *et al.* (2003) also noted that the MyD88 P200H mutation has no effect on MyD88-TLR4 interaction (consistent with their model in which the BB loop of MyD88 does not form a point of contact with the TLR TIR domain). However, one cannot rule out the possibility that MyD88 was recruited to the mutant receptor *via* endogenous Mal should the latter bind at a distinct surface.

The MyD88 P200H mutation does not affect ability of MyD88 to activate NFκB. A46R did not interact with the MyD88 P200H mutant, which was consistent with A46R not inhibiting MyD88 PH mediated NFκB activation (Figure 3.15). The TIR mutant of MyD88 does not activate NFκB and can be used as a dominant negative (Fitzgerald *et al.*, 2001). Consistent with this, peptides derived form the MyD88 TIR domain are very effective in inhibiting homodimerisation of MyD88 with either full-length MyD88 or its isolated TIR domain (Loiarro *et al.*, 2005). TIR MyD88 can inhibit both MyD88 and MyD88 PH induced NFκB activation (Figure 3.15), suggesting that the MyD88 Pro200 is not required for MyD88 TIR domain homotypic interactions. A46R interacts with the TIR domain of MyD88. The TIR-TIR interaction is presumably how A46R interacts with full-length MyD88, with MyD88's conserved Pro200 being required for the interaction to occur. It was therefore surprising to discover that A46R also interacted with the DD of MyD88. The fact that IRAK-2, which contains a DD, interacts with Mal, which has little more than a TIR domain, suggests that DD and TIR domains are capable of interaction (Fitzgerald *et al.*, 2001).

Another VV protein, A52R, which is related to A46R, has been shown previously to block the activation of NFκB by IL-1, IL-18 and TLR4, with little inhibitory effect on the activation of NFκB by TNF-α (Bowie *et al.*, 2000). Further investigation revealed that induction of NFκB by overexpression of CD4-TLR5, or a combination of either CD4-TLR2 and 1 or CD4-TLR 2 and 6 was inhibited by co-expression of A52R (Harte *et al.*, 2003). A52R was found to interact with TRAF6 and IRAK2, and interestingly, to disrupt signalling complexes containing these proteins. As the expression of A52R increased, the abilities of TRAF6-TAB1 and IRAK2-Mal to form complexes were severely impaired. Considering these data, I investigated whether or not A46R also behaves in such a way. MyD88 associates with the IL-1RAcP, and this interaction was not disrupted by A46R (data not shown). However, the formation of the MyD88-TRAF6 complex was slightly impaired by the presence of A46R (Figure 3.19). Compared to the dramatic effect of A52R on the TRAF6-TAB1 and IRAK2-Mal complexes in which the interaction is completely abrogated at the highest dose of A52R (Harte *et al.*, 2003), the effect of A46R on the MyD88-TRAF6 complex was very subtle and did not appear to be strong enough to account for the potent inhibition of MyD88 signalling by A46R. It would be interesting to examine whether the presence of A46R has an inhibitory effect on the interaction of MyD88 with either IRAK-1 or IRAK-2, both of which are important in MyD88-dependent signal transduction, especially because the MyD88-TRAF6 interaction may not be direct. MyD88 does not have a TRAF binding motif (Mansell *et al.*, 2004), and probably associates with TRAF6 *via* IRF7, IRAK-1 or IRAK-2.

As yet unstudied, is the possibility that when interacting with MyD88, A46R might mask the intermediate domain (ID) of MyD88, thus inhibiting NFκB activation.

MyD88s, an alternatively spliced version of MyD88 which lacks the ID, cannot recruit IRAK-4; hence IRAK-1 is not phosphorylated and NFκB is not activated (Burns *et al.*, 2003). Another possibility, again not yet investigated, is that rather than disrupting signalling complexes, A46R might actively prevent the dissociation of signalling molecules. For example in the case of IL-1 induced NFκB activation, phosphorylated IRAK-1 dissociates from the IL-1R-MyD88 complex and associates with TRAF6 at the plasma membrane (Jiang *et al.*, 2002). Therefore, the subtle effect of A46R on the MyD88-TRAF6 complex might actually reflect its inhibitory action.

Chapter Four

A46R Targets Multiple TIR Adapter Molecules & Inhibits TLR Signalling to NFκB and MAPKs

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4.1 Introduction

In the previous chapter, I demonstrated that A46R inhibited IL-1 signalling by targeting MyD88, and that TNF-α signals were unaffected. However, MyD88 is essential not only for IL-1 signalling, but also for responses against a broad range of microbial components *via* TLRs. MyD88-deficient mice do not produce TNF or IL-6 when exposed to IL-1 or microbial components that are recognised by TLR2, TLR4, TLR5, TLR7 or TLR9 (reviewed by Takeda *et al.*, 2003), implying that MyD88 was the only TIR adapter molecule involved in these signals and thus suggesting that A46R might target these signals. However, it was found that LPS-induced activation of NFκB and JNK, although delayed, still occurred in MyD88-deficient mice (Kawai *et al.*, 1999), as does DC maturation (Kaisho *et al.*, 2001), implying that an additional adapter was required for a subset of LPS-inducible, TLR4 dependent signals.

The first of these additional receptors to be described was MyD88 adapter-like (Mal) or TIR adapter protein (TIRAP), (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001) which, like MyD88, activated NFκB, JNK and ERK1/2 when overexpressed. A dominant negative form of Mal inhibited NFκB which is activated by TLR4 or LPS, but not NFκB activated by IL-1R or IL-18R (Fitzgerald *et al.*, 2001). Mal interacted with MyD88, TLR4 (Fitzgerald *et al.*, 2001) and TLR2 (A. Dunne, personal communication). Mal also interacted with both IRAK-2 (Fitzgerald *et al.*, 2001) and TRAF6 (Mansell *et al.*, 2004).

When Mal knockout mice were generated they were found to have delayed kinetics in the activation of NFκB and MAPKs in response to LPS, and were entirely resistant to LPS-induced shock (Yamamoto *et al.*, 2002a; Horng *et al.*, 2002). They responded

normally to the TLR3, TLR5, TLR7 and TLR9 ligands, as well as to IL-1 and IL-18, but were severely impaired in their responses to ligands for TLR2. Thus for TLR4 and TLR2, the phenotype of Mal deficient mice was very similar to that displayed by MyD88 deficient mice. These results showed that Mal is not specific to TLR4 signalling but has a crucial role in the MyD88-dependent pathway shared by TLR2 and TLR4 (Yamamoto *et al.*, 2002a; Horng *et al.*, 2002).

Two further adapters, TRIF (TIR-domain containing adapter Inducing IFN-β) (Yamamoto *et al.*, 2002b) and TRAM (TRIF related adapter molecule) (Fitzgerald *et al.*, 2003a) have been found to play a role in TLR4 signalling. Mice with a frameshift error in *Trif* are markedly resistant to the toxic effects of LPS, and compound homozygosity for mutations at *Trif* and *MyD88* loci ablates all response to LPS (Hoebe *et al.*, 2003; Yamamoto *et al.*, 2003a). Instead of the usual conserved proline in its BB-loop sequence, TRAM contains a cysteine residue at this position (Bin *et al.*, 2003). When this cysteine was mutated to a histidine LPS-induced NFκB was specifically inhibited (Fitzgerald *et al.,* 2003a), implying a TLR4-specific role for TRAM which was confirmed by Yamamoto and colleagues (2003b) when their TRAM-deficient mice showed normal responses to ligands for TLR2, TLR3, TLR7, TLR9 and IL-1β, but defective macrophage responses to LPS in terms of NFκB activation, cytokine production and B cell activation. It has been suggested that TRAM acts as a physical bridge between TLR4 and TRIF and functionally transmits LPS-TLR4 signalling to TRIF (Oshiumi *et al.*, 2003b). TRIF has been found to not interact with TLR4, lending credence to this theory (Oshiumi *et al.*, 2003a). A fifth TIR adapter molecule, SARM (sterile α and armadillo motifs), has recently been identified but has yet to be implicated in mammalian TLR signalling (Couillault *et al.*, 2004; Liberati *et al.,* 2004).

In this chapter, I show that A46R inhibits every murine TLR pathway to NFκB activation known to involve MyD88 in RAW264.7 cells. A46R was also found to inhibit TLR4 induced NFκB and MAPK activation in HEK 293 cells. Inhibition of TLR4 signalling by A46R was especially potent. Consistent with this observation, A46R was shown to interact with Mal, TRIF and TRAM as well as MyD88, and to inhibit NFκB activated by all of these molecules. A46R did not interact with SARM, a TIR adapter molecule which is not involved in TLR signal transduction.

4.2 Results

4.2.1 A46R inhibits many MyD88-dependent signals

Apart from its role in IL-1 signalling, MyD88 is also utilised by murine TLRs 1, 2, 4, 5, 6, 7 and 9 (reviewed by Takeda *et al.*, 2003). Therefore, I tested the effect of A46R on signalling *via* these TLRs in RAW264.7 cells, a murine macrophage cell line that expresses most TLRs (Applequist *et al.*, 2002). Stimulation of RAW264.7 cells with MALP-2, which activates NFκB *via* a TLR2/6 heterodimer, Pam3Cys, which activates NFκB *via* a TLR2/1 heterodimer, LPS (TLR4), Flagellin (TLR5), R-848 (TLR7), CpG DNA (TLR9), or PMA led to induction of the NFκB-dependent reporter gene, albeit to different extents (Figure 4.1). Ectopic expression of A46R caused strong inhibition of MyD88-dependent NFκB activation, while having no suppressive effect on control levels or on PMA-induced NFκB activation.

TLR8 is non-functional in mice (Jurk *et al.*, 2002). Therefore RAW 264.7 cells could not be used to examine the effect of A46R on TLR8-induced NFκB activation. Instead, HEK 293 cells which stably express TLR8 (HEK 293-TLR8) were used. Stimulation of HEK 293-TLR8 cells for 6 hours with R-848, a known human TLR8 ligand (Jurk *et al.*, 2002), resulted in NFκB activation. A single dose of A46R inhibited this NFκB activation (Figure 4.2). When HEK 293 cells which stably express pcDNA3.1 were stimulated with R-848, NFκB activation did not occur (data not shown).

4.2.2 A46R inhibits TLR4 signalling pathways

A chimeric version of TLR4, comprising the murine CD4 extracellular domain fused to the cytoplasmic domain of human TLR4 has been shown to render TLR4 constitutively active (Medzhitov *et al.*, 1997). Overexpression of CD4-TLR4 activates an NFκB

reporter gene and this signal was dose-dependently inhibited by A46R (Figure 4.3.A). In fact, the highest concentration of A46R-expressing plasmid almost completely prevented TLR4-induced NFκB activation, while not suppressing basal levels of reporter gene activity. A single dose of A46R inhibited CD4-TLR4-induced p65 transactivation (Figure 4.3.B), which provides a rationale for its inhibition of the NFκB-dependent reporter gene. A46R inhibited other TLR4 signalling pathways also. Expression of CD4-TLR4 activated ERK and p38 MAP kinases, as assayed by Pathdetect reporter gene assays (see Section 3.2.3). These signals were inhibited by a single dose of A46R (Figure 4.4). Despite many attempts using a variety of conditions, overexpression of CD4-TLR4 was not found to activate JNK as measured by a Pathdetect reporter gene assay.

4.2.3 A46R interacts with Mal

Given that TLR4 induced NFκB, p38 and ERK are only partially MyD88-dependent (Kawai *et al.*, 1999), it was difficult to account for the potent effects of A46R on these signals by an interaction with MyD88 alone. I therefore assessed the ability of A46R to target other TIR domain-containing proteins involved in the TLR4 receptor complex. The ability of A46R to interact with Mal was first tested. A46R was found to be capable of being co-immunoprecipitated with Mal (Figure 4.5.A), and this interaction was confirmed by a GST-pulldown experiment (Figure 4.5.B).

Because Mal acts as a bridging adapter for MyD88 in TLR4 signalling pathways (Horng *et al.*, 2001), it was possible that A46R may not have been directly interacting with Mal, but rather Mal was appearing to be coimmunoprecipitated in the experiment *via* its interaction with endogenous MyD88. However, evidence that the interaction

was direct was obtained from experiments using purified recombinant Mal (rMal) (prepared as described in Sections 2.10.1 and 2.10.3), and immobilised GST-A46R or GST. Figure 4.6 shows that A46R could bind rMal directly in an *i vitro* assay (Figure 4.6).

To examine how exactly A46R interacts with Mal, two Mal mutants were used. Both of these Mal mutants, namely, a truncated protein containing the TIR domain of Mal alone (TIR Mal), and the Mal Pro125His (Mal PH) have been found to function as dominant negatives for TLR4 and TLR2 signalling (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). In co-immunoprecipitation experiments, A46R interacted with TIR Mal (Figure 4.7.A), but not with Mal PH (Figure 4.8.A), demonstrating that A46R interacts with Mal *via* its TIR domain and that the conserved proline residue which is vital for Mal signalling is also required for the A46R-Mal interaction. Using GST-pulldown experiments, the TIR Mal-A46R (Figure 4.7.B) interaction and the lack of interaction between A46R and Mal PH (Figure 4.8.B) were confirmed.

4.2.4 Exploring the functional consequences of the A46R-Mal interaction

Having confirmed that A46R interacts directly with Mal, I tried to elucidate the significance of this interaction with respect to the inhibition of TLR4 signalling by A46R. Mal interacts with TLR4 directly (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001) and I hypothesised that A46R might impair the formation of this complex. A46R could interact with TLR4 in both co-immunoprecipitation and GST-pulldown experiments (Stack *et al.*, 2005), but the effect, if any, of A46R on the Mal-TLR4 interaction proved too difficult to examine. Instead, I investigated the effect of increasing amounts of A46R-expression plasmid on the association of overexpressed Mal and TLR2. Figure

4.9 shows that the interaction was unaffected at even the highest dose of A46R. Due to misadventure, much of the sample in lane 1 of the uppermost panel in Figure 4.9 was lost. This is why it appears that the presence of A46R enhances the Mal-TLR2 interaction, but that is not actually the case. Therefore, for the purpose of examining the effects of increasing A46R concentration, lanes 3, 4 and 5 should be compared.

Mal and MyD88 heterodimerise (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001), and I examined the possibility that perhaps A46R might interfere with this interaction, thus explaining its inhibition of TLR4 and TLR2 signalling. However, as can be seen in Figure 4.10, A46R does not affect the Mal-MyD88 interaction. At first glance, it might appear that the presence of 2 μg A46R expression plasmid severely affected the formation of the Mal-MyD88 heterodimer (lane 3 of uppermost panel), but on closer examination this proved not to be the case. For some reason sample 3 is much more dilute than the others (compare lane 3 to lane 4 in middle panel).

4.2.5 A46R inhibits Mal-dependent signals

As A46R has been shown to interact with Mal, its ability to interfere with Mal-dependent signalling was next investigated. Overexpression of Mal leads to activation of signals such as NFκB, p65 (Figure 4.11), and MAPKs (Figure 4.12). A46R dose-dependently inhibited Mal-dependent NFκB activation (Figure 4.11.A). A single dose of A46R expression plasmid was capable of inhibiting Mal-dependent p65 transactivation (Figure 4.11.B), which correlated with its effect on Mal-induced NFκB. Mal-dependent activation of ERK, JNK and p38 were all potently inhibited by ectopic expression of a single dose of A46R (Figure 4.12).

4.2.6 Exploring how A46R inhibits Mal-dependent signalling

I subsequently attempted to resolve how A46R was inhibiting Mal-dependent signalling. Mansell and colleagues (2004) have recently shown that Mal has a TRAF binding domain and can interact with TRAF6. From their data, they infer a specific role for Mal in TLR2 and TLR4 signalling in regulating NFκB-dependent gene transcription *via* this interaction. In order to examine whether A46R would affect this interaction, I first confirmed that Mal and TRAF6 could interact (data not shown). When increasing amounts of A46R expression plasmid were co-transfected with the interacting molecules (Figure 4.13), the interaction was slightly impaired (compare lanes 2 and 1 in the uppermost panel). Mal (middle panel) and TRAF6 (data not shown) expression levels were unaffected by the increasing expression levels of A46R (lower panel).

4.2.7 A46R interacts with the TIR adapter molecules, TRIF and TRAM

TRAM and Mal have similar roles in TLR4 signalling, in that they each act as bridging adapters between TLR4 and TRIF or MyD88, respectively (Oshiumi *et al.*, 2003b; Fitzgerald *et al.*, 2001). Considering that A46R interacts with both Mal and MyD88, which are responsible for early-phase TLR4 NFκB activation, I investigated whether A46R targeted the mediators of late-phase TLR4 NFκB activation, namely TRAM and TRIF. Using co-immunoprecipitation and GST-pulldown experiments, I found that A46R could interact with both TRAM (Figure 4.14) and TRIF (Figure 4.16). Interestingly, when the TIR domains of either TRIF or TRAM alone were expressed, the ability to interact with A46R was lost. Assuming that, like Mal and MyD88, the TIR domains of TRIF and TRAM are required for the interaction with A46R, these data

imply that other portions of TRIF and TRAM are also required for an interaction with A46R.

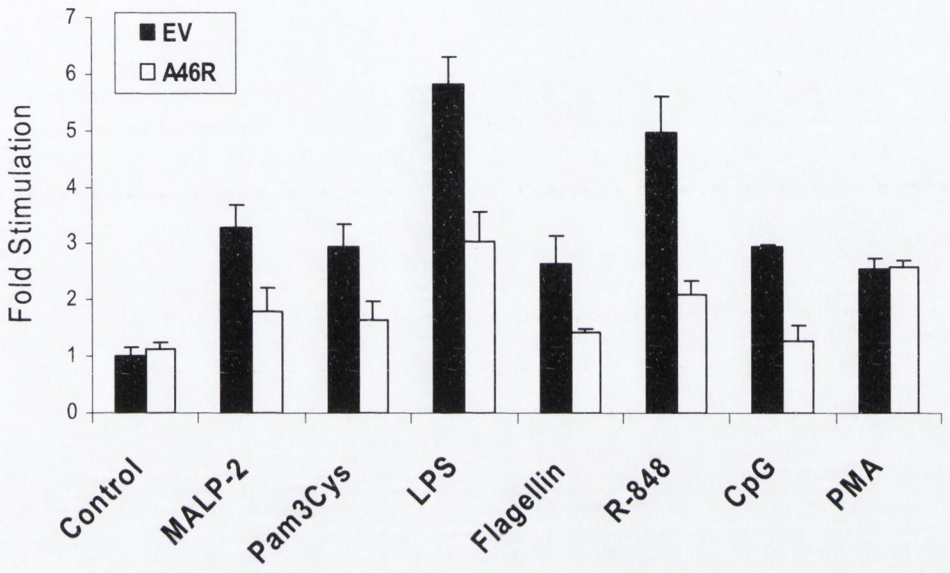
4.2.8 A46R inhibits TRIF and TRAM induced NFκB activation

Overexpression of TRIF and TRAM can both induce activation of an NFκB reporter gene construct (Yamamoto *et al.*, 2002b; Fitzgerald *et al.*, 2003a), similar to that observed with MyD88. Here, a single dose of A46R expression plasmid potently inhibited both TRIF- and TRAM-dependent NFκB activation (Figure 4.18).

4.2.9 A46R does not interact with SARM

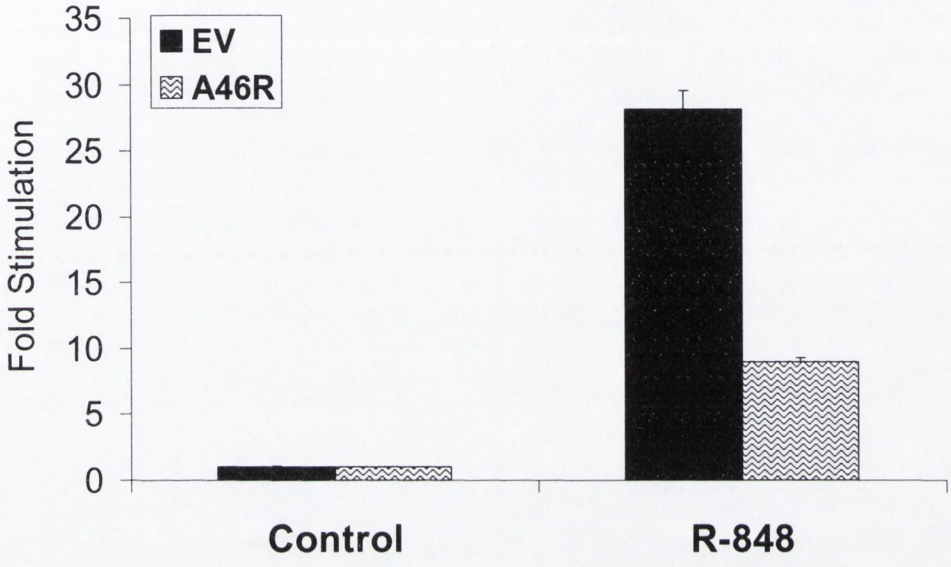
Because A46R interacted with so many TIR-containing molecules, a concern arose that perhaps it might interact with any overexpressed TIR-containing protein, which would question the physiological significance of the interactions. SARM is a cytosolic TIR molecule which does not play a positive role in TLR signalling to NFκB (Liberati *et al.*, 2004), and therefore should not represent a target for A46R. When A46R and Flag-tagged SARM were co-expressed and immunoprecipitation experiments carried out, no interaction between them could be observed (Figure 4.19.A). As a control, an experiment in which A46R and Flag-tagged Mal were co-expressed and immunoprecipitated was carried out in parallel. As expected, A46R and Flag-Mal interacted (data not shown). A GST-pulldown experiment confirmed the lack of interaction between A46R and SARM (Figure 4.19.B).

Figure 4.1: A46R inhibits MyD88-dependent, but not PMA-induced, NFκB activation in RAW 264.7 cells



RAW 264.7 cells (4 x 10^4 cells per well) were transfected with an NFκB luciferase construct and a Renilla-luciferase internal control, together with 100 ng empty vector (EV) or cDNA encoding A46R. Cells were stimulated for 6 hours with 125 nM MALP2, 5 μg/ml Pam3Cys, 1000 ng/ml LPS, 250 ng/ml Flagellin, 1 μM R-848, 5 μg/ml CpG or 10 ng/ml PMA, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

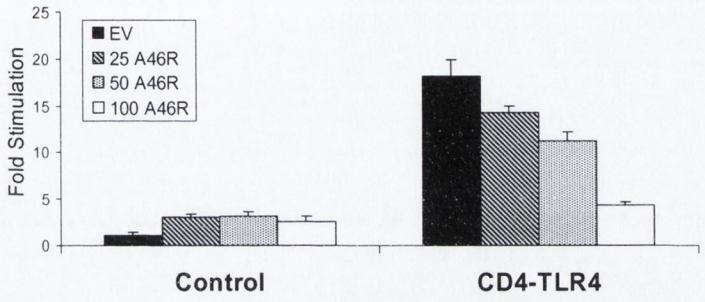
Figure 4.2: A46R inhibits TLR8-mediated NFκB activation



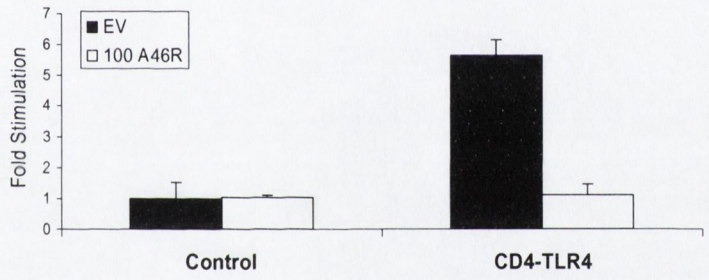
HEK 293 cells which stably express TLR8 (2 x 10^4 cells per well) were transfected with an NFκB luciferase construct and a Renilla-luciferase internal control, together with 150 ng empty vector (EV) or cDNA encoding A46R. Cells were stimulated with 1 μM R-848 for 6 h, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

Figure 4.3: A46R inhibits CD4-TLR4-mediated NFκB activation and p65 transactivation

A: NFκB



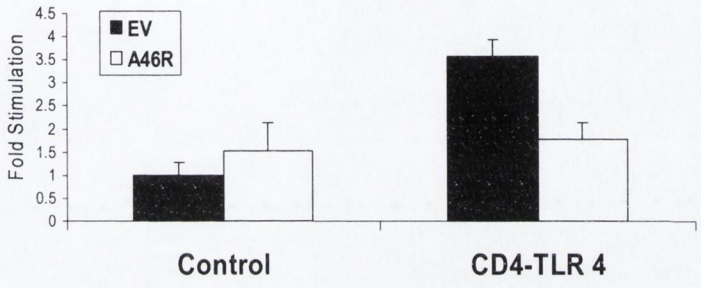
B: p65



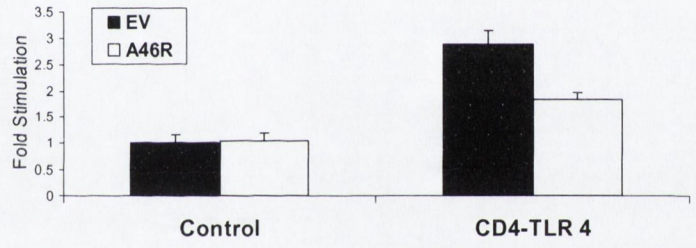
HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and either the κB-luciferase construct (A) or the pFR-luciferase reporter and a plasmid encoding Gal4 fused to p65 (B), in the presence or absence of 50 ng CD4-TLR 4, together with the indicated amount (ng) of A46R cDNA. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 4.4: A46R inhibits CD4-TLR4-mediated MAPK activation

A: ERK



B: p38



HEK 293 cells (2 x 10^4 cells per well) were transfected with 50 ng cDNA encoding CD4-TLR 4, in the presence or absence of 100 ng A46R expression vector, together with the pFR-luciferase reporter, a Renilla-luciferase internal control and plasmids encoding Gal4 fused to Elk1 (for ERK1/2) (A) or CHOP (for p38) (B). Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 4.5: A46R interacts with Mal

A: Coimmunoprecipitation

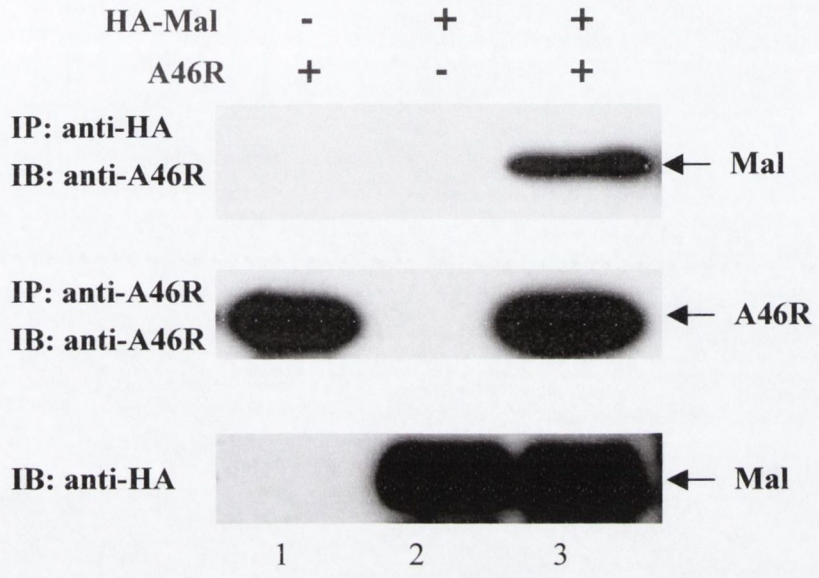
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The band in lane 3 of the upper panel indicates an interaction between Mal and A46R.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding HA-Mal and henceforth treated as described in Figure 3.10. The band in lane 3 indicates an interaction between Mal and A46R.

Figure 4.5: A46R interacts with Mal

A: Coimmunoprecipitation



B: GST Pulldown Assay

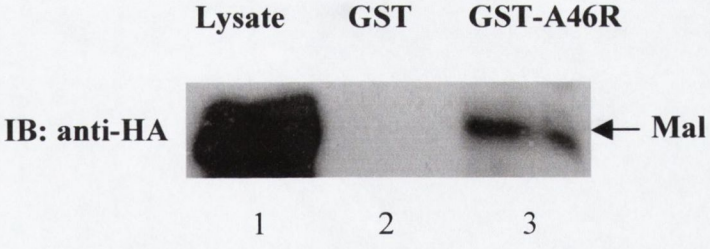


Figure 4.6: A46R interacts directly with Mal, as shown using recombinant Mal (rMal)

Upper panel: 1 μl rMal was incubated with 30 μl either immobilised GST or GST-A46R for 2 h at 4 °C. The sample was washed three times in lysis buffer and once with lysis buffer without NP-40, and resuspended in 30 μl 3X sample buffer. The samples were then analysed by Western blot.

Lower panel: GST and GST-A46R which were not incubated with rMal are ran on a gel, Western blotted and probed with the Mal antibody

Figure 4.7: A46R interacts with the TIR domain of Mal

A: Coimmunoprecipitation

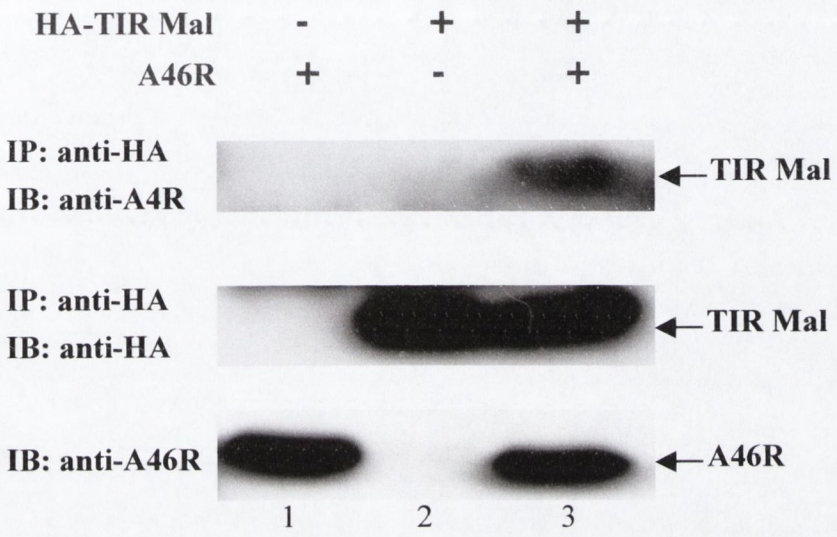
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The band in lane 3 of the upper panel indicates an interaction between the TIR domain of Mal and A46R.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding HA-TIR Mal and henceforth treated as described in Figure 3.10. The band in lane 3 indicates an interaction between the TIR domain of Mal and A46R.

Figure 4.7: A46R interacts with the TIR domain of Mal

A: Coimmunoprecipitation



B: GST Pulldown Assay

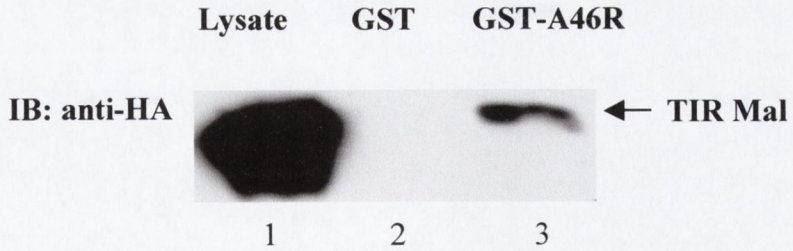


Figure 4.8: A46R does not interact with the Mal P125H mutant

A: Coimmunoprecipitation

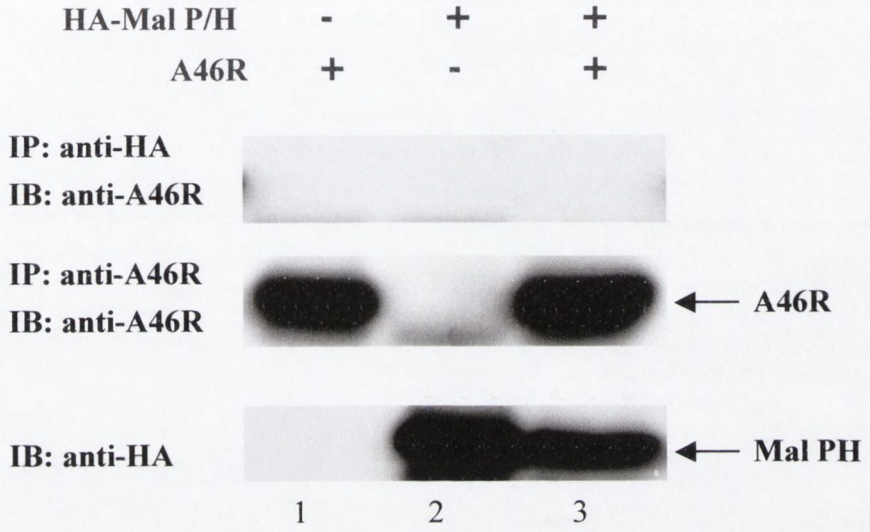
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The absence of a band in lane 3 of the upper panel indicates that no interaction occurred.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding HA-Mal PH and henceforth treated as described in Figure 3.10. The absence of a band in lane 3 indicates that no interaction occurred.

Figure 4.8: A46R does not interact with the Mal P125H mutant

A: Coimmunoprecipitation



B: GST Pulldown Assay

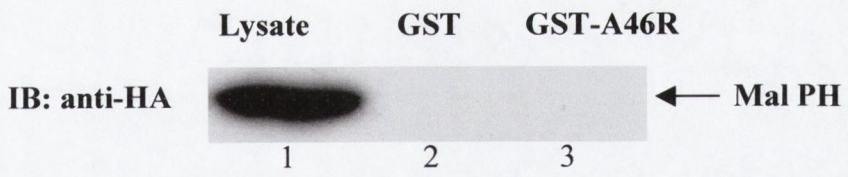
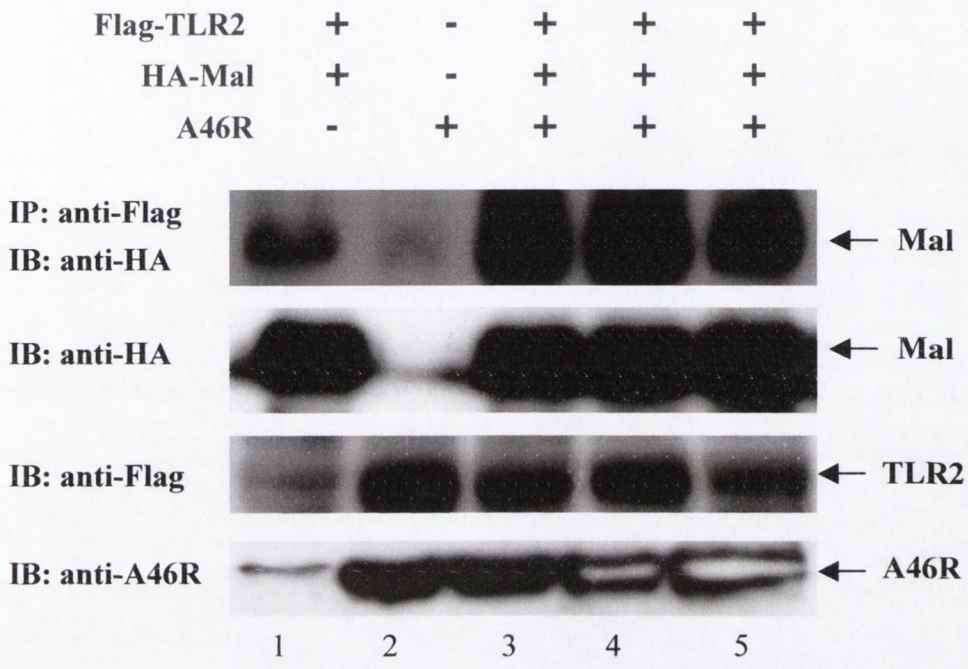
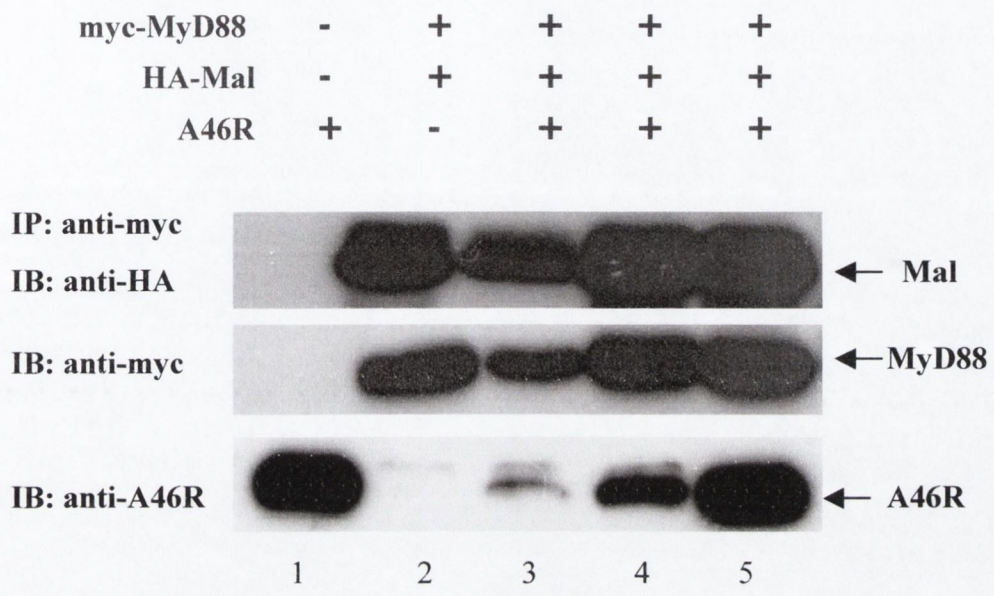


Figure 4.9: A46R does not affect the Mal-TLR2 interaction



HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection. 2 μg of both AU1-MyD88 and Flag-TRAF6 were transfected, in the presence of increasing amounts of A46R (2, 4 and 6 μg). Where only one construct was expressed the total amount of DNA (10 μg) was kept constant by supplementation with vector DNA. Cells were harvested 24 h post transfection and the immunoprecipitation experiment carried out as described. The top panel corresponds to lysates immunoprecipitated with anti-Flag antibody, and blotted with anti-HA antibody, to determine whether the interaction between the two proteins has been affected by the presence of A46R. The lower two panels correspond to lysates directly blotted for the expression of Mal and A46R.

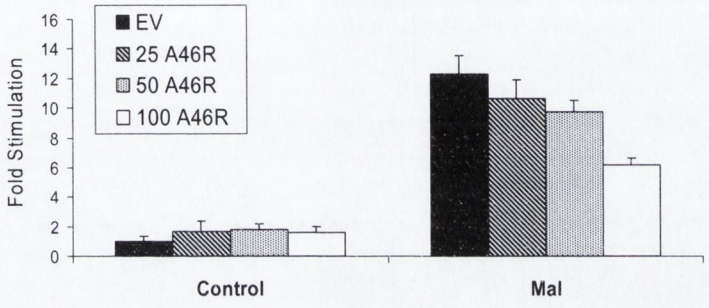
Figure 4.10: A46R does not prevent the MyD88-Mal interaction



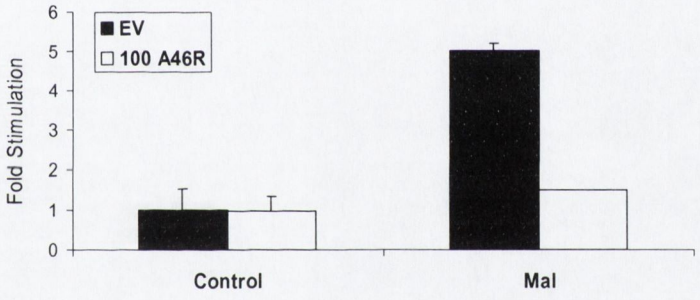
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection. 2 μg of both myc-MyD88 and HA-Mal were transfected, in the presence of increasing amounts of A46R (2, 4 and 6 μg). Where only one construct was expressed the total amount of DNA (10 μg) was kept constant by supplementation with vector DNA. Cells were harvested 24 h post transfection and the immunoprecipitation experiment carried out as described. The top panel corresponds to lysates immunoprecipitated with anti-myc antibody and blotted with anti-HA antibody, to determine whether the interaction between the two proteins has been affected by the presence of A46R. The lower two panels correspond to lysates directly blotted for the presence of myc-MyD88 and A46R.

Figure 4.11: A46R inhibits Mal-dependent NFκB activation

A: NFκB



B: p65



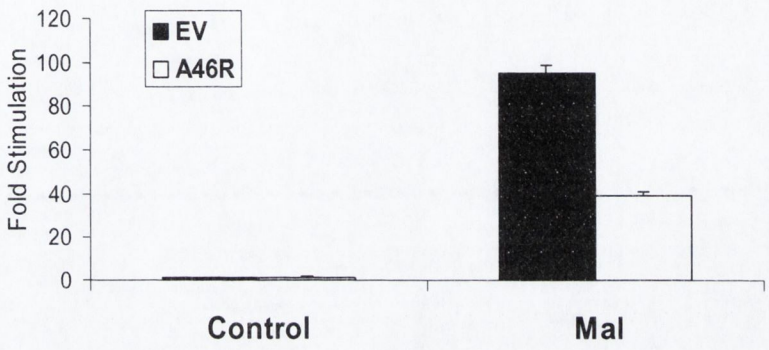
HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and either the κB-luciferase construct (A) or the pFR luciferase construct and a plasmid encoding Gal4 fused to p65 (B), in the presence or absence of 25 ng Mal, together with empty vector (EV) or the indicated amount (ng) of A46R cDNA. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 4.12: A46R inhibits Mal-dependent MAPK activation

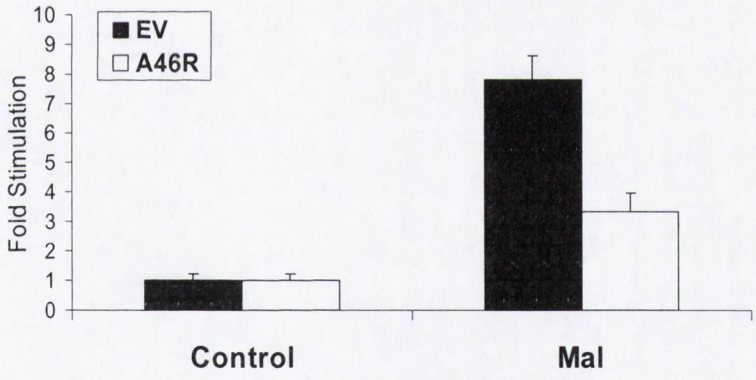
HEK 293 cells (2 x 10^4 cells per well) were transfected with 25 ng cDNA encoding Mal, in the presence or absence of 100 ng empty vector (EV) or A46R expression vector, together with the pFR luciferase construct, a Renilla-luciferase internal control and plasmids encoding Gal4 fused to Elk1 (for ERK1/2) (A), c-Jun (for JNK) (B) or CHOP (for p38) (C). Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 4.12: A46R inhibits Mal-dependent MAPK activation

A: ERK



B: JNK



C: p38

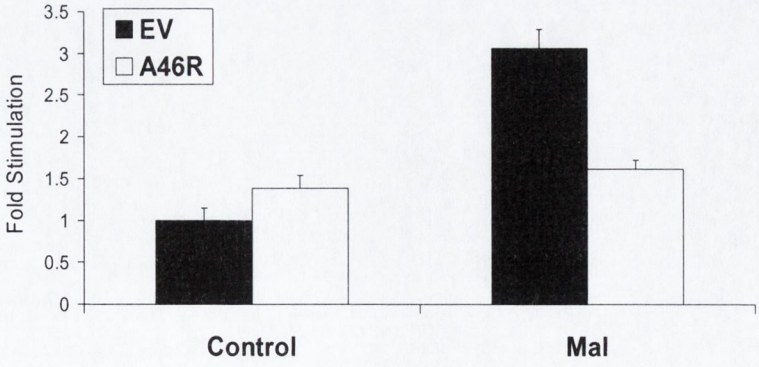
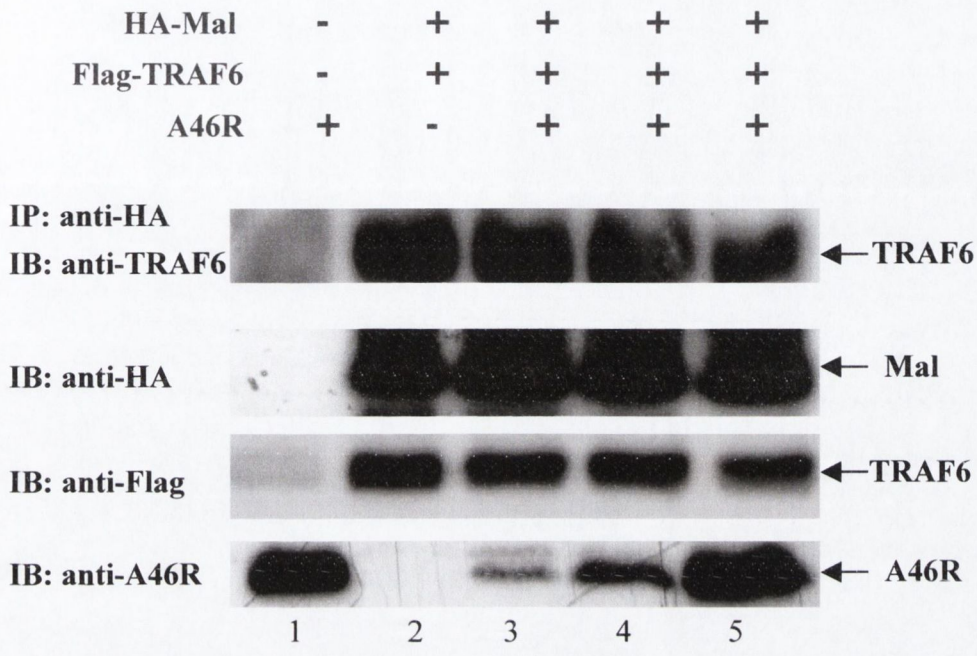


Figure 4.13: A46R slightly inhibits the Mal-TRAF6 interaction



HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection. 2 μg of both HA-Mal and Flag-TRAF6 were transfected, in the presence of increasing amounts of A46R (2, 4 and 6 μg). Where only one construct was expressed the total amount of DNA (10 μg) was kept constant by supplementation with vector DNA. Cells were harvested 24 h post transfection and the immunoprecipitation experiment carried out as described. The top panel corresponds to lysates immunoprecipitated with anti-HA antibody, and blotted with anti-TRAF6 antibody, to determine whether the interaction between the two proteins has been affected by the presence of A46R. The lower two panels correspond to lysates directly blotted for the expression of Mal and A46R.

Figure 4.14: A46R interacts with TRAM

A: Coimmunoprecipitation

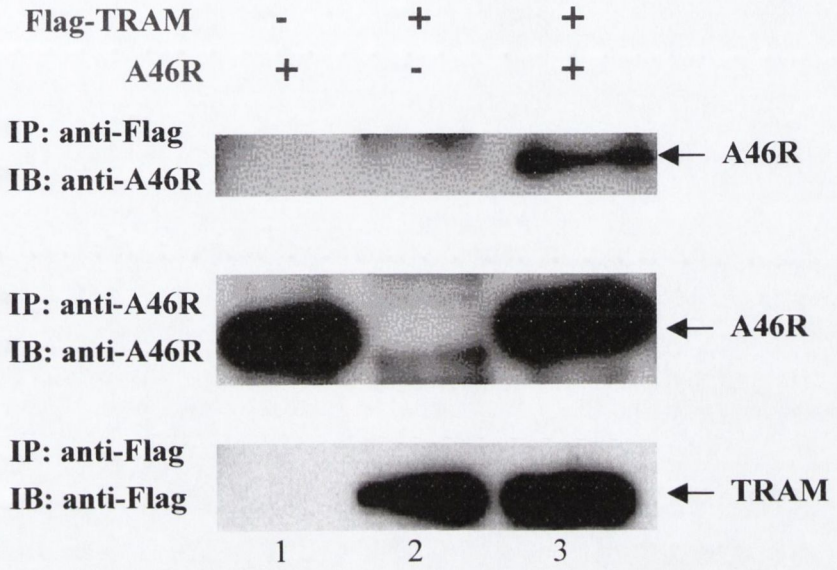
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The band in lane 3 of the upper panel indicates an interaction between TRAM and A46R.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding Flag-TRAM and henceforth treated as described in Figure 3.10. The band in lane 3 indicates an interaction between TRAM and A46R.

Figure 4.14: A46R interacts with TRAM

A: Coimmunoprecipitation



B: GST Pulldown Assay

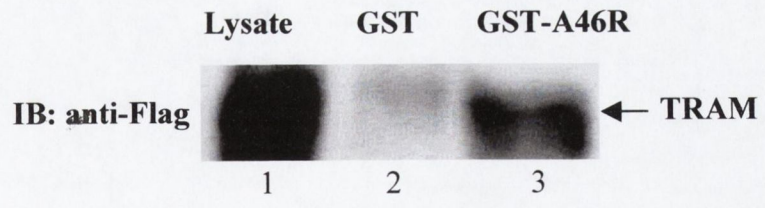


Figure 4.15: A46R requires more than just the TIR domain to interact with TRAM

A: Coimmunoprecipitation

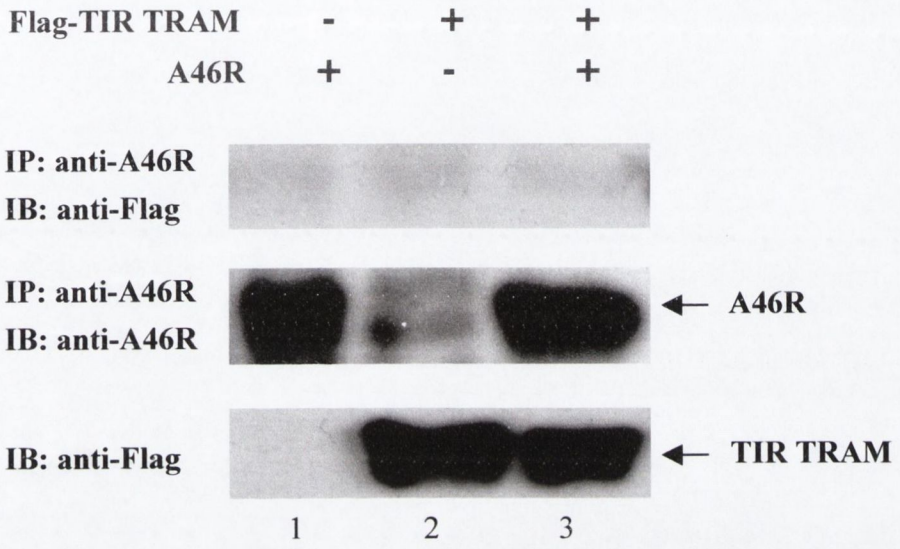
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The absence of a band in lane 3 of the upper panel indicates that no interaction occurred.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding Flag-TIR TRAM and henceforth treated as described in Figure 3.10. The absence of a band in lane 3 indicates that no interaction occurred.

Figure 4.15: A46R requires more than just the TIR domain to interact with TRAM

A: Coimmunoprecipitation



B: GST Pulldown Assay

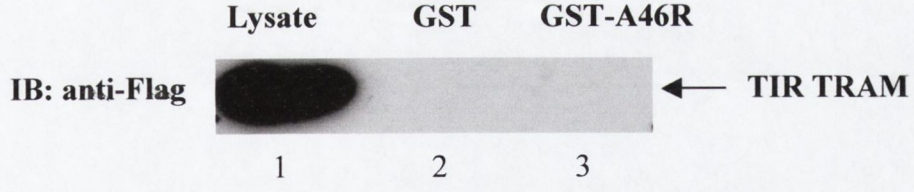


Figure 4.16: A46R interacts with TRIF

A: Coimmunoprecipitation

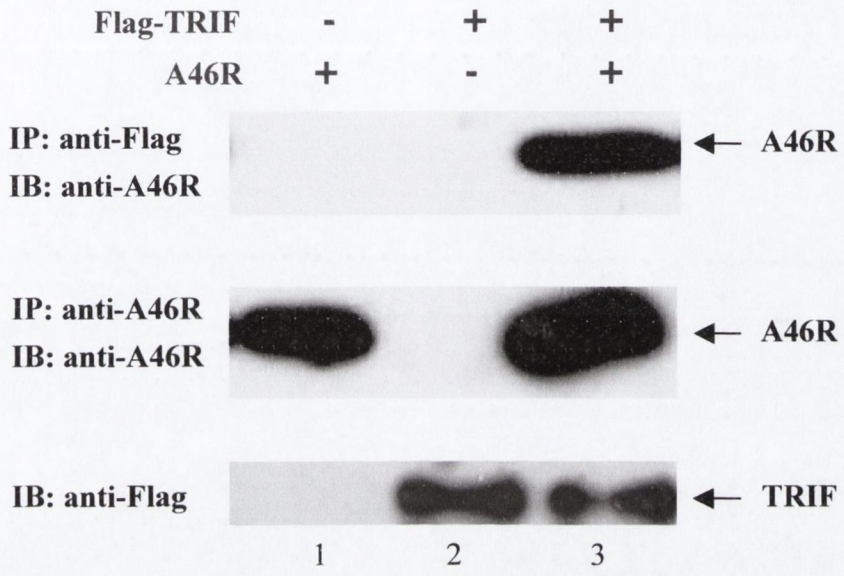
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The band in lane 3 of the upper panel indicates an interaction between TRIF and A46R.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding Flag-TRIF and henceforth treated as described in Figure 3.10. The band in lane 3 indicates an interaction between TRIF and A46R.

Figure 4.16: A46R interacts with TRIF

A: Coimmunoprecipitation



B: GST Pulldown Assay

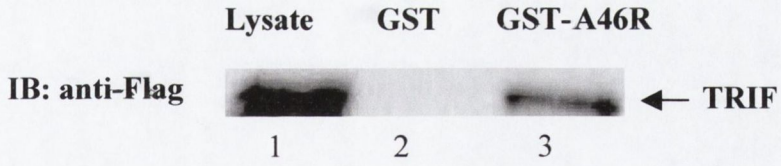


Figure 4.17: A46R requires more than just the TIR domain to interact with TRIF

A: Coimmunoprecipitation

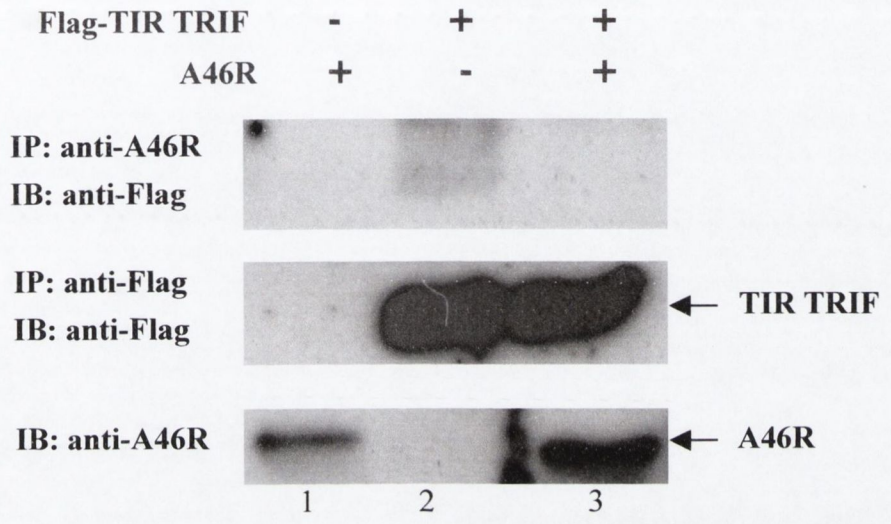
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The absence of a band in lane 3 of the upper panel indicates that no interaction occurred.

B: GST Pulldown Assay

HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding Flag-TIR TRIF and henceforth treated as described in Figure 3.10. The absence of a band in lane 3 indicates that no interaction occurred.

Figure 4.17: A46R requires more than just the TIR domain to interact with TRIF

A: Coimmunoprecipitation



B: GST Pulldown Assay

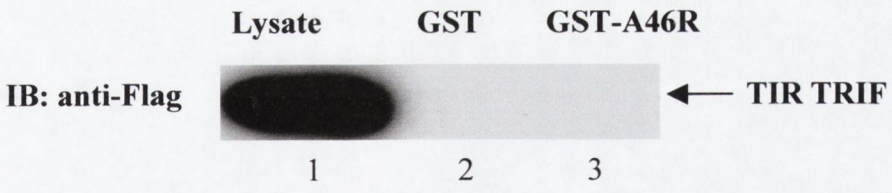
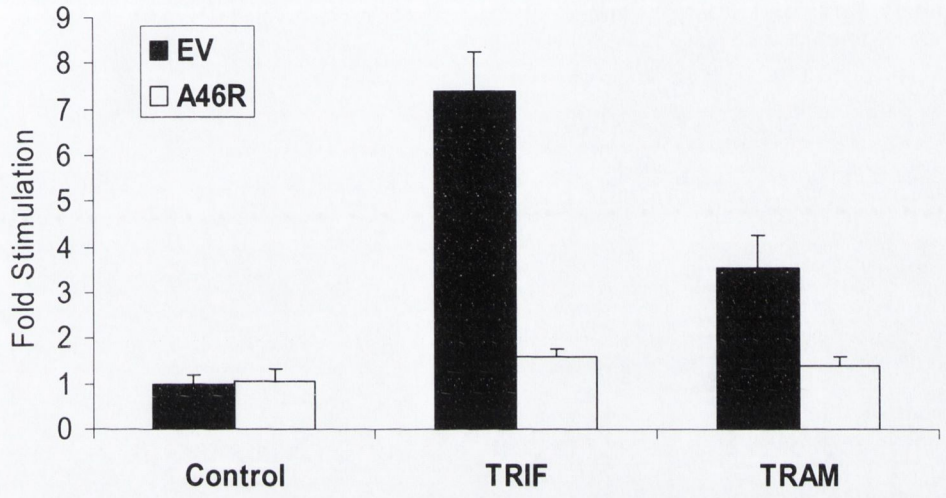


Figure 4.18: A46R inhibits the activation of NFκB by TRIF and TRAM in HEK 293 cells



HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and a κB-luciferase construct in the presence or absence of 100 ng empty vector (EV) or A46R cDNA, along with either 25 ng TRIF or 10 ng TRAM. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 4.19: A46R does not interact with SARM

A: Coimmunoprecipitation

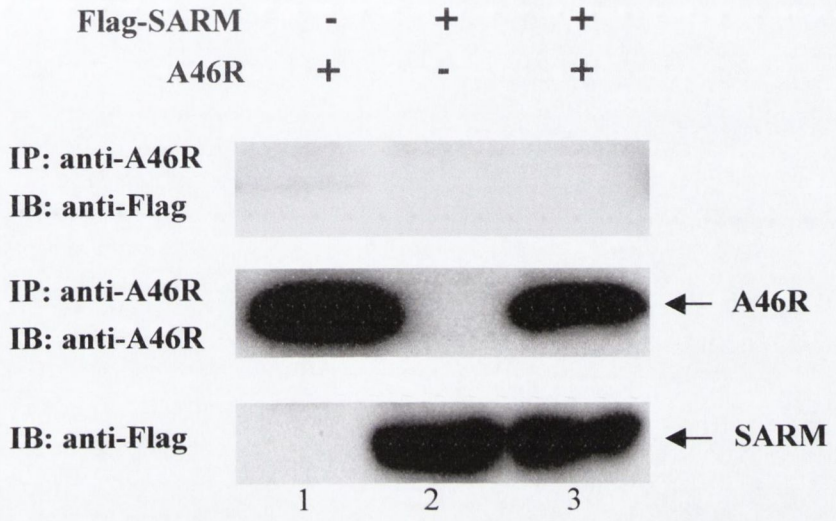
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection and treated as described in Figure 3.10. The absence of a band in lane 3 of the upper panel indicates that no interaction occurred.

B: GST Pulldown Assay

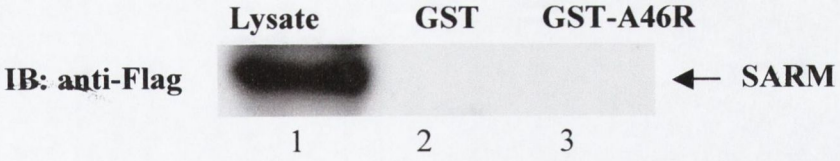
HEK 293T cells were seeded into 100 mm dishes (1.5 x 10^6 cells) 24 h prior to transfection, transfected with 8 μg of plasmid encoding Flag-SARM and henceforth treated as described in Figure 3.10. The absence of a band in lane 3 indicates that no interaction occurred.

Figure 4.19: A46R does not interact with SARM

A: Coimmunoprecipitation



B: GST Pulldown Assay



4.3 Discussion

MyD88 is a TIR adapter molecule involved in IL-1 signal transduction but not that of TNF-α. Many TLR signalling pathways also depend on MyD88. All TLR2- and TLR9-mediated responses seem to be abolished in *MyD88*$^{-/-}$ cells (Takeuchi *et al.*, 2000; Schnare *et al.*, 2000) and MyD88-null mice did not respond to flagellin, the known ligand for TLR5 (Hayashi *et al.*, 2001). R-848, a small anti-viral compound, is recognised in a MyD88-dependent fashion by TLR7 (Hemmi *et al.*, 2002) and by TLR8 also (Heil *et al.*, 2003). Early phase TLR4-induced NFκB activation also involves MyD88 (Kawai *et al.*, 1999). In the previous chapter I showed that A46R inhibits IL-1 and not TNF-α signalling, and that it does so by targeting MyD88. When I examined whether or not A46R could inhibit other MyD88-dependent TLR signalling pathways, I observed inhibition of TLR2, 4, 5, 7, 8 and 9 mediated NFκB activation. Presumably, A46R inhibits these signals by targeting MyD88. PMA activates NFκB using a distinct signalling pathway that does not require MyD88, but instead utilises protein kinase C (PKC) (Kim *et al.*, 1996). PMA-induced NFκB activation was unaffected by A46R.

Because TLR4 uses other TIR adapter molecules besides MyD88 in its signalling, I was interested to observe that TLR4-induced NFκB activation was as strongly inhibited in the RAW 264.7 cells as the other TLR signals. When I examined the effect of A46R on TLR4 signals such as p65 transactivation and activation of the MAPKs ERK and p38, I found that they too were inhibited. The downstream consequences of the inhibitory effects of A46R on TLR4 signalling were to suppress gene induction. LPS stimulation of HEK293 cells stably expressing TLR4 led to the release of IL-8. Transient transfection of these cells with A46R suppressed this release by approx. 50%. This is suggestive of a very potent effect on IL-8 induction given that only a portion of

the cell population stimulated by LPS to release IL-8 would be expected to be expressing A46R in this transient transfection system (Stack *et al.*, 2005).

The fact that A46R inhibited all TLR4 signals tested, together with the fact that A46R inhibited TLR4-mediated NFκB activation more potently than it did IL-1-induced NFκB activation (compare Figures 3.2 and 4.3.A), led to an examination of the ability of A46R to target the other TIR adapters important for TLR signalling. The A46R-Mal interaction was demonstrated by co-immunoprecipitation and GST-pulldown assays, and was shown to be direct by both yeast-two-hybrid pairwise assay (Stack *et al.*, 2005) and by using rMal in an *in vitro* immunoprecipitation assay. The physiological relevance of the Mal-A46R interaction has been confirmed since Mal was shown to co-immunoprecipitate with A46R in VV infected cells (Stack *et al.*, 2005). Mal is also involved in TLR2 signalling (Yamamoto *et al.*, 2002a; Horng *et al.*, 2002), and its targeting by A46R could explain why A46R can inhibit both MALP2 and Pam3Cys signals, which are mediated by TLRs 2 and 6, and TLRs 2 and 1 respectively. TLR2 can form heterodimers with TLR1 or TLR6 (Takeuchi *et al.*, 2001); thus Mal might bind to TLR1 or TLR6 to facilitate the association. Mal associates with both TLR2 and TLR1 (K. Fitzgerald, unpublished); however, the ability of Mal to interact with TLR6 has not been tested (Vogel *et al.*, 2003).

As was the case with the MyD88-A46R interaction, A46R was found to interact with Mal *via* the TIR domain of Mal. Mal has a conserved proline at position 125 in its BB loop, which when mutated to a histidine causes Mal to act as a dominant negative of TLR4-induced NFκB activation (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001), perhaps by sequestering TLR4. A46R could not interact with Mal P125H, showing that this highly

conserved proline is essential for the Mal-A46R interaction, as was the case with MyD88.

An initial theory was that A46R might inhibit TLR signalling pathways by sequestering Mal and preventing it from interacting with the receptor. Initially I wanted to examine the Mal-TLR4 interaction, but I could not consistently co-immunoprecipitate the two proteins. For that reason, I examined the effect of increasing A46R concentrations on the Mal-TLR2 interaction which was reproducibly detected and found that A46R did not affect the Mal-TLR2 association. Therefore, this is not the mechanism by which A46R inhibits TLR2 signalling. One could reason that A46R probably does not interfere in the TLR4-Mal association either, as it is thought that both receptors interact with Mal in a similar fashion. Because signals induced by overexpression of Mal and MyD88 were potently inhibited by A46R, it was more likely that A46R was acting not at the level of the TLR-adapter interaction, but rather at a point further downstream.

It was possible that A46R disrupted TLR4 signalling pathways by impairing the association of Mal and MyD88. However, even very high concentrations of A46R could not prevent the Mal-MyD88 interaction. This may be explained by the fact that A46R interacts with both adapters at the BB loop. Dunne *et al.* (2004) showed that Mal P125H could still interact with MyD88, and that MyD88 P200H could still interact with Mal. This suggests that the BB loops of the adapters do not form a point of contact with each other during Mal-MyD88 heterodimerisation, and therefore A46R may bind to them without interfering in their association.

Recently, a TRAF binding motif was identified in Mal by Mansell and colleagues (2004), and on further investigation, they found that Mal and TRAF6 could interact. When the TRAF binding motif is mutated in Mal by changing the glutamic acid at position 190 to alanine, it was discovered that the mutant, Mal E190A, inhibits TLR4 and TLR2 induced NFκB activation, and can no longer induce the same signals as wild type Mal, namely NFκB, p65 or MAPKs. From this data, a specific role for Mal in TLR2- and TLR4-induced p65 transactivation *via* this interaction was proposed. Surprisingly, however, it can still interact with TRAF6.

It is possible that the inhibition effects of Mal E190A have nothing to do with the mutation in the TRAF binding domain *per se*, but are instead due to the fact that the mutation is in the TIR domain of Mal and has perhaps rendered the TIR domain non-functional. However, the fact that the Mal-TRAF6 interaction appears to be a target for VV immune evasion, *via* A46R, implies that it may be an important signalling complex after all. I observed that the association between Mal and TRAF6 was slightly impaired in the presence of A46R, thus providing a convenient and plausible explanation for the inhibition of Mal-dependent signals, and hence some TLR4 signalling pathways, by A46R.

However, Mal and MyD88 are only responsible for early-phase TLR4-mediated NFκB activation (Kawai *et al.*, 1999; Horng *et al.*, 2002; Yamamoto *et al.*, 2002a). Two other TIR adapter molecules, TRIF and TRAM, are involved in late phase TLR4-induced NFκB activation. As a further explanation for the potent effect of A46R on TLR4-mediated NFκB activation, A46R was found to interact with both TRIF and TRAM, and to inhibit their induction of an NFκB-dependent reporter gene. The A46R-TRIF

interaction has been found to be direct in a yeast-two-hybrid pairwise assay (Stack *et al.*, 2005). In an attempt to determine whether or not the A46R-TRAM interaction was also direct, I tried to generate rA46R using the same conditions as described in Sections 2.10.1 and 2.10.3. However, I simply could not cleave GST-A46R, despite using a wide range of temperature and incubation conditions and different thrombin concentrations. Work is ongoing to generate recombinant TRAM and investigate whether A46R interacts directly with TRAM. I have also attempted to generate recombinant TRIF, but was unable to express GST-TRIF in *E. coli*, possibly due to the high molecular weight of the fusion protein (over 100 kDa).

However, A46R might interact differently with TRIF and TRAM than it did with MyD88 and Mal, as the plasmids encoding the TIR domains alone of each were insufficient to observe an interaction with A46R. This could be due to the TIR domains of TRIF and TRAM not being folded properly when expressed on their own. Overexpression of a plasmid encoding the TIR domain of TRIF inhibits TLR2, TLR3, TLR4, and TLR7-mediated NFκB activation (Yamamoto *et al.*, 2002b). However, studies using the TRIF knockout mouse imply a role for TRIF in TLR3 and TLR4 signalling alone (Yamamoto *et al.*, 2003a; Hoebe *et al.*, 2003). This suggests that either the TIR-TRIF dominant negative construct acts non-specifically, or that TRIF plays an as yet unappreciated role in these diverse signalling pathways. TIR-TRIF may, when overexpressed, sequester other TIR-containing proteins. Unusually, TIR TRAM does not act as a dominant negative for any signals (Fitzgerald *et al.*, 2003a). The failure to interact with the TIR domains alone of TRIF and TRAM could be due to the fact that A46R needs other portions of the protein to interact with also (i.e. residues from distant parts of the sequence may form part of the A46R binding pocket).

To determine exactly how A46R interacts with TRIF and TRAM, it would be prudent to use versions of TRIF and TRAM where highly conserved residues in the BB loop of their TIR domains are mutated. When TRIF was mutated by changing its conserved proline at position 434 to histidine, it was discovered that the mutant, TRIF P434H, inhibited TLR4- and TLR3-induced NFκB activation, and could no longer induce the same signals as wild type TRIF, namely NFκB or IRF3 (Oshiumi *et al.*, 2003a). TRAM has a cysteine instead of a proline in the conserved Φ_2 position and a proline in the Φ_1 spot of its BB loop. TRAM-C113H inhibited LPS-induced NFκB activity in HEK 293-TLR4-MD2 cells and did not interact with TLR4 (Fitzgerald *et al.*, 2003a). Also, it could not induce NFκB activation. If A46R did not interact with either TRIF P434H or TRAM C113H, it would be a very strong indication that A46R does actually interact with TRIF and TRAM *via* their TIR domains as expected. Considering that disruption of the BB loop impairs the signalling capabilities of most TIR adapters, it makes sense that A46R might bind to the TIR adapters at this region.

SARM is a 690 amino acid protein with a TIR domain, two sterile-α motif (SAM) domains and an Armadillo repeat motif (ARM). The protein was previously annotated as SARM for 'SAM and ARM protein' (O'Neill *et al.*, 2003) and has yet to be implicated in TLR signalling. Unlike other TIR adapters, overexpression of SARM does not activate either NFκB or IRF3 as measured by reporter gene assay (Liberati *et al.*, 2004) Because of its non-functionality in TLR signalling, it is logical that SARM would not represent a target for A46R, and therefore the fact that A46R did not interact with SARM was an important control for specificity. SARM and A46R have also been shown not to interact using a yeast-two-hybrid pairwise assay (M. Carty, personal communication).

TRIF and TRAM are critical for TLR4-induced activation of IRF3 as well as for late activation of NFκB (Yamamoto *et al.*, 2003a; Yamamoto *et al.*, 2003b; Fitzgerald *et al.*, 2003a). TRIF also plays a major role in TLR3-mediated NFκB activation (Yamamoto *et al.*, 2003a). Indeed, TRIF is thought to be the only TIR adapter molecule involved in TLR3 signal transduction. In Chapter 5, I examine the effects of A46R on other TRIF- and TRAM-dependent signals.

Chapter Five

The Effect of A46R on TLR-Induced Interferon Regulatory Factor Activation

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5.1 Introduction

So far, TLR4 signalling is the most complex of all the TLRs and is mediated *via* all four TIR adaptor molecules. MyD88-Mal signalling and TRIF-TRAM signalling define separate rami of a bifurcated pathway responsible for all signals that emanate from TLR4. Wild-type macrophages induce 148 genes in response to LPS. However, macrophages lacking both MyD88 and TRIF do not upregulate any genes in response to LPS (Hirotani *et al.,* 2005). MyD88 and Mal are involved in early activation of NFκB and MAP kinases (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001; Yamamoto *et al.*, 2002a; Horng *et al.*, 2002). The induction of proinflammatory cytokines was critically governed by these molecules. However, MyD88 and Mal were dispensable for the expression of interferon-inducible genes as well as for the maturation of dendritic cells in response to LPS (Kaisho *et al.*, 2001; Yamamoto *et al.*, 2002a). In contrast, TRIF and TRAM are critical for late activation of NFκB as well as for activation of IRF3 and induction of type I interferon (Yamamoto *et al.*, 2003a; Yamamoto *et al.*, 2003b; Fitzgerald *et al.*, 2003a).

TRIF knockout mice were defective in both TLR3-mediated IFN-β expression and IRF3 activation. Also, poly(I:C)-induced NFκB activation was severely impaired in TRIF deficient cells, indicating that TRIF plays a major role in TLR3-mediated NFκB activation (Yamamoto *et al.*, 2003a; Hoebe *et al.*, 2003). Indeed, TRIF is thought to be the only TIR adapter molecule involved in TLR3 signal transduction.

Type I interferons, a family of cytokines comprising more that 12 IFN-α and 1 IFN-β species, are important for the defence against viral infections. It has been known for a long time that interferons are produced and secreted by virus-infected cells, providing

self-defence and protection to neighbouring cells, and more recently their production in response to TLR ligands has been discovered (reviewed in (Akira and Hemmi, 2003). Hence, TLR-mediated type I interferon production is a very likely target for viral interference.

The transcription factor IRF3 is required for expression of type I interferons and the chemokine RANTES in response to viral infection (Hiscott *et al.,* 1999; Genin *et al.,* 2000). In unstimulated cells, IRF3 is present in the cytoplasm. Viral infection results in the phosphorylation of IRF3 by two IκB kinase-related kinases, IKK-ε and TBK1 (Sharma *et al.*, 2003; Fitzgerald *et al.*, 2003b), leading to its dimerisation and interaction with its co-activators CREB-binding protein (CBP) and p300 (Hiscott *et al.,* 1999). The IRF3 complex than translocates to the nucleus where it activates promoters containing IRF3-binding sites, such as the interferon stimulated response element (ISRE).

Previous studies have shown that both IRF3 and IRF7 are required for efficient type I interferon production (Servant *et al.*, 2002; Zhang and Pagano, 2002). Although IRF3 is ubiquitously expressed, most cells do not express or weakly express IRF7, but inducibly express IRF7 after type I interferon stimulation (Lin *et al.,* 2000; Sato *et al.,* 2000). IRF7 is then activated and transactivates multiple IFN genes, leading to robust production of interferons in response to viral infection (Servant *et al.*, 2002; Zhang and Pagano, 2002). However, plasmacytoid dendritic cells constitutively express IRF7 and rapidly produce IFN-α in response to viral infection, TLR7 and TLR8 ligands (ssRNA and imidazoquinolines) and TLR9 ligand (CpG DNA) (Hemmi *et al.,* 2003; Kerkmann *et al.,* 2003). IFN-α induction by these TLR ligands is abolished in MyD88-deficient cells and is controlled by a MyD88-TRAF6-IRF7 complex (Kawai *et al.,* 2004).

IRF5 has been recently discovered to play a more general role than expected in TLR signalling. Using IRF5-deficient mice Takaoka and colleagues (2005) showed that IRF5 is involved downstream of the TLR-MyD88 signalling pathway for gene induction of proinflammatory cytokines, such as IL-6, IL-12 and TNF-α, and does not participate in type I interferon induction. However, Schoenemeyer *et al.* (2005) demonstrated a positive role for IRF5 in type I interferon induction in that stimulation of IRF5-deficient cells with R-848 did not result in the induction of type I interferons and IRF5 siRNA impaired R-848 induced IFN-α induction. Work is ongoing to clarify the apparent discrepancies between these two studies.

The VV protein, A52R, which is related to A46R, has been shown previously to block the activation of NFκB by multiple TLRs, including TLR3, with little inhibitory effect on the activation of NFκB by TNF-α (Bowie *et al.*, 2000; Harte *et al.*, 2003). N1L is another VV immunomodulatory protein, which is not essential for viral replication but strongly affects viral virulence *in vivo* (Kotwal *et al.*, 1989; Bartlett *et al.*, 2002). Because of N1L's sequence similarity to A52R, it was hypothesised by DiPerna *et al.* (2004) that N1L also inhibited signalling to NFκB. On further investigation, N1L was found to inhibit signalling to NFκB by multiple inducers by acting at the level of the IKK complex (DiPerna *et al.,* 2004).

In this chapter, I found that A46R inhibited multiple TRAM- and TRIF-dependent signals emanating from TLR4, including the activation of the IFN-β and RANTES promoters, IRF3, IRF7 and the ISRE. A46R also inhibits TLR3-mediated RANTES production, activation of the IFN-β promoter, the ISRE, IRF3 and IRF7. Activation of IRF7 and IRF5 by TLR7 and TLR9, which are dependent on MyD88 and TRAF6, were

inhibited by A46R. When the activities of A46R and A52R were compared, I discovered that while A46R could inhibit poly(I:C)-induced activation of IRF3, A52R could not. On further investigation, A52R was found to have a potent inhibitory effect on poly(I:C)-induced NFκB activation, but this signal was unaffected by increasing concentrations of A46R. A46R could also inhibit other interferon signals activated by TLR3. Both N1L and A46R have inhibitory effects on TLR4-mediated NFκB activation, but while A46R targets the TIR adapter molecules, N1L acts at the level of the IKK complex.

5.2 Results

5.2.1 A46R inhibits TLR4-mediated IRF3 activation by targeting TRIF and TRAM

LPS induces type I interferon gene expression *via* the adapter molecules TRAM and TRIF and the transcriptional regulator IRF3 (Fitzgerald *et al.*, 2003a; Fitzgerald *et al.*, 2003b; Yamamoto *et al.*, 2003a; Hoebe *et al.*, 2003). I was interested in examining the effect, if any, of A46R on TLR4-mediated IRF3 activation. To this end, I employed an *in vivo* reporter assay that utilises a fusion protein consisting of the yeast Gal4-DNA binding domain fused to IRF3 lacking its own DNA binding domain, together with a reporter plasmid with a Gal4 upstream activation sequence (Fitzgerald *et al.*, 2003a; Fitzgerald *et al.*, 2003b). Reporter gene expression from this plasmid requires IRF3 activation through phosphorylation. Stimulation of RAW 264.7 cells with LPS or transfection of HEK 293 cells with CD4-TLR4 led to activation of IRF3 (Figures 5.1.A and 5.1.B). A46R potently inhibited both of these TLR4-mediated IRF3 signals. TLR4 uses both TRIF and TRAM to activate IRF3, and TBK1 interacts with TRIF to transmit the signal further downstream. I reasoned that A46R inhibited TLR4-mediated IRF3 activation at the level of the TIR adapter molecules involved. Consistent with this, when TRIF, TRAM and TBK1 were used to activate IRF3, A46R was seen to inhibit TRIF- and TRAM-, but not TBK1-, dependent IRF3 activation (Figure 5.1.C).

5.2.2 A46R inhibits TLR4-mediated activation of the ISRE promoter

Stimulation of RAW 264.7 cells with LPS or transfection of HEK 293 cells with CD4-TLR4 led to activation of the ISRE reporter construct (Figures 5.2.A and 5.2.B). A46R potently inhibited both of these TLR4-mediated signals. When TRIF, TRAM and

TBK1 were used to activate ISRE, A46R was seen to inhibit TRIF- and TRAM-, but not TBK1-, mediated ISRE activation (Figure 5.2.C).

5.2.3 A46R inhibits TLR4-mediated activation of the IFN-β promoter

Activation of IRF3 leads to induction of type 1 interferons such as IFN-β, which can be examined using an IFN-β promoter reporter gene construct. Stimulation of RAW 264.7 cells with LPS or transfection of HEK 293 cells with TRIF led to activation of the IFN-β promoter reporter gene construct (Figures 5.3.A and 5.3.B). A46R potently inhibited both of these signals. As previously reported, TRAM, Mal and MyD88 did not activate the IFN-β promoter (Yamamoto *et al.*, 2002b).

5.2.4 A46R inhibits TLR4-mediated activation of the RANTES promoter

The transcription factor IRF3 is required for expression of the chemokine RANTES in response to viral infection, which can be measured using a RANTES promoter reporter gene construct. Stimulation of RAW 264.7 cells with LPS or transfection of HEK 293 cells with TRIF led to activation of the RANTES promoter reporter gene construct (Figures 5.4.A and 5.4.B). A46R inhibited both of these signals.

5.2.5 A46R inhibits TLR4-mediated IRF7 activation

To measure IRF7 activation, I employed an *in vivo* reporter assay that utilises a fusion protein consisting of the yeast Gal4-DNA binding domain fused to IRF7 lacking its own DNA binding domain, together with a reporter plasmid with a Gal4 upstream activation sequence (Fitzgerald *et al.*, 2003a). Reporter gene expression from this plasmid requires IRF7 activation through phosphorylation. Transfection of HEK 293 cells with CD4-TLR4 or stimulation of RAW 264.7 cells with LPS led to activation of

IRF7 (Figures 5.5.A and 5.5.B). A46R inhibited these TLR4-mediated signals. TLR4 uses both TRIF and TRAM to activate IRF7 (Fitzgerald *et al.*, 2003a). Therefore, it was likely that A46R inhibited TLR4-mediated IRF7 activation at the level of the TIR adapter molecules involved. TRIF and TRAM both activated IRF7, albeit to different extents, and A46R inhibited those signals (Figure 5.6).

5.2.6 A46R inhibits TLR3-mediated IRF3 and IRF7 activation

TRIF is the only TIR adapter molecule involved in TLR3 signalling (Yamamoto *et al.*, 2003a; Hoebe *et al.*, 2003). RAW 264.7 cells were stimulated with poly(I:C) for six hours and this led to activation of IRF3, as measured by reporter gene assay (Figure 5.7). A46R inhibited TLR3-induced IRF3 activation potently. HEK 293 cells transfected with TLR3 and RAW 264.7 cells were stimulated with poly(I:C) for six hours and this led to activation of IRF7, as measured by reporter gene assay (Figure 5.8). In both systems, TLR 3-mediated IRF7 activation was inhibited by A46R.

5.2.7 A46R inhibits poly(I:C)-induced gene activation

Stimulation of HEK 293 cells transfected with TLR3 with poly(I:C) for six hours led to activation of the ISRE reporter construct (Figure 5.9.A) and the IFN-β promoter reporter construct (Figure 5.9.B). A46R inhibited activation of the IFN-β promoter slightly more potently than it inhibited ISRE activation (Compare Figures 5.9.A and 5.9.B).

Induction of RANTES was measured by ELISA. When HEK 293-TLR3 cells were stimulated with poly(I:C) for 24 h, RANTES production was induced. A46R inhibits

poly(I:C) induced RANTES production in a dose-dependent manner (Figure 5.10), achieving a maximum of approximately 50% inhibition when 230 ng A46R was used.

5.2.8 A46R inhibits TLR7- and TLR9-mediated IRF7 activation

TLR7, TLR8 and TLR9 use MyD88 and TRAF6 to activate IRF7 (Kawai *et al.,* 2004; Honda *et al.*, 2004). Because A46R has already been shown to target MyD88 and to inhibit all MyD88 signals tested, I examined the effect, if any, of A46R on TLR7- and TLR8-mediated IRF7 activation. RAW 264.7 cells were stimulated with either 1 μM R-848 or 5 μg/ml CpG for 6 hours, and IRF7 activation was measured by reporter gene assay as described in section 5.2.5. A46R inhibited TLR7 and TLR9-mediated IRF7 activation (Figure 5.11). Overexpression of MyD88 and TRAF6 activated IRF7 in a reporter gene assay (Figure 5.12), and as expected, both of these signals were inhibited by A46R.

5.2.9 A46R inhibits MyD88- and TRAF6-dependent IRF5 activation

MyD88, IRAK-1 and TRAF6 activate IRF5 in the TLR7 signalling pathway (Schoenemeyer *et al.,* 2005). To measure IRF5 activation, I employed an *in vivo* reporter assay similar in principle to those described for IRF3 and IRF7 activation in sections 5.2.1 and 5.2.5 respectively. Overexpression of MyD88 and TRAF6 activated IRF5 in a reporter gene assay (Figure 5.12). Both of these signals were inhibited by A46R.

5.2.10 A46R and A52R target different TLR3-mediated signalling pathways

Analysis of NFκB and IRF3 activation by TLR3 demonstrated a clear difference between A46R and A52R in terms of their effects on TLR3 signalling. Figure 5.14

shows that IRF3 activation induced by poly(I:C)/TLR3 was sensitive to A46R, but not A52R. In contrast, Figure 5.15 shows that A52R could potently block poly(I:C)/TLR3-induced NFκB activation as previously demonstrated (Harte *et al.*, 2004), while A46R had no effect on this signal. Stimulation of cells transfected with empty vector alone with poly(I:C) did not lead to induction of either the Gal4-IRF3 reporter or the NFκB reporter (data not shown). Hence A46R and A52R are not functionally redundant in that both may be required to effectively shut down TLR3 signalling.

5.2.11 A46R and N1L inhibit TLR-induced NFκB activation by targeting different components of the signalling pathway

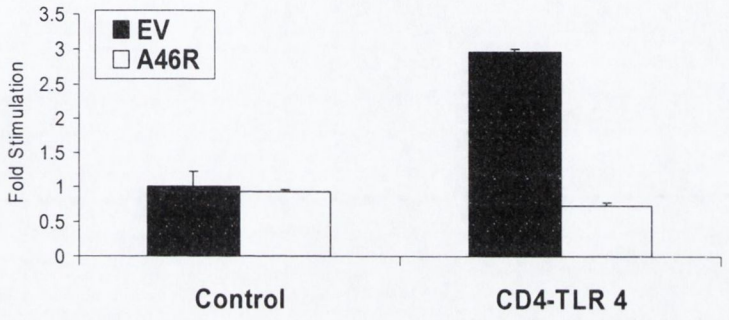
During the course of this study, N1L, a known virulence factor, was shown to inhibit NFκB activated by a wide variety of inducers, such as TLR3 and TLR4 (DiPerna *et al.*, 2004). This is in contrast to A46R which does not inhibit TLR3-mediated NFκB activation (Figure 5.16). It has also been demonstrated that N1L acts at the level of the IKK complex (DiPerna *et al.*, 2004). Again this is in contrast to A46R, which acts at the level of the TIR adapter molecules and has little if any inhibitory effect on NFκB activation mediated by IKK-α, IKK-β or TBK-1 (Figure 5.17).

Figure 5.1: A46R inhibits IRF3 activation in both HEK 293 and RAW 264.7 cells

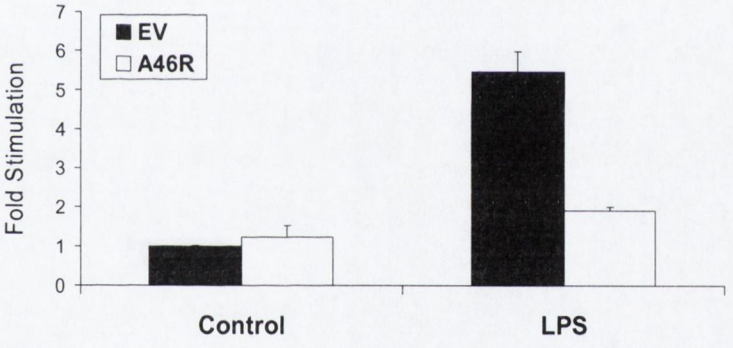
HEK 293 cells (2 x 10^4 cells per well) or RAW 264.7 cells (4 x 10^4 cells per well) were transfected with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF3. In Figure 5.1.A, 50 ng CD4-TLR4 was transfected, along with 100 ng either empty vector (EV) or A46R expression vector. In Figure 5.1.B, cells were stimulated with 1000 ng/ml LPS for 6 hours. In Figure 5.1.C, 10 ng TRIF, 50 ng TRAM or 50 ng TBK1 was transfected, along with 100 ng either empty vector or A46R. In all cases, cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.1: A46R inhibits IRF3 activation in both HEK 293 and RAW 264.7 cells

A: HEK 293



B: RAW 264.7



C: HEK 293

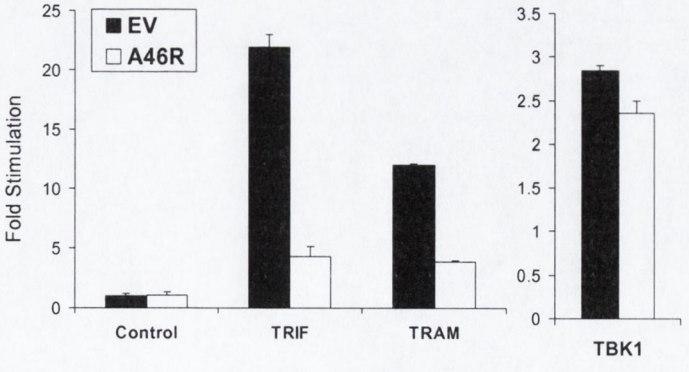
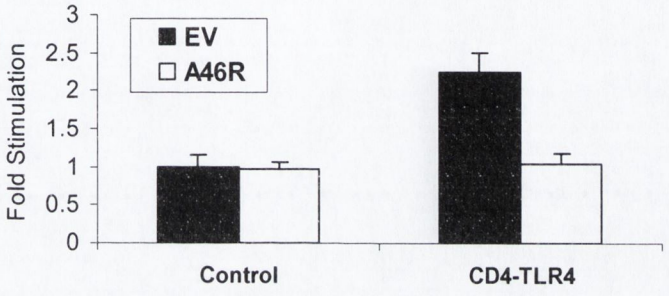


Figure 5.2: A46R inhibits ISRE activation in both HEK 293 and RAW 264.7 cells

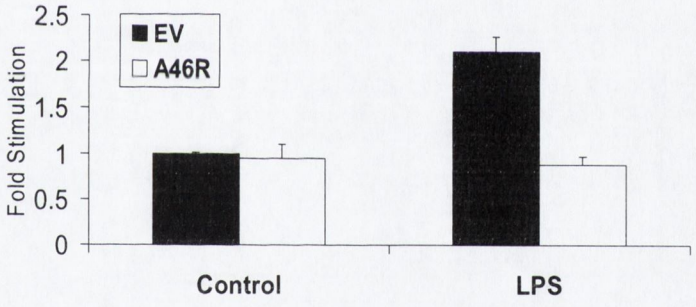
HEK 293 cells (2 x 10^4 cells per well) and RAW 264.7 cells (4 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and an ISRE luciferase construct. Figure 5.2.B, 50 ng CD4-TLR4 was transfected, along with 100 ng either empty vector (EV) or A46R expression vector. In Figure 5.2.B, cells were stimulated with 1000 ng/ml LPS for 6 hours. In Figure 5.2.C, 10 ng TRIF, 50 ng TRAM or 50 ng TBK1 was in the presence of 100 ng empty vector or A46R. In all cases, cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.2: A46R inhibits ISRE activation in both HEK 293 and RAW 264.7 cells

A: HEK 293



B: RAW 264.7



C: HEK 293

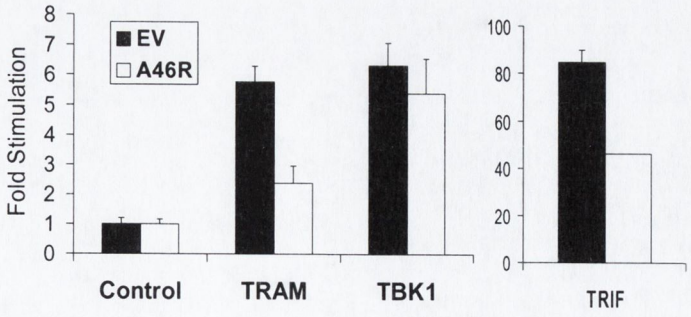
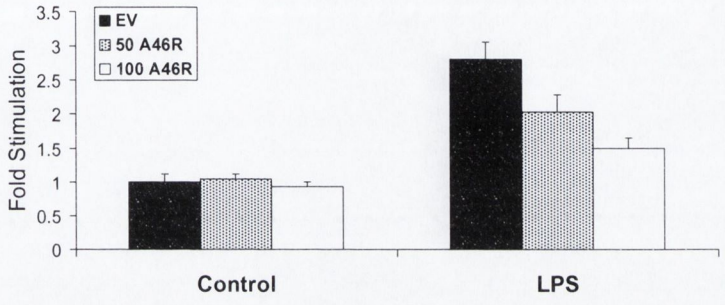
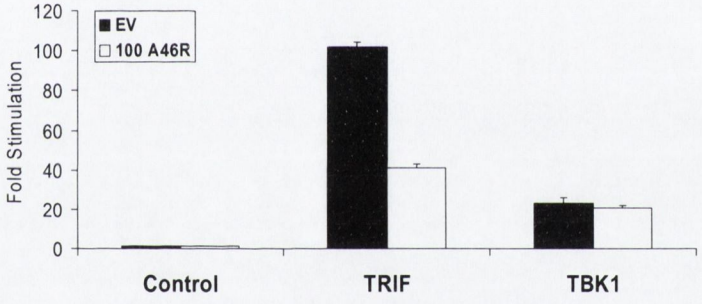


Figure 5.3: A46R inhibits both TLR4- and TRIF-mediated activation of the IFN-β promoter

A: RAW 264.7



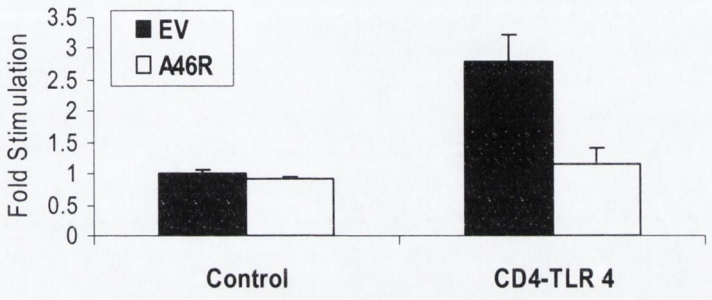
B: HEK 293



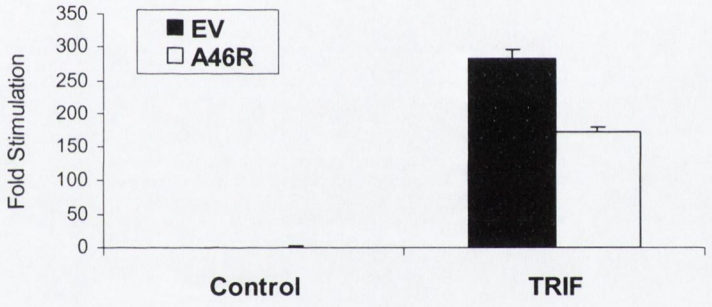
HEK 293 cells (2 x 10^4 cells per well) and RAW 264.7 cells (4 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and an IFN-β promoter luciferase construct along with either empty vector (EV) or the indicated amount (ng) of A46R. In Figure 5.3.A, cells were stimulated with 1000 ng/ml LPS for 6 hours. In Figure 5.3.B, the cells were also transfected with either 10 ng TRIF or 50 ng TBK1. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.4: A46R inhibits both TLR4- and TRIF-mediated activation of the RANTES promoter in HEK 293 cells

A: CD4-TLR 4



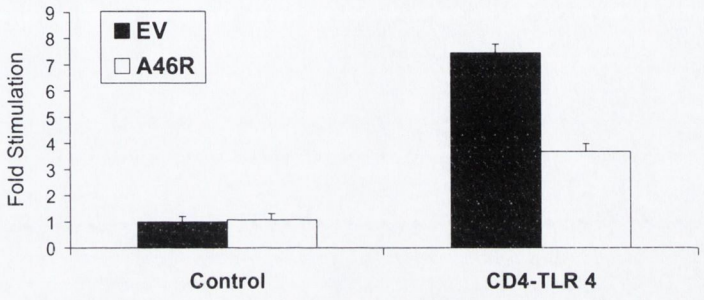
B: TRIF



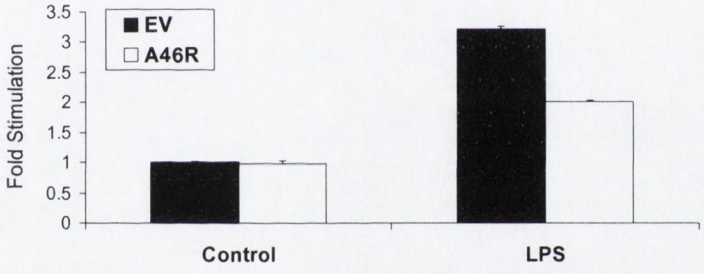
HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and a plasmid encoding a RANTES promoter luciferase construct, together with either 50 ng CD4-TLR 4 (A) or 10 ng TRIF (B), in the presence of 100 ng empty vector (EV) or A46R. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

Figure 5.5: A46R inhibits TLR4-mediated IRF7 activation in both HEK 293 and RAW 264.7 cells

A: HEK 293

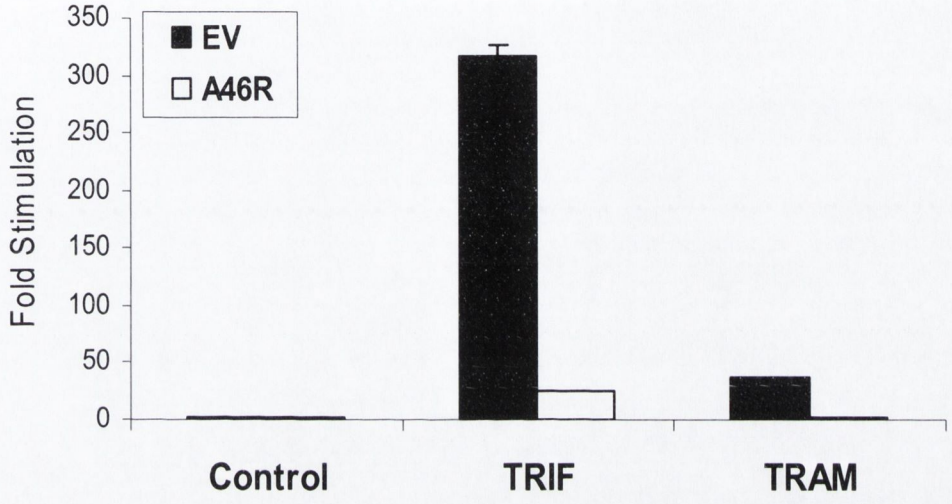


B: RAW 264.7



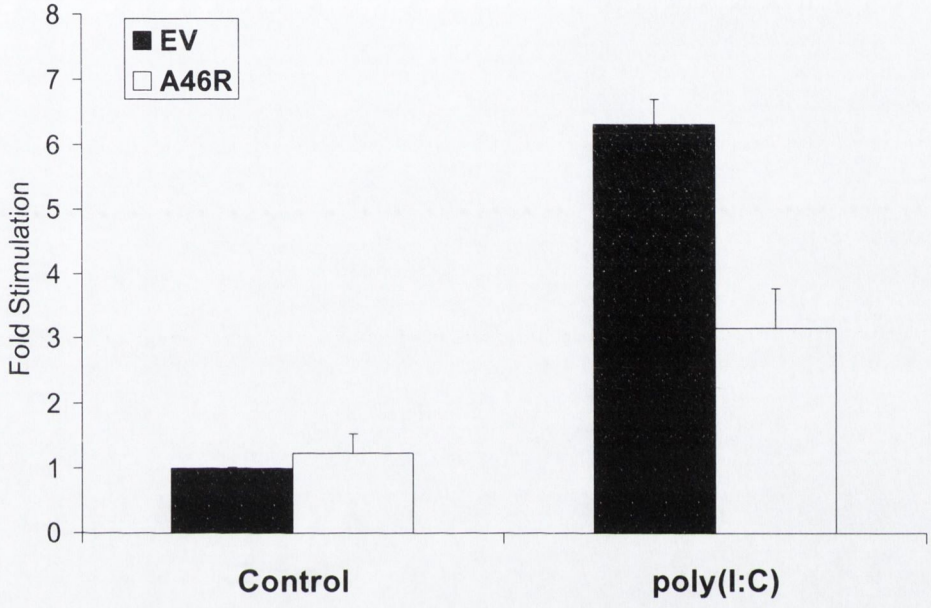
HEK 293 cells (2 x 10^4 cells per well) and RAW 264.7 cells (4 x 10^4 cells per well) were transfected with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF7, along with 100 ng empty vector (EV) or cDNA encoding A46R where indicated. In Figure 5.3.A, the cells were also transfected with 50 ng CD4-TLR 4. In Figure 5.5.B, cells were stimulated with 1000 ng/ml LPS for 6 hours. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.6: TRIF- and TRAM- dependent IRF7 activation is inhibited by A46R in HEK 293 cells



HEK 293 cells (2 x 10^4 cells per well) were transfected with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF7, together with either 10 ng TRIF or 50 ng TRAM in the presence of 100 ng empty vector (EV) or A46R expression vector. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

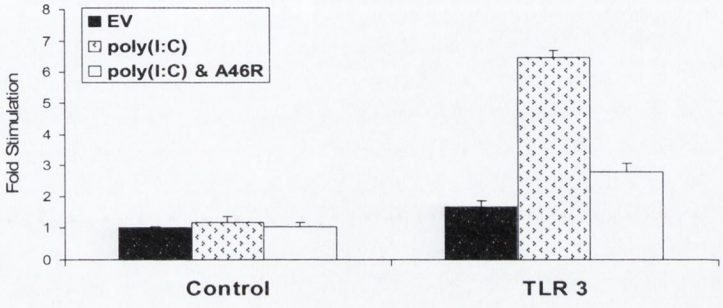
Figure 5.7: TLR3-mediated IRF3 activation is inhibited by A46R in RAW 264.7 cells



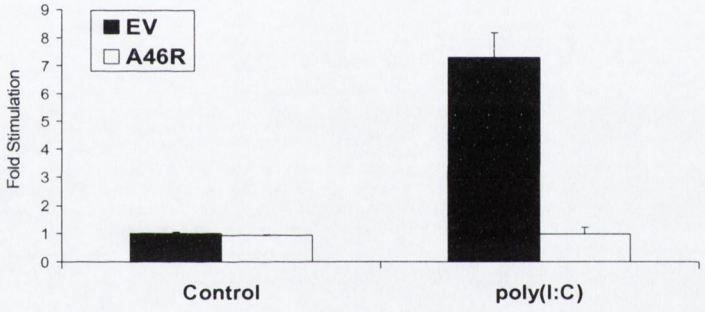
RAW 264.7 cells (4 x 10^4 cells per well) were transfected with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF3, together with 100 ng either empty vector (EV) or A46R expression vector. Six hours prior to harvesting, the cells were stimulated with 25 mg/ml poly(I:C). Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from two separate experiments, each performed in triplicate.

Figure 5.8: A46R inhibits TLR3-mediated IRF7 activation

A: HEK 293



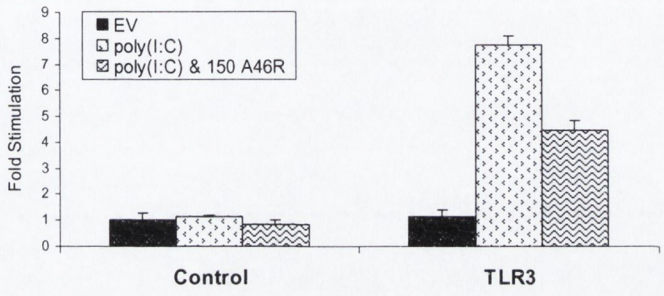
B: RAW 264.7



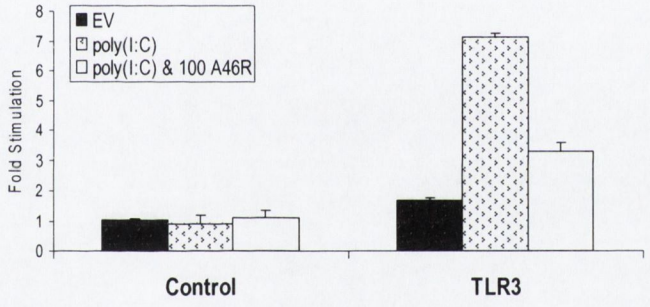
HEK 293 cells (2 x 10^4 cells per well) and RAW 264.7 cells (4 x 10^4 cells per well) were transfected with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF7, in the presence of 100 ng empty vector (EV) or A46R expression plasmid. The HEK 293 cells were also transfected with 0.5 ng TLR 3 (A). Six hours prior to harvesting, the cells were stimulated with 25 mg/ml poly(I:C). Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.9: A46R inhibits TLR3-mediated activation of ISRE and of the IFN-β promoter

A: ISRE

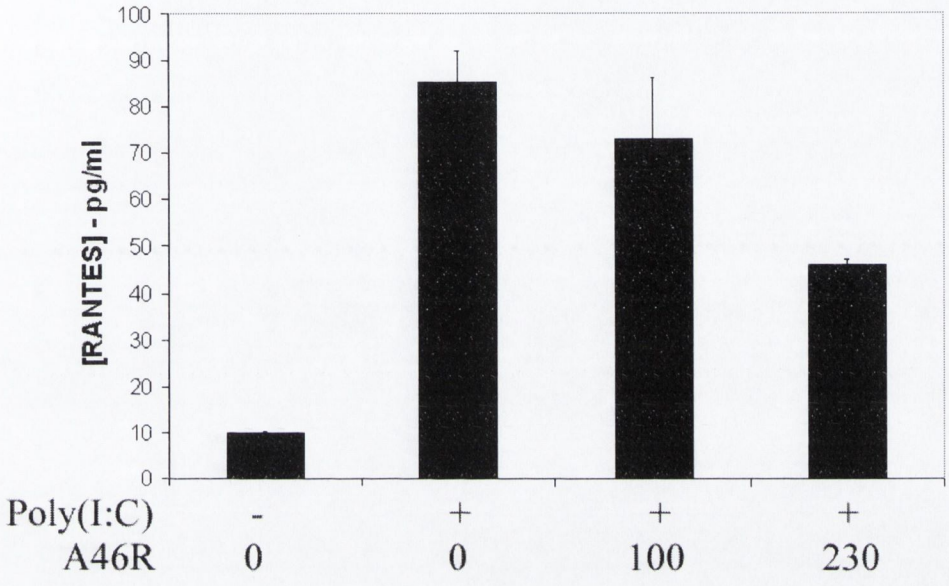


B: IFN-β



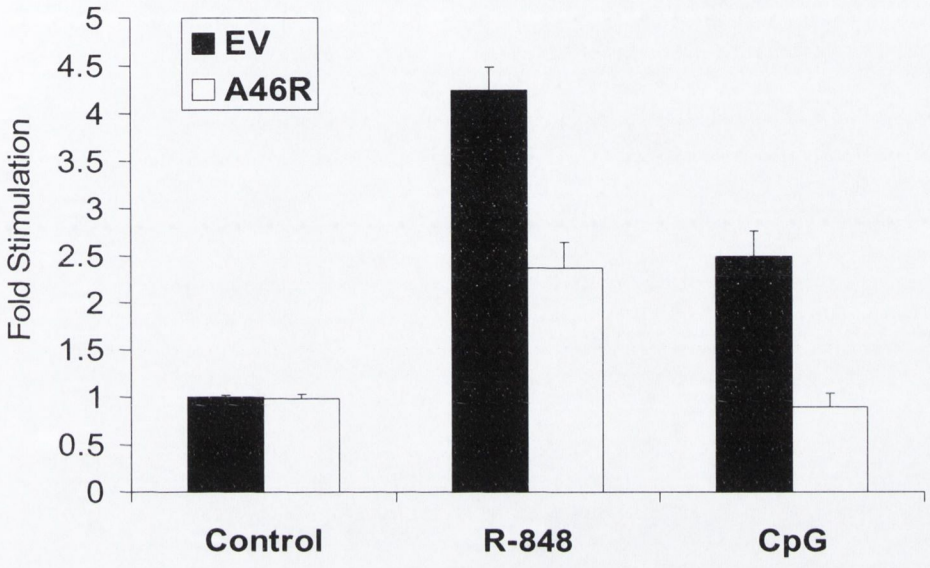
HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and either an ISRE (A) or an IFN-β promoter (B) luciferase construct in the presence of 0.5 ng TLR3, together with empty vector or the indicated amount (ng) of A46R cDNA. Cells were harvested 6 hours after stimulation with 25 mg/ml poly(I:C), and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.10: A46R inhibits poly(I:C)-induced RANTES production



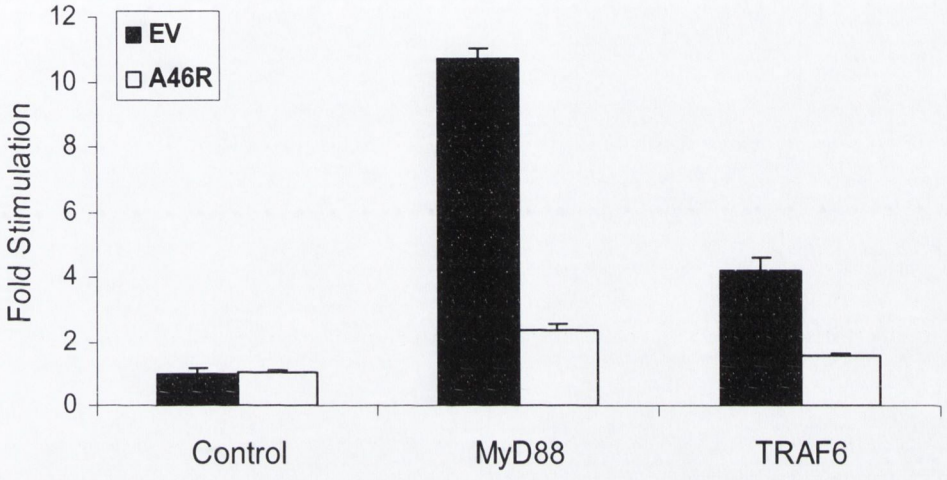
HEK 293 cells stably expressing TLR3 (2 x 10^4 cells per well) were seeded into 96-well plates and transfected with the indicated amounts of A46R expression plasmid (ng) 24 h later. 24 h after transfection, cells were stimulated with 25 mg/ml poly(I:C). After 24 h, supernatants were harvested and the RANTES concentration was determined by ELISA (R&D Biosystems). The experiment was performed three times in triplicate and data is expressed as mean fold induction ± s.d. from one representative experiment.

Figure 5.11: A46R inhibits both TLR7- and TLR9-mediated IRF7 activation in RAW 264.7 cells



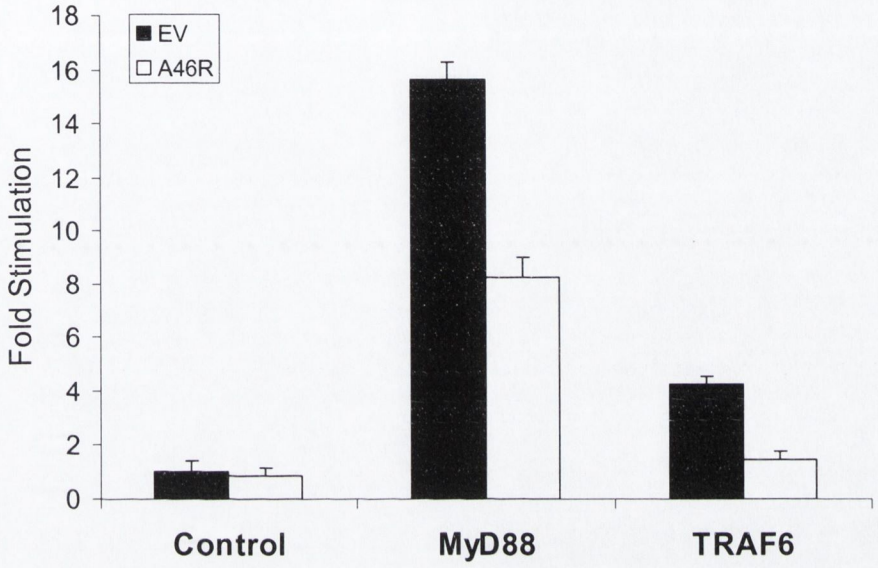
RAW 264.7 cells (4 x 10^4 cells per well) were transfected with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF7, in the presence of 100 ng empty vector (EV) or A46R expression plasmid. Six hours prior to harvesting, cells were stimulated with 1 μM R-848 or 5 μg/ml CpG. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.12: A46R inhibits both MyD88- and TRAF6-dependent IRF7 activation in HEK 293 cells



HEK 293 cells (2 x 10^4 cells per well) were transfected with either 25 ng MyD88 expression plasmid or 50 ng cDNA encoding TRAF6, in the presence of 100 ng empty vector (EV) or A46R expression plasmid, together with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF7. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.13: A46R inhibits both MyD88- and TRAF6-dependent IRF5 activation in HEK 293 cells



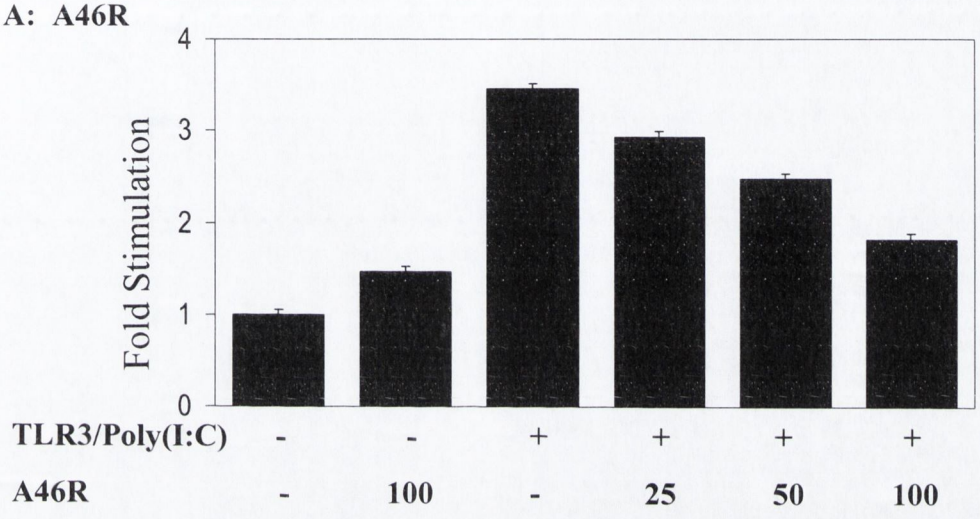
HEK 293 cells (2 x 10^4 cells per well) were transfected with either 25 ng MyD88 expression plasmid or 50 ng cDNA encoding TRAF6, in the presence of 100 ng empty vector (EV) or A46R expression plasmid, together with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF5. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.14: A46R inhibits poly(I:C)-induced IRF3 activation, A52R does not

HEK 293 cells (2 x 10^4 cells per well) were transfected with the pFR luciferase construct, a Renilla-luciferase internal control and a plasmid encoding Gal4 fused to IRF3 in the presence of 0.5 ng TLR3, together with the indicated amounts (ng) of either A46R cDNA (A) or A52R cDNA (B). Cells were harvested 6 hours after stimulation with 25 μg/ml poly(I:C), and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.14: A46R inhibits poly(I:C)-induced IRF3 activation, A52R does not

A: A46R



B: A52R

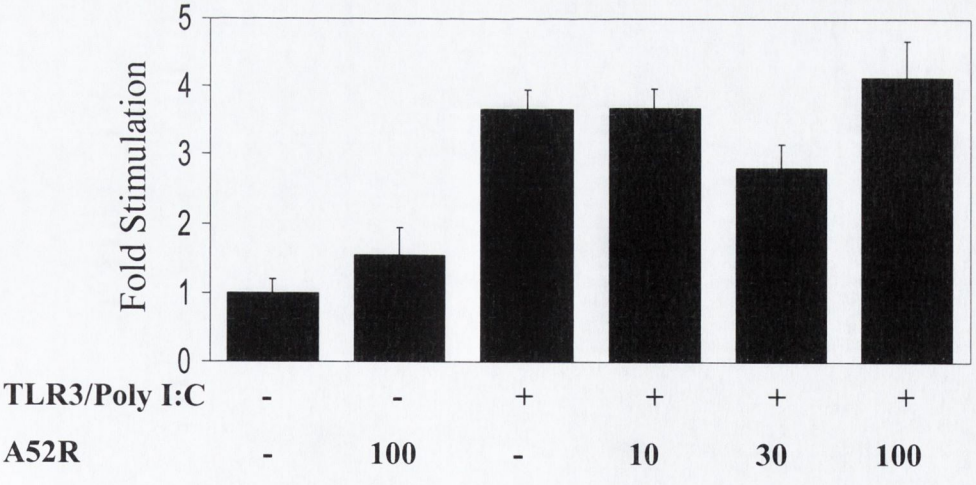
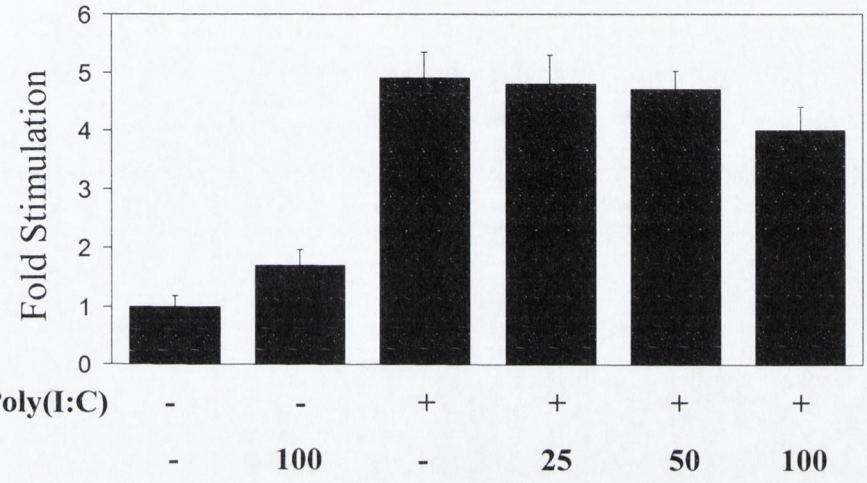


Figure 5.15: A52R inhibits poly(I:C)-induced NFκB activation, A46R does not

HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and a κB-luciferase construct in the presence of 0.5 ng TLR3, together with the indicated amounts (ng) of either A46R cDNA (A) or A52R cDNA (B). Cells were harvested 6 hours after stimulation with 25 μg/ml poly(I:C), and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Figure 5.15: A52R inhibits poly(I:C)-induced NFκB activation, A46R does not

A: A46R



B: A52R

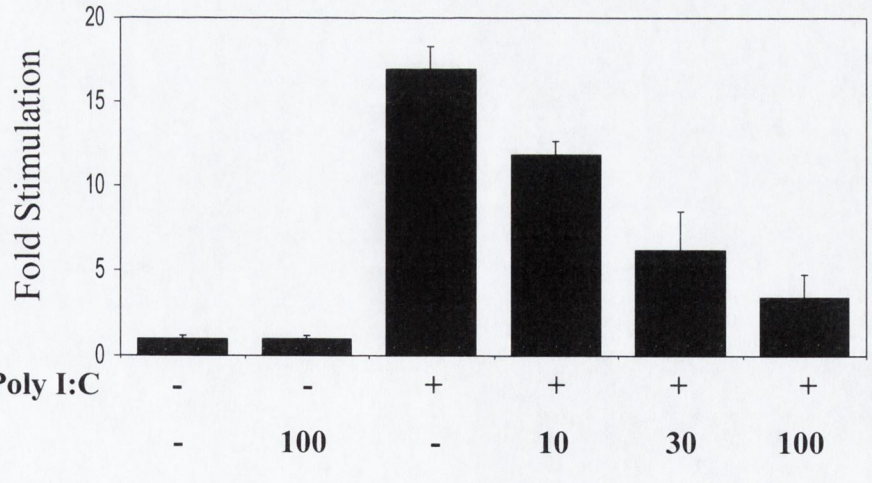
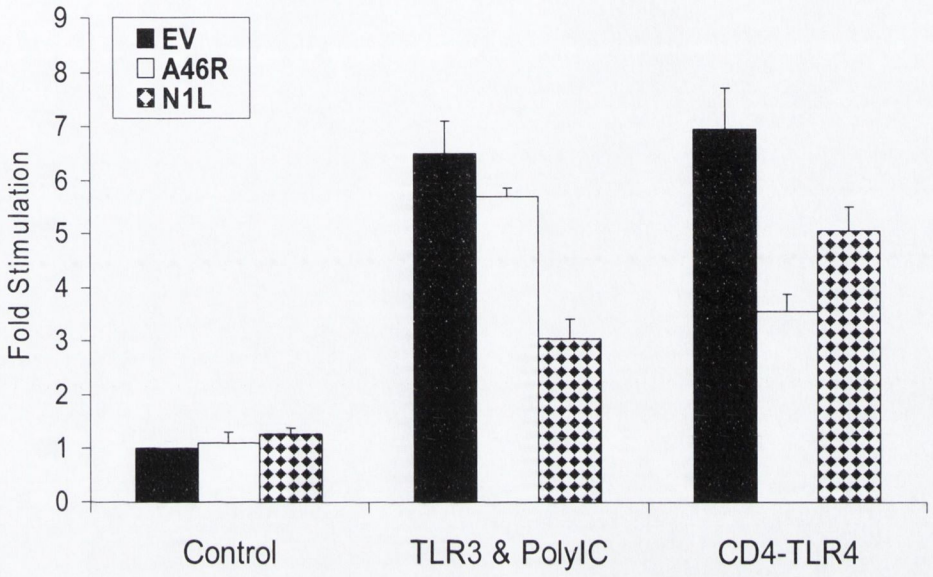
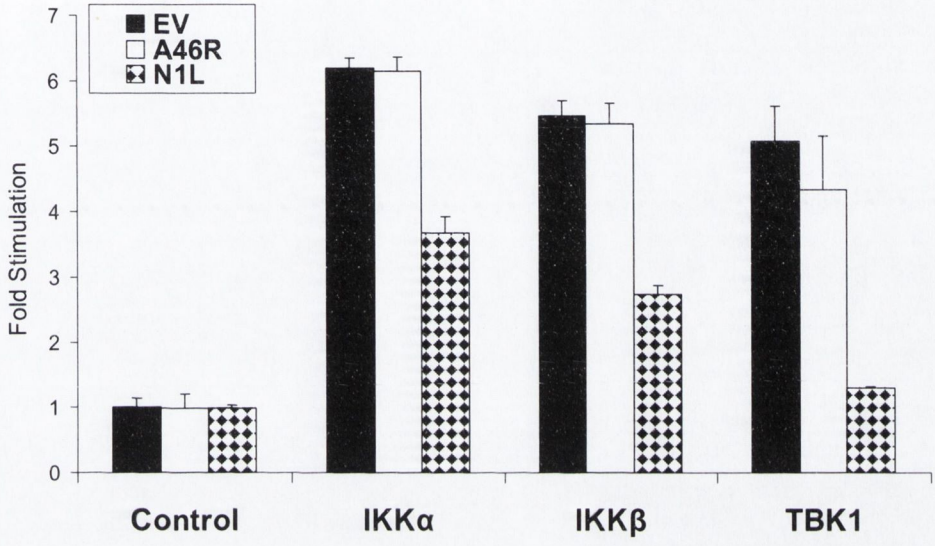


Figure 5.16: N1L inhibits TLR-mediated NFκB activation



HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and a κB-luciferase construct along with 100 ng of either A46R or N1L cDNA, together with either 5 ng TLR 3 or 50 ng CD4-TLR 4. Cells were harvested 6 hours after stimulation with 25 μg/ml poly(I:C), (24 hours after transfection), and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of two separate experiments, each performed in triplicate.

Figure 5.17: N1L inhibits NFκB activation by targeting the IκB kinases



HEK 293 cells (2 x 10^4 cells per well) were transfected with a Renilla-luciferase internal control and a κB-luciferase construct together with 100 ng of either A46R or N1L cDNA, along with 50 ng of either IKKα, IKKβ or TBK1. Cells were harvested 24 hours after transfection, and the reporter gene activity was measured. Data are expressed as mean fold induction ± s.d. relative to control levels, for a representative experiment from a minimum of two separate experiments, each performed in triplicate.

5.3 Discussion

TLR4 uses both TRIF and TRAM to activate IRF3 and hence induce type I interferon, as well as for late phase NFκB activation (Fitzgerald *et al.*, 2003a; Fitzgerald *et al.*, 2003b; Yamamoto *et al.*, 2003a; Hoebe *et al.*, 2003). A46R inhibited TRIF-, TRAM-, TLR4- and TLR3-mediated IRF3 activation. A46R appears to function at the level of the TIR adapters as their signalling is inhibited, but IRF3 activation induced by the molecule just downstream of TRIF, TBK1, is not. A46R may disrupt the formation of the TRIF-TBK1 signalling complex. Due to the difficulty of expressing TRIF, I was unsuccessful in my investigations to confirm this hypothesis.

It has previously been shown that the ISRE used in the reporter gene assays in this study serves primarily as an IRF3 read-out, and is not driven *via* a type I interferon feedback loop (Wietek *et al.,* 2003). Activation of the ISRE by TLR3, TLR4, TRIF and TRAM was also inhibited by A46R, due to its targeting of the IRF3 activation pathway at the level of the TIR adapter molecules. Consistent with this, TBK1-mediated ISRE activation was not affected. Wietek and colleagues (2003) showed that TLR4-induced activation of the ISRE requires the p65 subunit of NFκB as well as IRF3. Therefore, inhibition of TLR4-induced activation of the ISRE is probably due to the effects of A46R on TLR-4 induced IRF3 and NFκB activation. TLR3-induced ISRE activation does not require NFκB (Wietek *et al.*, 2003). This is consistent with the fact A46R did not inhibit TLR3-induced NFκB activation, but did inhibit TLR3-mediated activation of the ISRE.

It has been demonstrated that p65 is redundant for induction of the IFN-β promoter after poly(I:C) stimulation but is absolutely essential for LPS-induced IFN-β expression

(Peters *et al.*, 2002). Because TLR4-mediated activation of the IFN-β promoter involves both NFκB and IRF3, inhibition by A46R of the promoter probably involves targeting of both transcription factors. A46R inhibited poly(I:C)-induced IRF3 activation (Figures 5.7 and 5.14), and because of that, it also inhibited poly(I:C)-induced ISRE activation and activation of the IFN-β promoter (Figure 5.9). Again, A46R seems to be exerting its inhibitory effects on the IFN-β promoter at the level of the TIR adapters, as TRIF-, but not TBK1-, mediated activation of the IFN-β promoter was inhibited (Figure 5.3).

Because TRIF-dependent type I interferon induction is very important in containing VV replication, the ability of A46R to inhibit TRIF-mediated IRF3 activation may be of huge significance to VV infection. The response of macrophage cultures to infection by VV was demonstrably impaired when the *Trif* gene was disrupted, in that macrophage monolayers from *Lps2* homozygotes supported the replication of *Vaccinia* to a higher titre than did macrophages from normal mice (Hoebe *et al.*, 2003). Hoebe and colleagues (2003) also demonstrated that when infected with mCMV, robust production of type I interferon was observed in the serum of normal mice, but no type I interferon was detected in the serum of *Lps2* mutant homozygotes. Hence, the antiviral effect conferred by TRIF is very broad with regard to the virus species and is correlated with type I interferon production.

It has been reported that TRAM both does and does not activate the IFN-β promoter (Fitzgerald *et al.*, 2003a; Bin *et al.*, 2003). When I overexpressed TRAM with the IFN-β promoter in a reporter gene assay, I also found that TRAM did not activate the IFN-β promoter. I was surprised at this observation because in TRAM-deficient cells, TLR4-

mediated interferon-β production and activation of signalling cascades were abolished (Yamamoto *et al.*, 2003b), showing that TRAM is required for TLR4-mediated IFN-β production, and by implication, for activation of the IFN-β promoter. Other TIR adapters that have been found to be important for a TLR signalling pathway have also been shown to activate those pathway themselves on overexpression, for example TRIF is required for TLR4-induced IFN-β production (Yamamoto *et al.*, 2003a) and it can activate the IFN-β promoter by itself when overexpressed (Figure 5.3). However, TRAM may not be sufficient to activate the IFN-β promoter by itself; it may require cooperation with another TIR adapter molecule.

RANTES is a small protein of 68 amino acids and belongs to the chemokine family. RANTES production, which is generated predominantly by CD8+ T cells, epithelial cells, fibroblasts and platelets, is a particular feature of inflammation. Increased RANTES expression has been associated with a wide range of inflammatory disorders and pathologies, in which it is thought to act by promoting leukocyte infiltration to sites of inflammation (reviewed by Appay and Jones, 2001). Members of the NFκB and IRF transcription factor families participate in the virus-mediated activation of RANTES gene transcription. IRF3 plays a primary role in the Sendai virus-inducible activation of the human RANTES gene. A constitutively active form of IRF7 constitutes a strong activator of RANTES promoter (reviewed by Génin *et al.,* 2000). Use of dominant negatives of either IκB-α or IRF3 demonstrate that disruption of either pathway dramatically abolishes the ability of the other to and activate RANTES expression (Génin *et al.,* 2000). Furthermore, co-expression of IRF3, IRF7 and p65/p50 leads to synergistic activation of RANTES promoter transcription (Génin *et al.,* 2000).

TLR4-mediated activation of the RANTES promoter was potently inhibited by A46R (approximately 60%), as was TRIF-mediated RANTES promoter activation (Figure 5.4). This could be due to A46R acting on the NFκB or IRF3 pathways, or both. TRAM did not activate the RANTES promoter in a reporter gene assay. LPS did not induce RANTES production as measured by an ELISA, but poly(I:C) did (Figure 5.10). Poly(I:C)-induced RANTES production is TRIF dependent (Yamamoto *et al.*, 2003a). Transient transfection of HEK 293 cells stably expressing TLR3 with A46R suppressed poly(I:C)-induced RANTES release by approximately 50%. This is suggestive of a powerful effect on RANTES induction given that not all of the cell population stimulated by poly(I:C) to release RANTES would be expected to express A46R in this transient transfection system. Because A46R inhibits poly(I:C)-induced RANTES production but not poly(I:C)-induced NFκB activation (Figure 5.15), it is probable that this signal is more dependent on the IRF transcription factors than NFκB.

When overexpressed in HEK 293 cells, TRIF and TRAM both activate IRF7. These signals are inhibited by A46R (Figure 5.6). TLR4-mediated IRF7 activation is dependent on both TRIF and TRAM (Fitzgerald *et al.*, 2003a), and was inhibited by A46R (Figure 5.5). TLR3-mediated IRF7 activation is solely dependent on TRIF, and is also inhibited by A46R (Figure 5.8) IRF7 activation induced by TLRs 7, 8 and 9 involves MyD88, TRAF6 and IRAK-1 and IRAK-4 (Kawai *et al.*, 2004; Honda *et al.*, 2004), similar molecules that are involved in the MyD88-dependent NFκB activation induced by these TLRs. A46R can disrupt the MyD88-TRAF6 complex. This could be how A46R is inhibiting IRF7 activation by TLR7 and TLR9. The induction of type I interferon is very important in containing viral infection. The targeting of TLR9 signalling by A46R is interesting because TLR9, which recognises CpG motifs in

dsDNA, probably plays a role in recognising VV infection. Also, it is likely that both TLR3 and TLR4, which recognise dsRNA and various viral products respectively, play roles in detecting and combating VV infection. The results discussed above show that A46R inhibits the two arms of type I interferon induction from the TLRs i.e. the TRIF-IRF3/7 pathway and the MyD88/IRF7 pathway, possibly explaining why VV lacking A46R is less virulent than wild-type VV (Stack *et al.*, 2005).

IRF5 activation in response to TLR7 and 9 ligands is also MyD88/IRAK-1/TRAF6 dependent and is important in proinflammatory cytokine induction (Takaoka *et al.*, 2005; Schoenemeyer *et al.*, 2005). A46R inhibits MyD88- and TRAF6-mediated IRF5 activation (Figure 5.13), again probably by disrupting the complex in which MyD88 is associated.

A46R inhibited TLR3-mediated IRF3 activation, but A52R did not (Figure 5.14). However, A52R has previously been shown to inhibit TLR3-mediated NFκB activation quite potently (Harte *et al.*, 2003). In the previous chapters, I have showed that A46R could inhibit NFκB activation induced by many TLRs, including TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8 and TLR9. However, when I examined TLR3-mediated NFκB activation, I found that A46R did not inhibit this signal. This was quite surprising considering that A46R had been found to interact with TRIF, the only TIR adapter involved in TLR3 signal transduction. Also, A52R does not inhibit TLR3-mediated activation of the ISRE or IRF7 (data not shown), both of which signals are inhibited by A46R. These differing effects on distinct TLR3 pathways is one of several lines of evidence that A46R and A52R are not functionally redundant. A46R and A52R target distinct TLR signalling molecules (Harte *et al.*, 2003), which has already been discussed in Section 3.3. TLR4-mediated IRF3 activation and TLR7/9-mediated

IRF7, which are inhibited by A46R, are not inhibited by A52R (data not shown). From these results, it is clear that neither the TRIF-dependent nor MyD88-dependent IRF activation pathways are sensitive to A52R and that TLR-mediated IRF and interferon activation are key targets for A46R. Also, the effects of A46R and A52R on MAP kinase activation are very different. A52R has no inhibitory effect on MAP kinase activation (unpublished data), but A46R blocks MAP kinase activation.

TRIF interacts with TRAF6 through TRAF6-binding motifs found in the N-terminal portion of TRIF and the TRAF domain of TRAF6 (Sato *et al.*, 2003, Jiang *et al.*, 2004). Disruption of TRAF6 binding motifs of TRIF disabled it from binding TRAF6, and resulted in a reduction in the TRIF-induced activation of NFκB (Sato *et al.*, 2003; Jiang *et al.*, 2004). TBK1 associates with the N-terminal portion of TRIF to activate IRF3 (McWhirter *et al.,* 2004). Although TRAF6 and TBK1 probably do not associate with the same residues of TRIF, it seems that TRAF6 physically blocks the association between TRIF and TBK1 because the increased expression of the TRAF domain of TRAF6 repressed the association of TRIF-TBK1 (Sato *et al.*, 2003). RIP1 has recently been found to associate with TRIF *via* a C-terminus RIP homotypic interaction motif (RHIM) and to be important in TLR3-induced TRIF-mediated NFκB activation (Meylan *et al.,* 2004). In RIP1 deficient cells, poly(I:C)-induced NFκB activation was abolished, however, TRIF-mediated NFκB activation was impaired but not completely abrogated. From these data and other studies, it can be hypothesised that TRIF can induce NFκB activation using RIP1 and TRAF6 independently. However, TLR3-induced NFκB activation is only *via* RIP1 (Meylan *et al.,* 2004).

Because A46R does not inhibit TLR3-induced NFκB activation, it might prevent TBK1 associating with TRIF but not TRIF interacting with RIP1. Thus, it is possible that A46R did not inhibit TLR3-mediated NFκB activation, because it could not affect the TRIF-RIP1 association, but it inhibited NFκB activation triggered by TRIF overexpression by impairing TRIF's association with TRAF6. This could be the reason why A46R requires more than just the TIR domain alone of TRIF with which to interact i.e. it needs a portion of the TRIF N-terminus also. Again, it is clear that while A52R exerts a stronger influence on the TLR3-NFκB signal, activation of IRF3 is the key target for A46R. A52R has been shown to disrupt the IRAK-2/Mal and TRAF6/TAB1 signalling complexes (Harte *et al.*, 2003). However, its exact mechanism of inhibiting TLR3-mediated NFκB activation has not been determined. If, as I have just hypothesised, TLR3-mediated NFκB activation occurs predominantly *via* the TRIF-RIP1 and not the TRIF-TRAF6 interaction, it is likely that A52R does not inhibit this pathway by targeting TRAF6. It is possible that IRAK-2 may play an as yet unidentified role in TLR3 signalling, and is targeted by A52R. Jiang and colleagues (2003) have suggested that TLR3-induced NFκB activation is through an IRAK-1 independent pathway. However, IRAK-2 deficient cells were not tested. The generation of a IRAK-2 knockout mouse will be required to determine its exact role in anti-viral immunity and TLR signalling.

N1L inhibits signalling to NFκB by IL-1, TLR2, TLR4, TRIF, TRAM, MyD88 and Mal, all of which are also inhibited by A46R (DiPerna *et al.*, 2004). However, although some of the effects of A46R and N1L overlap, N1L inhibits a far wider range of inducers of NFκB than A46R does. For example, N1L inhibits signalling to NFκB by TNF-α, TLR3, TRAF2 (DiPerna *et al.*, 2004). N1L also inhibits NFκB activated by

members of the IKK family, including TBK1, IKK-α and IKK-β. N1L has been found to associate with IKK-α, IKK-β, IKK-γ and TBK1, and it is proposed that N1L exerts its inhibitory functions by targeting the members of, and possibly disrupting, the IKK complex (DiPerna *et al.*, 2004). Therefore, N1L functions as an inhibitor downstream of A46R, as evidenced by Figure 5.16, which shows that N1L, but not A46R, can inhibit NFκB activated by members of the IKK family. N1L can also inhibit TBK1-mediated IRF3 activation (data not shown), but A46R cannot. This again demonstrates that A46R and N1L target very different components of the signalling pathway in question. I have not tested the effects of N1L on TLR7- and TLR9-induced IRF7 activation. In response to TLR7 and TLR9 ligands, IRF7 is activated by members of the IRAK family (Kawai *et al.*, 2004; Honda *et al.*, 2004; Uematsu *et al.*, 2004) and I speculate that N1L would not inhibit these signals, because it has already been shown to activate at the level of the IKKs (DiPerna *et al.*, 2004) which are not involved in IRF7 activation induced by TLRs 7 and 9 (Honda *et al.*, 2004; Uematsu *et al.*, 2004).

The importance of blocking TLR signalling is demonstrated by the retention by VV of at least three distinct mechanisms of disrupting these pathways. Although the inhibitory effects of A46R, A52R and N1L overlap somewhat, it is nonetheless very clear that each has very specific target molecules and particular functions. Furthermore, the attenuated phenotypes seen in the absence of *A46R*, *A52R* or *N1L* is an indicator that none of these molecules are redundant in function and that they cannot compensate for each other. The attenuated phenotypes also provide evidence for a role for TLRs in containing VV infections.

Chapter Six

General Discussion and Future Perspectives

6.1 General Discussion

IL-1 signalling is an area that has been studied in great detail since IL-1 was first identified as an important mediator of inflammation. The identification of the TIR domain and the IL-1R/TLR superfamily has shown that many of these TIR-domain containing receptors utilise the same signalling components to activate downstream targets and transcription factors. Four adapter proteins – MyD88, Mal, TRIF and TRAM – transduce signals from all of the TLRs, activating protein kinases and then transcription factors that cause inflammatory effects. The identification of viral immune evasion strategies and the analysis of the molecular aspects of host-pathogen interactions are crucial to enhancing understanding of microbial pathogenesis and immunity to infection. During a database search for novel TIR-containing proteins, A46R from VV was found (Bowie *et al.*, 2000), which was potentially interesting because many viruses encode homologues of host factors for immunomodulatory purposes. To date, A46R is the only identified viral TIR domain-containing protein. This study has focused on the characterisation of the inhibitory effects of A46R on IL-1R and TLR signalling and on the determination of its intracellular targets.

Our initial investigations concerned the ability of A46R to inhibit IL-1-induced NFκB activation, without having an effect on TNF-α-induced NFκB activation. Because A46R also inhibited all IL-1 signals tested (IL-8 induction, MAPK activation and p65 transactivation), I reasoned that it must be acting very close to the receptor before the signals bifurcate. I showed that A46R interacted with MyD88 and inhibited many MyD88-dependent signals such as NFκB activated by TLRs 1, 2, 4, 5, 6, 7, 8, and 9. To determine how A46R was impairing MyD88-dependent signals, I examined the effect of A46R on the formation of the MyD88-TRAF6 complex and found that it was

being disrupted by the presence of A46R. These results were the first example of a viral protein targeting MyD88.

On closer examination, I realised that TLR4-mediated NFκB activation was much more potently inhibited than that induced by IL-1. This observation led to the discovery that A46R targets other TIR adapter molecules involved in TLR signalling to NFκB, namely Mal, TRIF and TRAM. A46R interacts with Mal, and inhibits Mal-dependent signalling. A46R can disrupt the Mal-TRAF6 interaction, which has been shown to be important for NFκB-dependent gene induction (Mansell *et al.*, 2004). A46R also interacts with TRIF and TRAM and inhibits their activation of NFκB.

The fact that A46R associated with all four TLR4 adaptors may suggest that TLR4 is a particularly important target for VV immunoevasion. Indeed, TLR4 has been purported to have a role in responding to F protein of respiratory syncytial virus (Kurt-Jones *et al.*, 2000; Haeberle *et al.*, 2002). TLR4 has also been shown to be activated by envelope proteins from both mouse mammary tumour virus and Moloney murine leukaemia virus (Rassa *et al.*, 2002). However VV may interact with other or multiple TLRs that also use these adaptors. Possible VV PAMPs detected by TLRs could be virion proteins (potentially detected by TLR2 or TLR4; Vaidya *et al.*, 2003; Rassa and Ross, 2003), intracellular dsRNA produced from the bi-direction transcription of the VV genome (potentially detected by TLR3; Alexopoulou *et al.*, 2001), or the dsDNA genome itself (potentially detected by TLR9 which responds to the dsDNA genome of herpes simplex virus, Krug *et al.*, 2004; Lund *et al.*, 2003). The role of TLRs in responding to VV PAMPs is currently being investigated.

A concern in using co-immunoprecipitation experiments to study intracellular signalling pathways is that overexpression of one or more signalling components might disrupt the physiologic stoichiometry and may favour, or even create, protein-protein interactions through TIR domains that might not occur under normal circumstances of agonist-driven stimulation. Alternatively, different proteins might be co-localised, but not physically associated, in membrane regions not disrupted by typical detergent solubilisation methods (e.g. lipid rafts) (Vogel *et al.*, 2003). However, there are several lines of evidence suggesting that the interactions between A46R and the TIR adapter molecules in this study are direct and specific. Firstly, VV-expressed A46R interacted with ectopically expressed Mal and MyD88. Second, the interaction of A46R with MyD88, Mal and TRIF were confirmed in yeast two-hybrid pairwise assays. 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 (Stack *et al.*, 2005) or SARM.

Exploring how A46R interacts with TIR adapter molecules has been studied in this work using mutants of the TIR adapter molecules themselves, such as isolated TIR domains and TIR adapters with mutations in their BB loops. This has demonstrated the importance of the TIR adapter BB loops for interacting with A46R. As yet, it is not known what portions of A46R are important for this interaction. This is being explored using mutagenesis studies. Mutant versions of A46R lacking either the N- or C-termini or both are being made. Also, A46R has two loops on either side of its AB loop that other TIR domains do not possess and the function of these residues are unknown. We have hypothesised that they may represent inhibitory loops. Mutant versions of A46R lacking one or both loops will be made, as will mutant A46R with point mutations

within its BB loop. The interaction and inhibition profiles of all these mutants will be determined.

Because A46R can interact with different and diverse TIR domains, elucidation of the crystal structure of A46R alone should provide important information of general relevance as to how TIR domains interact. So far the crystal structures of the TIR domains of TLR1 and TLR2 have been determined. However, only limited information regarding TIR-TIR interactions was acquired from these crystals. By generating crystals using highly purified recombinant A46R or failing that, GST-A46R, in a complex with purified rMal, it will be possible to obtain important information regarding the actual points of contact between these two TIR domains and the relative importance of the BB loop for the interaction. Also, analysis of the type of interactions between the TIR domains will be possible *i.e.* are they primarily linked by Hydrogen bonds?

Bowie and colleagues (2000) previously identified another VV TLR antagonist, A52R, which could inhibit NFκB activation *via* the TLRs and, like A46R, contribute to virulence (Harte *et al.*, 2003). In this study, I have demonstrated that A46R and A52R are not functionally redundant. Although they do have some overlapping effects (such as the inhibition of MyD88-dependent NFκB activation), their overall effects on TLR signalling are quite distinct. A46R blocks the activation of IRFs by all TLRs tested, whereas A52R blocks the activation of NFκB by all TLRs (Harte *et al.*, 2003). A46R, but not A52R, 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. Given that I have shown here that VV A46R targets human adaptors, together with the fact that the VV and variola A46R amino acid sequences differ by only eight residues (Figure 1.15), it is possible that variola A46R would have an important role in interactions between variola and the human TLR system. Also the fact that A46R, a potent inhibitor of TLR-induced IRF activation, is conserved in variola, whereas the strong NFκB inhibitor that is A52R is not, suggests that perhaps subversion of the interferon response is more important for viral virulence than interference in NFκB.

N1L, a known intracellular VV virulence factor (Kotwal *et al.*, 1989; Bartlett *et al.*, 2002), has recently been shown to function by antagonising many signalling pathways, including TLR signals (DiPerna *et al.*, 2004). N1L acts at a point even further downstream than A52R, at the level of IκB kinases and related kinases involved in NFκB and IRF3 activation (DiPerna *et al.*, 2004). Thus the importance to VV of blocking TLR signalling is demonstrated the retention of the virus of at least three distinct mechanisms of disrupting these pathways. Further the attenuated phenotypes seen in the absence of either *A46R*, *A52R* or *N1L* provides further evidence for a role for TLRs in containing VV infections, and demonstrates that these proteins are not redundant.

Recently, K1L, another VV protein, has been shown to inhibit NFκB activation *via* its inhibition of IκB-α degradation. VV lacking K1L is attenuated and induces NFκB (Shisler and Jin, 2004). This study did not examine whether or not this NFκB activation was TLR-dependent, but the fact that VV has at least four separate proteins

that specifically inhibit NFκB activation indicates that this is an important immune evasion strategy.

The ability of A46R to target intracellular TIR-dependent signalling 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 might account for the early enhanced levels of cells in lungs after infection in the absence of A46R expression (Stack *et al.*, 2005).

6.2 Future Work

- Further analysis of the effects of A46R on gene induction is warranted. Given that I have observed A46R inhibiting many pathways leading to the activation of IRF3, 5 and 7, it is important to correlate these results with an effect on type I interferon induction. It is predicted that A46R would inhibit these signals. VV has been shown to target IL-18 in a number of ways, and A52R can inhibit IL-18-induced NFκB activation (Harte *et al.*, 2003). The effect of A46R, if any, on IL-18 signalling should be investigated. In addition, to further define and clarify the distinct roles of A46R, A52R and N1L, the inhibition profiles of all three molecules on a wide variety of proinflammatory cytokines and interferon regulated genes should be compared.

- As yet the mechanism of inhibition of TLR signalling by A46R is unclear. I have demonstrated that A46R can disrupt some signalling complexes, but not as severely as that observed with A52R (Harte *et al.*, 2003). It would be

interesting to examine the effect of A46R on the formation of other signalling complexes, such as MyD88-IRAK-1. It is possible that A46R may modify the TIR adapter molecules with which it interacts, possibly by altering their phosphorylation patterns. Performing mass spectroscopy analysis on cells infected with wild type VV or VV lacking A46R may determine whether or not A46R causes modification of the TIR adapter, or even if another as yet unidentified protein is recruited to the complex.

- The A46R interactions with TIR domain containing proteins should be explored through A46R mutagenesis studies. Also, it is worth investigating whether A46R interacts with TRIF and TRAM *via* their TIR domains by performing co-immunoprecipitation and GST-pulldown experiments with TRIF P434H and TRAM C113H. In addition, investigating whether or not A46R and TRAM interact directly by generating recombinant TRAM and performing a GST-pulldown assay would be very worthwhile.

- Generating crystals of the A46R-Mal complex and their analysis using X-ray diffraction would be very valuable in terms of understanding how TIR domains interact and how TIR domain-containing proteins transmit their signals. These data should also provide information on how A46R inhibits TLR signalling pathways.

- Recently, a peptide derived from A52R was found to inhibit cytokine secretion in response to TLR-dependent signalling and to reduce bacterial-induced inflammation *in vivo* (McCoy *et al.*, 2005). This peptide may have application

in the treatment of chronic inflammation initiated by bacterial or viral infections. The therapeutic potential of A46R could be likewise examined by generating A46R peptides. The results of the ongoing A46R mutagenesis studies will indicate which portions of A46R have immunomodulatory properties. Any bioactive peptide should then be tested in an *in vivo* system.

Chapter Seven

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Chapter Eight

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

Chemicon Europe Ltd.	The Science Centre Eagle Close Chandlers Ford Hampshire SO53 4NF United Kingdom
Corning B.V. Life Sciences	European Office and Distribution Centre Koolhovenlaan 12 1119 NE Schiphol-Rijk The Netherlands
Dunnes Stores	Outlets Nationwide
Eurogentec Bel Sa	Parc Scientifique du Sort Tilman 4102 Seraing Belgium
Greiner GmbH	Maybachstrasse P.O. Box 1162 D-7443 Frichenhausen Germany
Invitrogen	PO Box 2312, 9704 CH Groningen, The Netherlands
Invivogen	3950 Sorrento Valley Blvd. Suite A San Diego CA 92121 USA
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

Sigma Chemical Company Ltd	Fancy Road Poole Dorset BH17 7BR United Kingdom
Stratagene Cloning Systems	11011 North Torrey Pines Road La Jolla CA 92037 USA
Turner Designs ***(Distributed by RS Aqua Ltd.)***	Units 4-6 Hurst Barns Privett Alton Hants GU34 3PL United Kingdom
Unitech	United Drug House Belgard Road Dublin 24 Ireland

APPENDIX II: List of Abbreviations

AcP	IL-1 Receptor Accessory Protein
AP	IL-18 Receptor Accessory Protein
APC	Antigen Presenting Cells
AP-1	Activator Protein 1
APS	Ammonium Persulphate
ARM	Armadillo Repeat Motif
ATP	Adenosine Triphosphate
bp	base pair
BP	Binding Protein
BSA	Bovine Serum Albumin
CARD	Caspase-Recruitment Domain
CBP	CREB-Binding Protein
CDC	Centres for Disease Control and Prevention
CEV	Cell-Associated Enveloped Virus
CrmA	Cytokine Response Modifier A
CRP	C-Reactive Protein
CTL	Cytotoxic T Lymphocyte
DBD	DNA Binding Domain
DC	Dendritic Cell
DD	Death Domain
D-Gal N	D-Galactosamine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide

DNA	Deoxyribonucleic Acid
DNA-PK	DNA-Dependent Protein Kinase
dsRNA	double stranded RNA
dToll	Drosophila Toll
DTT	Dithiothreitol
ECL	Enhanced Chemiluminence
ECM	Extracellular Matrix
ECSIT	Evolutionarily Conserved Signalling Intermediate in Toll Pathway
EDTA	Ethylenediaminetetraacetic Acid
EEV	Extracellular Enveloped Virion
eIF-2α	eukaryotic translation Initiation Factor 2α
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Regulated Kinase
EST	Expressed Sequence Tag
FADD	Fas-Associated Death Domain
FCS	Foetal Calf Serum
GAG	Glycosaminoglycan
GARG-16	Glucocorticoid Attenuated Response Gene 16
GPCR	G-Protein Coupled Receptor
GPI	Glycophosphoinositol
GST	Glutathione-*S*-Transferase
HCl	Hydrochloric Acid
HCV	Hepatitis C Virus

HEK	Human Embryonic Kidney
HSP	Heat Shock Protein
HSV-1	Herpes Simplex Virus Type-1
hToll	Human Toll
HZ	Hemozoin
ID	Intermediate Domain
IEV	Intracellular Enveloped Virion
IFN-α, β, γ	Interferon-α, β, γ
Ig	Immunoglobulin
IκB	Inhibitor of NFκB
IKK	IκB Kinase
IL-1, 6, 8, 10, 12, 18	Interleukin 1, 6, 8, 10, 12
IL-1 RI	type I IL-1 Receptor
IL-1 RII	type II IL-1 Receptor
IL-18 R	IL-18 Receptor
IL-18bp	IL-18 binding protein
IL-1 ra	Il-1 Receptor antagonist
IMS	Industrial Methylated Spirits
IMV	Intracellular Mature Virus
IP-10	Inducible Protein 10
IPTG	Isopropyl Thiogalactoside
IRAK	IL-1 Receptor Associated Kinase
IRF-3, 5, 7	IFN Regulatory Factor 3, 5, 7
IRG-1	Immune-Responsive Gene 1
ISRE	IFN Stimulatory Response Element

ITR	Inverted Terminal Repeat
JAK	Janus Kinase
JIP	JNK Interacting Protein
JNK	c-Jun Amino-Terminal Kinase
kbp	kilobase pairs
LAK	LPS-Activated Kinase
LAM	Lipoarabinomannan
LB	Luria-Bertani
LBP	LPS-Binding Protein
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeat
LTA	Lipoteichoic Acid
Mal	MyD88 Adaptor-Like
MALP-2	Macrophage-Activating Lipopeptide-2
MAPK	Mitogen-Activated Protein Kinase
MARCO	Macrophage Receptor with Collagenous Structure
MBL	Mannan-Binding Lectin
MEKK1	MAPK/ERK Kinase Kinase 1
MHC	Major Histocompatibility Complex
MKK3	MAP Kinase Kinase 3
MMTV	Mouse Mammary Tumour Virus
mRNA	messenger RNA
MV	Measles Virus
MyD88	Myeloid Differentiation Marker 88

MyD88s	MyD88 short form
NAK	NFκB Activating Kinase
NEMO	NFκB Essential Modulator
NES	Nuclear Export Signal
NETN	NaCl, EDTA, Tris, NP-40
NFκB	Nuclear Factor-κB
NIK	NFκB Inducing Kinase
NLS	Nuclear Localisation Signal
NOD	Nucleotide-Binding Oligomerization Domain
O.D.	Optical Density
OPV	Orthopoxvirus
ORF	Open Reading Frame
PACT	Protein Activator of PKR
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phospho-Buffered Saline
pDC	plasmacytoid Dendritic Cell
PGN	Peptidoglycan
PGRP	PGN Recognition Protein
PI3K	Phosphatidylinositol 3-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PKR	dsRNA Activated Protein Kinase
PMA	Phobol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride

poly(I:C)	Polyinosine-Polycytidylic Acid
PRR	Pattern Recognition Receptors
PVDF	Polyvinylidene Fluoride
Q	Ubiquinone
R-847	Imiquimod
R-848	Resiquimod
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RIG-I	Retinoic Acid-Inducible Gene I
RIP	Receptor Inhibitor Protein
RHD	Rel Homology Domains
RHIM	RIP homotypic interaction motif
rMal	Recombinant Mal
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
SAM	Sterile α Motif
SAP	Serum Amyloid Protein
SARM	SAM and ARM protein
SDS	Sodium Dodecyl Sulphate
SIGIRR	Single Immunoglobulin IL-1 Receptor Related Molecule
SLAM	Signalling Lymphocyte Activation Molecule
SOC	Super Optimal Catabolite
SPICE	Smallpox Inhibitor of Complement Enzymes
STAT	Signal Transducers and Activators of

	Transcription
T2K	TRAF2-Associated Kinase
TBK1	TANK-Binding Kinase-1
TANK	TRAF Family Member-Associated NFκB Activator
TAB-1, 2	TAK-1 Binding Protein 1, 2
TAK1	TGF-β-Activated Kinase 1
TEMED	N,N,N',N'-tetramethylenediamine
TICAM-1/2	TIR-Containing Adapter Molecule ½
TIGGIR	Three Immunoglobulin Domain-Containing IL-1R-Related
TIR	Toll/IL-1/Resistance
TIRAP	TIR-Domain Containing Adaptor Protein
TIRP	TIR-containing Protein
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TOLLIP	Toll Interacting Protein
TRAM	TRIF-Related Adapter Molecule
TRADD	TNF Receptor Associated Death Domain
TRAF-2, 6	TNF Receptor Associated Factor 2, 6
TRIF	TIR Domain-Containing Adapter Inducing IFN-β
UV	Ultra Violet
VCP	Viral Complement Control Protein
V	Volts
VV	Vaccinia Virus

WHO	World Health Organisation
WNV	West Nile virus
WR	Western Reserve
WT	Wild Type

Appendix III: List of Publications

DiPerna G., Stack J., Bowie A.G., Boyd A., Kotwal G., Zhang Z., Arvikar S., Latz E., Fitzgerald K.A., Marshall W.L. 2004. Poxvirus protein N1L targets the I-kappaB kinase complex, inhibits signaling to NF-kappaB by the tumor necrosis factor superfamily of receptors, and inhibits NF-kappaB and IRF3 signaling by toll-like receptors. *J. Biol. Chem.* **279**(35):36570-8.

Stack J., Haga I.R., Schroder M., Bartlett N.W., Maloney G., Reading P.C., Fitzgerald K.A., Smith G.L. and Bowie A.G. 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *J. Exp. Med.* **201**(6):1007-18.

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Poxvirus Protein N1L Targets the I-κB Kinase Complex, Inhibits Signaling to NF-κB by the Tumor Necrosis Factor Superfamily of Receptors, and Inhibits NF-κB and IRF3 Signaling by Toll-like Receptors*

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Poxviruses encode proteins that suppress host immune responses, including secreted decoy receptors for pro-inflammatory cytokines such as interleukin-1 (IL-1) and the vaccinia virus proteins A46R and A52R that inhibit intracellular signaling by members of the IL-1 receptor (IL-1R) and Toll-like receptor (TLR) family. *In vivo*, the TLRs mediate the innate immune response by serving as pathogen recognition receptors, whose oligomerized intracellular Toll/IL-1 receptor (TIR) domains can initiate innate immune signaling. A family of TIR domain-containing adapter molecules transduces signals from engaged receptors that ultimately activate NF-κB and/or interferon regulatory factor 3 (IRF3) to induce pro-inflammatory cytokines. Data base searches detected a significant similarity between the N1L protein of vaccinia virus and A52R, a poxvirus inhibitor of TIR signaling. Compared with other poxvirus virulence factors, the poxvirus N1L protein strongly affects virulence *in vivo*; however, the precise target of N1L was previously unknown. Here we show that N1L suppresses NF-κB activation following engagement of Toll/IL-1 receptors, tumor necrosis factor receptors, and lymphotoxin receptors. N1L inhibited receptor-, adapter-, TRAF-, and IKK-α and IKK-β-dependent signaling to NF-κB. N1L associated with several components of the multisubunit I-κB kinase complex, most strongly associating with the kinase, TANK-binding kinase 1 (TBK1). Together these findings are consistent with the hypothesis that N1L disrupts signaling to NF-κB by Toll/IL-1Rs and TNF superfamily receptors by targeting the IKK complex for inhibition. Furthermore, N1L inhibited IRF3 signaling, which is also regulated by TBK1. These studies define a role for N1L as an immunomodulator of innate immunity by targeting components of NF-κB and IRF3 signaling pathways.

Vaccinia virus is a poxvirus that was used over 20 years ago to eradicate variola virus infection (smallpox) (1). The variola and vaccinia orthopoxviruses express homologs of proteins encoded by the immune response genes of their hosts (1). These homologs include secreted decoy receptors for interleukins 1 and 18 (IL-1 and IL-18)[1] (1). Vaccinia also encodes proteins that inhibit intracellular signaling by the toll-like receptor family (TLRs) (2). *In vivo*, the TLRs mediate the innate immune response by serving as pathogen-associated pattern recognition receptors (3), whose oligomerized intracellular regions can cluster intracellular Toll/IL-1 receptor (TIR) domains. Clustering of homotypically associated TIR domain-containing adapters transduces signals that ultimately activate NF-κB and cytokine response pathways (4). Vaccinia virus encodes the A46R and A52R proteins, which have been shown to act as dominant negative inhibitors of TIR signaling. A46R has been shown to inhibit IL-1 signaling, whereas A52R interferes with IL-1, IL-18, and TLR signaling, thereby inhibiting both the innate and ultimately the adaptive immune response (2).

Signals initiated by several pro-inflammatory cytokines, or by Toll receptor engagement, lead to TIR clustering that recruits one or more TIR adapter proteins: Mal/TIRAP, TRIF/TICAM-1, MyD88, and TRAM/TIRP/TICAM-2 (5–12). Recruitment of these TIR domain-containing adapter proteins leads to the recruitment of IRAK family members, which subsequently activate TRAF6 (13). Activated TRAF6 is thought to autoubiquitinate and then bind and activate a transforming growth factor-β activated kinase 1-containing complex. Transforming growth factor-β activated kinase 1 then likely phosphorylates and activates the I-κB kinase (IKK) complex (14). The IKK complex is composed of two structurally similar kinases, IKK-α and IKK-β, as well as the noncatalytic regulatory subunit IKK-γ (also called NEMO). The activated IKK complex phosphorylates I-κBα on serines 32 and 36 leading to its polyubiq-

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[1] The abbreviations used are: IL-1, IL-18, interleukins 1 and 18; TLR, Toll-like receptor; TIR, Toll/IL-1 receptor; IKK, I-κB kinase; TNF, tumor necrosis factor; IRF3, interferon regulatory factor 3; HN, C-terminal 6× histidine-arginine tag; HA, hemagglutinin; LPS, lipopolysaccharide; PVDF, polyvinylidene difluoride; dsRNA, double strand RNA; TBK1, TANK-binding kinase 1; tK, thymidine kinase; TFN, tumor necrosis factor; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; ISRE, interferon-sensitive response element; TRAM, Toll-IL-1-resistance domain-containing adaptor-inducing IFN-β-related adaptor molecule.

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uitination and degradation by the proteasome. Degradation of I-κB permits nuclear translocation of NF-κB, ultimately driving transcription of NF-κB-responsive genes (reviewed in Refs. 15 and 16). Current data indicate that the IKK complex is critical for IκB degradation and NF-κB activation in response to pro-inflammatory signals.

Vaccinia virus was known to encode two proteins that inhibit the components of the Toll and IL-1 signaling pathways (2). Other poxviruses encode several uncharacterized homologs of these Toll/IL-1R signaling inhibitors (17). We conducted data base searches that detected a significant similarity among previously identified poxvirus inhibitors of Toll/IL-1R signaling (2) and the N1L protein of vaccinia virus. N1L is among the strongest determinants of vaccinia virus virulence (18, 19). Whereas N1L has modest effects on adaptive immunity, N1L suppresses initial host defenses against vaccinia virus challenge by a factor of 10^4 (18), suggesting that N1L inhibits innate immunity. However, the mechanism whereby the N1L virulence factor functions was previously unknown (18, 19).

Here it is shown that signaling via TNF-α, lymphotoxins, and Toll/IL-1R family members are fundamental immune response pathways inhibited by N1L. Signaling by inflammatory cytokines, TIR adapter molecules, and several downstream components of the NF-κB signaling pathway is inhibited by N1L. N1L co-immunoprecipitates along with members of the IKK complex. N1L also inhibited the activation of interferon regulatory factor 3 (IRF3) in response to double-stranded RNA stimulation of TLR3 or transfection of a TIR adapter that mediates signaling to IRF3. Taken together, these findings are consistent with the hypothesis that N1L disrupts the IRF3/NF-κB signaling pathways by targeting the IKK kinase complex, thereby establishing the role of N1L as a viral immunomodulator of innate immunity.

MATERIALS AND METHODS

Cell Culture and Cell Lines—Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. HEK 293 cell-stable transfectants were maintained in Dulbecco's modified Eagle's medium in the presence of 400 μg/ml Geneticin sulfate, 10% heat-inactivated bovine calf serum, 600 μg/ml L-glutamine, penicillin-streptomycin (Invitrogen), and 20 μg/ml ciprofloxacin. Wild type HEK cells were maintained in the identical medium without Geneticin. The HEK cells stably expressing CD14, MD2, and either TLR2, TLR3, or TLR4 have been described previously (6, 20, 21). For use in transfection assays, HEK 293 cells were typically seeded at 2×10^4 cells per milliliter in 96-well plates 24 h prior to transfection.

Vector Construction and Plasmids—An N1L construct was created by PCR amplification of the N1L open reading frame of vaccinia virus (Copenhagen strain) DNA donated by Francis Ennis and Masanori Terajima (University of Massachusetts Medical School, Worcester, MA). To create N1L-encoding vectors, N1L was amplified using the primers: 5′-ATGAGGACTCTACTTATTAGA-3′ and 5′-TTATTTTTCACCATATAGATC-3′. The PCR product was TA-cloned into pCR3.1 (Invitrogen). The resulting construct, pCR3.1N1L, was sequenced in both directions using bGH reverse and T7 forward primers. N1L cDNA was subcloned into the pDNR-dual vector, which encodes a C-terminal 6× histidine-arginine (HN) tag (BD Biosciences). pDNRN1L-6×HN underwent recombination with a hemagglutinin (HA)-acceptor vector yielding an HA-N1L-HN-tagged version of N1L. The resulting construct was also sequenced. pcDNA3.1N1L was constructed by ligating BamHI-NotI-digested pCR3.1N1L into pcDNA3.1(+) that had been identically digested. FLAG-tagged Mal, TRIF, IKKε, TBK1, and the pGL3–5XκB-luc plasmid were previously described (6). The ISG54 ISRE luciferase vector was purchased from Stratagene. (FLAG-tagged TRAM was also previously described (6).) N-terminally FLAG-tagged IKK-α (22), a C-terminally FLAG-tagged modification of IKK-β (23), FLAG-TRAF6, and FLAG-TRAF2 (24) were gifts of D. Goeddel (Tularik, Inc., San Francisco, CA). The plasmid encoding BZLF1 was a gift of Joan Mannick (University of Massachusetts Medical School). The plasmid thymidine kinase *Renilla* luciferase (pTk-*Renilla*) was purchased from Promega Inc. (Madison, WI). The plasmid encoding MyD88 was from M. Muzio (Mario Negri Institute, Milan, Italy). Chimeric TLR receptors CD4-TLR1, CD4-TLR2, CD4-TLR4, and CD4-TLR6, composed of the extracellular domain of CD4 fused to the transmembrane domain and cytosolic tail of the TLR, were gifts from R. Medzhitov (Yale University, New Haven, CT).

Reagents—Poly(dI·dC) was purchased from Amersham Biosciences. The *Mycoplasma fermentans*-derived membrane macrophage-activating lipopeptide of 2 kDa was generated as previously described (25) and was obtained both from P. F. Mühlradt (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) and from G. Rawadi (Institute Pasteur, Paris, France). Bacterial lipopolysaccharide (LPS) derived from *Escherichia coli* strain 011:B4 was purchased from Sigma-Aldrich and either used directly or purified as described before (26). Human IL-1-β and TNF-α were purchased from Cell Sciences (Woburn, MA).

Transient Transfection and NF-κB or IRF3 Reporter Assays—HEK 293 cells were transfected with vectors encoding components of either the TIR or the lymphotoxin signal transduction pathways. HEK cells were co-transfected with ISRE luciferase vector or NF-κB luciferase vector, Tk-*Renilla* luciferase vector, N1L or empty vector, and in certain cases with a component of the NF-κB signaling pathway. All HEK 293 cell transfections were performed using GeneJuice (Novagen, Madison, WI) according to the manufacturer's recommendations. Transfection efficiency was normalized by transfection of cells with a plasmid encoding *Renilla* luciferase. The amount of DNA transfected was equalized among experiments by the addition of various amounts of the appropriate empty vector plasmid. To assay firefly and *Renilla* luciferase activity, cells were lysed using passive lysis buffer (Promega), and luciferase activity was determined by standard protocols, using methods as described (7). Results are presented as mean ± S.E. and are consistent with the results of at least three separate experiments. Statistical analyses were via an unpaired, two-tailed *t* test performed using GraphPad Prism software.

Co-immunoprecipitation—HEK cells were co-transfected with HA/HN-tagged N1L and a FLAG-tagged component of the NF-κB signal transduction system, as indicated in the figure legends. For each transfection, a confluent T25 flask of HEK 293 cells was trypsinized and washed. Cells were then lysed on ice for 1 hour in 100 μl of a lysis buffer comprising 0.5% Nonidet P-40, 300 mM NaCl, 50 mM Tris, pH 7.6, and Complete™ protease inhibitor cocktail (Roche Applied Science). Lysates were centrifuged at 4 °C for 20 min at 14,000 × *g*, and lysates were immunoprecipitated with anti-HN antibody (BD Biosciences) bound to Protein A-Sepharose beads (Amersham Biosciences). Immunoprecipitated proteins were boiled in Laemmli buffer for 5 min and electrophoresed on a 15% SDS-PAGE gel. Transfer to a 0.45-μm PVDF membrane was followed by blocking, and then the blot was probed with anti-FLAG monoclonal antibodies (M2, Sigma-Aldrich) and later probed with a donkey anti-mouse horseradish peroxidase (Amersham Biosciences) for 30 min essentially as described (27). In certain experiments the primary antibody step was followed by incubation with a horseradish peroxidase-conjugated antibody that recognizes native rabbit IgG (TrueBlot™, eBioscience, San Diego, CA). Between the blocking and antibody incubation steps the PVDF membrane was washed repeatedly with phosphate-buffered saline/Tween 20. Resulting bands on the immunoblot were developed with the ECL developer system (Amersham Biosciences) and visualized on a Fuji Imager. Immunoprecipitation with anti-HN-coated Protein A-Sepharose beads followed by immunoblot with anti-FLAG antibody was used to confirm the specificity of the results.

In other experiments, anti-HN antibodies (Clontech) were used to co-immunoprecipitate HA-TANK and HA-IKKγ alongside HA-N1L-HN. Subsequently, an immunoblot with anti-HA antibody (Clontech) was performed as above. Finally, anti-IKKγ antibody (Cell Sciences) was used to immunoblot lysates from the indicated transfectants that were immunoprecipitated with anti-FLAG antibody. Presence of IKKγ in lysates was also confirmed.

In Vitro Transcription and Translation and Protein-Protein Interaction—In vitro transcription and translation were performed essentially according to the manufacturer's instructions as outlined in the TNT Quick kit from Promega (Madison, WI) using 0.5 μg of the indicated DNA template, a biotinylated lysine label (Pathdetect, Promega). After confirmation of protein expression with an aliquot of protein, aliquots of N1L and various IKK components were incubated in 20 mM HEPES, pH 7.9, 60 mM NaCl, 1 mM dithiothreitol, 6 mM $MgCl_2$, 8.2% glycerol, and 0.1 mM EDTA and Complete™ protease inhibitor (Roche Applied Science) at 4 °C for 1 h. FLAG-tagged proteins were immunoprecipitated with Protein G-Sepharose-coupled anti-FLAG antibody, or HA-tagged proteins were immunoprecipitated with Protein A-Sepharose-coupled anti-HA antibody and then washed four times in hypotonic lysis buffer (see above). Samples were then analyzed by SDS-PAGE and blotted

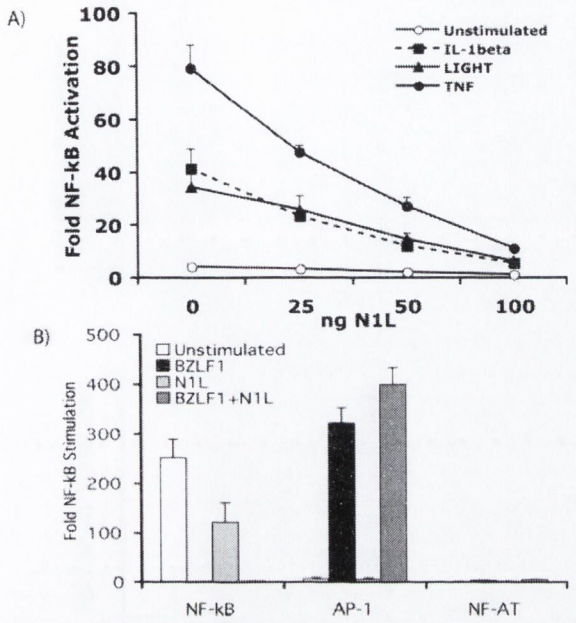

FIG. 1. **N1L inhibits signaling by IL-1, TNF, and the lymphotoxin LIGHT.** *A*, HEK cells were co-transfected with NF-κB-luciferase and *Renilla*-luciferase reporters and 0, 50, or 100 ng/well N1L. At 24 h following transfection, cells were stimulated with 10 ng/ml IL-1β, LIGHT, or TNF-α for 4–6 h. Luciferase activity was measured as described before (2) in triplicate wells in three separate experiments. Results were normalized to *Renilla* luciferase intensity in the same well and are expressed as a ratio to the lowest relative luminescence unit value. These results are representative of at least four individual experiments. *B*, N1L does not inhibit AP-1 luciferase activity. HEK 293 cells were transfected with one of three reporter constructs: NF-κB luciferase (*NF-κB*), AP-1 luciferase (*AP-1*), or NFAT luciferase (*NFAT*). Constructs were co-transfected with *Renilla* luciferase and either empty vector or BZLF1 and analyzed as above. These results are representative of three individual experiments.

onto PVDF membranes, which were blocked in phosphate-buffered saline, 0.05% Tween 20 and subsequently incubated with streptavidin horseradish peroxidase. Labeled proteins on the blot were detected by chemiluminescence (Promega), and images were captured on a Fuji Imager.

RESULTS

The N1L Proteins from Vaccinia and Other Poxviruses Have Sequence Similarity to Inhibitors of NF-κB Signaling—The amino acid sequence of N1L is relatively conserved from sheeppox virus to vaccinia, and N1L amino acid sequence is almost identical among most vaccinia viruses (19). Vaccinia virus encodes two known inhibitors of IL-1 and TLR signaling that are similar in sequence (2), albeit functionally distinct inhibitors of NF-κB signaling (27). Several poxvirus proteins have been noted as potential inhibitors of NF-κB signaling based upon their sequence similarity (17, 28) to known poxvirus inhibitors of NF-κB stimulation (2, 27). Our data base searches demonstrate that N1L is very similar (PHI-BLAST $e = 3 \times 10^{-12}$) to A52R, which has been shown to inhibit Toll, IL-1β, and IL-18 signaling to NF-κB (2). Because of this sequence similarity, we hypothesized that N1L inhibited signaling to NF-κB.

N1L Inhibits Signaling to NF-κB by IL-1β, TNF-α, and Lymphotoxins—Because N1L has sequence similarity to poxvirus inhibitors of signaling by IL-1 and TLRs (2), we first tested the hypothesis that N1L inhibits IL-1 function. Stimulation of NF-κB activity by IL-1β was significantly suppressed by N1L (Fig. 1*A*, $p = 0.0084$). N1L also inhibited stimulation of NF-κB by TNF-α signaling (Fig. 1*A*, $p = 0.015$). Next, the effect of N1L on stimulation by the lymphotoxin family member, LIGHT (29), was examined. In a simultaneous experiment (Fig. 1*A*), N1L inhibited LIGHT-mediated NF-κB signaling 5-fold compared with vector-transfected cells, which was significant ($p = 0.02$). The finding that N1L also inhibited signaling by TNF-α and LIGHT (Fig. 1*A*), whereas A52R does not block TNF-α (2), indicated that N1L was a functionally distinct vaccinia virus inhibitor of signaling to NF-κB.

To determine the specificity of the inhibition of NF-κB activity by N1L, the activity of N1L on reporter constructs other than NF-κB was tested. N1L was transfected with one of three reporter constructs: NF-κB luciferase, AP-1 luciferase, or NFAT luciferase. Reporter constructs were co-transfected with the Epstein-Barr virus transactivator BZLF1, which both binds to and stimulates transcription from AP-1 sites (30) while inhibiting NF-κB activity in lymphocytes (31). Despite 2-fold inhibition of basal NF-κB activity, N1L did not inhibit AP-1 luciferase activity (Fig. 1*B*). BZLF1 suppressed NF-κB activity, consistent with results in lymphocytes (31). NFAT luciferase activity was not observed. Thus, the inhibitory effect of N1L is specific to the NF-κB and not the AP-1 pathway. In separate experiments, no cytotoxicity was evident in N1L-transfected cells based upon Annexin V staining or flow cytometric analysis (data not shown). Overall, these data indicate that N1L specifically down-modulates signaling by NF-κB that occurs in response to several inflammatory cytokines.

N1L Inhibits Signaling to NF-κB by Toll Receptors TLR2, TLR3, and TLR4—The hypothesis that N1L inhibits Toll receptor signaling was considered for several reasons. First, N1L possesses sequence similarity to vaccinia virus inhibitors of toll-like receptor (TLR) signaling (2). Second, N1L inhibits IL-1β signaling (Fig. 1*A*), suggesting that N1L may interact with a component of the Toll/IL-1 signaling pathway. Finally, whereas N1L does not appreciably inhibit adaptive immunity, such as antibody response and cytotoxic T lymphocyte activity, N1L significantly increases the ability of vaccinia virus to overcome host defenses to initial viral challenge (18), suggesting that N1L inhibits innate immunity. Therefore, we hypothesized that N1L is involved in inhibition of the Toll receptors that mediate an antiviral innate immune response.

To test whether N1L inhibited NF-κB activation by TLR2, TLR3, or TLR4, we used two different approaches: a ligand-dependent system utilizing stable transfection of HEK 293 cells and a transient, constitutively active TLR expression system. HEK 293 cells stably expressing either TLR2, TLR3, or TLR4 were treated with their respective ligands: lipopeptide, the dsRNA mimetic, poly(dI·dC), or bacterial lipopolysaccharide (LPS) (8, 32, 33) and analyzed as in Fig. 1. Chimeric versions of the TLRs, comprising the murine CD4 extracellular domain fused to the cytoplasmic domain of a given human TLR, have proved useful in probing TLR signaling pathways (34). Some TLR cytoplasmic domains can induce gene expression as homodimers (TLR4 and TLR5), whereas others signal as heterodimers (TLR1, TLR2, and TLR6) (35). The extracellular domain of CD4 renders the TLR chimera constructs constitutively active. Using these chimeras, TLR signaling can be examined in the absence of an exogenous activator.

Signaling by TLR2 was first analyzed in stable HEK 293 transfectants. N1L inhibits TLR2-dependent signaling in response to lipopeptide agonists (Fig. 2*A*, *top panel*, $p = 0.0014$ for unpurified LPS, $p = 0.044$ for membrane macrophage-activating lipopeptide of 2 kDa). Use of transient co-transfection of N1L and constitutively active chimeric CD4-TLR1, CD4-TLR2, CD4-TLR4, and CD4-TLR 6 demonstrates N1L-mediated inhibition of TLR2 signaling in conjunction with TLR1 (Fig. 2*A*, *middle panel*, $p = 0.0246$) or TLR6 (Fig. 2*A*, *bottom panel*, $p = 0.0017$).

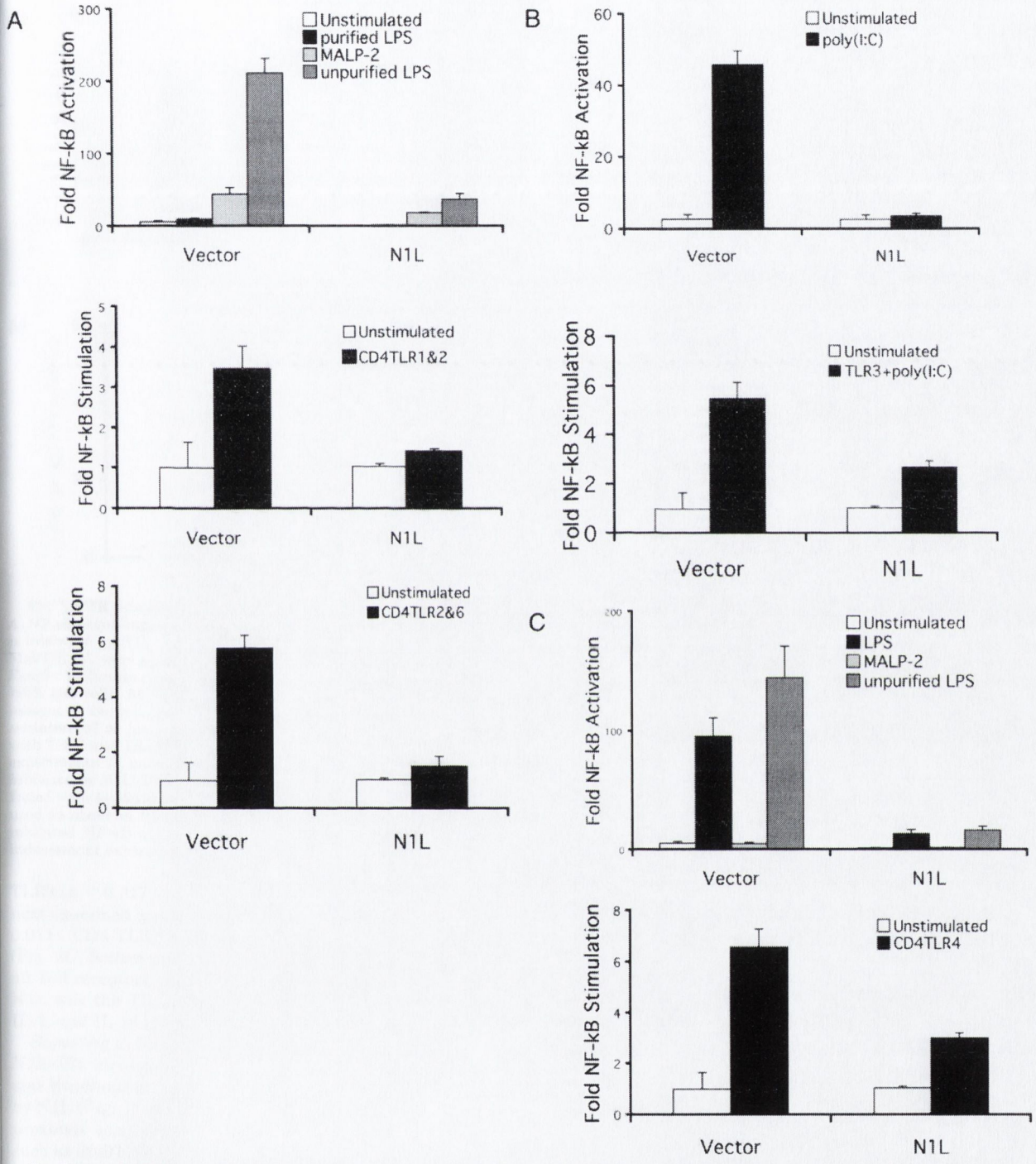

FIG. 2. **N1L inhibits signaling by several Toll receptors.** *A*, N1L inhibits TLR2-dependent signaling in response to lipopeptide agonists. HEK 293 cells transfected with TLR2 were transiently co-transfected with luciferase reporters as above, and N1L or control vector. At 24 h following transfection, cells were stimulated with the indicated ligands. 6 h later luciferase activity was assayed as above. *Middle panel*, N1L inhibits signaling by transiently co-transfected, constitutively active chimeric CD4-TLR1 and CD4-TLR2. *Bottom panel*, N1L inhibits signaling by transiently co-transfected CD4-TLR1 and CD4-TLR6. These results are representative of three independent experiments. *B*, N1L inhibits stimulation of stable TLR3 transfectants with poly(dI·dC). In five independent experiments, HEK 293 cells stably transfected with TLR3 were transfected with N1L and luciferase reporters, stimulated with the indicated ligand, and analyzed 18 h later as above. *Bottom panel*, N1L inhibited signaling induced by poly(dI·dC) treatment of HEK cells transiently transfected with TLR3. These results are representative of three independent experiments. *C*, N1L inhibits the response of TLR4 transfectants to LPS. *Top panel*, HEK 293 cells transfected with TLR4/MD2 were transfected with N1L and luciferase reporters, stimulated with the indicated ligand, and analyzed 18 h later as above. *Bottom panel*, signaling by transiently co-transfected CD4-TLR4 was inhibited by N1L. These results are representative of three independent experiments.

TLR3, which is known to interact with viral dsRNA, was analyzed next. The TLR3 ligand poly(dI·dC) was used to stimulate stable TLR3 transfectants. 12-fold inhibition by N1L of HEK 293 cells stably expressing TLR3 was observed (Fig. 2*B*, *top panel*, $p = 0.0004$). N1L inhibits signaling induced by poly(dI·dC) treatment of HEK cells transiently transfected with

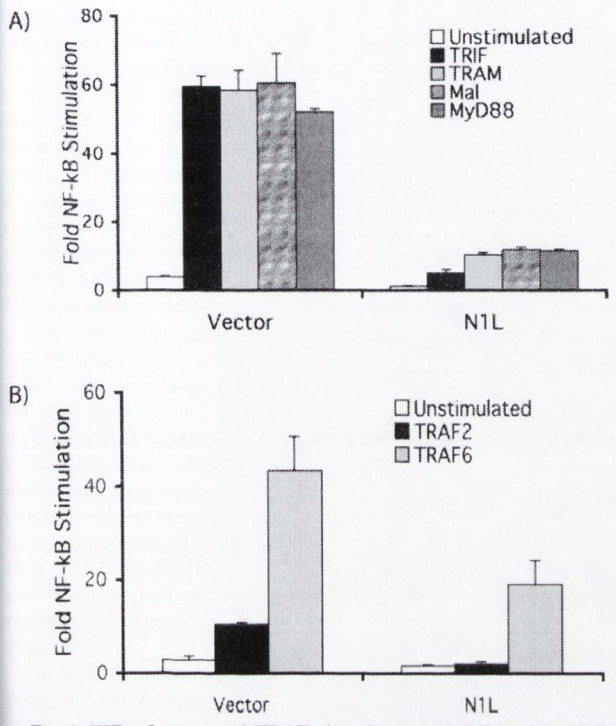

FIG. 3. **TIR adapter and TRAF signaling are inhibited by N1L.** *A*, NF-κB signaling of the TIR adapters MyD88, TRIF, TRAM, and Mal is inhibited by N1L. Several TIR adapters, TRIF, TRAM, MyD88, and Mal/TIRAP, were separately co-transfected with NF-κB luciferase, Tk-*Renilla* luciferase reporters, and N1L or control empty vector (*EV*) into HEK 293 cells. At 24 h following transfection, luciferase activity was assayed in the cell lysates (2). Results with MyD88 and Mal are representative of at least five independent experiments, whereas results with TRIF and TRAM are representative of at least three independent experiments. *B*, induction of NF-κB signaling by TRAF2 and TRAF6 is inhibited by N1L. TRAF2 and TRAF6 cDNAs were separately co-transfected with luciferase reporters as above. NF-κB activation was measured as above in the presence or absence of co-transfected N1L. N1L inhibited NF-κB activation induced by TRAF2 and TRAF6 in three independent experiments.

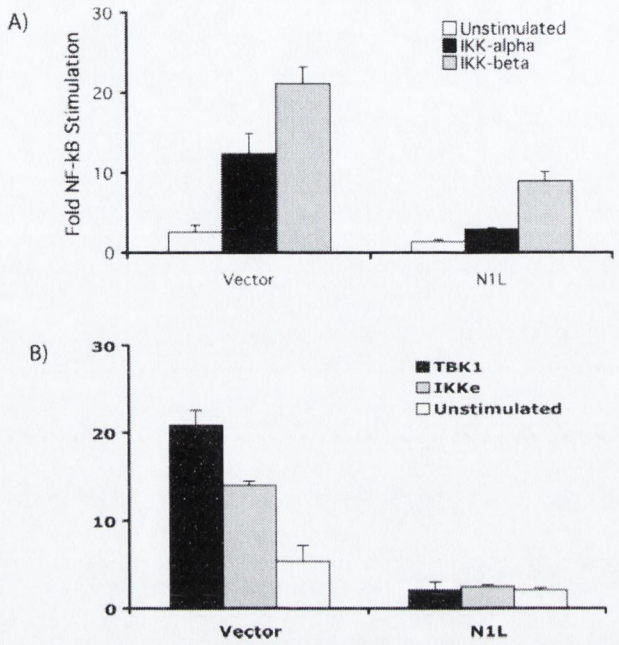

FIG. 4. **N1L inhibits NF-κB activation by TBK1, IKK-α, and IKK-β.** *A*, cDNAs encoding IKK-α and IKK-β were separately co-transfected with N1L into HEK 293 cells with luciferase reporters and analyzed as above. N1L inhibited NF-κB activation induced by ectopic expression of IKK-α and IKK-β in at least five independent experiments. *B*, cDNAs encoding IKK-ε and TBK1 were transfected with N1L or vector control and analyzed as above. N1L inhibited NF-κB activation induced by ectopic expression of TBK1 and IKK-ε in three independent experiments.

TLR3 ($p = 0.0174$) (Fig. 2*B*, *bottom panel*). TLR4 function was next examined and is inhibited by N1L (Fig. 2*C*, *top panel*, $p = 0.011$). CD4-TLR4 signaling is inhibited by N1L ($p = 0.0146$) (Fig. 2*C*, *bottom panel*). Given that N1L inhibits signaling by all Toll receptors tested, one logical candidate for inhibition by N1L was the TIR adapters that transduce signals from Toll, IL-1, and IL-18 receptors (4, 36).

Signaling to NF-κB by TIR Adapter Molecules Is Inhibited by N1L—To identify the mechanism whereby Toll, IL-1, TNF-α and lymphotoxin signal transduction pathways are inhibited by N1L (Fig. 1), we studied the effect of N1L upon membrane-proximal components of the Toll/IL-1R signaling pathways such as Mal/TIRAP, TRIF/TICAM-1, MyD88 (7–11) and TRAM (5, 6) (Fig. 3*A*). Signaling via TRIF/TICAM-1 is especially relevant to innate immunity against vaccinia virus since mutations in TRIF are associated with a 20-fold rise in vaccinia virus production in infected macrophages (37). N1L inhibits TRAM and MyD88 activation of NF-κB 4-fold, which is significant ($p < 0.002$). N1L inhibits Mal/TIRAP and TRIF activation of NF-κB by 6-fold (Fig. 3*A*, $p < 0.005$). In general, the N1L-induced inhibition of the TIR adapters is comparable, suggesting that a common signaling component downstream in the Toll/IL-1R signaling pathway might be inhibited. This suggested the hypothesis that TRAF family members or components of the IKK complex might be targeted by N1L. This hypothesis that the IKK complex is the target of N1L is consistent with the fact that TIR-independent signaling, such as signaling induced by TNF and lymphotoxins, is inhibited by N1L (Fig. 1*A*).

NF-κB Signaling by TRAF2 and TRAF6 Is Inhibited by N1L—Signals initiated by pro-inflammatory cytokines or Toll receptor engagement lead to the activation of a family of serine/threonine kinases, the IRAK family, and TRAF6, ultimately activating the I-κB kinase (IKK) complex and leading to NF-κB translocation (15). Furthermore, because of the known interaction between A52R and TRAF6 (27) and because N1L has sequence similarity to vaccinia A52R, functional interactions between N1L and TRAF6 were examined next.

Fig. 3*B* demonstrates that signaling by ectopic overexpression of TRAF2 or TRAF6 is inhibited by N1L. In Fig. 3*B*, 4-fold inhibition of TRAF6 signaling and a 5-fold inhibition of TRAF2 signaling were observed, which is significant ($p = 0.0002$ and $p = 0.05$, respectively). Although it is formally possible that N1L individually targets both TRAF2 and TRAF6 for inhibition, the data of Fig. 3 suggest that inhibition by N1L may occur downstream of these TRAF proteins.

NF-κB Signaling by TBK1, IKK-α, and IKK-β Is Inhibited by N1L—Several lines of evidence pointed to the IKK complex as being central to the function of N1L. Signaling by IL-1β, TNF-α, Toll, and LIGHT converged at the IKK complex and was inhibited by N1L (Figs. 1 and 2). Furthermore, both IKK-α and IKK-β signal downstream of the distinct TRAF2 and TRAF6 signaling pathways, which are inhibited by N1L (Fig. 3). Finally, viral modulation of signaling by the IKK complex occurs via the adenovirus 14.7-kDa protein (38), which was reported to be similar to N1L (18).

To test the hypothesis that N1L-mediated inhibition of NF-κB signaling occurs at (or distal to) the IKK complex itself, ectopic overexpression of IKK-α and IKK-β was performed in

cells transfected with N1L or empty vector (Fig. 4*A*). The function of IKK-α and IKK-β is significantly inhibited by N1L (Fig. 4*A*, $p < 0.02$ for IKK-α and $p < 0.0066$ for IKK-β). Two related kinases, IKKε (also designated IKK-i) and TANK-binding kinase 1 (TBK1) (alternatively designated NAK and T2K), have been found to be part of a multiprotein complex that also contains IKK-α, IKK-β, IKK-γ, and the TRAF family member-associated NF-κB activator, TANK (39). The ability of N1L to inhibit these kinases was tested in Fig. 4*B*. N1L inhibits TBK1 stimulation of NF-κB ($p = 0.003$) and IKKε ($p = 0.01$). Thus, N1L inhibits the NF-κB stimulatory function of four kinases in the IκB kinase complex.

Co-immunoprecipitation of N1L and Certain NF-κB Signaling Components of the IKK Complex—Poxvirus protein A52R physically associates with IRAK2 and TRAF6, thereby inhibiting Toll and IL-1 signaling (27). Many other inhibitors of the TLR and IL-1R pathway physically associate with the components that they inhibit (4, 32). Because N1L inhibited NF-κB signaling components at and/or upstream of the IKK complex (Figs. 2–4), we tested for a physical association between N1L and components of the IKK complex such as IKK-α and IKK-β (Fig. 5*A*). Two related kinases, IKKε and TBK1, have also been shown to be part of the IKK complex (39). Therefore, we also examined the interactions of N1L with IKKε and TBK1 (Fig. 5, *B* and *C*).

To identify which kinases of the IKK complex associated with N1L, a 6×HN-tagged N1L fusion protein was co-expressed in HEK cells with several FLAG-tagged components of the NF-κB signal transduction system. Immunoprecipitation by anti-6×HN antibody and immunoblot with anti-FLAG antibody was performed, which revealed an association between N1L and IKK-α and IKK-β (Fig. 5*A*, *top panel*). The *bottom panel* is an anti-HN blot of the lysates used in the immunoprecipitation, and it demonstrates approximately equal loading of HA-N1LHN-tagged protein. Thus, Fig. 5*A* indicates that N1L can co-immunoprecipitate IKK-α and IKK-β. When the same lysate was immunoprecipitated with anti-FLAG antibody, only a minimal amount of N1L fusion protein was detected, suggesting only a minimal association between IKK-α and IKK-β on the reverse immunoprecipitation (data not shown).

TBK1 and IKKε co-immunoprecipitate with N1L (Fig. 5, *B* and *C*). Because the association between N1L and TBK1 occurs whether either N1L or TBK1 is the protein being immunoprecipitated, these interactions in the immunoprecipitations provide further evidence that N1L and TBK1 are specifically associated. Fig. 5 suggests an association between N1L and components of the IKK complex. IKK-α and IKK-β have been shown to form a large complex with IKKε, TBK1, TANK, and members of the TRAF family. IKK-γ forms an oligomeric structure with IKK-α and IKK-β (40), associating with TANK, and thereby, TBK1 and IKKε (39). Furthermore, several other viral proteins that modulate signaling to NF-κB specifically target IKK-γ. Therefore, we examined interactions between N1L and IKK-γ (Fig. 6*A*). Fig. 6A indicates that HA-IKK-γ and HA-TANK co-immunoprecipitate with N1L.

Because HA-IKK-γ co-immunoprecipitates with N1L, we considered whether N1L might disrupt the association of IKK-γ with IKK-α/β by binding to sites common to their point of physical association. Peptides containing a C-terminal motif of IKK-α/β, which is required for IKK-γ binding, have been shown to disrupt formation of IKK-γ·IKK-α/β complexes (41). Thus, another hypothetical mechanism of action of N1L would be to disrupt the association of IKK-γ with IKK-α/β. If N1L bound IKK-α/β directly at the IKK-γ·IKK-α/β binding site, formation of IKKγ·IKK-α/β complexes would be inhibited. This is not seen in Fig. 6*B*, where the association between IKKβ·IKKγ

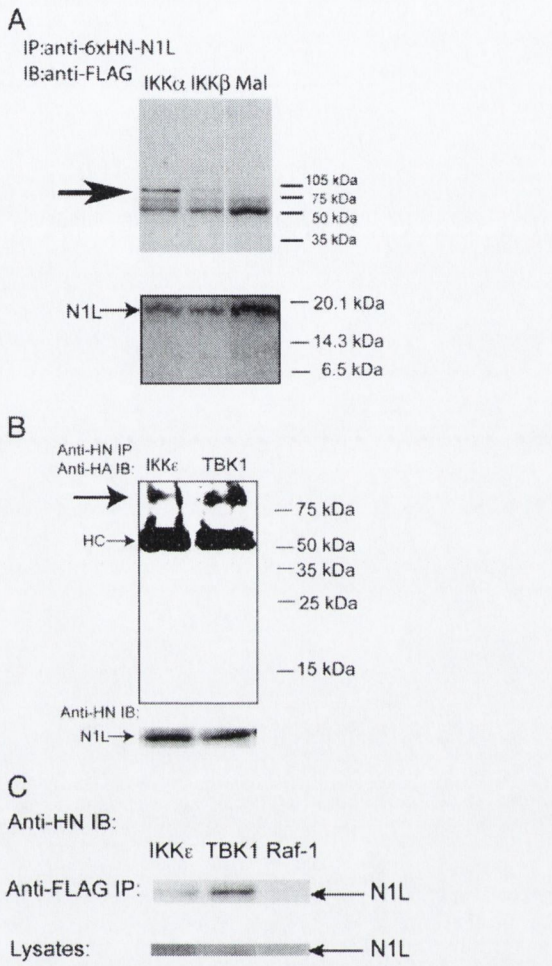

FIG. 5. **N1L associates with components of the IKK signaling complex.** *A*, IKK-α and IKK-β associate with N1L. *Top panel*, an N1L-HN fusion protein was co-expressed in HEK cells with FLAG-tagged: IKK-α (*lane 1*), IKK-β (*lane 2*), and Mal (*lane 3*). Proteins immunoprecipitated with anti-HN were detected on the blot with anti-FLAG-antibody. The *arrow* at the *left* denotes the approximate size of IKK-α and IKK-β. Control, FLAG-tagged Mal does not co-immunoprecipitate with N1L-6×HN. The *bottom panel* denotes loading of the N1L-6×HN fusion protein. *B*, TBK1 associates with N1L. *Top panel*, an N1L-HN fusion protein was co-expressed in HEK cells with FLAG-tagged: IKKε (*lane 1*) and TBK1 (*lane 2*). Proteins immunoprecipitated with anti-HN were detected on the blot with anti-FLAG-antibody. The *arrow* at the *left* denotes the approximate size of IKK-ε and TBK1. Control, FLAG-tagged TRAF6 does not co-immunoprecipitate with N1L-6×HN. The *bottom panel* denotes loading of the N1L-6×HN fusion protein. *C*, N1L associates with TBK1. *Top panel*, FLAG-tagged IKK-ε (*lane 1*), FLAG-tagged TBK1 (*lane 2*), and endogenous raf-1 (*lane 3*) were immunoprecipitated and probed on the blot with anti-HN antibody. The *arrow* at the *left* denotes the size of HAN1L-6×HN. The *bottom panel* demonstrates the amount of N1L-6×HN fusion protein in 5% of the lysate from each sample. *IP*, immunoprecipitation; *IB*, immunoblot.

persists in the presence of N1L. N1L also associated with TANK (Fig. 6*A*), which suggested that TANK, as well as TBK1, might together be direct targets of N1L. Because other poxvirus inhibitors target tandem members of the NF-κB signaling pathway (27), we sought to test whether N1L directly associated with TANK, TBK1, and IKK-γ.

In Fig. 7, protein-protein interactions are compared between *in vitro* transcribed and translated N1L and either TBK1, IKK-γ, and TANK. Aliquots of N1L and various IKK complex components were incubated, subsequently immunoprecipi-

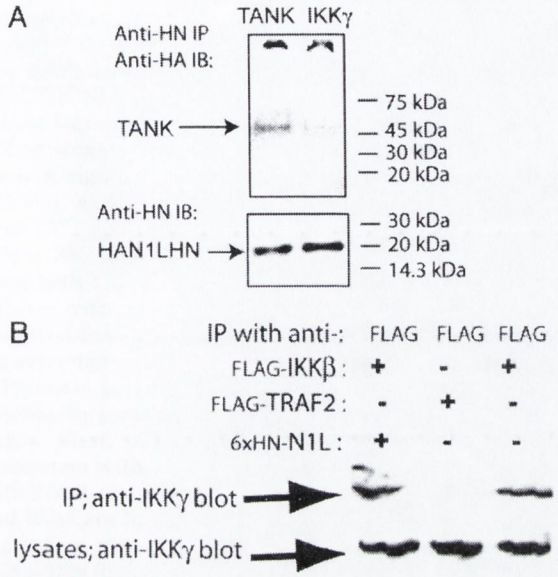

FIG. 6. **TANK and IKKγ associate with N1L.** *A*, HEK cells were co-transfected with either HA-TANK or HA-IKKγ and N1L-6×HN. Lysates were immunoprecipitated as denoted in the figure, and proteins were detected with anti-HA antibody. *B*, IKK-β·IKKγ associations persist in the presence of N1L. A N1L-HN fusion protein was co-expressed in HEK cells with FLAG-IKK-β or FLAG-TRAF2. Lysates were immunoprecipitated as denoted in the figure, and proteins were detected with anti-IKKγ antibody.

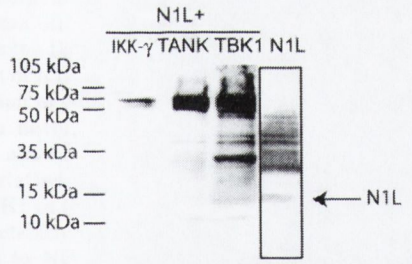

FIG. 7. **N1L interacts with TBK1 *in vitro*.** Protein-protein interactions were assessed using *in vitro* transcribed and translated N1L (*lane 4*) and either IKK-γ (*lane 1*), TANK (*lane 2*), or TBK1 (*lane 3*). Following binding *in vitro* the proteins were immunoprecipitated and then analyzed by SDS-PAGE. The *arrow* at the *right* denotes a 15-kDa band in the *N1L* and *TBK1 lanes*. A band of ~50 kDa is present in the *TANK* and *IKK-γ lanes*, consistent with their molecular sizes.

tated, and then analyzed by SDS-PAGE. The results in Fig. 7 demonstrate a band in the N1L and TBK1 lanes at ~15 kDa, which corresponds to the anticipated size of N1L (18). This N1L band is not present in the lanes containing *in vitro* translated IKK-γ and TANK. *In vitro* translated/transcribed IKK-γ and TANK are each present in the lanes as indicated by a band of ~50 kDa. These data are consistent with the finding in Fig. 5 that TBK1 immunoprecipitates with N1L and N1L immunoprecipitates with TBK1. Furthermore, signaling to NF-κB is inhibited by N1L (Figs. 1–4), and N1L inhibits NF-κB signaling by overexpression of TBK1 (Fig. 4*B*). Overall, the results of Fig. 7 suggest that N1L directly targets TBK1 to inhibit activation of NF-κB.

N1L Inhibits Signaling by the Interferon Response Pathway—The host innate immune system responds to pathogens via the interferon response pathway, which can be induced by TLR3 and TLR4 signaling in response to viruses, double-stranded RNA, and LPS (43). The TIR adapters TRIF and TRAM have been shown to mediate signaling via the IRF3

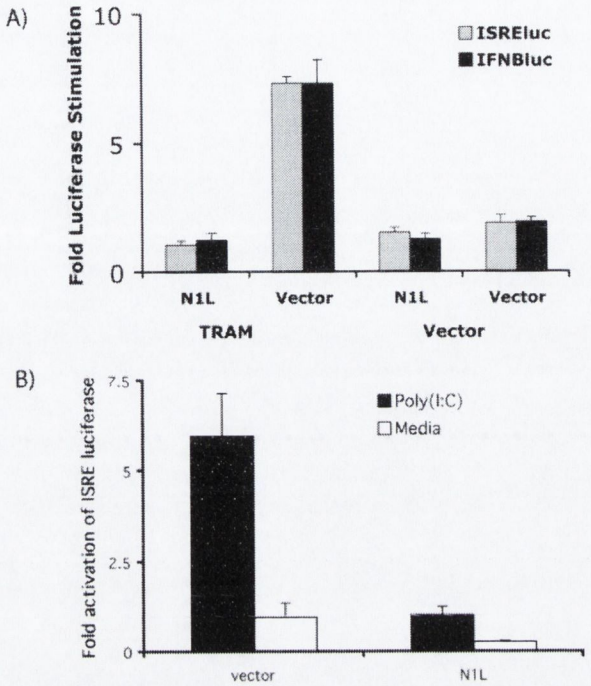

FIG. 8. **N1L inhibits IRF3 signaling.** *A*, N1L inhibits activation of the ISRE and the IFN-β promoter induced by transfection the TIR adapter, TRAM. HEK cells were co-transfected with *Renilla*-luciferase and either ISRE- or IFN-β-promoter luciferase, either 25 ng/well vector or TRAM, and either 100 ng/well N1L-encoding plasmid, or empty vector to a total of 200 ng/well. Luciferase activity was measured at 24 h as described (2). Results are consistent with three experiments. *B*, N1L inhibits IRF3 signaling induced by double-stranded RNA. HEK cells stably expressing TLR3 were co-transfected with *Renilla*-luciferase, ISRE-luciferase, and either 100 ng/well N1L-encoding plasmid or empty vector to a total of 200 ng/well. Luciferase activity was measured at 24 h as described (2). Results are consistent with three experiments.

pathway in response to dsRNA (poly(dI·dC)) or LPS stimulation of TLR3 and -4, respectively (44). The downstream kinases involved in signaling include TBK1 (42) and IKKε (6), which phosphorylate the IRF3 transcription factor. Phosphorylation permits IRF3 activation and translocation to the nucleus where IRF3 binds co-ordinately with NF-κB to promoters such as the IFN-β promoter driving the production of cytokines such as IFN-β (6).

The observation that N1L physically targets TBK1 and IKKε (Fig. 5) suggested the hypothesis that N1L would inhibit TIR adapter stimulation driving the ISRE promoter. Because N1L inhibits signaling to NF-κB, we hypothesized N1L would inhibit the activity of promoters containing both NF-κB and ISRE elements, such as the interferon β (IFN-β) promoter. To test this hypothesis, N1L was co-transfected with plasmids encoding ISRE and IFN-β promoter elements driving a luciferase construct. The *top panel* in Fig. 8 demonstrates N1L-mediated inhibition of ISRE ($p = 0.0002$) and IFN-β ($p = 0.0046$) luciferase activity. The *bottom panel* in Fig. 8 demonstrates that N1L inhibits IRF3 signaling induced by the dsRNA ligand, poly(dI·dC) in TLR3 expressing HEK cells ($p = 0.01$). Taken together these data indicate that N1L associates with the IKK complex and that N1L inhibits NF-κB and IRF3 signaling.

DISCUSSION

The virulence factor N1L down-modulates host cytokine responses and dysregulates innate immune signaling by IL-1R, TLRs, and members of the TNF receptor superfamily. The data presented here demonstrate that N1L functions, at least part, by associating with, and inhibiting, the IKK complex. The

demonstration that N1L associated with several IKK complex kinases provides a mechanistic explanation for the N1L-mediated inhibition of NF-κB-activating stimuli such as cytokines (LIGHT, TNF-α, and IL-1 in Fig. 2) or overexpression of intracellular signaling components such as TRAF2, TRAF6, and the TIR adaptors (Figs. 2 and 3). All these pathways converge on the IKK signaling complex (23, 45, 46), which is inhibited by N1L (Fig. 4). Because IKK-α and IKK-β are associated with the large IKK multiprotein complex via their regulatory subunit IKK-γ (39), IKK-α and IKK-β may be indirectly targeted because both kinases associate with IKK-γ, which physically associates with N1L (Fig. 6*A*). Other viral proteins have been described that physically associate with IKK-γ, thereby altering signaling to NF-κB (38, 47, 48).

However, several lines of evidence suggest that N1L does not function by inhibiting the association of IKK-α or IKK-β with IKK-γ. First, indirect evidence against N1L functioning by association with IKK-γ is provided by the association of N1L with TBK1 and IKKε (Fig. 5*B*) and *vice versa* (Fig. 5*C*). TBK1 and IKKε are linked to IKK-α/β by IKKγ and TANK, suggesting that a proportion of the IKK-γ remains associated with IKK-α/β in the presence of N1L. Second, N1L does not interfere with the IKK-γIKK-β association (Fig. 6*B*), which further suggests that N1L does not target IKK-γ directly. The limited amount of HA-IKKγ associated with N1L (Fig. 6*A*) suggests that N1L may target another member of the IKK-containing multiprotein complex (39). The direct interaction of N1L with TBK1 (Fig. 7) suggests that a principal target of N1L is TBK1. Overall, these observations are consistent with the hypothesis that N1L targets the IKK complex by a mechanism that may be distinct from other viruses (38, 47, 48).

TBK1 is the IKK component that is most strongly implicated as a target of N1L (Fig. 8). TBK1 has been shown to be involved in activation of NF-κB-dependent transcription (49–51). Most recently a novel, TBK1-interacting IKK component, NAP-1, has been shown to mediate NF-κB activation by TBK1 via phosphorylation of the p65 subunit of NF-κB (52). However, the role of TBK1 in NF-κB activation is still unclear, because some of the effects attributed to TBK1 deficiency cannot reproducibly be linked to NF-κB and are instead hypothesized to relate primarily to effects on signaling by interferon regulatory factor 3 (IRF3) (42). TBK1 and IKKε mediate the type I interferon antiviral response (6, 42). The data presented here indicate that TBK1 associates with N1L (Figs. 5*B*, 5*C*, and 7), and IKKε associates with N1L less strongly (Figs. 5*B*, 5*C*, and 7). These findings suggested the hypothesis that, in addition to inhibiting NF-κB activation, N1L might also inhibit IRF3 signaling that is mediated by TBK1 and IKKε. In Fig. 8, N1L inhibits IRF3 signaling. Future studies will be needed to further test the hypothesis that the association of N1L with TBK1 interrupts the type I interferon antiviral response that is mediated in part by IRF3.

N1L-mediated inhibition of signaling by TNF superfamily members is a distinct, and perhaps vital, viral immune evasion mechanism. The lymphotoxins (LTs), LT-α, LIGHT, and LT α1β2 (LT-β), are part of the TNF superfamily (53). The activation of lymphotoxin receptors stimulates antiviral responses (54–56), NF-κB translocation (57), and lymphoid organogenesis. Furthermore, TNF-α itself has potent antiviral effects and can induce programmed cell death in certain virus-infected cells (reviewed in Refs. 54 and 58). Several viral mechanisms for avoiding immune responses mediated by TNF-α have been described (54, 58). Although certain poxviruses encode decoy TNF-α receptors, only certain decoy receptors neutralize LT-α, and no other poxviral lymphotoxin antagonist has been identified to date (59). Thus, N1L-mediated inhibition of the lymphotoxin and TNF-α antiviral pathways may influence the outcome of poxviral infections.

Poxviral inhibitors of signaling inhibit a wider array of cellular anti-viral genes than is possible with a single, virus-encoded soluble decoy receptor. For example, N1L targets signaling by IL-1β, TNF-α, lymphotoxins, and Toll receptors (Figs. 1 and 2). Thus, targeting of the IKK complex identifies N1L as a unique vaccinia virus inhibitor of certain antiviral signaling pathways. Simultaneously, N1L is one of several poxvirus proteins that target distinct points in the innate antiviral signaling pathway (17, 28), all of which converge at the IKK complex. Furthermore, N1L inhibits signaling by the innate immune pathways that simultaneously suppress vaccinia virus replication (37) and activate the adaptive immune response (3, 4, 60). We hypothesize that the much less virulent, yet almost equally immunogenic, phenotype of N1L-deficient vaccinia virus (18) reflects suppression of innate immune signaling by N1L (Figs. 1–4, and 8), resulting in increased virulence (18, 19) and an inhibited adaptive immune response during wild type vaccinia virus infection *in vivo* (18). Overall, identification of NF-κB and IRF3 signaling pathways as a target of N1L identifies the role of N1L as a viral protein with novel immunomodulatory properties.

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JEM ARTICLE

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 κB. 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, extracellular signal–regulated kinase; IRAK, IL-1 receptor–associated kinase; IRF3, interferon regulatory factor 3; JNK, c-Jun NH_2-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-κB 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-κB (19, 20). MyD88 is involved in NF-κB 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-κB 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-κB 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-κB 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-κB activation induced by IL-1, whereas TNF-induced NF-κB 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-κB–dependent reporter gene activation was confirmed (Fig. 1 a). To measure IL-1–induced transactivation of NF-κB specifically, an assay was performed using an expression plasmid encoding the transactivation domain of the p65 subunit of NF-κB 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-κB–dependent reporter gene. In a similar assay (23), A46R blocked both c-Jun NH_2-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

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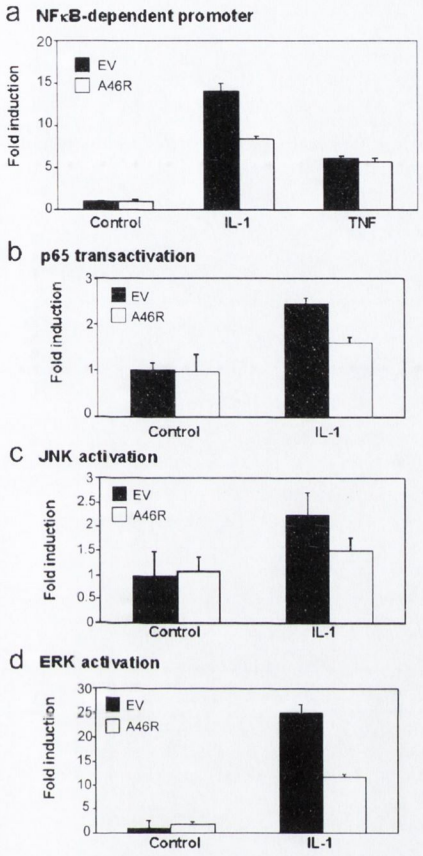

Figure 1. A46R inhibits multiple IL-1–dependent signals. HEK 293 cells were transfected with 100 ng A46R or pcDNA3.1 (EV) and the NF-κB (a), p65 (b), JNK (c), or ERK (d) reporter plasmids as described in Materials and methods. 6 h before harvesting, the cells were stimulated with either 100 ng/ml IL-1 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 (vΔA46R), 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), Pam_3Cys (TLR2 and 1), LPS (TLR4), flagellin (TLR5), R848 (TLR7), or CpG DNA (TLR9) led to induction of the NF-κB–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-κB, 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-κB 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-κB, 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-κB 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-κB 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

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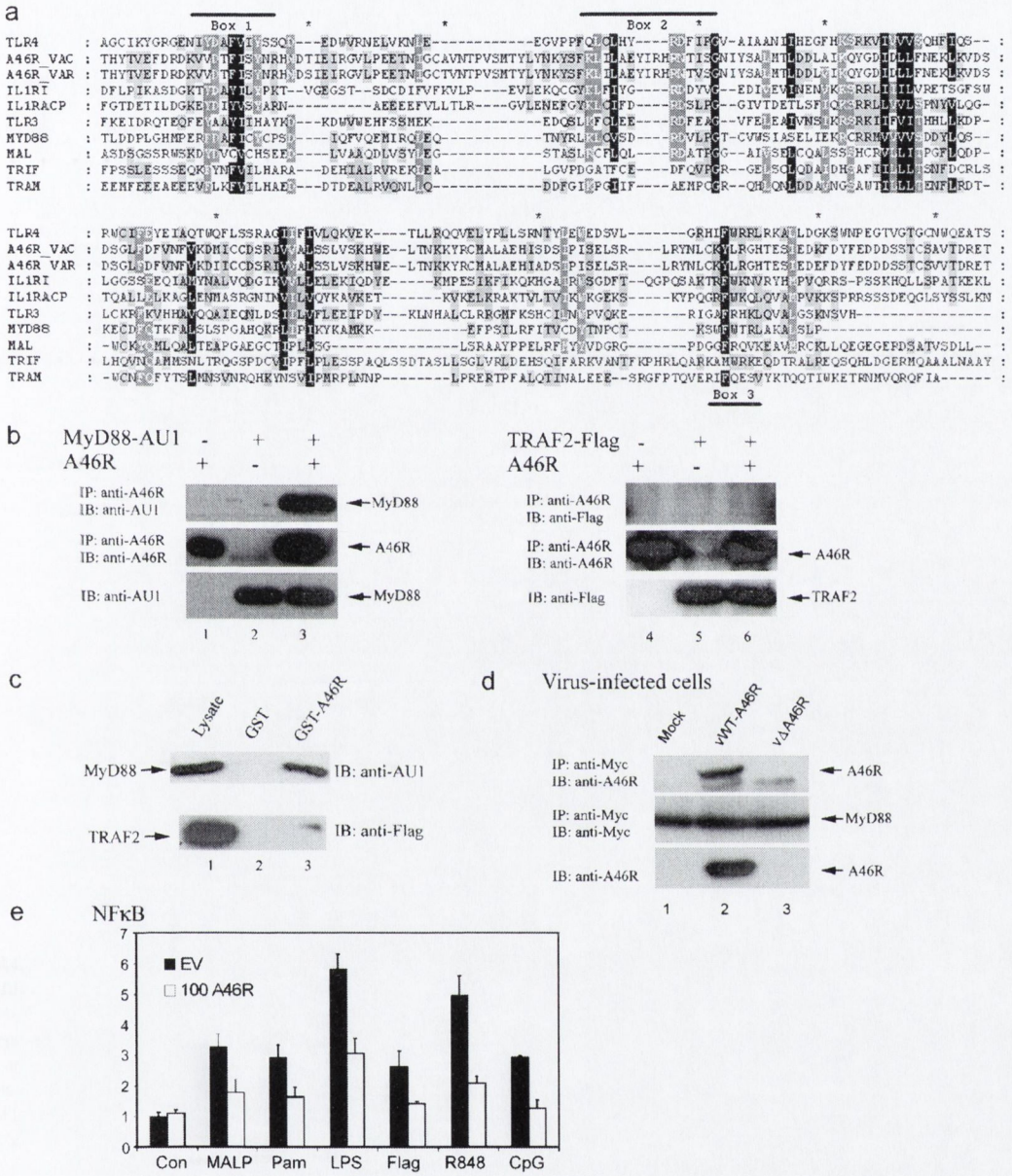

Figure 2. A46R associates with MyD88 and blocks MyD88-dependent signaling. (a) Alignment of A46R with human TIR domains. The conserved motifs Box 1, Box 2, and Box 3 are indicated by a solid line. The eight differences between VV and variola virus A46R sequences are indicated by an asterisk. For A46R, 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 μg 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 293 cells were transfected with 4 μg of myc-MyD88. After 24 h, cells were infected with viruses either containing (vWT-A46R) or not (vΔA46R) 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 phRL-TK reporter gene and the NF-κB 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 μg/ml Pam3Cys (Pam), 1 μg/ml LPS, 250 ng/ml Flagellin (Flag), 1 μM R-848, or 5 μg/ml CpG DNA. Cells were harvested 24 h after transfection and the reporter gene activity was measured.

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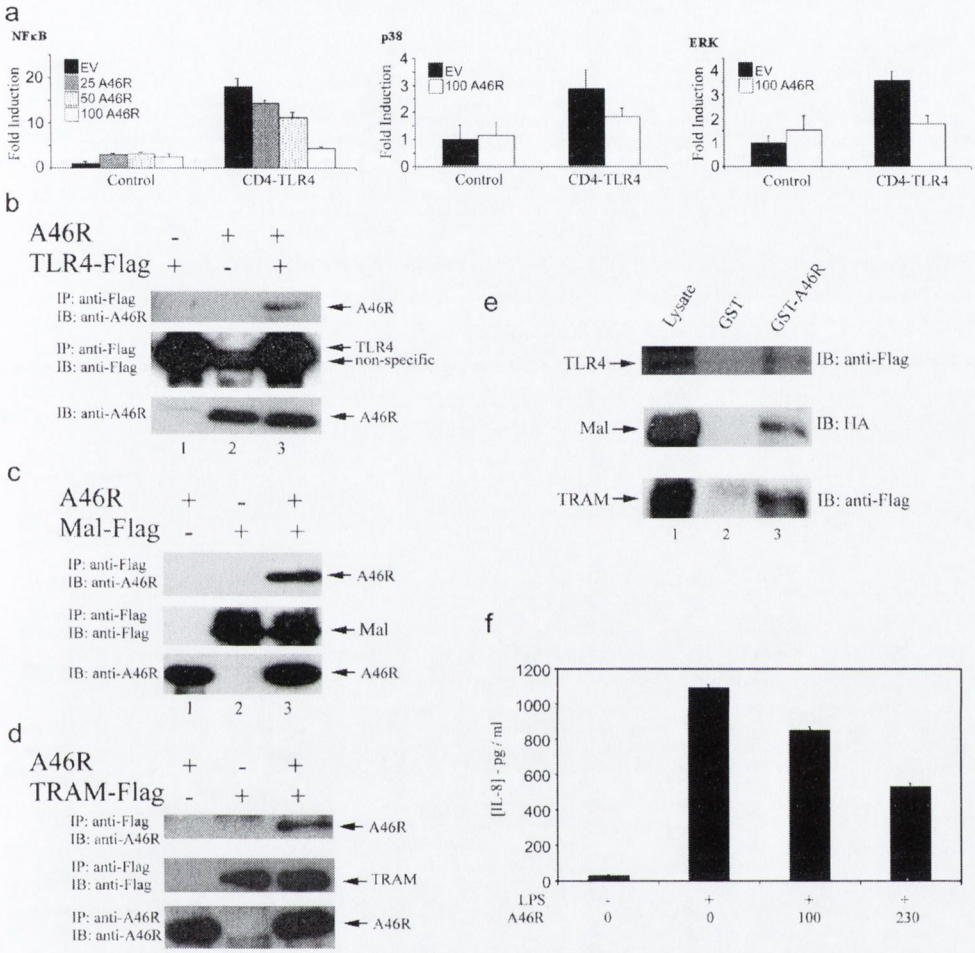

Figure 3. A46R inhibits TLR4 signaling and interacts with TLR4, Mal, and TRAM. (a) HEK 293 cells were transfected with 50 ng CD4-TLR4, 25–100 ng A46R, or pcDNA3.1 (EV) and the NF-κB (left), p38 (middle), or ERK (right) reporter plasmids as indicated. Cells were harvested 24 h after transfection, and 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 μg 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 μg/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-κB 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-pulldown 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

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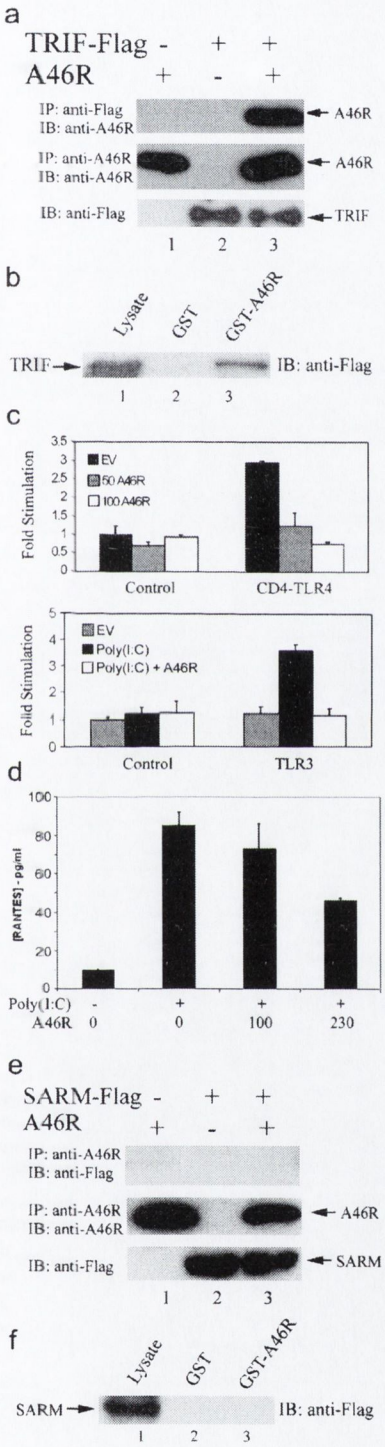

Figure 4. A46R associates with TRIF and inhibits TRIF-dependent signaling and gene induction. (a) HEK 293T cells were transfected with A46R and Flag-TRIF as indicated. After 24 h, lysates were subject to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies. (b) HEK 293T cells were transfected with 8 μg 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 μg/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 μg/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.

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-κB 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

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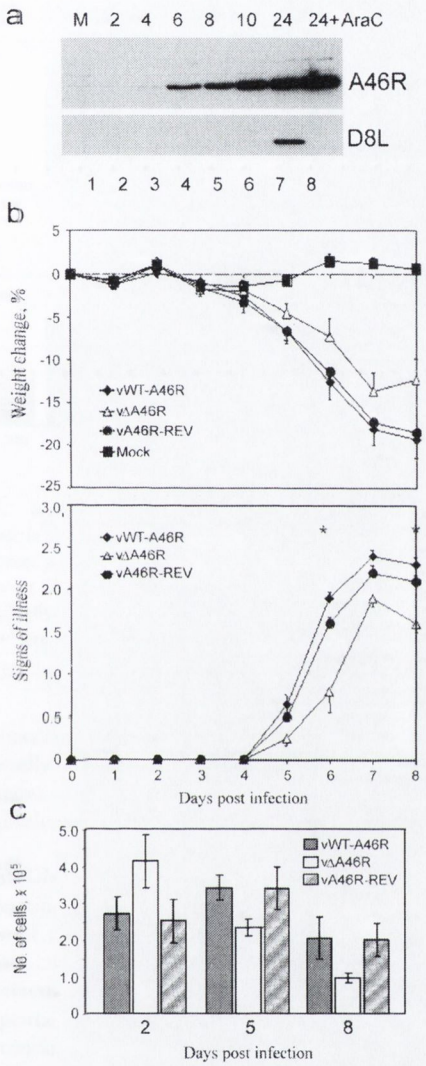

Figure 5. A46R is expressed early during virus infection and contributes to virus virulence. (a) BSC-1 cells were mock infected (M) or infected with VV Western reserve (MOI = 5) in the absence or presence of 40 μg/ml cytosine β-D-arabinofuranoside (AraC). Cells were harvested at the indicated times (h) 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, vΔA46R, 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 ± 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 (vΔA46R). As a control, a revertant virus was also constructed in which the A46R gene was reinserted into the vΔA46R mutant (vA46R-REV). Western blot analysis confirmed expression of A46R in wild-type virus (vWT-A46R) and vA46R-REV, but not in vΔA46R (Fig. 2 d and not depicted). Mice were infected intranasally with vΔA46R, vA46R-REV, or vWT-A46R and were weighed and assessed for signs of illness daily. Fig. 5 b shows that vΔA46R 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 vΔA46R 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 vΔA46R on day 5 was statistically significant ($P < 0.05$). The p-value for the difference between vWT-A46R and vΔA46R on day 8 was 0.09, whereas the value for the difference between vA46R-REV and vΔA46R 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-κB 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-κB 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-κB 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-

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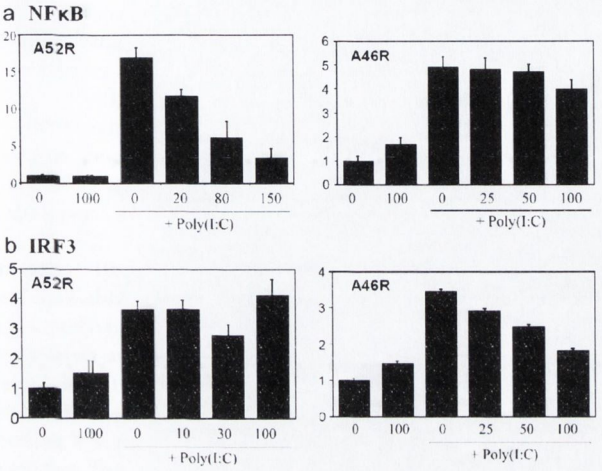

Figure 6. A46R and A52R target different TLR3-mediated signaling pathways. (a and b) HEK 293 cells were transfected with 0.5 ng TLR3 and the indicated amounts (ng) of either A52R or A46R, together with the NF-κB (a) or IRF3 (b) reporter plasmids as described in Materials and methods. Cells were stimulated with 25 μg/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-κB, 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-κB activation known to involve MyD88 in a mouse macrophage cell line, together with TLR4-mediated NF-κB, p38, and ERK activation, and IL-8 induction in human 293 cells.

Inhibition of TLR4-dependent NF-κB 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-κB 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-κB 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 bidirection transcription of the VV genome (potentially detected by TLR3; reference 11), or the dsDNA genome it-

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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 downsstream 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-κB 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-κB activation), their overall effects on TLR signaling are quite distinct. Although A52R is a good NF-κB 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-κB 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-κB because these two pathways 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 IκB 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-1α 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 Pam_3Cys-Ser-$(Lys)_4$ (Pam3Cys; Invivogen), and macrophage-activating lipopeptide 2 kD (MALP-2; Qbiogene).

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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 phRL-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 2000 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 ± SD relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

For NF-κB assays, 60 ng of a κB-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 μg of each construct was transfected. Cells were harvested after 24 h in 850 μl 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-1000, 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 WV 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 μg/ml cytosine β-D-arabinofuranoside (Sigma-Aldrich). Cell lysates were analyzed by immunoblotting using anti-A46R or anti-ID8L 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 μl 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 μg/ml LPS or 25 μg/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 ± SD from one representative experiment.

Recombinant VV viruses. A VV mutant (strain WR) lacking 93.5% of the *A46R* gene (vΔA46R) 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 μl 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-μm 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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