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Modulation of Innate Immunity by the Vaccinia Virus protein K7 and its target DDX3

by
Orla Mulhern

Doctorate in Philosophy

Submitted to the University of Dublin, Trinity College, February 2010
Declaration
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Abstract

Vaccinia virus (VACV) has many mechanisms to subvert and modulate the host immune response. One well characterised VACV protein that does this is A52. K7 was found from a search of the VACV genome looking for genes with sequence similarities to A52. A52 is known to modulate IL-1 and TLR signalling and contributes to virulence. Like A52, K7 can inhibit interleukin-1 (IL-1) and toll-like receptor (TLR) signalling leading to NFκB activation. In addition both K7 and A52 can induce IL-10, an anti-inflammatory cytokine, to modulate the host response to VACV. These activities of K7 and A52 can be explained by these VACV proteins targeting the host proteins, IRAK2 (Interleukin-receptor associated kinase) and TRAF6 (TNF receptor associated factor). A52 and K7 target IRAK2 in order to inhibit IL-1/TLR-induced NFκB activation. While TRAF6 is likely targeted by these two proteins to induce IL-10 production via p38 MAP kinase.

K7 was found to be a very potent inhibitor of p65 function. Furthermore, unlike A52, K7 can inhibit non-TLR induced NFκB activation, specifically TNF induced NFκB. K7 has an additional host interaction partner, namely DDX3. However I tested DDX3 for its involvement in NFκB activation in response to proinflammatory signals and found it to have no role.

In contrast, DDX3 was found to have a role in cytokine and DNA mediated chemokine and interferon induction. A lot of interest recently has been generated in the host response to cytosolic DNA. The precise signalling components involved in this response has yet to be determined. Previous reports have shown that DDX3 is
part of the machinery involved in Sendai virus induced type I interferon production. Given the consensus that the kinases involved in Sendai virus induced type I interferon production are also involved in DNA response, I also tested DDX3 in the host response to cytosolic DNA.
Acknowledgements

Firstly I would like to thank Prof. Andrew Bowie for the chance to work in his lab. Thank you for all your guidance and encouragement. Obviously I could not have done this without you and it has been a pleasure working for you.

To the members of the Bowie lab past and present. I have had such fun with you all. I may go on to bigger things but will struggle to go to better things. Martina, Tatyana, Sinead Keating, Sinead Flannery, Kiva, Michael, Leonie, Geraldine, Jules, Claudia, Marcin and Ismar. I could not have asked for better colleagues.

To all the fantastic friends I have made along the way. Suzanne you have been brilliant and it was great to go through this with you. Thank you! Susan, you are great source of strength and calmness. I am very grateful to both of you for your friendships. Thanks to Clare, Murial and all the ‘Vit. Chicks’. Clare and Annmaire- ‘You just get it’

To all the other members of the School of Biochemistry and Immunology who have helped me. Thanks to Vinnie’s lab and Amir’s lab especially. Thank you to Kate Fitzgerald and all the crew in UMASS who made the last three months not only bearable, but rewarding and fun.

Finally thanks to my family and wonderful parents. I dedicate this thesis to you. Rahim, my rock, hero and soul mate: Thank you, this is partially yours.
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<td>ADAR</td>
<td>Adenosine deaminase acting on RNA</td>
</tr>
<tr>
<td>AIM2</td>
<td>Absent in melanoma 2</td>
</tr>
<tr>
<td>ARM</td>
<td>HEAT/Armadillo</td>
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<tr>
<td>AP-1</td>
<td>Activator protein1</td>
</tr>
<tr>
<td>ATF</td>
<td>Activation transcription factor</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>Bp</td>
<td>Basepair</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase Recruitment Domain</td>
</tr>
<tr>
<td>CARDIF</td>
<td>CARD adaptor inducing IFNβ</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional DC</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding protein</td>
</tr>
<tr>
<td>CEV</td>
<td>Cell associated envelope virus</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>ChPV</td>
<td>chordopoxvirus</td>
</tr>
<tr>
<td>cIAP-2</td>
<td>cellular inhibitor of apoptosis 2</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CREB</td>
<td>cAMP reponse element-binding</td>
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<tr>
<td>CTD</td>
<td>C-terminal domain</td>
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CTLD
CTR
Da
DAI
DBD
DC
DC-SIGN
DD
dsDNA
dsRNA
EEV
eIF
EMCV
FADD
GlcNac
GPI
HDAC
HEK
HIV
HSV
IEV
IFN
lg
IκB
IKK

C-type lectin-like domain
CC-type lectin receptor
Dalton
DNA-dependent activator of IFN-regulatory factors
DNA binding domain
Dendritic cell
Dendritic cell-specific ICAM-3-guarding non-integrin
Death domain
Double stranded DNA
Double stranded RNA
Extracellular enveloped virus
Eukaryotic initiation factor
Encephalomyocarditis virus
Fas-associated death domain
N-acetyl glucosamine
Glycosylphosphatidylinositol
Histone deacetylase
Human Embryonic Kidney
Human immunodeficiency virus
Herpes simplex virus
Intracellular enveloped virus
Interferon
Immunoglobulin
Inhibitor of NFκB
IκB kinase
<table>
<thead>
<tr>
<th>Abbr</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Inflammatory monocyte</td>
</tr>
<tr>
<td>IMV</td>
<td>Intracellular mature virus</td>
</tr>
<tr>
<td>IPS-1</td>
<td>Interferon β promoter stimulator-1</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-receptor associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<tr>
<td>ISG</td>
<td>IFN-stimulated gene</td>
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<td>IV</td>
<td>Immature virus</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<td>JEV</td>
<td>Japanese encephalitis virus</td>
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<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>Kb</td>
<td>Kilobase</td>
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<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
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<td>LGP2</td>
<td>Laboratory of genetics and physiology 2</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeat</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-Activated Protein</td>
</tr>
<tr>
<td>MALP2</td>
<td>Macrophage activating lipopeptide 2kDa</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signalling</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
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<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryonic fibroblast</td>
</tr>
<tr>
<td>MITA</td>
<td>Mediator of IRF-3 activation</td>
</tr>
<tr>
<td>MKK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mammary tumour virus</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MurNac</td>
<td>N-acetyl muramic acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NALP</td>
<td>NACHT, LRR and PYD containing proteins</td>
</tr>
<tr>
<td>NAP</td>
<td>NAK (spell out) associated protein 1</td>
</tr>
<tr>
<td>ND</td>
<td>Newcastle disease</td>
</tr>
<tr>
<td>NIK</td>
<td>NFκB-inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequence</td>
</tr>
<tr>
<td>Nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerisation domain</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PG</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>Pol III</td>
<td>Polymerase III</td>
</tr>
<tr>
<td>poly I:C</td>
<td>Polyinosinic acid:cytidylic</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>Ra</td>
<td>Receptor antagonist</td>
</tr>
<tr>
<td>RAcP</td>
<td>Receptor accessory protein</td>
</tr>
</tbody>
</table>
RHIM  RIP1 homotypic interaction motif
RIG-I  Retinoic acid-inducible gene-I
RIP    Receptor interacting protein
RLH    RIG-like helicases
RLR    RIG-I like receptors
RNF    Ring finger protein
RSV    Respiratory syncitial virus
SAM    Sterile alpha motif
SARM   SAM and ARM-containing protein
SeV    Sendai virus
SIGIRR Single Ig IL-1 related receptor
SINTBAD Similar to NAP1 TBK1 adaptor
siRNA  Small interfering RNA
SMRT   Silencing mediator for retinoic acid receptor and thyroid hormone receptor
ssRNA  Single stranded RNA
STING  Stimulator of interferon genes
STAT   Signal transduction and activators of transcription
TAB    TAK binding protein
TAK1   Transforming growth factor β activated kinase 1
TANK   TRAF-family member associated NFκB activator
TBK1   TRAF family member associated NFκB activator
(TANK)-binding kinase
TGN    trans-Golgi network
TICAM-1 TIR-containing molecule-1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TIM</td>
<td>TRAF interacting motif</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R/resistance</td>
</tr>
<tr>
<td>TIRAP</td>
<td>(TIR) domain containing adaptor protein</td>
</tr>
<tr>
<td>TIRP</td>
<td>TIR domain containing protein)</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>TRADD</td>
<td>TNFRI-associated death domain protein</td>
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<td>TRAF6</td>
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<td>TRIF-related adaptor molecule</td>
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<td>TIR domain containing adaptor inducing IFNβ</td>
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<tr>
<td>TRIM</td>
<td>Tripartite motif-containing</td>
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<td>Vacinnia virus</td>
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<tr>
<td>VARV</td>
<td>Variola virus</td>
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<tr>
<td>VISA</td>
<td>Virus-induced signalling adaptor</td>
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<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<td>West Nile virus</td>
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<tr>
<td>5'-ppp</td>
<td>5' triphosphate</td>
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1.1 The Immune System

The immune system is a highly complex system that recognises and responds to invading pathogens and antigens. The immune system is also capable of differentiating self from non-self, ensuring inappropriate responses do not occur. In higher vertebrates the immune response can be divided into innate and adaptive components.

1.2 The Innate Immune System

The innate immune response is capable of discriminating the origin of an organism. The system relies on highly conserved germ-line encoded receptors. These pattern-recognition receptors (PRRs) recognise pathogen-associated molecular patterns (PAMPs). PAMPS are highly conserved molecular structures that are found to be associated with pathogenic and nonpathogenic organisms, e.g.: viral DNA or bacterial proteins. These PAMPs are always non-self and are essential to the survival of the pathogenic organism. One of the essential functions of the innate immune response is to instruct lymphocytes about the nature of the PAMP. Once a PRR recognises its respective PAMP a signalling cascade occurs with the production of many immune mediators. The resultant immune mediators can signal to components of the adaptive immune response and modulate the adaptive immune response that is mounted to an antigen (Medzhitov and Janeway 1998).

1.3 The Adaptive Immune System

In comparison, the adaptive immune response relies on specific antigen receptors. These receptors are produced by gene-rearrangement during ontogeny of an individual organism. There are a huge number of these receptors, each specific for a
single antigen, that are clonally distributed on T and B cells. These T and B cells potentially recognise an antigen specific for a receptor present on a T or B cell, but are unable to distinguish the origin of the antigen.

1.4 Pattern Recognition Receptors

PRRs can be broadly divided into 5 classes: the Toll-like receptors (TLRs), the RIG-like helicases (RLHs), cytosolic DNA sensors and members of the NOD-like receptor (NLR) family and the C-type lectin receptors (CLRs)

Members of the C-type lectin super-family are defined by one or more C-type lectin-like domain (CTLDs). This CTLD is defined by a distinctive protein fold caused by linkages between conserved cysteine residues. Similar to other innate immune PRRs, despite a similar structure the CTLDs recognize diverse ligands. Classical C-type lectins recognize carbohydrate ligands, whereas the non-classical ligands recognize non-carbohydrate ligands. In addition to the recognition of bacteria and viruses, the CLRs have been shown to be involved in antifungal immunity. In particular the CLRs such as mannose receptor (MR), dectin-1, dectin-2, dendritic cell-specific ICAM-3-guarding non-integrin (DC-SIGN) and the collectins have been studied extensively in the field of antifungal immunity (Huysamen and Brown 2009).

The NLR family can be divided into two groups, the NODs (nucleotide-binding oligomerisation domain) and the NALPs [NACHT, LRR (leucine rich repeats) and PYD (pyrin domain) containing proteins]. All members of this family share a common structural organization based on three domains. The amino-terminal
CARD (Caspase Recruitment Domain)/PYRIN effector domain, a nucleotide-binding domain (‘NACHT’ domain) and finally a C-terminal LRR domain. This LRR domain is a common motif found in other PRRs that is vital for the recognition of ligands. NLRs are synthesised in a structurally inactive form involving the LRRs folding back on the remainder of the protein. This folding blocks NACHT mediated oligomerization. Upon activation as a result of ligand binding to the LRR, NACHT-dependent oligomerization occurs. This results in the formation of a complex referred to as the ‘inflammasome’. Formation of the inflammation results in activation of casapse 1 and the cleavage of pro-IL-1β to active IL-1β (Martinon, Mayor et al. 2009)

1.5 The TIR Receptor Family

The first member of the TIR family to be discovered was the interleukin-1 receptor (IL-1R). Gay et al subsequently showed that a protein involved in dorsoventral polarity in the embryo of Drosophila melanogaster shared sequence similarity with the human type I IL-1R (Gay and Keith 1991). This protein had previously been studied by Anderson et al and was given the name Toll (Anderson and Nusslein-Volhard 1984). In addition to Toll a transcription factor called Dorsal was also needed for correct development (Anderson and Nusslein-Volhard 1984). The homology between Toll and IL-1R was in the cytoplasmic region. This region was termed the Toll/IL-1R/resistance (TIR) domain or Toll/IL-1R domain. Later Medzhitov et al carried out a database search from a human foetal liver/spleen library to identify other TIR domain containing proteins (Medzhitov, Preston-Hurlburt et al. 1997). A novel protein was found and called human Toll (hToll). From an alignment it was found that the homology between human Toll and the
Drosophila Toll was higher than the homology between Drosophila Toll and the IL-1R. hToll and Drosophila Toll had homology throughout the entire protein. As in Drosophila Toll, hToll consisted of an extracellular domain of repeated leucine rich repeats (LRR) separated by a non LRR region followed by an intracellular TIR domain. In contrast IL-1R has extracellular Ig domains coupled to the intracellular TIR domain (Medzhitov 2001). The Toll pathway is also important in plant host defence and several TIR domain containing proteins are found in plants. It is likely that this pathway arose before the divergence of plants and animals in defence against pathogens. Thus the TLR family of proteins and the IL-1 receptor family were grouped together to form the IL-1/TLR receptor superfamily. See figure 1.1, Alignment of IL-R/TLR superfamily

1.5.1 The IL-1R subfamily

The IL-1 receptor family has 10 members, of which most have three immunoglobulin G-like domains (IgG-like) domains in the extracellular portion of the proteins. The one exception being single Ig IL-1 related receptor (SIGIRR). Table 1.1 summarises the members of this family and their functions. After ligand binding to the IL-1RI, a second co-receptor is necessary for signalling called the IL-1RAcP. This co-receptor joins with IL-1/IL-1RI to form a complex leading to induced proximity of the TIR domains of both receptor chains (Arend, Palmer et al. 2008). From crystallization studies it has been shown that IL-1RI undergoes a conformational change upon binding ligand and allows the IL-1RAcP to form a
heretodimer (Dinarello 2009). This then results in activation of a signalling cascade that activates nuclear factor κB.

SIGIRR functions as a negative regulator of IL-1α, IL-1β and TLR agonists. Studies have shown that when SIGIRR is expressed as a chimeric molecule with IL-1RαC, IL-1 and TLR mediated NFκB activation is suppressed (Wald, Qin et al. 2003). SIGIRR serves as a competitive inhibitor of the adaptor molecule that causes signal transduction upon ligand binding to receptor. As was mentioned in Table 1 the receptor for IL-1α and IL-1β is IL-1RI. IL-1RI-deficient mice display a reduced inflammatory response to a sterile abscess when compared to wild type mice (Dinarello 2009). IL-1 receptor antagonist (IL-1ra) is a third ligand for IL-1RI. The IL-1ra is related structurally to the other IL-1 ligands but has differences that render it incapable of interacting with IL-1RαC. Therefore IL-1ra functions as a specific inhibitor of IL-1.

There are two forms of IL-1R: IL-1RI and IL-1RII. IL-1RII contains a short intracellular domain and is biologically inert. Colotta et al showed that IL-1RII is a decoy receptor (Colotta, Re et al. 1993). Signal transduction is unable to occur due to this truncation in the cytoplasmic domain of this protein (Dinarello 2009). IL-1RII may negatively regulate IL-1 activation by competing for ligand with the biologically active IL-1RI. In addition IL-1RII can be released from cells where it can bind to soluble IL-1 and thus inhibit IL-1 signal transduction (Chau, Gioia et al. 2008). Other receptors which belong to this family include the receptor for IL-18 (IL-18R). More recently the IL-1R/TLR family members, ST2 and IL-1RrP have been shown to be the receptors for novel IL-1-like cytokines. ST2 serves as a
receptor for IL-33 and IL-1RrP serves as a receptor for IL-1F6, 8 and 9 (Chau, Gioia et al. 2008). Finally the function of the member of this family TIGIRR remains to be elucidated. See figure 1.2 for illustration of IL-1R family members.

1.5.2 TLRs

To date there are ten members of the TLR family in humans (TLR1-TLR10) and twelve members in mice (TLR1-TLR9, TLR11-TLR13) (Takeda and Akira 2007). As was already mentioned, the first Toll receptor was identified in Drosophila and was essential for dorso-ventral patterning in the developing embryo, Lemaitre and Hoffman then showed that Drosophila toll was important in anti-fungal immunity (Lemaitre, Nicolas et al. 1996). The first human TLR was identified by Medzhitov in 1997 based on its homology to Drosophila toll (Medzhitov, Preston-Hurlburt et al. 1997). The name of this protein has been changed from hToll to TLR4. Following this, several proteins were discovered that were related to TLR4 and thus the TLR family of proteins were revealed. The cytoplasmic portion of TLRs shows high similarity to the IL-1 receptor family.

The extracellular domain of TLRs bind to ligands and is comprised of LRRs. Each extracellular LRR is 25-29 amino acids in length and contains the motif (XLXXLXLXX). There are typically 19-25 repeats of this motif in the extracellular domain of each TLR. Each LRR consist of a β strand and an α helix connected by loops. Initially it was believed that the LRR domain of each TLR would form a horseshoe configuration with the ligand binding to the concave surface. Conversely with the solving of the crystal structure of the LRR domains of TLR3 it appears that
the ligand of TLR3 binds to the outer convex structure (Choe, Kelker et al. 2005; Akira, Uematsu et al. 2006).

The TLRs are expressed on a variety of immune cells including macrophages, dendritic cells (DCs), B cells and certain types of T cells (Akira, Uematsu et al. 2006). In humans TLR family surface expression seems to be low among monocytes and immature DCs (Visintin, Mazzoni et al. 2001). TLR expression is observed in a variety of other cells (vascular endothelial cells, adipocytes, cardiac myocytes and intestinal epithelial cells) (Takeda and Akira 2007). Some TLRs are expressed on the cell surface (TLR 1,2,4,5 and 6) while others are expressed almost exclusively in intracellular compartments such as endosomes (TLR 3, 7, 8 and 9).

TLRs can be classified according to the ligands or PAMPs that they are capable of recognising. The TLRs that recognise nucleic acids are TLR3 (dsRNA), TLR9 (CpG DNA), TLR7 and TLR8 (ssRNA). The TLRs that recognise proteins are TLR5 (Flagellin) and TLR11. Finally TLR4 and TLR11 are capable of recognising bacterial derived products (LPS and uropathogenic bacteria respectively) (Akira, Uematsu et al. 2006).

1.5.2.1 TLR4

The first TLR to be discovered was TLR4 (Medzhotov, Preston-Hurlburt et al 1997; reviewed in Kawai and Akira 2005). TLR4 recognises a fundamental component of the outer membrane of Gram-negative bacteria called lipopolysaccharide (LPS). Using the C3H/HeJ mouse strain, that is hyporesponsive to LPS, it was discovered that TLR4 is required for LPS signalling. This mouse strain contained a mis-sense point mutation within the tlr4 gene region, leading to a highly conserved change in a
proline to histidine (Poltorak, He et al. 1998). This region encoded the cytoplasmic tail of TLR4. An additional molecule is required for TLR4-mediated LPS signalling. LPS that has been released from Gram-negative bacteria binds to LPS binding protein (LBP). This complex then binds to a glycosylphosphatidylinositol (GPI) linked protein called CD14. CD14 is expressed on the cell surface of phagocytes. The LPS is then transferred to MD-2 (Akira, Uematsu et al. 2006). MD-2 co-localises within the extracellular domain of TLR4 on the cell surface (Shimazu, Akashi et al. 1999). Following association of MD-2, TLR4 can also be activated in response to a select few viral, fungal and parasitic PAMPs. The first such PAMP was shown to be the fusion (F) protein of the respiratory syncitial virus (RSV). Despite this finding, the importance of TLR4 in the murine response to RSV remains to be clarified (Ehl, Bischoff et al. 2004). Other viral PAMPs recognised by TLR4 include the envelope protein from mouse mammary tumour virus (MMTV) (Kumar, Kawai et al. 2009). Fungal PAMPs sensed by TLR4 comprise Mannan from Candida albicans (Kumar, Kawai et al. 2009). TLR4 can also detect glycoinositolphospholipids from Trypanosoma (Gazzinelli, Ropert et al. 2004). Finally host proteins are subject to TLR4 recognition. Heat-shock protein 60 and 70 (Akira, Uematsu et al. 2006), fibrinogen, hyaluronic acid, β-defensin and extracellular domain A in fibronectin are all examples of host proteins that have been proposed to stimulate TLR4 activation (Takeda and Akira 2007).

1.5.2.2 TLR3

Double stranded RNA (dsRNA) is an important PAMP that is produced in the life cycle of many RNA and DNA viruses. dsRNA may be generated by ssRNA viruses as a replication intermediate or by DNA viruses as a by-product of symmetrical
transcription (Akira, Uematsu et al. 2006). It has long been established that dsRNA is a potent activator of innate immune cells and induces a strong type I IFN response. Double stranded RNA-dependent protein kinase (PKR) was long believed to be the sole receptor for dsRNA. Studies showing that mice deficient in PKR still responded to dsRNA suggested the existence of another receptor which was shown to be TLR3 (Alexopoulou, Holt et al. 2001). Polyinosinic : cytidylic acid [poly (I:C)] is also recognised by TLR3 and is often used as a synthetic alternative to viral dsRNA. Using TLR3 knock out mice it was shown that cells from these mice showed reduced responses to dsRNA and poly(I:C) (Alexopoulou, Holt et al. 2001).

TLR3 has been implicated in the recognition of dsRNA derived from both dsRNA viruses (reovirus) and ssRNA viruses (West Nile virus) (Edelmann, Richardson-Burns et al. 2004) (Wang, Town et al. 2004). Interestingly, TLR3 does not contain the proline residue in the TIR BB loop that is conserved in the other TLRs. This finding hinted that the TLR3 signalling mechanism might be different from the other TLRs (Applequist, Wallin et al. 2002). This proves to be the case and will be explained later. TLR3 is a PRR recognising nucleic acids and unsurprisingly is found in endosomal compartments like TLR7 and 9 (Akira, Uematsu et al. 2006).

Expression of TLR3 can be seen in conventional DCs (cDCs), macrophages, B-cells, NK cells and non-immune cells such as epithelial cells. In addition TLR3 expression is high in the brain (Lafon, Megret et al. 2006). The generation of TLR3 knockout mice has led to some confusion over the role that TLR3 plays in viral infection. It was found using these mice that responses to RNA viruses such as MCMV (Murine cytomegalovirus), VSV (Vesicular stomatitis virus), lymphocytic choriomeningitis virus (LCMV) and RSV (Respiratory syncitial virus) displayed similar vulnerabilities to these viruses when compared to wild type cells (Edelmann,
Richardson-Bums et al. 2004). Therefore it is possible that TLR3 is dispensable for an immune response against these viruses. However when infected with a lethal dose of WNV, wild type mice showed more lethality then TLR3 knock out mice (Daffis, Samuel et al. 2008). Wang et al proposed that TLR3 causes a disruption of the blood-brain barrier, thus enabling the virus to enter the brain (Wang, Town et al 2004)

1.5.2.3 TLR 7 and 8

Initially TLR7 was found to recognise imidazoquinoline derivatives (imiquimod and resiquimod/R-848) and guanine analogues e.g.: loxoribine. Soon after it was found that guanosine or uridine rich ssRNA derived from human immunodeficiency virus (HIV) and the influenza virus were ligands for TLR7 (Hemmi, Kaisho et al. 2002; Heil, Ahmad-Nejad et al. 2003). TLR7 is also capable of recognising synthetic poly (U) RNA and a select few small interfering RNAs (Diebold, Kaisho et al. 2004). TLR8 is very similar to TLR7 and human TLR8 mediates recognition of ssRNA derived from HIV and also R-848. On the contrary, mice deficient for TLR8 have normal responses to these molecules (Heil, Hemmi et al. 2004). This suggests that mouse TLR8 may not be functional (Akira, Uematsu et al. 2006).

1.5.3.4 TLR9

Unmethylated CpG dinucleotides are present in large amounts in bacterial DNA and DNA viruses. In particular the DNA virus herpes simplex virus (HSV) and murine cytomegalovirus (MCMV) contain CpG rich genomes (Hochrein, Schlatter et al. 2004). In comparison mammalian DNA is undermethylated. This serves as one
basis for how the mammalian immune system can recognise non-self DNA. Cells lacking TLR9 are hypo-responsive to synthetic CpG DNA, along with a select few DNA viruses (Hemmi, Takeuchi et al. 2000; Krug, French et al. 2004). It has been shown that CpG DNA enters the cell through endocytic pathways where it encounters TLR9 in phagosome-like vesicles. The DNA-dependent protein kinase (DNA-PK) enzyme is also essential for the response to CpG DNA (Akira, Takeda et al. 2001). Previously it was believed that hemozoin from *Plasmodium falciparum* (a protein that digests host haemoglobin) was also recognised by TLR9. More recently it was shown by Parroche et al that hemozoin simply acts as a carrier for malaria DNA (Parroche, Lauw et al. 2007). Therefore hemozoin is not a natural ligand for TLR9 and instead targets malaria DNA to the endosomes. The effects of TLR9 signalling depend largely on the nature of the ligands. There are 3 classes of CpG DNA. Conventional CpG DNA (also called K-type CpG DNA or CpG-B) has multiple CpG motifs. K/B-type CpG has the ability to strongly activate B cells and induce the production of cytokines from macrophages. D-type CpG (also called CpG-A) can also activate B cells and macrophages although less potently than K/B-type CpG. D/A-type CpG contains a single CpG motif. Finally C-type CpG, which consists of a phosphorothioate backbone with multiple CpG motifs and a TCG dimer at the 5' end has B-cell activating abilities (Kaisho and Akira 2006)

1.5.3.5 TLR 2/1/6

Gram positive bacteria do not contain LPS, but do have innate immuno-stimulatory properties. This is due to lipoproteins and peptidoglycan which are found in both gram positive and gram negative bacteria. TLR2 functions as a PRR for lipoproteins. Lipoproteins are proteins containing a lipid that is covalently linked to
the NH$_2$ terminal cysteine. This lipoylated NH$_2$ terminal is responsible for the lipoprotein immunogenic activity (Aliprantis, Yang et al. 1999; Takeuchi, Kaufmann et al. 2000). In addition TLR2 acts as a PRR for a diverse number of pathogenic products (yeast cell walls, whole mycobacteria, mycobacterial lipoarabinomannan, whole gram positive bacteria, peptidoglycan and the Trypanosome cruzi glycoprophatidylinositol anchor) (Akira, Uematsu et al. 2006). This broad range of PAMPs that TLR2 is capable of recognising is partially explained by the fact that TLR2 forms heterodimers with TLR1 and TLR6. TLR2 interacts physically and functionally with TLR1 and TLR6 (Akira, Uematsu et al. 2006). From studies using dominant negative TLR2 and TLR6 it was found that TLR6 and TLR2 are both needed to detect gram positive bacteria, peptidoglycan and Zymosan. Using TLR2 and TLR6 knock out cells it was found that TLR2 and TLR6 cooperate to recognise MALP-2 (macrophage activating lipopeptide 2kDa). MALP-2 is diacylated, compared to most lipoproteins that are triacylated (Akira, Takeda et al. 2001). In addition, TLR2 functions as a PRR for atypical LPS produced by Leptospira interrogans and Porphyromonas gingivitis (Medzhitov 2001). Peptidoglycan (PG) consists of long linear sugar chains of alternating N-acetyl glucosamine (GlcNac) and N-acetyl muramic acid (MurNac) that are linked by peptide bridges. It has been proposed that TLR2 recognises PG but controversy surrounds this. Studies have used components that have been purified from bacteria. Therefore this perceived ability of PG to activate TLR2 could simply be as a result of contamination from other TLR2 ligands, therefore synthetic analogues of active compounds would prove fruitful (Akira, Uematsu et al. 2006). TLR2 and TLR1 have been shown to elicit response to CMV. Boehme et al showed that two glycoproteins from the envelope of the virus, gB and gH, were responsible for the
response (Boehme, Guerrero et al. 2006). In addition, both were capable of co-immunoprecipitating with TLR2 and TLR1 (Boehme, Guerrero et al. 2006). The hemagglutinin protein from measles virus can also activate TLR2 (Bieback, Lien et al. 2002). Finally HSV and Varicella-zoster virus have been implicated in TLR2 activation (Akira, Uematsu et al. 2006).

1.5.3.6 TLR5

TLR5 recognises a 55-kDa protein monomer called flagellin. Flagellin is obtained from the flagella of bacteria. TLR5 recognises bacterial flagellin from both Gram positive and negative bacteria. From the crystal structure of Salmonella flagellin it was observed that the N and C terminals form helical chains (D0), there is a central α helix chain (D1) and a hypervariable central region with β sheets (D2 and D3) (Yonekura, Maki-Yonekura et al. 2003). TLR5 is capable of recognising the D1 region which is conserved among species (Hayashi, Smith et al. 2001). Using tandem mass spectrometry flagellin was identified from the supernatants of purified Listeria monocytogenes as the agent responsible for causing signalling activity in Chinese hamster ovary cells (CHO cells) over-expressing human TLR5. Expression of L. monocytogenes flagellin in nonflagellated E. coli conferred the ability to activate TLR5. In addition, when the flagellin genes from Salmonella typhimurium were deleted this removed the organism’s TLR5 stimulating abilities (Akira, Takeda et al. 2001). TLR5 is highly expressed on the basolateral side of the intestinal epithelium. More recently Uematsu et al. showed that flagellin is the true natural ligand for TLR5 using TLR5 knock out cells (Uematsu, Jang et al. 2006). In particular this group demonstrated that a group of intestinal cells, called lamina propria cells (LPCs) preferentially express TLR5 and detect pathogenic bacteria in
the gut (Uematsu, Jang et al. 2006). TLR5 is also highly expressed in the lungs, where it has been associated with defence against pathogens in the respiratory tract (Hawn 2003).

1.5.3.7 TLR11

It has been shown that expression of TLR11 was strong only in the liver, kidney and bladder of the mouse (Zhang, Zhang et al. 2004). However TLR 11 is not expressed in humans due to a stop codon in the gene (Lauw, Caffrey et al. 2005). This expression pattern is quite unique among the TLRs and hinted that TLR11 might have a specific role in these organs. Further studies went on to demonstrate that TLR11 induces signalling in response to uropathogenic bacteria. Uropathogenic *E. coli* strains showed dramatic activation of NFκB when compared to non-pathogenic strains. In addition, it was found that knock out mice for TLR11 were more susceptible to uropathogenic *E. coli*. More recently it was shown that *T. gondii* profilin stimulated TLR11 induced NFκB activation in a dose-dependent manner and that TLR11 knockout DCs failed to produce IL-12p40 (Yarovinsky, Zhang et al. 2005). See figure 1.3 for TLR ligands.

1.6 Generic Signalling by the IL-1R/TLR Receptor family

As was previously mentioned, in 1991 Gay et al. found that the Drosophila protein Toll had a cytosolic domain that was highly homologous in sequence to IL-1RI (Gay and Keith 1991). This domain is now referred to as the TIR domain. Toll was
found to be important in determining dorsoventral polarity in *Drosophila*. Later Toll was shown to be important for antifungal immunity (Chau, Gioia et al. 2008).

Importantly Toll could regulate a transcription factor called Dorsal through a protein kinase called pelle (O'Neill 2002). In 1994 Hultmark et al. showed that a protein involved in myeloid differentiation, adaptor molecule myeloid differentiation primary response gene 88 (MyD88) was also related to the cytoplasmic domains of IL-1R and Toll (Hultmark 1994). Cao et al. showed that MyD88 binds a serine/threonine kinase called interleukin-receptor associated kinase (IRAK) and that the IL-1R cytoplasmic domain mediated association of IRAK with the receptor (Cao, Henzel et al. 1996). The presence of a death domain in MyD88 mediates the interaction with the IRAKs. Tube is the functional homologue of MyD88 in *Drosophila* and like MyD88 has a death domain involved in the recruitment of a downstream kinase. Pelle in *Drosophila* is homologous to IRAK in the IL-1 signalling pathway and serves as the downstream kinase in *Drosophila* (O'Neill 2008). Upon IL-1 engaging its receptor the adaptor molecule MyD88 is recruited to the IL-1R. Induced proximity of two receptor chains exposes the TIRs to facilitate receptor/MyD88 interactions. MyD88 interacts with the IL-1R through TIR-TIR domain interactions. In addition to a TIR domain, MyD88 has a death domain. Following MyD88 binding to the IL-1R, IRAK molecules continue to transduce the signal. This all results in the eventual activation of NFκB. NFκB is the name for a family of transcription factors that regulate the expression of a large number of genes involved in the immune system and pro-inflammatory response. Based on homology, Dorsal in *Drosophila* is a member of the NFκB family. (O'Neill 2008). The TLRs signal in a basically similar manner to the IL-1R. Overall signalling by the IL-1R/TLR family can be simplified as is depicted in figure 1.4
1.6.1 IL-1 Receptor Signalling

Upon binding of IL-1β to IL-1RI conformational changes occur which allow IL-1RAcP and IL-1RI to form a heterodimer. (Arend, Palmer et al. 2008). The IL-1RAcP is only recruited to IL-1RI when IL-1 is present and the two TIR domains act as a signalling platform to recruit MyD88 (O'Neill 2008). MyD88 was the first of the TIR domain containing adaptor molecules to be characterised (Lord, Hoffman-Liebermann et al. 1990). There are five TIR domain containing adaptor molecules, which will be described later. MyD88 contains a TIR domain in its C-terminus and a death domain in its N-terminus (Muzio, Ni et al. 1997). MyD88 is recruited to a dimerised form of the IL-1R via its TIR domain. It is believed that the dimerisation of the two TIR domains is necessary in order to recruit MyD88 (O'Neill 2006). IRAK-4 (a serine-threonine kinase) is then recruited to the signalling/receptor complex via a death domain interaction with MyD88. IRAK4 was shown to be essential for IL-1R induced signals (Lye, Mirtsos et al. 2004; Qin, Jiang et al. 2004). In addition to IRAK4, IRAK1 is also recruited. The close proximity of IRAK1 to IRAK4 at the receptor complex facilitates IRAK4 mediated phosphorylation of IRAK1 (Li, Strelow et al. 2002). This stimulates IRAK1 kinase activity and its ensuing autophosphorylation (Kollewe, Mackensen et al. 2004). Hyperphosphorylated IRAK1 then interacts with an additional adaptor protein called TNF receptor associated factor 6 (TRAF6). An additional set of proteins, called the Pellinos which serve primarily as scaffold proteins also join the complex. TRAF6 is a RING domain containing protein. This RING domain contains E3 ubiquitin ligase activity. TRAF6 is capable of K63-specific ligase activity (Li and
K63-linked ubiquitination is an important mechanism for scaffold building, which is often necessary for complex formation and signal transduction. In comparison, K-48 linked ubiquitination chains are important in the mechanism leading to degradation of the associated protein. Thus, K-63 linked ubiquitination forms non-degradative ubiquitin chains. To date the mode of action of TRAF6 is thought to involve the homo-oligimerisation of TRAF6 molecules. The RING finger domain (with E3 ligase activity) complexes with K63-specific E2 conjugating enzymes (Ubc13/Uev1a or UbcH7). This results in the attachment of non-degradative ubiquitin chains to TRAF6 substrates and to TRAF6 itself (Deng, Wang et al. 2000; Geetha, Jiang et al. 2005). This then causes the recruitment of TAB2 (TAK-1 binding protein). TAB 2 and TAB1 contain zinc finger domains with binding affinity for K63-ubiquitin chains (Kanayama, Seth et al. 2004). TAB2 then brings TAB1 and TAK1 (Transforming growth factor β activating kinase 1) to TRAF6 (Wang, Deng et al. 2001). TAK1 is phosphorylated causing its activation of the IKK complex and MKKs. Recently Walsh et al. showed that the RING domain of TRAF6 is required for activation of TAK1, but is not needed for the interaction between TRAF6 and the TAK1-TAB1/2 complex (Walsh, Kim et al. 2008). In addition the authors showed that TRAF6 autoubiquitination was not needed for both the interaction and activation of TAK-1. Therefore autoubiquitination was not needed for IL-1 signalling but the RING domain was. This suggested an unidentified factor ‘X’ which interacts with TRAF6 (independent of autoubiquitination) and may serve as an interaction partner for TAB2. Also recruitment of TAK1 to TRAF6 is insufficient for TAK-1 activation (but does require RING finger activity). Therefore the authors suggested that an unknown factor ‘Y’ may serve as a substrate for TRAF6 ubiquitination. Factor ‘Y’ would
then promote TAK1 phosphorylation and activation, thus leading to MAPK and IKK α/β activation (Walsh, Kim et al. 2008) (Martin and Falk 1997; O'Neill 2002; Li and Qin 2005). See figure 1.4 for illustration of IL-1R signalling.

There is a lot of literature linking TAK1 to NFκB activation by IL-1. RNAi for TAK1 inhibited IL-1 signalling to NFκB (Verstrepen, Bekaert et al. 2008). Also in MEFs (murine embryonic fibroblasts) an inactive form of TAK1 lead to impaired IL-1 signalling to NFκB (Sato, Sanjo et al. 2005). In contrast Shim et al. showed that TAK1+/− MEFs were only partially defective in signalling of IL-1 (and the TLRs) to NFκB. Therefore there exists a possible TAK1-independent signalling pathway to NFκB. Yao et al. suggested that mitogen activated protein kinase kinase kinase 3 (MEKK3) might be the contender in TAK1 independent signalling to NFκB (Yao, Kim et al. 2007). The authors found that in MEKK3+/− cells, signalling to NFκB by IL-1 and TLR4 was impaired. In addition, IL-1 induced phosphorylation of the inhibitor of NFκB (IκBα, discussed later) was only completely inhibited in the absence of both TAK1 and MEKK3. Thus the authors proposed that TAK1 dependent signalling led to phosphorylation of Iκκβ and activation of NFκB. The TAK-1 independent signalling, caused by MEKK3, led to Iκκα activation. This then resulted in phosphorylation of IκBα its disassociation for NFκB but not degradation (Yao, Kim et al. 2007; Verstrepen, Bekaert et al. 2008).

As well as being involved in the activation of NFκB, the TRAF6/TAK1 complex can activate MAP (mitogen activated protein) kinases. TAK1 can phosphorylate MKK6 (MAP kinase kinase) which in turn activates the p38 pathway. (Wang, Deng et al. 2001). In mammals there are four p38 MAP kinases: p38 α, p38 β, p38 γ (ERK6, SAPK3) and p38 δ (SAPK4). The most characterised of the p38 isoforms is
p38α (Cuenda and Rousseau 2007). p38α was first described as a 38kDa protein that was tyrosine phosphorylated in response to LPS stimulation. The activation of p38 is observed in response to a variety of biological stimuli such as inflammatory cytokines, growth factors, UV light, heat and osmotic shock. Downstream signalling from p38 involves the eventual activation of a variety of transcription factors. Many transcription factors have been shown to be phosphorylated and activated by p38. These include activation transcription factor 1, 2 and 6 (ATF-1/2/6), p53, C/EBPβ (CCAAT-enhancer-binding protein) and indirectly AP-1 (Activating protein 1) (Ashwell 2006). Another MAPK pathway that is activated by MKKs downstream of TAK1 is Jun N-terminal kinase (JNK). There are three forms of JNK, namely JNK1, JNK2 and JNK3. The transcription factor AP1 (which is composed of Jun and Fos members) is a major target of JNK (Wagner and Nebreda 2009).

1.6.2 TLR signalling

Dimerisation of TLRs occurs after recognition of a specific PAMP. The TLRs then undergo conformational changes required for the recruitment of TIR containing adaptor proteins. The adaptor molecules engage with the TIR domains of TLRs. All the TLRs have been shown to signal through TIR domain containing adaptor proteins. The differential responses mediated by each TLR is believed, in part to be, due to the discriminating usage of these adaptor molecules by the TLRs. The five TIR domain containing adaptors are called MyD88, Mal, TRIF (TICAM-1), TRAM and SARM. The eventual outcome of TLR signalling is the activation of the
transcription factors from the NFκB and interferon regulatory factor (IRF) families.
(Akira, Uematsu et al. 2006)

1.6.2.1 MyD88 and Mal

In MyD88-deficient mice it was found that signalling did not occur in response to
TLR2 ligands (peptidoglycans), TLR5 ligands (flagellin), TLR9 ligands (CpG
DNA) and IL-1 stimulation (Adachi, Kawai et al. 1998). Therefore, MyD88 is
essential for the signalling of the IL-1 receptor and the TLRs 2, 5 and 9. From these
studies it was also found that some signalling was still intact for TLR4 and the next
TLR adaptor, Mal (TIRAP) was discovered (Fitzgerald, Palsson-McDermott et al.
2001; Homg, Barton et al. 2001). From the study of Mal knock out mice and an
anti-Mal peptide it was found that Mal did not have a role in the MyD88
independent pathway (discussed below), but did have a role in the response to TLR2
and TLR4 ligands (Horng, Barton et al. 2001; Yamamoto, Sato et al. 2002).
Signalling was impaired in Mal-deficient mice in response to TLR2 ligands (using
TLR1 and TLR6 co-receptors). In addition to the observation that TLR2 signalling
was abolished in the MyD88 knockout mice it was concluded that both MyD88 and
Mal are needed for TLR2 signalling. Mal is required for TLR4 signalling, as Mal
deficient mice are resistant to the toxic effects of LPS (Yamamoto, Sato et al. 2002).
Signalling in Mal deficient cells was totally intact in response to IL-1 and TLR5, 7
and 9 ligands confirming that MyD88 is the only adaptor used by these receptors
(McGettrick and O'Neill 2004). MyD88 is used by all the TLRs except TLR3. In a
similar manner to IL-1 signalling, MyD88 contains a C-terminal TIR domain and an
N-terminal death domain (DD). MyD88 is recruited to the dimerized TLR complex
via TIR domain interactions. IRAK4 and IRAK1 are then recruited to MyD88 via
DD interactions. IRAK4 phosphorylates IRAK1 which then causes recruitment of TRAF6. TRAF6 becomes K63 ubiquitinated, which then causes the recruitment of TAB2. TAB2 activates TAK1 which in turn activated the IKK complex. TAK1 is also involved in the activation of AP-1 through MKK6 phosphorylation of JNK and p38. Mal serves as a bridging adaptor for MyD88 in TLR2 and TLR4 signalling. In Mal deficient cells TLR4 activation of NFκB and MAPK shows delayed kinetics and impaired TNF and IL-6 production. Mal has been shown to undergo numerous phosphorylation events that are important for downstream signalling. One such kinase important in Mal phosphorylation is Bruton’s tyrosine kinase (Btk) (Carpenter and O’Neill 2009).

The IRF (Interferon Regulatory Factor) transcription family consists of IRF1-9 (Mamane, Heylbroeck et al. 1999; Akira, Uematsu et al. 2006), all of which contain a highly conserved N-terminal DNA binding domain (DBD). Of the IRFs, IRF1, 3, 5 and 7 have been shown to be positive regulators of type I IFN, a group of important cytokines involved in anti-viral immunity. MyD88 activation by TLR7/8 and 9 results in the activation of IRF7 and the induction of IFNα in plasmacytoid dendritic cells (pDCs) (Watters, Kenny et al. 2007). In pDCs upon activation of TLR7, 8 or 9, IRF7 is recruited to the MyD88/TRAF6/IRAK4/IRAK1 complex. IRAK4 phosphorylates IRAK1 which in turn phosphorylates IRF7 leading to its dimerisation and translocation to the nucleus, resulting in transcriptional activation (Uematsu, Sato et al. 2005). It was found in IRF5 deficient mice that IFNα production was not altered, but production of pro-inflammatory cytokines was impaired. IRF5 interacts with MyD88 and TRAF6. Upon activation IRF5 translocates to the nucleus where it induces the induction of inflammatory cytokines. (Negishi, Fujita et al. 2006; Watters, Kenny et al. 2007)
1.6.2.2 TRIF

In MyD88-deficient mice NFκB and MAPK activation still occurred in response to LPS and polyI:C. This indicated the existence of a MyD88 independent pathway which was not Mal-dependent (Watters, Kenny et al. 2007). Very soon the third TIR domain containing adaptor protein was discovered. This novel adaptor molecule was named TIR domain containing adaptor inducing IFNβ (TRIF) (Yamamoto, Sato et al. 2002) or TIR-containing molecule-1 (TICAM-1) (Oshiumi, Matsumoto et al. 2003). It was shown by Yamamoto et al that over expression of TRIF/TICAM-1 could activate NFκB, but more interestingly it was shown that TRIF could activate the interferon-β-dependent (ifnβ) promoter (which requires both NFκB and IRF3) (Yamamoto, Sato et al. 2002). TRIF was also found to be an interaction partner for TLR3 (Oshiumi, Matsumoto et al. 2003). TRIF also co-immunoprecipitated with IRF3 (Yamamoto, Sato et al. 2003). Using siRNA TRIF expression was removed and in parallel TLR3 induced activation of the IFN-β promoter was blocked (McGettrick and O'Neill 2004). Oshiumi et al showed that TLR3 could bind to TRIF but not Mal or MyD88 (Oshiumi, Matsumoto et al. 2003). Using TRIF-deficient mice and a population of mice with a chemically induced mutation in the trif gene it was demonstrated that IRF3 activation failed to occur in response to TLR3 and TLR4 ligands. These findings hinted that TRIF might be involved in the MyD88-independent pathway. In addition, in response to LPS in TRIF-deficient cells it was found that pro-inflammatory cytokine production was severely affected in both TRIF and MyD88-deficient mice. It was also shown that in the TRIF deficient mice, IRAK1 and early phase NFκB activation and MAP kinase activation by LPS was normal. In contrast, MyD88 deficient mice showed impaired activation of IRAK1, late-phase NFκB activation and MAP kinase activation. (McGettrick and
O'Neill 2004). TRIF leads to the activation of IRF3 and NFκB in TLR3 and TLR4 signalling in a MyD88 independent manner. (Oshiumi, Matsumoto et al. 2003; Yamamoto, Sato et al. 2003; Watters, Kenny et al. 2007). TBK1 (TRAF family member associated NFκB activator (TANK)-binding kinase) mediates IRF3 activation by TRIF. TBK1 can associate with the N-terminus of IRF3 (Sato, Sugiyama et al. 2003; McWhirter, Fitzgerald et al. 2004). TRAF3 and NAP1 (NAK associated protein 1) intercede the activation of TBK1 by TRIF (Sasai, Oshiumi et al. 2005; Hacker, Redecke et al. 2006; Oganesyan, Saha et al. 2006). It was thought that TRAF6 was important in the TRIF mediated activation of NFκB as the N-terminus of TRIF can interact with TRAF6. However this was disputed from the finding that TRAF6 deficient mice still showed activation of NFκB by TLR3 and TLR4. TRIF can interact with RIP1 (receptor interacting protein 1) This interaction is based on a C-terminal RIP1 homotypic interaction motif (RHIM). In the absence of RIP1, TLR3 did not activate NFκB. This, therefore, suggested that RIP1 is important in TRIF induced NFκB activation. In addition LPS could not activate NFκB in MyD88/RIP1 double knockout cells. Therefore RIP1 is also important in TRIF dependent NFκB activation by TLR4 signalling.

1.6.2.3 TRAM

TRIF is now known to be the sole adaptor used by TLR3 for the activation of the IFN-β promoter and NFκB (O’Neill and Bowie 2007). As was previously mentioned, TRIF is utilized by TLR4. Bin et al showed in 2003 a new TIR domain containing adaptor protein that was called TRAM (TRIF-related adaptor molecule)/TIRP (TIR domain containing protein) (Bin, Xu et al. 2003; Fitzgerald, Rowe et al. 2003). Oshiumi et al showed in 2003, in contrast to previous reports by
Bin et al, by yeast-two hybrid that TRAM could bind to TLR4 and weakly to TLR3 but not TLR 2,5,6,7,8 and 9 (Oshiumi, Sasai et al. 2003). It was shown that TRAM could homodimerise and then form heterodimers with TRIF. In contrast TRAM could not interact with MyD88 or Mal. In addition it was shown by co-immunoprecipitation studies that TRAM could interact with TLR4 but not TLR3. Overexpression of TRAM in HEK293T cells resulted in mild activation of NFκB and the IFN-β promoter. Using siRNA and a dominant negative form of TRAM it was found that NFκB and the IFN-β promoter was inhibited in response to LPS but that there was no change in response to TLR3 ligands. All of these studies lead to the belief that TRAM acts upstream of TRIF in the TLR4 signalling cascade and may act as a bridge between TLR4 and TRIF in the activation of IRF3 in a MyD88-independent manner. (McGettrick and O'Neill 2004). TRAM is only used by TLR4. TRAM serves as a bridging adaptor for TRIF in TLR4 signalling. TRAM deficient cells showed that the MyD88 independent pathway in TLR4 signalling was removed. (Watters, Kenny et al. 2007). See figure 1.5 for TLR signalling.

1.6.2.4 SARM

The final TIR domain-containing adaptor molecule to be discovered was SARM [for SAM (sterile alpha motif) and ARM-containing protein]. SARM has a TIR domain and HEAT/Armadillo (ARM) motifs. The function of SARM remained elusive until recently when it was shown by Carty et al that SARM functioned to negatively regulate TLR cascades. This study showed that SARM expression down
regulated TRIF-dependent NFκB activation and also that SARM inhibited induction of TLR4 and TLR3-dependent genes. It was shown that SARM associated with and inhibited TRIF and that this inhibition was dependent on the sterile alpha motifs and the TIR domains that are present in SARM (Carty, Goodbody et al. 2006). In contrast to the findings by Carty et al, Kim et al found no difference in cytokine levels in macrophages from SARM−/− mice when compared to wild-type mice in response to TLR4 and TLR3 ligands. Given the fact that Carty used human cells this discrepancy could be due to species difference. In addition, mechanism may be in place in the knock-out mice to compensate for the loss of SARM (Kim, Zhou et al. 2007).

1.7 Role of IRAK2 in NFκB by the TLRs

The classical theory of IRAK involvement in IL-1/TLR signalling has centred around IRAK1 and IRAK4 being the critical IRAKs needed for full activation of NFκB by IL-1 and the TLRs. Consistent with this, IRAK4−/− mice were completely resistant to LPS treatment and also showed reduced IL-1R and TLR-2, -3 and -9 cytokine responses. (Suzuki, Suzuki et al. 2002). Recently, Keating et al showed that IRAK-2 may play an important role in TLR signalling (Keating, Maloney et al 2007). IRAK-2 siRNA inhibited TLR8 and TLR4 induced activation of NFκB in human cell lines. The authors also showed that in primary human cells, LPS mediated production of IL-8 (an NFκB-dependent gene) was inhibited by siRNA
against IRAK-2. Furthermore, IRAK-2 and not IRAK-1 could trigger the polyubiquitination of TRAF6 which is a marker of its E3 ligase activity that is essential for NFκB activation. Additionally a residue (Glu528) was identified in IRAK-2 that was important for TRAF6 stimulation, which when mutated, prevented IRAK2 from inducing TRAF6 polyubiquitination. Finally the authors showed that siRNA against IRAK-2 could inhibit polyIC-induced TLR3 mediated NFκB activation (Keating, Maloney et al. 2007). Interestingly IRAK-2 was shown to be part of the TLR-3 complex (in stimulated and unstimulated cells). The authors proposed that IRAK-1 may be more important for TLR signalling to the IRFs (Keating, Maloney et al. 2007). It is still unclear the role that IRAK2 plays in TRIF activation of NFκB since direct recruitment of TRAF6 and RIP-1 to TRIF stimulated TAK1 activation, resulting in NFκB and MAPK activation (Sato, Sugiyama et al. 2003; Meylan, Burns et al. 2004). Determining the exact role that IRAK2 plays in innate immune signalling has been complicated by the presence of 4 isoforms of IRAK2 in mice (some of which are inhibitory). In contrast, there is only 1 known isoform in humans (Hardy and O'Neill 2004). Previous studies have shown that in IRAK2^−/− mice only late TLR signalling to NFκB was inhibited (Kawagoe, Sato et al 2008) while another report showed a post-transcriptional role for IRAK2 in TLR induced cytokine induction (Wan, Xiao et al. 2009). However, in wild-derived mice, which are more genetically heterogeneous than the classical inbred strain of laboratory mice, TLR-induced NFκB and MAPK activation was strongly inhibited when cells from these mice were treated with siRNA against IRAK2. (Conner, Smirnova et al. 2009)
1.8 TNF-R signalling to NFκB

TNF is a proinflammatory cytokine and like IL-1 and TLR signalling TNF can activate NFκB and MAPKs. Unlike IL-1, TNF can induce apoptosis. TNF binding to its receptor causes trimerisation of the receptor. TNF can bind to TNF-R1 or TNF-R2 (Verstrepen, Bekaert et al. 2008). Upon trimerisation by the receptor, the adaptor protein TRADD (TNFR1-associated death domain protein) is recruited to the receptor complex. This association occurs via homotypic DD interactions with the receptor, and TRADD then recruits TRAF2 (Hsu, Huang et al. 1996; Hsu, Shu et al. 1996). TRAF5 is also required for TNF-regulated NFκB activation. Using double knockout cells for TRAF2 and TRAF5 it was found that both of these TRAF proteins are necessary for TNF-induced NFκB activation (Tada, Okazaki et al. 2001). TRAF2, and not TRAF5, can interact with TRADD, whereas TRAF5, and not TRAF2, can interact with RIP1. Using cells deficient for RIP1 it was found that RIP1 is necessary for TNF-induced NFκB activation (Kelliher, Grimm et al. 1998). RIP1 most likely functions as an adaptor that enhances IKK recruitment to the activated receptor through an interaction with polyubiquitinated RIP1 and IKKγ (Karin and Gallagher 2009). IKKγ is an essential adaptor of the NFκB activating complex (see below). Upon association with the activated TNF-receptor complex, the IKK complex becomes activated by phosphorylation, causing activation of NFκB (see below).

It has been shown that TAK1 is also involved in IKK activation upon TNF stimulation. RIP1-deficient cells fail to recruit TAK1 and IKKγ to the TNFR upon stimulation (Ea, Deng et al. 2006). It was found that TAK1 associated with RIP1 via TAB1/2. (Ea, Deng et al. 2006). It was shown that polyubiquitinated RIP1
associates with TAB2 (Kanayama, Seth et al. 2004). In addition stimulation of TAB1-deficient cells with TNF resulted in no activation of TAK1 (Mendoza, Campbell et al 2008). Another kinase involved in TNF induced NFκB activation is MEKK3. MEKK3 can interact with RIP1 and MEKK3-deficient fibroblast display reduced NFκB activation upon TNF stimulation (Karin and Gallagher 2009). It is thought that RIP1 acts as an adaptor that recruits MEKK3 to the TNFR1. MEKK3 then phosphorylates the activation loops of IKKα and IKKβ (Karin and Gallagher 2009). The role that these two putative IKK kinases (TAK1 and MEKK3) play in TNF-induced NFκB activation may be cell type specific. These IKK kinases may also amplify the IKK signalling following initial IKK activation (Karin and Gallagher 2009).

1.9 NFκB family of Transcription factors

Thus IL-1, TLR and TNF signalling all lead to activation of NFκB. The NFκB family of transcription factors are key regulators of immune and inflammation signalling. They were first discovered by Sen and Baltimore in 1986 as a protein that bound to the DNA sequence (5'-GGGACTTTCC-3') within the intronic enhancer of the immunoglobulin kappa light chain gene in mature B and plasma cells (Sen and Baltimore 1986). The NFκB family consists of 5 different proteins p100/p52, p105/p50, p65 (RelA), Rel B and c-Rel. Both p105 and p100 are inactive for DNA binding and serve as precursors for p52 and p50 respectively. The first NFκB subunits to be purified were p65 and p50 using the NFκB DNA binding motif κB in DNA affinity chromatography (Kawakami, Scheidereit et al. 1988; Ghosh, Gifford et al. 1990; Zabel, Schreck et al. 1991). These two subunits were shown to form heterodimers with one another and it later turned out that all the NFκB family
members appear in cells as homo or heterodimers. Additional studies added three more members, p52, c-Rel and RelB, to the NFκB family to make 5 members. All members of the NFκB/Rel family contain a Rel homology domain (RHD). This domain is highly conserved and consists of around 300 amino acids in the N-terminal domain of the protein. This Rel homology domain is responsible for binding to the NFκB consensus sequence (5'-GGRNNYYCC-3, where R=purine, Y=pyrimidine and N=any base) and also for homo- or heterodimerisation of family members (Kunsch, Ruben et al. 1992). The 5 members of the NFκB/Rel family can be broken down into two subclasses based on structure of their C-terminal domains. The first class, which contain one or more transactivation domains in the C-terminus include RelA (p65), RelB and cRel. The second class, p105 and p100, do not have transactivation domains. Instead p105 and p100 contain one glycine rich region and a series of ankyrin repeats in their C-terminal regions. Both p105 and p100 are processed into their active forms by proteolysis. The site of this proteolysis for p105 and p100 is between the glycine rich region and the ankyrin repeats (May and Ghosh 1997).

1.9.1 IκB family

Around the time NFκB was discovered, extracts were found in the cytoplasm that were capable of inhibition of NFκB DNA binding activity (Ghosh and Baltimore 1990). It was proposed that this was due to an inhibitor of NFκB (IκB) which would become inactive upon initiation of NFκB activity. Initially it was found that IκB was in fact two proteins, termed IκBα and IκBβ, that were capable of associating with NFκB (May and Ghosh 1997). On additional studies and with the help of cloning the number of IκB proteins grew and a new family of proteins was discovered. The
various IκB molecules show different specificity of binding to NFκB. For example, it appears that IκBα and IκBβ bind to homo or heterodimers of RelA and c-Rel and dimers of p50 of p52 complexed with RelA or c-Rel (Kunsch, Ruben et al. 1992; Oeth, Parry et al. 1994). All proteins of the IκB family contain multiple copies of ankyrin repeats. These ankyrin repeats are important for interaction with NFκB (Whiteside and Israel 1997).

It is classically believed that NFκB is kept in the cytoplasm in an inactive form by IκB proteins. IκB is thought to conceal the nuclear localization sequence (NLS) of NFκB. This prevents NFκB interacting with nuclear import machinery and therefore it is retained in the cytoplasm. In addition, IκB contains a NLS, within a second ankyrin repeat, that is concealed once it is bound to NFκB proteins (Birbach, Gold et al. 2002). In order for NFκB to translocate to the nucleus, IκB must be degraded. Each IκB contains a pair of serine residues in their N-terminal region. In the case of IκBα these serines are amino acids 32 and 36. These serine residues become phosphorylated by a number of proteins (Birbach, Gold et al. 2002). The IKK (IκB kinase) complex is a key kinase complex that phosphorylates IκB proteins. The kinase activity of IKK resides in two catalytic subunits, IKKα, IKKβ. IKKγ/NEMO is an essential scaffold protein of the complex. IKKα and IKKβ share 52% amino acid identity and both have an N-terminal catalytic domain, a leucine-zipper motif (involved in homodimers and heterodimer formation) and a C-terminal helix-loop-helix domain. A NEMO-binding domain within IKKβ interacts with IKKγ/NEMO to regulate the formation of the IKK complex. IKKγ/NEMO has no catalytic function but is essential for stimulus induced activation of the IKK complex (Yamamoto and Gaynor 2004). See figure 1.6 for a picture of members of the NFκB and IκB family.
1.10 Activation of NFκB

Activation of NFκB can occur via at least three distinct pathways, the canonical or classical pathway, the non-canonical pathway and through DNA damage. The canonical pathway is induced by TLRs, proinflammatory cytokines such as TNF and IL-1 and antigen receptor ligation (Birbach, Gold et al. 2002; Bonizzi and Karin 2004; O'Neill 2006). The second, non-canonical pathway is induced by B-cell activating factor (BAFF), lymphotixin β, CD40 ligand and some viruses (Claudio, Brown et al. 2002; Coope, Atkinson et al. 2002; Gloire, Dejardin et al. 2006). This second pathway function by enhancing NFκB-inducing kinase (NIK)-and IKKα-dependent processing of p100 into p52. The third pathway is classified as atypical as it is dependent on the proteasome but is independent of IKK activation.

1.10.1 Canonical Pathway

IL-1 and TNF activate NFκB via the canonical pathway, therefore I will now focus on the canonical pathway leading to NFκB activation. A very large number of stimuli can activate NFκB in the canonical pathway, but all the signalling pathways converge at the level of the IκB kinases (IKKs). Activation of the IKKs by a variety of factors, including IL-1 and TNF, results in the phosphorylation of specific serine residues within the activation loop of IKKα and IKKβ (Burns, Martinon et al. 1998). For IκBα, activated IKK phosphorylates Ser\(^{32}\) and Ser\(^{36}\). In the case of IκBβ, Ser\(^{19}\) and Ser\(^{23}\) are phosphorylated (DiDonato, Mercurio et al. 1996). The ankyrin containing and inhibitory molecule p105 is phosphorylated on Ser\(^{927}\) and Ser\(^{932}\) (Kishore, Huynh et al. 2002). This phosphorylation is required for the subsequent processing of p105 into the NFκB protein p50. The phosphorylation of IκB proteins leads to the binding of the β-TrCP-SCF complex (E3\(^{κB}\) ubiquitin ligase complex)
(Tojima, Fujimoto et al. 2000). In the case of IκBα this results in IκBα being a substrate for ubiquitination, mainly at lysines 21 and 22. This ubiquitination targets the protein for degradation by the 26S proteasome (Brown, Gerstberger et al. 1995). NFκB is now free to enter the nucleus and carry out its transcriptional activities. More recently it has been proposed that both IκB and NFκB can shuttle in and out of the cell in non-stimulated cells and that the process by which IκB can inhibit NFκB is not solely down to the fact that IκB can sequester NFκB in the cytoplasm. Schmid et al proposed that IκB also functions in the nucleus to inhibit p65 binding to DNA and shifts the steady state distribution of NFκB-IκB complexes to the cytosol (Schmid, Birbach et al. 2000) (Birbach, Gold et al. 2002).

1.10.2 IKKs function beyond IκB phosphorylation

It was found using cells lacking kinases that are not vital for IκB degradation that kinases are required not only for the degradation of inhibitory molecules but also for optimal NFκB activation (Bonnard, Mirtsos et al. 2000). In these cells there was a normal profile of IκB phosphorylation and degradation despite there being defects in NFκB activation (Viatour, Merville et al. 2005). These phosphorylations occur in the cytoplasm or in the nucleus. The NFκB protein p65 is phosphorylated on Ser\(^{536}\) by a variety of kinases. In the majority of cases, Ser\(^{536}\) phosphorylation results in increased p65 transactivational potential (Viatour, Merville et al. 2005). There are numerous kinases that are responsible for this phosphorylation. Another important p65 serine phosphorylation site is Ser\(^{276}\). This phosphorylation enhances the ability of p65 to recruit histone acetyltransferases such as cAMP reponse element-binding (CREB)-binding protein (CBP) and p300 (Zhong, Voll et al. 1998). In addition
Ser\textsuperscript{276} also allows p65 to displace a p50-histone deacetylase (HDAC)-1 complex from DNA (Zhong, May et al. 2002). There are numerous other phosphorylation sites on p65 and other NFκB proteins and the differential phosphorylation of these NFκB proteins may be responsible for whether there is transcription of a gene. Some of these phosphorylation events are summarised in table 1.2.

It has been demonstrated that IKK subunits can enter the nucleus. While in the nucleus the IKKs regulate lots of aspects of NFκB-dependent and independent gene expression. The nuclear role of IKKα has been studied extensively. One nuclear function of IKKα was reported by Hoeberg et al. This group showed that IKKα mediates derepression of cIAP-2 (cellular inhibitor of apoptosis 2) and IL-8 gene promoters (Hoberg, Yeung et al. 2004). This derepression is necessary before NFκB transcription can occur (Gloire, Dejardin et al. 2006). In unstimulated cells promoters on NFκB binding sites are repressed by p50 and p52 homodimers that are transcriptionally inactive. These p50 or p52 homodimers recruit repressor complexes, for example: SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor) and HDAC3. These repressors must be removed in order for transcription to occur (Hoberg, Popko et al. 2006). IKKα was shown to phosphorylate SMRT. This promotes its nuclear export (along with HDAC). Once this occurs SMRT is degraded via the proteosome. This allows transcriptionally active p50/p65 heterodimers to initiate transcription (Hoeberg, Popko et al. 2006). The next nuclear role of IKKα involves phosphorylation of p65 that is bound to chromatin on Ser\textsuperscript{536} (Buss, Dorrie et al. 2004). This partially prevents HDAC3 being recruited to chromatin and enables p300 to acetylate p65 at lysine 310. This acetylation of p65 is also vital for full transcription (Gloire, Dejardin et al. 2006).
1.11 The IRF transcription factor family

There are 9 members of the IRF transcription family, IRF 1-9 (Mamane, Heylbroeck et al. 1999; Tamura, Yanai et al. 2008). Members of this family have highly conserved N-terminal DNA binding domains (DBD) with five tryptophan repeats (Veals, Schindler et al. 1992; Tamura, Yanai et al. 2008). From the crystal structure of IRF 1 it was found that the DBD bound to positive regulatory domains (consensus sequence 5' -GAAA-3') of the IFNβ enhancer. Further analysis revealed that the consensus sequence for IRF recognition is 5' -AANGAAA-3'. The 5' flanking AA sequence is vital for IRF recognition, hence IRFs do not bind to the NFκB binding site which contains a GAAA core sequence (Fujii, Shimizu et al. 1999). Of the IRFs, IRF-1, -3, -5 and -7 have been shown to be positive regulators of type I IFN.

1.11.1 Kinases involved in IRF activation.

The canonical activation of NFκB requires the degradation of IκBα (through the kinases IKKα and IKKβ). In contrast activation of the transcription factors IRF3 and IRF7 simply requires the phosphorylation of these proteins in their C-terminal region. There are two kinases responsible for these phosphorylation events. TBK1 and IKKe (also called IKKi) (Shimada, Kawai et al. 1999). TBK1 was identified in a yeast-2-hybrid screen using TANK as bait (Bonnard, Mirtsos et al. 2000). Very soon after, IKKe was identified by Peters et al through a database search of IKKα/IKKβ related proteins (Peters, Liao et al. 2000). TBK1 and IKKe have 61% sequence identity (Fitzgerald, McWhirter et al. 2003). MEFs lacking TBK1 or IKKe show normal IκBα degradation and NFκB binding in response to IL-1, LPS and TNF. (Claudio, Brown et al. 2002; Sasai, Oshiumi et al. 2005). Several stimuli
induce TBK-1 and IKKe phosphorylation of IRF3 and IRF7. IRF3 and 7 are very important in type I interferon induction. After phosphorylation of IRF 3 and 7, dimerisation and translocation to the nucleus occurs with the result of the expression of anti-viral genes such as IFN-β (Tamura, Yanai et al. 2008). IRF5 is also involved in type I interferon production and is also phosphorylated by TBK1/IKKε. (Chau, Gioia et al. 2008; Clement, Meloche et al. 2008)

Just like in the canonical pathway leading to NFκB activation the kinases IKKα and IKKβ require the scaffold protein NEMO, so too do the kinases involved in IRF activation require a scaffold protein. The first TBK1/IKKε scaffold identified was TANK [TRAF-interacting protein (I-TRAF)]. TANK constitutively binds to IKKε and TBK1 through its N-terminal domain. TANK was thought to be involved in some TLR-dependent IRF-activating pathways, however the TANK<sup>−/−</sup> mouse recently showed that its function is restricted to the NFκB and not IRF pathway (Kawagoe, Takeuchi et al. 2009). NAP-1 (NAK associated protein) was identified as a TBK-1 interacting protein and shares strong protein motifs with TANK. Like TANK, NAP-1 constitutively binds TBK1 and is required for IRF3 phosphorylation by TLR3 and RIG-I signalling (discussed later). Finally SINTBAD (similar to NAP1 TBK1 adaptor) also constitutively binds TBK-1 and IKKε. SINTBAD shares conserved TBK-1 and IKKε binding domains with TANK and NAP1 (Chau, Gioia et al. 2008; Clement, Meloche et al. 2008).

1.12 Type I IFN system

The interferon family of proteins can be divided into three groups. Type I interferons comprise the largest subfamily and include IFNα (13 subtypes in
humans) and IFNβ. IFNβ controls a positive feedback loop leading to the production of IFNa. Type II IFNs comprise IFN-γ and type III IFNs (IFNλ) contain three members. All interferons exert antiviral activity. PRRs recognise PAMPs and induce the activation of transcription factors from the IRF family. These transcription factors regulate the expression of type I IFNs (among other cytokines). PRRs are responsible for the early production of IFNβ. Secreted IFNβ binds to the IFN receptor (IFNAR1/2) and enhances type I IFN production (Levy, Marie et al. 2003; Honda, Takaoka et al. 2006). IFN binding to the receptor causes tyrosine phosphorylation of the receptor. This phosphorylation is caused by members of the JAK family of protein kinases (Janus kinases). The phosphorylated receptor is now a docking site for STAT proteins (signal transduction and activators of transcription). These STATs become phosphorylated and dissociate from the receptor, homo-or-heterodimerise and migrate to the nucleus. In the nucleus, the STATs bind to elements on the promoter region of IFN-stimulated genes (ISGs). This results in the transcription of more than 300 ISGs, many of which are involved in anti-viral immunity (Bowie and Unterholzner 2008).

1.13 Cytosolic Pattern Recognition Receptors of Nucleic Acid

The endosomal TLRs 3, 7, 8 and 9 all serve as PRRs capable of recognising nucleic acid species. In order for the immune system to detect exogenous nucleic acid from pathogens in the cytoplasm a different set of receptors is used.

1.13.1 PKR
PKR was the first PRR shown to recognise a product of viral replication, namely dsRNA. PKR is critical in the host anti-viral defence mechanism and is induced by IFN (Garcia, Meurs et al. 2007). PKR was discovered in cells pre-treated with IFN. In these cells it was found that the translation of viral mRNA was blocked (Metz and Esteban 1972; Garcia, Meurs et al. 2007). Viral, cellular and synthetic dsRNAs that are longer then 30 nucleotides can activate PKR as can ssRNAs with internal dsRNA structure (Katze, DeCorato et al. 1987). More recently ssRNA with a 5’-ppp (5’ triphosphate) was shown to activate PKR (Nallagatla, Hwang et al. 2007). The main function of PKR is to inhibit translation. This is achieved through PKR-mediated phosphorylation of eIF-2α. This phosphorylation renders eIF-2α inactive and thus inhibits translation. PKR can also affect the transcription factors from the STAT, NFκB and IRF family. In addition PKR can signal to p53 and the JNK and p38 MAPK pathways (Verma, Stevenson et al. 1995; Williams 2001; Garcia, Meurs et al. 2007). Unactivated PKR is monomeric in its latent state. Upon recognition of an RNA substrate, conformational changes result in PKR homodimerisation and autophosphorylation. Phosphorylation of eIF2α then occurs at Ser\(^{51}\), resulting in inhibition of cellular translation of both host and viral mRNA (Clemens 1997; Garcia, Meurs et al. 2007).

Cytosolic RNA is also recognised by the RIG-I like family of receptors (RLRs)

### 1.13.2 RIG-I like receptors.

Cytosolic RNA is also recognised by the RIG-I like family of receptors (RLRs). In 2004 a cDNA clone was isolated that could activate IRF-regulated reporter gene expression in response to transfection with synthetic dsRNA (polyI:C) (Yoneyama, Kikuchi et al. 2004). This protein was called retinoic acid-inducible gene-I (RIG-I)
and belonged to the family of DEXD/H box-containing RNA helicases. RIG-I contained two caspase recruitment domains (CARD) in the N-terminus of the protein and a helicase domain in the C-terminus. The CARD domains were necessary for downstream signalling (Cui, Eisenacher et al. 2008; Takahasi, Yoneyama et al. 2008). In addition substitution of an important residue in the ATP binding domain in the helicase domain resulted in a dominant negative of RIG-I. Therefore ATP is also necessary for signalling. (Takahasi, Yoneyama et al. 2008). Using knockout mice it was shown that RIG-I is essential for NDV (Newcastle disease virus), SeV (Sendai virus) and VSV (Vesicular stomatitis virus) induced type I IFN expression in fibroblast and cDCs (Kato, Sato et al. 2005). From the study of the mammalian genome two other genes highly related to RIG-I were discovered. Melanoma differentiation-associated gene 5 (MDA5) was identified by treating cells with a compound that induces IFN and PKC activity in a melanoma cell line (Kang, Gopalkrishnan et al. 2002). MDA5 showed 23% amino acid similarity in the N-terminal CARD domain and 35% amino acid similarity in the helicase domain when compared to RIG-I (Saito, Hirai et al. 2007). MDA5 was shown to be a positive regulator of type I interferons like RIG-I. In addition generation of Mda5 knockout mice demonstrated the essential role of MDA5 in virus induced innate immunity in vivo (Gitlin, Barchet et al. 2006; Kato, Takeuchi et al. 2006). RIG-I and MDA5 knockout mice also showed that these PRR could detect different types of viruses. The early presumption that there is probably a preferential RNA structure recognised by RIG-I and MDA5 proved to the case (discussed later) (Kato, Sato et al. 2005; Kato, Takeuchi et al. 2006). Laboratory of genetics and physiology 2 (LGP2) was identified as a gene adjacent to the STAT3/5 locus (Cui, Li et al. 2001). LGP2 displayed 41% amino acid similarity to the
helicase domain of RIG-I and 31% amino acid similarity to the helicase domain of MDA5. LGP2 had no N-terminal CARD domain. LGP2 can inhibit RIG-I and MDA5 signalling (Reviewed Yoneyama 2009 (Yoneyama and Fujita 2009). Although it was noted that LGP2 can play a positive role in EMCV infection (Yoneyama and Fujita 2009).

From knockout studies it was shown that RIG-I is involved in the response against ND (Newcastle disease), SeV (Sendai virus), VSV, Influenza virus and JEV (Japanese encephalitis virus), whereas no defects in signalling were found in MDA5 knockout cells when challenged with viruses mentioned above. From MDA5 knockout mice it was found that EMCV (Encephalomyocarditis virus), Theliers virus and Mengo virus innate responses were inhibited in the absence of this protein but independent of RIG-I (Kato, Takeuchi et al. 2006). Collectively then, most ssRNA viruses are detected by RIG-I, whereas Picornaviridae and murine norovirus are detected by MDA5. (Kato, Takeuchi et al. 2008; Loo, Fornek et al. 2008; Yoneyama and Fujita 2009).

Differential recognition by RIG-I and MDA5 is based on preferences for substrate RNA. Hornung et al and Pichlmair et al both showed that 5'-triphosphate (5'-ppp) containing viral RNA was recognised by RIG-I (Hornung, Ellegast et al. 2006). This moiety is present in most viral RNA virus genomes and is masked in host transcripts by the maturation process of RNA or by the capping of this moiety. (Hornung, Ellegast et al. 2006; Pichlmair, Schulz et al. 2006). Saito et al went on to show that short (25bp) dsRNA with at least 1 phosphate at the 5’ or 3’ end can serve as a substrate for RIG-I (Saito, Hirai et al. 2007; Takahasi, Yoneyama et al. 2008). MDA5 serves as the PRR for poly(I:C), which is a long synthetic dsRNA species.
Interestingly poly(I:C) could be converted to a RIG-I substrate after digestion with an enzyme. Therefore recognition of poly(I:C) by RIG-I or MDA5 is dependent on the length of the RNA. Other RNA species recognised by MDA include long dsRNAs from EMCV infection and long segments of the dsRNA Reovirus genome. Therefore long dsRNAs, that are typically not observed in self RNA, are substrates for MDA5. (Yoneyama and Fujita 2009). More recently a more detailed study of the exact substrates of RIG-I and MDA5 was carried out. It appears that 5’-ppp short blunt ended dsRNA results in optimal activation of RIG-I (Schlee, Roth et al. 2009). In contrast, a higher-ordered structure RNA species that contains single and double stranded RNA stimulates MDA-5 (Pichlmair, Schulz et al. 2009).

The 3-dimensional structure of the C-terminal domain (CTD) of RIG-I was revealed by NMR and X-ray crystallography. This revealed that one side of the CTD had a cleft like surface with positive amino acids and this region was possibly responsible for RNA binding. The opposite side had a convex acidic surface (Cui, Eisenacher et al. 2008; Takahasi, Yoneyama et al. 2008). In addition residues on the basic surface were preferentially titrated out in the presence of 5’-ppp ssRNA or dsRNA. Finally site directed mutagenesis revealed that the basic residues are essential for substrate recognition and substrate signal transduction.

1.13.2.1 Signal transduction of RLRs

In 2005, four groups independently identified the CARD domain containing adaptor molecule that is responsible for RLR signalling. This protein is referred to as interferon β promoter stimulator-1 (IPS-1), mitochondrial antiviral signalling (MAVS), virus-induced signalling adaptor (VISA) and CARD adaptor inducing
IFNβ (CARDIF) (Kawai, Takahashi et al. 2005; Meylan, Curran et al. 2005; Seth, Sun et al. 2005, Xu, Wang et al. 2005). Overexpression of IPS-1 lead to the induction of an IFN signal. In addition IPS-1<sup>−/−</sup> cells showed an impaired response to RIG-1 or MDA5 ligands (Kumar, Kawai et al. 2006; Sun, Sun et al. 2006). IPS-1 contains a single N-terminal CARD motif and a proline rich region (PRR) in the middle of the protein. There is also a transmembrane region at the C-terminus of IPS-1. It was shown by Seth et al that IPS-1 is expressed on the outer membrane of the mitochondria (Seth, Sun et al. 2005). This demonstrates that the mitochondria plays an important role in RLR signalling. Forced localisation of IPS-1 to the plasma membrane or ER or a truncation at the C-terminus where the transmembrane region is located all results in a loss of signalling by the RLRs (Seth, Sun et al. 2005). Activated RIG-I and MDA5 are proposed to interact with IPS-1 via CARD domain interactions (Yoneyama and Fujita 2009). TRAF3 is also involved in the signal transduction of RLR signalling. TRAF3 interacts with a TRAF interacting motif (TIM) in IPS-1 (Oganesyan, Saha et al. 2006). TRAF3<sup>−/−</sup> mice showed reduced responses to RLR-dependent viral signals. IPS-1 can also interact with TRAF2 and TRAF6 (Xu, Wang et al. 2005). The TRAFs can then signal to the IKKs. IPS-1 and TRAF3 can signal to both the IKKa/IKKβ and TBK-1/IKKe complexes, while TRAF2/6 are probably needed for NFκB signal transduction (Xu, Wang et al. 2005; Saha, Pietras et al. 2006). Fas-associated death domain (FADD) and RIP-1 are also involved in IPS-1 signalling complexes (Balachandran, Thomas et al. 2004; Kawai, Takahashi et al. 2005). These proteins interact with the C-terminus of IPS-1 and activate NFκB and caspases 8 and 10 (Kawai, Takahashi et al. 2005; Takahashi, Kawai et al. 2006). TRADD can also form a complex with IPS-1, TRAF3, TANK, FADD and RIP-1 to induce NFκB and IRF3 activation (Michallet, Meylan et al.)
A critical scaffold for IRF3 activation was found by Zhong et al. and Ishikawa et al. This protein was named mediator of IRF-3 activation (MITA) and stimulator of interferon genes (STING) respectively. STING can interact with IPS-1 and IRF3. In addition STING positively regulates viral dependent recruitment of TBK-1 to the IPS-1 complex on the mitochondria (Ishikawa and Barber 2008; Zhong, Yang et al. 2008). Another group showed that STING is expressed in the ER (Ishikawa and Barber 2008). Another protein involved in IPS-1 signalling is TRIM25. TRIM 25 contains E3 ligase activity and ubiquitinates the CARD domain of RIG-I. Ubiquitiniation of RIG-I is necessary for signal transduction and the production of type I interferons (Gack, Shin et al. 2007). Signal termination is achieved by another E3 ligase, RNF125. This protein leads to the ubiquitination and proteasomal degradation of RIG-I (Arimoto, Takahashi et al. 2007). See figure 1.7 for an illustration of RLR signalling.

1.13.3 DNA sensing cytosolic receptors

The phenomena of DNA triggering the innate immune system is well known. TLR9 serves as a DNA sensing PRR, but recently there has been mounting evidence for a TLR-9 independent system. For example, in TLR9^/- mice, DNA viruses (such as HSV-1) and intracellular bacteria (such as Listeria monocytogenes), induced a DNA-dependent type I interferon response. (Hochrein, Schlatter et al. 2004; Stetson and Medzhitov 2006). Transfection of synthetic DNA can also induce a type I interferon response that is independent of TLR9 (Ishii, Coban et al. 2006; Stetson and Medzhitov 2006).
1.13.3.1 DAI

Initial excitement surrounded the first DNA sensing cytosolic receptor to be found. This receptor was named DAI (DNA-dependent activator of IFN-regulatory factors), previously known as DLM-1 or ZBPI (Takaoka, Wang et al. 2007). DAI was identified from a cDNA microarray analysis for IFN-inducible genes that have DNA binding domains. DAI is expressed in the cytosol and overexpression in L929 cells led to increased IFN-α and IFN-β, IL-6, CXCL10 levels by poly(dA:dT) treatment (a ds synthetic form of B-type DNA that contains random stretches of As and Ts) (Ishii, Coban et al. 2006; Takaoka, Wang et al. 2007). siRNA to DAI in L929 cells led to decreased innate immune response upon stimulation with B-DNA and bacteria or viral sources. DAI contains a D3 region (DNA binding region) that is important for the induction of type I interferon. Therefore DAI serves as a cytosolic DNA sensor (Yanai, Savitsky et al. 2009)

TBK1 and IKKe are recruited to the C-terminal region of DAI in a DNA dependent manner (Ishii, Coban et al. 2006; Takaoka, Wang et al. 2007; Ishii, Kawagoe et al. 2008). DAI can also activate NFκB. This is possibly through RIP-1, as RIP-1 can interact with DAI. In addition siRNA to RIP-1 prevents NFκB activation by B-DNA stimulation (Kaiser, Upton et al. 2008). DAI possibly has RHIM domains crucial for its interaction with RIP-1. (Kaiser, Upton et al. 2008). More recently Redsamen et al showed that there was indeed RHIM domains in DAI. This domains recruited RIP1 and RIP3. Using siRNA the authors showed that knockdown of RIP1 and RIP3 affected DAI-induced NFκB activation (Rebsamen, Heinz et al. 2009)
Redundant Mechanisms of DNA sensing in the cytosol.

Despite the initial excitement upon the discovery of DAI, it was found that DAI is possibly not the only cytosolic DNA sensing receptor. Although RNAi treatment to DAI lead to inhibition of type I interferon response in L929 cells, there was no such effect seen in MEFs (Takaoka, Wang et al. 2007; Wang, Choi et al. 2008). More evidence of this redundant system can be seen from the observation that interferon stimulatory DNA induced type I IFN genes in the MEFs, but this induction was much lower in L929 cells (Wang, Choi et al. 2008). In addition, DAI−/− mice showed no defect in DNA-mediated activation of the innate immune response (Ishii, Kawagoe et al. 2008).

One DAI-independent pathway leading to IFNβ induction by DNA actually involves RIG-I. As was previously mentioned, RIG-I detects cytosolic RNA. Recently Chiu et al showed that in HEK293T cells, poly (dA:dT) is able to activate RIG-I and induce IFN-β. siRNA against RIG-I and IPS-1 (the adaptor used in RIG-I signalling) inhibited this DNA induced INF-β response. In addition the authors showed that the ideal form of DNA recognised by this pathway is DNA that is A-T rich and that is 30-50 nucleotides in length. The authors identified RNA-polymerase III (Pol III) as part of this mechanism for signalling. Finally it was shown that Pol III transcribes DNA into an RNA intermediate which is then subject to recognition by Pol III (Chiu, Macmillan et al. 2009). This signalling process was confirmed by Ablasser et al (Ablasser, Bauernfeind et al. 2009). However DAI and RIG-I cannot fully account for DNA responses in monocytes, macrophages and DCs so further DNA sensors remain to be discovered (Ablasser, BauernFeind et al. 2009).
It would seem logical for there to be mechanisms in place to inhibit the duration and magnitude of cytosolic DNA-induced immune response? A protein has been identified that might play this role in the cell. This protein is a 3' repair exonuclease (Trex1) (Stetson, Ko et al. 2008). Trex1 is ISD-inducible (interferon stimulatory DNA) and is also an ISD binding protein. Trex1<sup>−/−</sup> mice are lethal due to autoimmunity accompanied by high levels of type I IFN and auto-antibodies (Stetson, Ko et al. 2008). These symptoms were suppressed by genetic ablation of IRF3 or type I IFN receptor genes (Crow, Hayward et al. 2006). Mutations of Trex1 in the human genome have been associated with autoimmune diseases. Therefore Trex1 prevents the triggering of self-DNA by cytosolic DNA detecting receptors (Yanai, Savitsky et al. 2009). Another protein that is involved in negative regulation of type I IFN production by cytosolic DNA is ADAR1 (adenosine deaminase acting on RNA 1). This protein is IFN inducible and also has DNA binding domains (Herbert, Alfken et al. 1997). ADAR<sup>−/−</sup> MEFs show increased IFN-β production upon B-DNA stimulation or HSV-1 infection. Presumably ADAR1 inhibits the cytosolic DNA response. Reviewed (Yanai, Savitsky et al. 2009)

### 1.14 Poxvirus

One DNA virus family likely to sensed by PRRs for nucleic acid is the poxviridae. Poxviruses encompass a big family of viruses that contain a large, linear dsDNA genome. The genome size of the different poxvirus species is variable (130-360 kb)(Goebel, Johnson et al. 1990). Poxviruses replicate in the cytoplasm. The linear dsDNA poxvirus genome contains inverted terminal repeats of variable sizes (Baroudy, Venkatesan et al. 1982). These viruses usually contain more that 150 genes, only 49 of which are present in all of the fully sequenced poxviruses. The
poxviridae are classified based on host range into two families. The two families are Chordopoxvirinae and Entomopoxvirinae. Members of the Chordopoxvirinae subfamily infect vertebrates and members of the Entomopoxvirinae infect insects.

There are eight genera of Chordopoxvirinae (Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capropoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus). The best characterised genus is the Orthopoxvirus which includes variola virus (VARV) and vaccinia virus (VACV) (Yen, Golan et al. 2009). VARV is the most infamous member of the poxvirus family, which is the causative agent of smallpox. VACV virus is a naturally attenuated virus that was used as a vaccine to eradicate smallpox worldwide (Yen, Golan et al. 2009).

There are 90 genes that are common to all the chordopoxviruses (ChPVs). These genes, which are involved in key functions such as replication, transcription and virion assembly are located in the central region of the genome and those genes that are host or species specific are located towards the ends of the genome (Gubser, Hue et al. 2004). Many of these terminal genes encode for proteins that are involved in host immune evasion. These proteins function to disrupt processes such as apoptosis, antigen presentation and recognition, interferon functions and immune signalling processes in favour of the virus. These genes have been given the general term ‘virulence genes’ (Moss and Shisler 2001).

In most poxvirus genomes, the genes tend to be arranged in groups that are transcribed in the same direction. The poxvirus genome is non-infectious. Replication requires the viral transcription complex within the core particle that, after a virion enters the host cell and undergoes a preliminary uncoating, transcribed
a large set of early genes into mRNA (Goebel, Johnson et al. 1990; Moss and Shisler 2001)

The morphogenesis and structure of poxvirus virions are distinctive among viruses as they lack the symmetry and structure that are associated with virions (e.g.: helical icosahedral capsids or nucleocapsids). In contrast poxvirus virions appear as brick shaped or ovoid membrane-bound particles with a complex internal structure featuring a walled, biconcave core flanked by lateral bodies (Cyrklaff, Risco et al. 2005).

1.15 Vaccinia Virus

There are numerous strains of VACV of which twelve have been sequenced (www.orthopox.org). These all have a highly conserved central region of roughly 100kb. Again the terminal regions are more variable and contain deletions, transpositions and mutations. Some of these mutations leave genes inactive.

1.15.1 VACV genome

The whole VACV genome consist of 191,636 bp with an A +T content of 66.6% (Goebel, Johnson et al. 1990). Both ends of the genome contain a 12 kb inverted terminal repeat. The terminal repeats also contain smaller repeated elements. In addition, each of these terminals are composed of an incompletely base-paired hairpin of 101 nucleotides (Goebel, Johnson et al. 1990). Throughout the central region of the genome there are numerous HindIII sites that are used as markers to create a relative position of genes of transcripts in the genome (Mackett and
Archard 1979). Post digestion with *Hind*III each fragment that is produced is given a letter. Open reading frames are identified according to the name of the *Hind*III fragment in which the first in-frame ATG is situated. Within a fragment open reading frames are then numbered from left to right according to the position of the first base (Cyrklaff, Risco et al. 2005). Each reading frame is then given the suffix ‘L’ or ‘R’ (left and right respectively) indicating the orientation of the open reading frame. For example, the open reading frame K7R is within the K fragment of the genome and is the 7th open reading frame with the K fragment and is transcribed to the right.

1.15.2 Structure

Using techniques such as electron cryo-tomography the structure of the most abundant infectious form of VACV, the intracellular mature virus (IMV), has been studied in detail (Cyrklaff, Risco et al. 2005). The virions are roughly brick shaped with the outer dimensions of the virion being approximately 360 x 270 x 250nm. This outer layer has numerous layers. The innermost layer forms a dumbbell-shaped centre. This core is studded with spikes 8nm in ‘height’ that are arranged in small two-dimensional hexagonal arrays. The core membrane seems to be interrupted randomly with 7nm openings that are thought to be used to export *in situ-* transcribed RNA that encodes for viral enzymes in the host cell plasma. The core is dumbbell shaped with condensed DNA around its edges (Cyrklaff, Risco et al. 2005).
1.15.3 VACV Life cycle

Viral life cycle begins with the attachment of virions to the cell surface. There are two forms of the virus that are infectious, called the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). These two forms are structurally and antigenically diverse (Boulter and Appleyard 1973). VACV replicates and assembles in the preinuclear compartments within the cytoplasm. These compartments are named virosomes or viral factories (Moss and Shisler 2001). The first stage in viral morphogenesis is the production of the spherical immature virus (IV). These IVs are formed from the assembly of membrane crescents around electron-dense material containing viral DNA and core proteins (Ichihashi, Matsumoto et al. 1971). The IV is then transformed into the oval/brick shaped intracellular mature virus (IMV). This occurs through a series of proteolytic cleavages and condensation of the viral core and results in the first infectious form of VACV (Moss and Rosenblum 1973). IMV represent the majority of produced virions and remains trapped inside the cell until its lysis. A small portion of the IMV moves to the trans-Golgi network (TGN) from the virosomes via the microtubule network (Sanderson and Smith 1999). Here the IMV acquires two extra membranes and is transformed into the intracellular enveloped virus (IEV). The IEV now moves to the cell surface again along the microtubule networks and buds through the plasma membrane. The budding process produces a cell associated envelope virus (CEV). Actin tails are induced by the CEV that propel the viral particles that are now referred to as extra-cellular enveloped virions (EEV)(Geada, Galindo et al. 2001; Krauss, Hollinshead et al. 2002). There has been varying reports to explain
the origin of the membranes that constitute the virus. Several groups have now reported that proteins that are originally found in the TGN are found to be virion components (Sodeik, Doms et al. 1993; Schmelz, Sodeik et al. 1994; Krauss, Hollinshead et al. 2002).

1.15.4 Immune Detection of VACV

Detection of intracellular VACV is mediated through many of the PRRs mentioned. Unsurprisingly TLRs function in the detection of VACV. TLR4 has recently been implicated in the host response to VACV and this role has been shown to be of one of protection. Of all the TLRs, TLR2 plays the most obvious role. Zhu et al and Delaloye et al have both independently shown that TLR2 is critical for a successful host response against TLR2 (Zhu, Martinez et al. 2007; Delaloye, Roger et al 2009). Continuing on from this observation, Barbalat et al showed that TLR2 could induce a type I interferon response upon stimulation with VACV. This response was limited to inflammatory monocytes (IMs) (Barbalat, Lau et al. 2009). More evidence for the importance of TLR2 in VACV was shown by Quigely et al. This study showed that TLR2-MyD88 signalling was important in order for an intact host adaptive response (Quigley, Martinez et al. 2009). dsRNA from VACV infected cells can induce MDA5-dependent IFNα and IFNβ production when transfected into cells. Thus MDA5 is capable of detecting VACV infection (Pichlmair, Schulz et al. 2009). More recently a novel cytosolic DNA receptor was identified by Hornung et al (Hornung, Ablasserr et al. 2009). This receptor, called AIM2, contains a HIN200 domain that can bind to DNA. The PYRIN domain can associate with the adaptor
molecule ASC. This results in the activation of NFκB and also the activation of caspase1 (which results in IL-1β secretion). Finally the authors showed that knockdown of AIM2 inhibited caspase-1 activation in response to VACV (Hornung, Ablasser et al. 2009). Therefore AIM2 can additionally detect VACV infection.

1.15.5 Immune Evasion by VACV

VACV, along with most other viruses, has numerous genes that are involved in the evasion of the host immune system. Broadly these genes that code for immune evasion proteins can be divided into two groups (Haga and Bowie 2005). One group of proteins are secreted and have the ability to bind to various cytokines, chemokines and complement proteins. For example, B18R is capable of binding to and inhibiting type I IFNs. Another gene encoding an IFN binding protein is B8R. B8R binds IFNγ. Both A53R and K3R can bind and inhibit TNFα. Other cytokine binding gene products include B15R and C12R which bind to IL-1β and IL-18 respectively. Chemokine binding proteins include B29R and C23L, both of which bind CC chemokines. Of the complement binding proteins C21L binds C3ba and C4b complement proteins which results in the inhibition of the classical and alternative complement pathways (Smith, Symons et al. 1997; Alcami and Koszinowski 2000). The second group of immune evasion proteins in VACV are intracellular and often act on intracellular signalling pathways. Examples of extensively studied VACV virus proteins that are capable of blocking signalling are E3, A52, A46 (Haga and Bowie 2005) and K7 (Schroder, Baran et al. 2008).
E3 is required for VACV pathogenesis and for efficient VACV replication in several cell lines (Brandt, Yao et al. 2001). The E3L gene is expressed early in infection and the protein is present in both the cytoplasm and the nucleus. E3 has two domains, an N-terminal Z-DNA binding domain and a C-terminal double stranded RNA-binding domain. Both the N-terminal and C-terminal domains are required for normal pathogenesis in a mouse model (Brandt, Yao et al. 2001). It also has been shown that viral pathogenicity requires E3 binding Z-DNA (Kim, Muralinath et al. 2003). The N-terminal half of E3 is highly conserved among distantly related poxviruses (Ha, Lokanath et al. 2004). It has been suggested that the N-terminal domain of E3L is involved in direct inhibition of PKR activation, nuclear localisation and Z-DNA binding. VACV lacking E3L induced apoptosis in HeLa cells. Furthermore it has been reported that E3L inhibits dsRNA induced apoptosis and has some oncogenic properties (Yuwen, Cox et al. 1993). It should be noted that E3L has a DAI-like DNA binding domain and can inhibit B-DNA-mediated stimulation of IFNβ in MEF cells (Wang, Choi et al. 2008). Therefore it is possible that VACV can use E3 to inhibit cytosolic DNA-sensing receptors (Yanai, Savitsky et al. 2009).

A46 was found from carrying out a database search of novel TIR domain containing proteins. A46 is expressed early in infection and a deletion of A46R from VACV causes attenuation in the murine intranasal model (Stack, Haga et al. 2005). A46R was found to be an intracellular inhibitor of multiple TLR signals. Stack et al found that A46 was capable of interacting with the four TIR domain containing adaptors MyD88, Mal, TRIF and TRAM, but not SARM (Stack, Haga et al. 2005).
Another VACV intracellular inhibitor of signalling that is closely related to A46R is A52R. A52R is also able to block TLR signalling (in particular LPS and poly(I:C)) (Maloney, Schroder et al. 2005). Despite the fact that A46 and A52 are highly homologous there is a distinct lack of redundancy between these proteins. Both A52 and A46 target different signalling molecules. Each have distinct overall effects on TLR signalling. A46 inhibits MAP kinase activation, but in contrast, A52 drove MAPK activation via TRAF6 to produce IL-10, an immunoregulatory cytokine (Maloney, Schroder et al. 2005). In addition, A52 potently blocked TLR-3 mediated NFκB activation, whereas A46 had no such effect (Stack, Haga et al. 2005). Finally A46 inhibited IRF activation whereas A52 had no effect (Stack, Haga et al. 2005). Whereas A46 can target TIR adaptors, A52 can interact with TRAF6 and IRAK2 (Harte, Haga et al. 2003). A52 can inhibit all TLR pathways leading to NFκB activation (Bowie, Kiss-Toth et al. 2000; Harte, Haga et al. 2003; Keating, Maloney et al. 2007). This suggested that a target of A52 was common to all TLR pathways to NFκB. As was previously mentioned, this led to the observation that IRAK2 had an important role in NFκB activation (Keating, Maloney et al. 2007).

1.16 K7

K7 was discovered from the study of the VACV genome and was found to have approximately 50% homology and 25% amino acid identity to A52. Despite being an immunomodulator, A52 is not expressed by VARV, however K7 is. K7 is conserved among other orthopoxviruses (Schroder, Baran et al. 2008). Previously it
was found that K7 inhibited TLR-induced NFκB activation and gene expression (Schroder, Baran et al. 2008). Like A52, K7 was found to interact with TRAF6 and IRAK2 (Schroder, Baran et al. 2008). Similar to A46, K7 inhibited TLR dependent activation of IRFs (Schroder, Baran et al. 2008). In addition and unlike A52, K7 has broad effects on PRR signalling. K7 inhibits TLR-dependent and independent signalling pathways leading to both NFκB and IRF activation, including the RIG-I pathway (Schroder, Baran et al. 2008). Schroder et al showed that K7 could target DDX3, a host RNA helicase, to inhibit type I IFN production. It was through this study of K7 that the exact role that DDX3 plays in IFNβ induction was determined. DDX3 was shown to interact with IKKe and exert a positive role on ifnβ promoter (Schroder, Baran et al. 2008).

K7 has now joined the Pfam family of proteins that contain the ‘N1-like domain’. Other VACV proteins that are members of this family include A52, A46, C6, C16/B22 and B14. Initially it was believed that members of this family would take on a TIR domain-type fold. Indeed this it was through a search of TIR-like proteins in the VACV genome that certain members of this family were discovered. The VACV protein N1L was the first member of this protein family to be shown to take on the bcl-2 like fold (Aoyagi, Liddington et al 2006). Following on from this both A52 and B14 both showed a similar fold (Graham, Bahar et al 2008). K7 is the last member of this family to be confirmed via structural studies to also take on this fold (Kalverda, Thompson et al 2009). The other members of this family are believed to also fold in a bcl-2-like manner through structural alignments and bioinformatical studies. Figure 1.7 shows a structural alignment of family members. Initially it was reported that A52 and A46 were members of the TIR family (Bowie, Kiss-Toth et al 2000). However, it is now clear that this is not the case for A52 (Graham, Bahar et
al 2008). The structure of A46 remains to be resolved but it would appear to be the most TIR like of this family, but it has bcl-2 like structural features (Personal communication, Julienne Stack). Future studies will confirm the exact structural nature of A46. See figure 1.8 for an alignment of this VACV family of proteins.

1.17 DDX3

DDX3 (also known as DBX) is a member of the DEAD-box family of RNA helicases. Members of this family contain an Asp-Glu-Ala-Asp (DEAD) motif. RIG-I and MDA5 are also part of this family. DDX3 is ubiquitously expressed in a variety of cells (Kim, Lee et al. 2001). In addition to VACV, numerous viruses target DDX3. The HCV core protein interacts with DDX3 via the C-terminus of DDX3 (Owsianka and Patel 1999; You, Chen et al. 1999). Co-expression of DDX3 and the core protein enhanced the induction of a luciferase construct. Therefore it is likely that HCV activates DDX3 function in favour of the virus (You, Chen et al. 1999). It should be noted that core protein expression resulted in co-localisation of the DDX3-core complex to the cytoplasm. Therefore the HCV core protein may influence a cytoplasmic role of DDX3 also (Schroder, Baran et al. 2008). In contrast to HCV, DDX3 has a protective role in HBV infection. Since DDX3 can block HBV replication (Chang, Chi et al. 2006). In the case of HIV, DDX3 is up regulated in infected cells (Yedavalli, Neuveut et al. 2004). The Rev protein from HIV can interact with DDX3. It appears that HIV utilises DDX3 to transport its viral mRNAs from the nucleus to the cytoplasm (via CRM1)(Yedavalli, Neuveut et al. 2004).
DDX3, along with other members of the DEAD-box family are involved at almost every level of gene induction (Schroder 2009). DDX3 has been shown to associate with the IFNβ-promoter (Soulat, Burckstummer et al. 2008). In this case DDX3 served as a positive regulator of IFNβ induction. DDX3 can also promote the activation of the p21<sup>waf</sup> promoter in cooperation with the Sp1 transcription factor (Chao, Chen et al. 2006). In contrast, DDX3 lead to down regulation of the E-Cadherin promoter (Botlagunta, Vesuna et al. 2008). The yeast homologue of DDX3 (Ded1) can unwind dsRNA (Yang and Jankowsky 2006), In addition Ded1 may be involved in the splicing of pre-mRNAs. The C-terminus of DDX3 can interact with splicesome (Merz, Urlaub et al. 2007) and also shows homology to other splicing factors (Owsianka and Patel 1999). DDX3 can interact with the TAP and the CRM1 proteins (Kohler and Hurt 2007), both of which are involved in the export of mRNA and proteins from the nucleus (Kohler and Hurt 2007; Lai, Lee et al. 2008). In addition DDX3 can associate with mRNAs (Lai, Lee et al. 2008).

Ded1 plays an important role in translation initiation (Berthelot, Muldoon et al. 2004; Marsden, Nardelli et al. 2006). DDX3 can compensate for this loss in Ded1<sup>−/−</sup> mice, thus demonstrating that DDX3 is likely to be involved in translation initiation. DDX3 has been shown to interact with numerous translation initiation proteins, e.g.: eIF4e (Shih, Tsai et al. 2008), eIF4a, eIF2α, PABP (Lai, Lee et al. 2008) and eIF3 (Lee, Dias et al. 2008). DDX3 has been described as both a positive (via an interaction with eIF3) (Lee, Dias et al. 2008) and negative regulator (by blocking the binding of eIF4e to eIF4G) (Shih, Tsai et al. 2008). Other studies have observed no effects on global protein levels, but these studies have not ruled out the possibility that DDX3 can exert certain influences on specific mRNAs (Lai, Lee et al. 2008).
Figure 1.1 a) Alignment of the IL-1R/TLR superfamily, b) Consensus sequence of the IL-1R subgroup TIR domain c) Consensus sequence of the TIR domain for the entire IL-1R/TLR superfamily. (Taken from Subramaniam et al, 2004)
Figure 1.2: Members of the IL-1R family

Structural representation of members of the IL-1R family. All members of this family share similar domains, but vary in the number of domains and exact size.
Upon recognition of its respective PAMP by a TLR, signal transduction occurs resulting in altered gene profile. Members of the Toll-like receptor (TLR) family and the pathogen-associated molecular patterns (PAMP) that are recognized by each TLR. Figure 1.3: TLR recognition of PAMPs

**Figure 1.3: TLR recognition of PAMPs**

Domain = LRR
Domain = TIR
Endosomal

**Domain**

Polyl. C
dsRNA
TLR3

**Cytoplasmic**

**Extracellular**

Lipide for TLR9
Mannan Ceridias Alexis
Envelop protein M1V
TLR4

T. C. Glu anchor
96.8k from CMV, Ha from MV

M. Mycobacteria, Lipopolysaccharide

Yeast cell wall

Urophagogenic

TLR11

Bacteria

TLR2/6

Fig 3

TLR5
Figure 1.4: Archetype signalling by the IL-1R/TLR Receptor family.

Toll, being the founding member of the TLR family displays homologous signalling mechanism to the IL-1R signalling pathway. Upon receptor activation by Toll and IL-1, the transcription factors Dorsal and NFκB are respectively activated.
Detailed representation of the downstream proteins involved in the signal transduction upon IL-1 engagement with its receptor.

Figure 1.4: IL-1 receptor signaling leading to NF-kB activation.
The activation of transcription factors. Proteins involved in the downstream signaling upon TLR activation. Many of the TLRs use common proteins resulting in

**Figure 15**: Signaling by the TLRs

**NFκB activation**

**Type I IFN**

**IRF3**

**TBK1**

**RIP1**

**TRIF**

**Endosomal domain**

**TLR3**

**TIRAF6**

**IRF5**

**IRF7**

**Cytosolic domain**

**TLR4**

**TLR2/1, TLR 2/6**

**TLR1**

**TLR5**

**Extracellular domain**
Figure 1.6: Members of the NFκB family

Domain structure and length members of the NFκB family. Members of this family include the NFκB proteins and the IκB proteins.
Proteins involved in RLR signalling and the transcription factors that are activated by this signalling pathway. The RLRs use the adaptor IPS-1 which is located on the mitochondria outer membrane.
The members of the K7 family of proteins were aligned. A62 and B14 are aligned on a structural base and show a high degree of structural similarity.

Figure 1.8: Alignment of the K7 family of proteins.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Function/Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1R1</td>
<td>IL-1α / IL-1β / IL-1ra</td>
</tr>
<tr>
<td>IL-1R2 (IL-1RⅡ)</td>
<td>IL-1α / IL-1β / IL-1ra</td>
</tr>
<tr>
<td>IL-1R3 (IL-1RAcP)</td>
<td>Signalling component</td>
</tr>
<tr>
<td>IL-1R4 (ST-2/Fit/TL/DER4)</td>
<td>IL-33</td>
</tr>
<tr>
<td>IL-1R5 (IL-18Ra/IL-Rrp)</td>
<td>IL-18 / IL-1F7</td>
</tr>
<tr>
<td>IL-1R6 (IL-1 Rrp2/IL-1RL2)</td>
<td>IL-1F9 /IL-1F5</td>
</tr>
<tr>
<td>IL-1R7 (IL-18Rβ/IL-18AcRL)</td>
<td>Signalling component</td>
</tr>
<tr>
<td>IL-1R8 (TIGIRR-1)</td>
<td>?</td>
</tr>
<tr>
<td>IL-1R9 (TIGIRR-2)</td>
<td>?</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>Negative regulator</td>
</tr>
</tbody>
</table>

**Table 1.1** Members of the IL-1 receptor family

First Column: Various IL-1R family members, Second Column: Respective functions of the various members
<table>
<thead>
<tr>
<th>Site of Phosphorylation</th>
<th>Kinase responsible</th>
<th>Function</th>
<th>Stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser276</td>
<td>PKAc, MSK1 (mitogen stress - activated protein kinase-1), p38?</td>
<td>Increased recruitment of CBP, p300. Displacement of HDAC-1 from DNA, Increased transactivation potential</td>
<td>After IkB degradation</td>
</tr>
<tr>
<td>Ser311</td>
<td>PKCζ (protein kinase C ζ)</td>
<td>Increased p65 interaction with CBP and recruitment with polymerase II on IL-6 promoter</td>
<td>TNF</td>
</tr>
<tr>
<td>4 sites within amino acids 354-551</td>
<td>GSK3β</td>
<td>Increased transactivation potential</td>
<td>Constitutive</td>
</tr>
<tr>
<td>Ser468</td>
<td>?</td>
<td>?</td>
<td>T-cell stimulation</td>
</tr>
<tr>
<td>Ser529</td>
<td>CK2</td>
<td>Increased transactivation potential</td>
<td>TNF/IL-1</td>
</tr>
<tr>
<td>Ser536</td>
<td>Variety of kinases (E.g.: IKKα, IKKβ, Tpl2, PKCθ)</td>
<td>Increased transactivation potential</td>
<td>Proinflammatory cytokines/T-cell receptor stimulation/DNA damaging agents</td>
</tr>
</tbody>
</table>

**Table 1.2:** Some known p65 phosphorylation events

Column 1: Some of the well characterised sites on p65 that undergo phosphorylation, column 2: known kinases involved in the respective phosphorylation of p65, column 3: the known function of the various phosphorylation events, column 4: stimuli responsible for the respective phosphorylation events
2.1 Materials

Human IL-1β and TNF were a gift from the National Cancer Institute. LPS was purchased from Alexis Biochemicals. Poly (dA:dT) was purchased from Sigma. Malaria DNA was a gift from Prof. Kate Fitzgerald. Anti mouse and anti rabbit IgG peroxidase conjugate antibodies were both purchased from Sigma. The competent E. coli strain, Novablue, the UltraMobius™ 1000 plasmid purification kits and GeneJuice™ were all purchased from Novagen (Merck Biosciences). Agar and Yeast extract were purchased from Chromatrin. All cell culture materials (DMEM, gentamycin, trypsin and L-glutamine) were purchased from Sigma. Plastics for cell culture were purchased from Greiner. Foetal calf serum was purchased from LabTech. The human embryonic kidney cell line 293 (HEK293) and 293 cells stably transfected with IL1R1 (HEK293R1) were gifts from Tularik Inc, San Francisco, CA 94080. The human HEK293 cells stably expressing TLR4 were a gift from Prof. Kate Fitzgerald (University of Massachusetts. MA) The HEK 293FT cells were purchased from Invitrogen (Biosciences). The mouse leukaemic monocyte-macrophage cell line, RAW 264.7, were purchased from the European collection of animal cell cultures (Salisbury, U.K.). All ELISA kits were purchased from R & D Biosystems. Coelentrazine was purchased from Insightbio. Passive lysis buffer was purchased from Promega. Broad range prestained protein markers were sourced from New England BioLabs Ltd. siRNA oligonucleotides were purchased from Invitrogen (Biosciences). General laboratory chemicals were purchased from Sigma-Aldrich (poole, U.K.).
2.2 Expression and Reporter constructs and siRNA oligonucleotides

The NFκB luciferase reporter construct with 5 kB elements was a gift from R. Hofmeister. The IFNβ luciferase construct was a gift from Dr. Taniguchi (University of Tokyo, Japan). The phRL-TK vector contains the herpes simplex virus thymidine kinase promoter upstream of the synthetic Renilla luciferase gene (Promega). The components for the PathDetect™ p38 assay were obtained from Stratagene. The human IL-10 reporter construct was a gift from Prof. Ziegler-Heitbrock (university of Munich, Munich, Germany). The IFN-β luciferase promoter was a gift from Prof. Kate Fitzgerald (University of Massachusetts).

Plasmids encoding for A52, K7, A52-GFP, K7-GFP, K7\(^{1-123}\) and DDX3-myc were generated by PCR amplification from Western Reserve DNA by Dr. Schroder. The wild-type IL-8 luciferase promoter and mutant IL-8 luciferase promoters were gift from Prof. Kate Fitzgerald. IκB-C-Tag was a gift from Prof. Ron Hay. The IKKβ-flag construct was a gift from Greg Cadwell. For silencing of human DDX3 Stealth™ RNAi oligonucleotides were purchased from Invitrogen (5’-GGGAGAAAUUAUCAUGGGAAACAUU-3’). A control oligonucleotide of a matched GC content (36% GC) was also purchased from Invitrogen.

2.3 Methods

2.3.1 Cell Culture

HEK293T, HEK293R1, HEK293FT and RAW264.7 cell lines were all cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% (v/v) foetal calf serum (FCS), 100μg/ml of gentamicin and 2mM L-glutamine. The HEK293 cells
stably expressing TLR4 were cultured in the same medium plus 500μg/ml of the neomycin analogue G-418 as a selection agent. The TBK1\(^{+/−}\), STING\(^{+/−}\) and matched wild type control immortalised macrophages were cultured in DMEM containing 10% FCS, 10μg/ml of ciprofloxacin and 2mM L-glutamine. Cells were maintained at 37°C in a humified atmosphere of 5% CO\(_2\). Prior to seeding HEK293 cells were removed from the surface of a flask first by aspiration of the medium from the flasks, next the cells were washed by adding warm PBS gently to the cells. The PBS was removed and enough trypsin-EDTA (0.5mg/mL) was added to flask to form a thin layer on the cells. The cells were incubated with the trypsin-EDTA for 5miuntes at 37°C. Complete medium was then added to the cells. The contents of the flask were then transferred to a 30ml sterlin and centrifuged at 1000 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in 1ml of complete medium. The density was determined by counting the cells in a 10μl sample of the cell suspension with a haemocytometer and a light microscope.

HEK293 cells were seeded at 1 \times 10^5 \text{cells/ml} with 200μl/well for a 96 well plate, 2ml/well for a 6 well plate and 15ml/dish for a 100mm dish. For siRNA studies, HEK293T cells were plated at 1.5 \times 10^5 \text{cells/ml}. RAW264.7 cells and TBK1\(^{+/−}\), STING\(^{+/−}\) and the matched wild type cells were removed from the flask by scraping. The entire contents of the flask were transferred to a 30ml tube and were spun down and counted in the same manner as the HEK293 cells. The RAW264.7, TBK1 and the matched wild type cells were seeded at 2 \times 10^4 \text{cell/ml} with 200μl/well for a 96 well plate and 3 \times 10^5 \text{cells/ml} with 15ml per 100mm dish. For continuing HEK293 cell lines, the cells were seeded at 1 \times 10^5 \text{cells/ml} and sub-cultured two or three times a week. For RAW264.7 cells and TBK1\(^{+/−}\), STING\(^{+/−}\) and the matched wild
type the cells were seeded at $2 \times 10^5$ cells/ml and sub-cultured two or three times at week.

### 2.3.2 Cryo-Preservation of Cells

Cells were grown to 50-80% confluence, harvested and counted as previously described (Section 2.2.1). The cells were centrifuged at 1000 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 $3 \times 10^6$ cells/ml. Aliquots (1ml) of this suspension were placed in 1.5ml cryotubes. These aliquots were placed at -20°C for 2 hours and then frozen at -80°C for 3 hours or overnight before being stored in liquid nitrogen.

### 2.3.4 Plasmid Purification for Transient Transfection.

#### 2.3.4.1 Plasmid Transformation

The required number of tubes of the competent *E. coli* strain, NoveBlue (Novagen) were left to thaw on ice and mixed gently to ensure the cells were evenly suspended. 1μl of the DNA solution was added to 25μl of competent cells. The mixture was stirred gently and left on ice for 5 minutes. The tubes were heat-shocked for 30 seconds at 42°C in a water bath and cooled on ice for 2 minutes. 125μl of room temperature SOC medium was added to each tube. 50μl aliquots were plated out onto Luria-Bertani (LB) agar plates, containing a selection antibiotic specific for the plasmid, and grown overnight (16-18 hours) at 37°C. Individual colonies were selected for plasmid purification.

#### 2.3.4.2 Plasmid DNA purification
Plasmids were purified using Novagen UltraMobius™ 1000 plasmid purification system. 100ml of LB broth (containing a selection antibiotic specific for the plasmid) was inoculated with a single colony from an agar plate. Cells were grown to an optical density of (OD) 600 at 37°C in a shaking incubator at 200rpm overnight (12-16 hours). 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 was discarded. Pellets were resuspended in 8ml of bacterial resuspension buffer. Cells were lysed for 5 minutes in 8ml of bacterial lysis buffer, on ice. The reaction was stopped using 8ml of neutralisation buffer. The sample was incubated on ice for 5 minutes. The neutralised lysates were centrifuged at 10,000 x g for 2 minutes to remove the bulk of the insoluble material. The cleared supernatant lysates was then decanted into a ClearSpin™ Filter unit and centrifuged in a benchtop centrifuge at 2000 x g for 3 minutes to filter the lysates. 2.4ml of Detox Agent was added to the clarified and neutralised lysates, mixed gently and incubated on ice for 15 minutes. The lysates were transferred to an equilibrated Mobius 1000 Column and the entire volume allowed to flow through by gravity. The column was washed with 20ml of Mobius Wash Buffer. The DNA was precipitated using 0.7 volumes of room temperature isopropanol and collected by centrifugation at 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 (0.25-0.5ml). Plasmid DNA quality and concentration was determined using a UV spectrophotometer to measure the A\textsubscript{260} and A\textsubscript{280} values, and by running the samples on a 1% agarose gel stained with ethidium bromide.

2.3.4.3 Transient Transfection using GeneJuice™
GeneJuice™ transfection reagent (Novagen) was used for transfection of all HEK293 cell lines. For 96 well plate transfections, the cells were seeded as previously described (section 2.2.1) and grown overnight. Cells were transfected in triplicate with a total of 230ng of DNA per well per transfection. In all cases, the total amount of DNA was kept constant using the appropriate amount of a relevant empty vector. For each transfection GeneJuice™ (0.8µl) was mixed with serum-free DMEM (9.2µl) (SFM) and left to incubate for 5 minutes at room temperature. 30µl of this mixture (thus triplicate amounts of GeneJuice™:SFM) was added to triplicate amounts of DNA and incubated for 15 minutes at room temperature. 10µl of this GeneJuice™:SFM and DNA mixture was added to each well of cells. The cells were harvested 24 hours post transfection and then harvested 6-24 hours post stimulation. For 6 well plate transfections, the total DNA used was 2.3µg/well per transfection. A GeneJuice™:SFM mixture was made with 8µl and 9.2µl respectively used per well of a 6 well plate. This mixture was incubated for 5 minutes at room temperature. This mixture was then added to the aliquotted DNA for each well and incubated for 15 minutes at room temperature and then added to the cells. Finally for a 100mm dish, the total amount of DNA per dish was 16µg. A GeneJuice™:SFM mixture of 15µl and 235µl respectively was used per dish. This mixture was incubated for 5 minutes, and then added to the DNA. This mixture was then incubated for 15 minutes and then added to the cells. For transfecting RAW264.7 cells the cells were plated as previously described (section 2.2.1) and left over night. The cells were transfected in the same manner to the HEK293 cell lines except total DNA was kept constant at 200ng/ml.
2.3.5 Luciferase Gene Reporter Assay

2.3.5.1 Preparation of Cellular Lysates

Cell lines were transfected as described above in 96 well plates. The cells were harvested 6 hours post stimulation. The medium was removed from the cells by inversion of the plate and the cells were lysed for 15 minutes in 50μl of passive lysis buffer (Promega, Madison, Wisconsin, U.S.A) with vigorous shaking. The lysates were analysed for Firefly luciferase and Renilla luciferase by taking 20μl of the lysates from each well and adding to two separate plates. For Firefly luciferase activity, 40μl of luciferase assay mix was used as a substrate [20mM tricine, 1.07mM (MgCO₃)₄Mg(OH)₂.5H₂O, 2.67mM MgSO₄, 0.1M EDTA, 33.3mM DTT, 270mM coenzyme A, 470mM Luciferin and 530mM ATP]. For Renilla luciferase activity, 40μl of coelentrazine (2μg/ml in 1 x PBS) was used as a substrate. The samples were analysed using a luminometer.

2.3.5.2 Reporter Gene Activity

For NFκB and IL-10 reporter gene assays, 60ng of κB-luciferase or IL-10-luciferase reporter genes were used per well per transfection. Both the NFκB and IL-10 reporter gene constructs, contain a luciferase gene downstream from the reporter of interest. Once activation of reporter occurs, activation of the luciferase gene occurs and luciferase activity is used as a measure for gene activity. The Stratagene PathDetect System™ was used for p38 MAP kinase reporter assays. A fusion trans-activator protein is used in this system that consists of the activation domain of the CHOP transcription activator fused with the DNA binding domain of the yeast Gal4 (residues 1-147). The transcription activator CHOP is phosphorylation and activated.
by p38. Therefore, the activity of CHOP reflects the *in vivo* activation of p38. The pFR-luciferase reporter plasmid contains a synthetic reporter with 5 repeats of the yeast Gal4 binding sites that control expression of the 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. Therefore activity of luciferase reflects the activation of p38. For p38 MAPK kinases assays, 60ng of pFR-luciferase and .5ng of CHOP were used per well, per transfection. In all cases 20ng of *Renilla*-luciferase internal control was used per well, per transfection.

2.3.6 ELISA

RAW 264.7 cells or HEK293T cells were seeded into 96 well plates as previously described. The cells were then transfected and stimulated as required. The supernatants were either frozen down or used immediately to detect levels of IL-10, MIP-2 and RANTES (for RAW cells) or IL-8 (for HEK293T cells). For detection of IL-10, MIP-2 and RANTES levels a mouse IL-10, MIP-2 and RANTES (respectively) ELISA kit was used (R&D) and the manufacturers instructions were followed. For detection of IL-8 a human IL-8 ELISA kit was used (R & D) and once again the manufactures instructions were followed. For detection of IL-10, supernatants were added neat to the 96-plate used for the ELISA. In the case of mouse MIP-2, mouse RANTES and human IL-8, the samples were diluted 1:5-1:20 in DMEM until an accurate reading of the cytokine levels was achieved.

2.3.7 Western Blotting
2.3.7.1 Preparation of Whole Cell Lysates

Cells were seeded at $1 \times 10^5$ cells/ml. After the necessary transfection and/or stimulation the cells were washed in phosphate buffered saline (PBS) and lysed in 100μl of 3 x sample buffer [62.5mM TRIS pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 50mM DTT, 10% (v/v) Glycerol, 0.1% (w/v) Bromophenol Blue]. Samples were then sonicated for 5 minutes at 80% strength using a DAWESONIprobe and boiled for 5 minutes.

2.3.7.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were resolved on a sodium dodecylsulphate (SDS) polyacrylamide gel using a constant voltage of 100V. In addition prestained protein markers were resolved in parallel to the samples of interest as a reference for protein size. Samples were first run through a stacking gel [1ml 30% (w/v) bisacrylamide mix, 0.75 ml 1M Tris pH 6.8, 60U1 10% (w/v) ammonium persulphate and 6μl TEMED made up to 6ml with H$_2$O] and then resolved according to size using 8-15% polyacrylamide resolving gel, depending on the size of the protein (see table 2.1). Samples were run with pre-stained protein markers (New England Biolabs) as molecular weight standards.

<table>
<thead>
<tr>
<th></th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>6.9 ml</td>
<td>5.9 ml</td>
<td>4.9 ml</td>
<td>3.4 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>3.8 ml</td>
<td>3.8 ml</td>
<td>3.8 ml</td>
<td>3.8 ml</td>
</tr>
<tr>
<td>Acrylamide: Bisacrylamide</td>
<td>4 ml</td>
<td>5 ml</td>
<td>6 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
</tbody>
</table>
Table 2.1: Gel Formulations

<table>
<thead>
<tr>
<th>TEMED</th>
<th>6 µl</th>
<th>6 µl</th>
<th>6 µl</th>
<th>6 µl</th>
</tr>
</thead>
</table>

2.3.7.3 Transfer of Protein to Membrane

The resolved proteins were transferred to Immobilon polyvinylidene difluoride (PVDF) membrane (Sigma) using a wet transfer system, with all the components pre-soaked in transfer buffer [25mM 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 the PVDF membrane. A second piece of filter paper was placed on top, followed by a second sponge. The apparatus was then placed in a cassette, the chamber filled with transfer buffer and a constant voltage of 100V was applied for 15-65 minutes depending on the size of the protein.

2.3.7.4 Antibody Blotting

Membranes were blocked for an hour at room temperature in blocking buffer [5% (w/v) non-fat dried milk, Marvel™ in 1% (v/v)PBS-Tween]. The membrane was washed for 5 minutes in PBS-Tween. The membrane was then incubated for 2-4 hours at room temperature or overnight at 4°C with the relevant primary antibody. The membrane was then washed for 5 minutes in PBS-Tween, three times. The membrane was then incubated with the appropriate secondary horseradish peroxidise linked enzyme for 1-2 hours at room temperature.

<table>
<thead>
<tr>
<th>Antibody/source</th>
<th>Primary antibody dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Incubation Conditions</td>
<td>Dilution/Incubation Conditions</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>K7/Inbiolabs</td>
<td>1:2000 (2 hours rm. Temp)</td>
<td>1:5000 rabbit (2 hours rm. Temp)</td>
</tr>
<tr>
<td>Ha/Sigma</td>
<td>1:1000 (2 hours rm. Temp)</td>
<td>1:5000 mouse (2 hours rm. Temp)</td>
</tr>
<tr>
<td>IκBα/Gift from Prof Ron Hay University of St. Andrews, Scotland)</td>
<td>1:2000 (overnight/2 hr rm. Temp)</td>
<td>1:5000 mouse (2 hours rm. Temp)</td>
</tr>
<tr>
<td>pIκBα/Cell Signalling</td>
<td>1:1000 (overnight in 3% BSA)</td>
<td>1:3000 rabbit (2 hours rm. Temp)</td>
</tr>
<tr>
<td>p65/Santa Cruz</td>
<td>1:1000 (4 hours rm. Temp)</td>
<td>1:3000 mouse (2 hours rm. Temp)</td>
</tr>
<tr>
<td>p65 Ser^{36}/Cell signalling</td>
<td>1:1000 (overnight in 3% BSA)</td>
<td>1:3000 rabbit (2 hours rm. Temp)</td>
</tr>
<tr>
<td>Lamin/Upstate</td>
<td>1:2000 (overnight)</td>
<td>1:3000 rabbit (2 hours rm. Temp)</td>
</tr>
<tr>
<td>Tubulin/Upstate</td>
<td>1:5000 (2 hours rm temp)</td>
<td>1:5000 mouse (2 hours rm. Temp)</td>
</tr>
<tr>
<td>GFP/Santa Cruz</td>
<td>1:2000 (2 hours rm Temp)</td>
<td>1:5000 mouse (2 hours rm. Temp)</td>
</tr>
<tr>
<td>Myc/Sigma</td>
<td>1:2000 (2 hours rm Temp)</td>
<td>1:5000 mouse (2 hours rm. Temp)</td>
</tr>
<tr>
<td>Flag/Sigma</td>
<td>1:2000 (2 hours rm Temp)</td>
<td>1:5000 mouse (2 hours rm. Temp)</td>
</tr>
<tr>
<td>Antibody</td>
<td>Dilution</td>
<td>Incubation</td>
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<td>----------</td>
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</tr>
<tr>
<td>DDX/Bethyl Labatories</td>
<td>1:2000 (4 hours rm Temp)</td>
<td>1:3000 rabbit (2 hours rm Temp)</td>
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<tr>
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<td>1:1000 (overnight)</td>
<td>1:3000 mouse (2 hours rm Temp)</td>
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<tr>
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<td>1:3000 rabbit (2 hours rm Temp)</td>
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<td>1:1000 (overnight)</td>
<td>1:3000 rabbit (2 hours rm Temp)</td>
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<td>β-actin/Sigma Aldrich</td>
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<td>1:5000 mouse (2 hours rm Temp)</td>
</tr>
<tr>
<td>Total JNK/Cells Signalling</td>
<td>1:2000 (4 hours rm Temp)</td>
<td>1:3000 mouse (2 hours rm Temp)</td>
</tr>
</tbody>
</table>

Table 2.2: Conditions of antibody blotting used. Unless stated, all dilutions are in 5% non-fat dried milk in PBS/1% Tween and incubations overnight were at 4°C.

2.3.8 Confocal Microscopy

2.3.8.1 Seeding and transfecting HEK293T cells for Confocal work

HEK293T or R1 cells were seeded and transfected in 6 well plates as described above. Just before seeding of cells cover-slips were added into each well. Wells were transfected with the necessary plasmids using GeneJuice™ as described above. 24 hours post transfection the cells were stimulated if required. After the
cells were stimulated the medium was removed and the cells were washed 3 times in ice-cold PBS, pH 7.2 to remove serum derived proteins. When washing the cells on the coverslips, great care was taken not to wash the cells off. Pasteur pipettes were used to gently add the PBS to the side of the well, and the PBS was gently swirled around the cover-slip.

2.3.8.2 Permeabilization of HEK293T cells

The cells on coverslips were fixed in 4% Paraformaldehyde in PBS, pH 7.2 for 15 minutes on ice. The cells were washed 3 times in ice-cold PBS, again taking care not to wash the cells off the cover-slips. 1ml of 0.05% (v/v) Triton-X 100/PBS was added to each well, ensuring that each cover-slip was covered. The cells were left to permeabilize for 30 minutes on ice. The cells were washed again and blocked in 5% (w/v) BSA, 0.05% (v/v) Tween-20/PBS for 1 hour at room temperature in the dark.

2.3.8.3 Staining for endogenous p65

The cells were then stained for endogenous p65 using the p65 antibody (Santa Cruz) at 1:1000. The primary antibody was diluted in blocking buffer, for 3 hours at room temperature. The cells were washed in PBS three times and probed with the appropriate fluorochrome-coupled secondary antibody for 2 hours at room temperature in the dark. The cells were washed in PBS three times.

2.3.8.4 DAPI Staining

DAPI was used to stain for the nucleus. The stock DAPI (10.9mM, Sigma) was diluted to a final concentration of 300nM in PBS. 200μl of final concentration DAPI was pipetted carefully on top of each cover-slip, ensuring that the cells were
completely covered. The cells were incubated with the DAPI for 3-5 minutes, at room temperature in the dark.

2.3.8.5 Mounting cover-slips for confocal viewing.

The cells were washed for a final time in PBS and mounted on slides with ‘anti-fade’/PBS [3μl of 10mM p-Phenylenediamine in 50% (v/v) glycerol/PBS]. In order to preserve the three-dimensional structure of the cells, a varnish spacer was constructed on each coverslip by brushing along each side of the slip with clear nail varnish. The coverslips were gently placed on clean slides and were affixed to the glass slide by applying a thin film of nail varnish to the edges of the coverslip. The slides were analysed by phase contrast and confocal microscopy with an Olympus Fluoview FV1000 Imaging system. Images were collected and processed with the Olympus Fluoview software (version 1.3c).

2.3.9 Immunoprecipitation

2.3.9.1 Antibody Pre-coupling

2μg of the relevant antibody was coupled to 30μl of a 50% slurry of either Protein A or Protein G sepharose (prewashed x 3 in PBS or appropriate lysis buffer), per immunoprecipitation sample, overnight at 4°C.

2.3.9.2 Immunoprecipitation

Cells were seeded into 100mm dishes as previously described. The cells were transfected/stimulated as necessary. The cells were harvested on ice by scraping into ice-cold PBS. The cells were then centrifuged at 5,000 x g for 5 minutes in a bench top centrifuge at 4°C. The supernatants were removed and the cells were washed
once more with 1ml of ice-cold PBS. The cells were centrifuged again as before and the supernatant was discarded. The cell pellet was resuspended in 850μl of lysis buffer [50mM HEPES, pH 7.5, 1mM EDTA, 100mM NaCl, 0.5% (v/v) NP40, 10% (v/v) glycerol] containing protease inhibitors (Aprotinin, 1mM PMSF and 1mM sodium orthovanadate) and left on ice for 45 minutes. After lysing the cells were spun down at 13,000 x g for 5 minutes using a benchtop centrifuge at 4°C. The supernatants were removed and placed in a fresh eppendorf; 50μl of the supernatants were retained and 25μl of 3 x sample buffer was added to the lysates for a later ‘lysate’ blot.

The remaining 800μl of lysates was split in two and used to immunoprecipitate the proteins of interest. The antibody specific for the protein of interest (pre-coupled to either Protein A or G beads) in 30μl of the 50% sepharose beads was added to the lysates. The lysates and antibody/sepharose mixture was incubated for 3-18 hours at 4°C with rolling.

After the incubation period the samples were centrifuged at 3,000 x g on a benchtop centrifuge, the supernatant was removed and the bead mixture was resuspended in 500μl of lysis buffer containing protease inhibitors. This was repeated twice more, for a total of 3 washes. The beads were then resuspended in 30μl of 3 x sample buffer. Both the lysates samples and the immunoprecipitation samples were boiled at 99°C for 5 minutes and resolved using a SDS containing gel.
2.10 Lentivirus Expression System

2.10.1 Generation of an Entry Vector

PCR primers were designed for the gene of interest to include a 4 base pair sequence (CACC) necessary for directional cloning on the 5' end of the forward primer. Using these primers and a proofreading DNA polymerase a blunt end PCR product was produced. A TOPO™ cloning reaction was set up using a 1:1 molar ratio of PCR product:TOPO vector. The reaction was mixed gently and incubated for 5 minutes at room temperature. The mixture was then placed on ice and used to transform One Shot™ chemically competent E.coli. The cells were spread on prewarmed selective plates and incubated overnight at 37°C. Colonies that grew on the plate were selected and screened for positives. See figure 2.1 for an illustration of the entry vector.

2.10.2 Generation of a Destination Vector

A LR recombination reaction was performed between the entry clone and the destination vector (pLenti4/TO/v5-DEST) using the enzyme LR Clonase™ II. The attL1 and the attL2 regions in the entry vector plasmid (flanking the gene of interest) recombined with the attR1 and attR2 regions in the destination plasmid. 150ng of both the entry vector (containing the gene of interest) and destination vector were mixed and 8μl of TE buffer (pH8.0) was added to the mixture along with 2μl of LR Clonase™ II enzyme mix. The reaction was incubated at 37°C for 1 hour. This mixture was then transformed into One Shot™ Stbl3™ competent E.coli. See figure 2.2 for an illustration of the destination vector.
2.10.3 Producing Lentivirus in 293FT Cells

In a sterile tube, 9μl of ViraPower™ Packaging Mix and 3μl of the destination vector were mixed in 1.5ml of Opti-MEM™ I medium without serum and mixed gently. In a separate tube, 36μl of Lipofectamine™ 2000 was diluted in 1.5ml of Opti-MEM™ I medium without serum, mixed gently and allowed to incubate for 5 minutes at room temperature. The DNA and Lipofectamine™ 2000 mixtures were mixed and incubated for 20 minutes. While the DNA-lipid complexes were forming, 293FT cells were trypsinized and counted. The cells were resuspended at a density of 1.2 x 10^6 cells/ml, in growth medium containing serum. After the 20 minute incubation was over the DNA-Lipofectamine™ 2000 complexes were added to a 100mm tissue culture plate containing 5ml of growth medium. 5ml of the 293FT suspension mix was added to the plate and mixed gently. The cells were incubated overnight at 37°C. The next day the medium containing the DNA-Lipofectamine™ 2000 complexes was removed and replaced with complete culture medium containing sodium pyruvate. After 48-72 hours the supernatant (containing virus) was harvested. The virus containing supernatant was centrifuged at 3,000 x g for 5 minutes at 4°C to pellet cell debris. The viral supernatants were frozen down at -80°C in 1ml aliquots.

2.10.3.1 Transduction of the Lentivirus construct

The cells of choice used for the transduction of the Lentivirus were trypsinized, counted and plated at 2 x 10^5 cells/ml, in 6-well dishes. The next day the Lentivirus stock was thawed and diluted to a final volume of 1ml in full culture medium if needed. The media was removed from the cells and 1ml of the raw or diluted viral construct was added to the cells. Polybrene™ was added to each well at a final
concentration of 6\mu l/ml. The following day the virus containing medium was removed and replaced with 2ml of full culture medium.

2.12 Cell Fractionation

HEK293T and R1 cells were seeded into 6 well plates as previously described. The cells were transfected 24 hours post seeding and stimulated 24 hours post transfection. The cells were harvested by scraping in ice-cold PBS and spun down in a table top centrifuge at 1,000 \times g for 5 minutes at 4°C. The supernatant was discarded and the cells were washed twice more in ice cold PBS. The washed cell pellet was resuspended in 200\mu l of buffer A (10mM HEPES, 1.5mM MgCl$_2$, 10mM KCl, 0.5mM DTT and 0.5mM EDTA) with protease inhibitors (Aprotinin, 1mM PMSF and 1mM sodium orthovanadate). The resuspended cells were allowed to stand on ice for 10 minutes to allow the cells to swell. The cells were dounced using a 2ml mortar and pestle at least 10 time for each sample. The dounced cells were centrifuged for 10 minutes at 2,800 \times g at 4°C using a table top centrifuge. The supernatant (cytoplasmic portion) was retained and SDS containing sample buffer was added. The remaining pellet was resuspended in 100\mu l of buffer B (20mM HEPES, 20 Glycerol, 150mM KCl, 0.5mM DTT, 0.5mM EDTA, 0.25% NP40 and 1mM PMSF). The second resuspended pellet (nuclear portion) was dounced as before. The samples were then centrifuged at 13,000 \times g for 10 minutes at 4°C using a table top centrifuge. SDS containing sample buffer was added to the samples. Both the cytoplasmic and nuclear portion were resolved using an SDS containing gel.
2.13 Oligopulldown

The ds oligonucleotides were created by purchasing oligonucleotides from Sigma that contained the NFκB consensus sequence (5’-AGT TGA GGG GAC TTT CCC AGG C-biotin  3’-TCA ACT CCC CTGAAA GGG TCC G-biotin). The oligonucleotides were resuspended to a concentration of 1μg/ml in TE buffer. Equal volumes of each of the oligonucleotides were mixed and incubated for 10 minutes at 95°C in a water bath. The samples were then cooled down slowly to create a 1μg/ml stock of biotinylated oligonucleotides.

HEK293T and R1 cells were seeded as previously described. The cells were transfected 24 hours after seeding and stimulated 24 hours after transfection. The cells were harvested by scraping into ice cold PBS and washed 2 times in ice cold PBS. The cells were lysed using 100μl of lysis buffer [50mM Tris (pH 7.9), 100mM EDTA, 10% Glycerol, 10mM NaF (pH 8), 1% NP40, 1μg/ml SPI, 1μg/ml aprotinin and 1mM of each of DTT, Na3VO4 and PMSF]. The cells were vortexed and lysed for 20 minutes at 4°C with rotation. After lysis 900μl of lysis buffer without NaCl was added to the samples. The samples were centrifuged for 5 minutes at 13,000 rpm at 4°C using a table top centrifuge. The supernatant was added to a new tube and 20μl of streptavidin-agarose beads (washed 3 times using lysis buffer without NaCl) were added to each sample and incubated for 15 minutes at 4°C with rotating. The samples were spun down at 6,500 x g for 5 minutes at 4°C using a table top centrifuge. The supernatant was added to a new tube and 30μl of washed streptavidin-agarose and 1μg of a 5’ biotinylated-double stranded oligonucleotide was added to each sample. The samples were rotated for 2 hours at 4°C. After the incubation period the samples were spun down at 6,500 rpm for 5 minutes at 4°C.
using a table top centrifuge. The supernatants were removed and discarded and the beads were washed. The beads were washed by centrifugation at 6,500 rpm for 5 minutes at 4°C 3 times using 1ml of lysis buffer without NaCl. After the final wash 50μl of 3 x SDS containing sample buffer was added to each sample. The samples were boiled for 5 minutes and the samples were resolved using a SDS-containing gel.

2.14 Transfection using siRNA:

2.14.1 96 well
The cells were plated into 96 well plates at a concentration of 1.5 x 10^5 cells per ml with 200μl per well. 24 hours post seeding the siRNAs were aliquoted in triplicate using a V-bottom 96 well. Therefore 1 well of a V-bottomed plate represents 3 wells of plated cells as is typical of a transfection. The siRNA is then diluted in 43.75μl of OptiMEM. Lipofectamine was diluted in OptiMEM with 0.2μl of lipofectamine in 12.3μl of OptiMEM per transfection, for triplicate this corresponds to 0.7μl lipofectamine and 43.05μl of OptiMEM. Therefore 43.75μl of this lipofectamine/OptiMEM solution was added to each V-bottomed well containing siRNA. The required amount of lipofectamine/OptiMEM was mixed and incubated for 5 minutes at room temperature, prior to adding to the aliquoted RNA. After this incubation, the lipofectamine/OptiMEM solution was added to each well. The V-bottomed plate was covered to prevent evaporation and left for 20 minutes at room temperature. After 20 minutes, using a multi-channel pipette 25μl of the transfection mix was added into each well of cells. 24 hours after the first hit of siRNA, the cells were given a second hit of siRNA using the same procedure as before. If using
reporter genes, these reporter genes were co-transfected with siRNA by simply adding the required DNA to the well containing siRNA. 24 hours after the second hit the cells were stimulated and lysed in 50µl of lysis buffer (promega) and reporter gene activity was detected. The remainder of the lysates (10µl) were pooled within a triplicate and SDS containing buffer was added to each sample. The samples were resolved using a SDS containing gel.

2.14.2 6 well

For siRNA studies in 6 well plates, the cells were seeded as previously described. 24 hours after seeding the cells were transfected with the siRNAs using lipofectamine. The siRNAs were aliquoted into eppendorfs and diluted in 125µl of SFM. 5µl of lipofectamine was diluted in 125µl of SFM, per transfection. This lipofectamine/SFM mixture was incubated for 5 minutes at room temperature. After incubation, the lipofectamine/SFM mixture was added to the aliquoted siRNAs and incubated for 20 minutes at room temperature. After this incubation, this mixture was added to the cells. 24 hours after the first hit of siRNA, the cells were given a second hit of siRNA. This procedure was identical to the first hit of siRNA. 24 hours after this second hit of siRNA the cells were stimulated if needed and the medium was removed from the cells. The cells were then gently washed in PBS. 100µl of SDS containing sample buffer was added to each well and the cells were scraped down in this buffer and added to eppendorfs. The samples were boiled for 5 minutes and resolved using SDS containing gels. The gels were transferred to a PVDF membrane and blotted for the protein of interest as previously described.
2.15 2D Gel Electrophoresis

The PI of the protein of interest was determined via a search of the literature. IPG strips that are within the range of the PI for the protein of interest were ordered (Biosciences). For initial experiments, a broad pH range strip was used. At a later date, narrower range strips were used.

HEK293R1 cells were plated into 100mm dishes as previously described. The cells were transfected using gene 24 hours after plating and stimulated 24 hours after transfecting. The cells were harvested by scraping the cells in ice-cold PBS. The cells were then spun down at 13,000 x g for 5 minutes at 4°C using a table top centrifuge. 100µl of lysis buffer (7M Urea, 2M Thiourea, 4% Chaps, 0.2% Biolytes 3/10, 0.5% Triton X-100, 2.5% Sodium-pyrophosphate, 1 mM Na3VO4) was added to the pelleted cells. (Amount of lysis buffer can be varied, as long as cells resuspended in lysis buffer can be sucked up in pipette tip. NB: Once lysis buffer has been added to the cell the sample was taken off ice. At this stage some portion of the samples can be aliquoted and frozen down at -80. A Bradford assay was carried out on the samples to determine the concentration of protein. For the Bradford, the BSA standards were dissolved in lysis buffer.

The IPG strips recommend loading a maximum of 30µg of protein sample. Using the Bradford this can be easily calculated. The maximum volume that can be loaded on the strips is 140µl. Of this 140µl there must be at least 50% running buffer. Therefore 30µg of sample was diluted to a final volume of 140µl in running buffer. If the running buffer was clear a small amount of bromophenol blue was added to
the buffer. 140µl of sample in running buffer was added into the chamber of an apparatus designed for holding IPG strips and left over-night. The sample was dispersed the length of the chamber to ensure maximum absorption of the sample with the strip. The IPG strips were placed on top of the sample and 1ml of mineral oil was added on top of the strip. (Note: Each IPG strip has an individual bar code. The number on the bar code is completely unique. It can be important to note the code on the strip if running more than one sample. Therefore the strip can be traced back to the sample at the end of the experiment)

The next day the samples were sufficiently absorbed onto the strips. The strips were then focused. The apparatus used to focus the strips is very similar to the apparatus used to store the strips overnight. The only difference was there were wires at either end of the apparatus that were used to conduct electricity. Wet paper wicks were used to cover the wires in the apparatus. The strips were placed, sample side down into a chamber in the apparatus. 1 ml of mineral oil was added on top of the strip in the chamber. The apparatus was then slotted into a compartment that is connected to a power pack with capabilities to focus the strip.

The strips were run as follows:

- **Step 1 = RAPID SLOPE** 30 min 300 Volts
- **Step 2 = LINEAR SLOPE** 30 min 1000 Volts
- **Step 3 = LINEAR SLOPE** 90 min 5000 Volts
- **Step 4 = RAPID SLOPE** 120 min 5000 Volts
- **Step 5 = WHOLE STEP** *(indefinite)* 50 Volts
*60 minutes is the maximum you can leave on the WHOLE STEP

2.16 Real Time PCR reaction

The cells were plated out into 6 well plates as previously described. 24 hours after plating, the cells were transfected as required. 24 hours after transfection the cells were stimulated. After the required stimulation the medium was removed from the cells and the cells were gently washed in ice cold PBS. 350μl of lysis buffer (supplied with Qiagen RNeasy® kit) was added to each well. 50μl of this mixture was retained and SDS containing sample buffer was added for later use to check for protein levels by Western blot. Using the remaining 300μl, RNA was isolated from the cells (Using Qiagen RNeasy® kit according to the manufactures instructions). cDNA was generated from this RNA using a QuantiTect® Reverse Transcription Kit from Qiagen and the manufactures instructions. The real time reaction was carried out using 1μl cDNA, 0.5 μl Forward Primer (5 pmol/ μl stock), 0.5 μl Reverse Primer (5 pmol/ μl stock), 5 μl SYBR green master mix and 3 μl H$_2$O (total volume). For convenience a master mix was set-up with all the components excluding the cDNA, with enough for the number of reactions required. 9μl of this master mix was added to the wells of a ABI Fast optical plate. 1μl cDNA was then added to each well. The same reaction was set-up, but this time β-actin primers were used. The plate was covered using ABI film and spun down using a centrifuge briefly. The plate was run using a programme specific for the SYBR green used (95°C for 20 seconds then 40 cycles of each of 95°C for 3 seconds and primer annealing temperature for 30 seconds). The results were analysed using the 7500
Fast System SDS software (Applied Biosciences) programme according to the manufactures instructions.

Suppliers

**Universal Biologicals (Cambridge) Ltd**, Passhouse Farmhouse, Papworth St.Agnes, Cambridge, CB3 8QU, England

**Alexis Biochemicals**, Nottingham, NG13 8LS, UK

**Biosciences**, 3 Charlemont Terr, Crofton Road, Dun Laoghaire

**Cell Signalling Technology**, Hitchin, Hertfordshire, SG4 0TY U.K.

**Chromatrin**, Unit G4, TCD Enterprise Centre, Pearse Street, Dublin2, Ireland

**Greiner**, Maybachstrasse, P.O. Box 1162 D-7443, Frichenhausen, Germany

**Insight Bio**, PO Box 520, Wembley, HA9 7YN, U.K.

**LabTech**, 1 Acorn House, The Broyle Ringmer, East Sussex, BN8 5NN, U.K.

**Merck Biosciences**, Padge Road Beeston, Nottingham, NG9 2JR, U.K.

**National Cancer Institute**, Frederick, WA, USA

**New England Biolabs Ltd**, Beverly, M.A, U.S.A.

**R & D Biosystems**, 19 Barton Lane, Abington Science Park, OX14 3NB, U.K.

**Promega**, Madison, WI, U.S.A.

**Santa Cruz Biotechnology**, Delaware Avenue, Santa Cruz CA, 95060 U.S.A

**Sigma**, Poole, Dorset, U.K.

**Upstate**, (Millipore Ireland) Tullagreen, Carrigtwohill, County Cork, Ireland
3.1 Chapter Introduction

The VACV ORFs A52R and A46R were originally described as potential members of the IL-1/TLR superfamily. Bowie et al (2000) carried out a search for unidentified novel members of the IL-1/TLR family based on sequence similarity to known TIR domains and identified A46 by bioinformatics. Following this A52 was identified by a BLAST search using A46. A46R and A52R were cloned from a laboratory strain of VACV (WR) into mammalian expression vectors. It was shown that when transfected into cells, A52 and A46 could inhibit IL-1 induced NFκB reporter gene activation (Bowie, Kiss-Toth et al. 2000).

A52 was shown to inhibit TLR2-, 3-, 4- and 5- induced NFκB activity in HEK293 cells (Harte, Haga et al. 2003). Following on from this Keating et al (2007) showed A52 could block ligand-induced NFκB activation through all the TLRs including TLRs 7 and 9 (Keating, Maloney et al. 2007). Harte et al (2003) originally showed that A52 could interact with both IRAK2 and TRAF6 and Keating et al (2007) went on to demonstrate that the interaction with IRAK2 accounted for the inhibitory effect that A52 has on NFκB. In addition to inhibiting NFκB, A52 was found to activate p38 and JNK MAP Kinase and potentiate LPS induced IL-10 production. A52 required TRAF6 to activate p38 and JNK. A52-induced p38 activation via TRAF6 enhanced LPS induced IL-10 production. Consistent with this, A52 expression led to activation of the IL-10 promoter (Maloney, Schroder et al. 2005).

Using A52 in a similar manner to A46 another as yet uncharacterised VACV protein was found. Schroder et al (2008) identified K7 from an analysis of poxvirus genomes. K7 was found to have 26% amino acid identity and 45% similarity within common regions of sequence common to both ORFs. Like A52, K7 was shown to
interact with IRAK2 and TRAF6 (Schroder, Baran et al. 2008)

In resting cells NFκB is retained in the cytoplasm where it is rendered inactive by members of the IκB family. Upon stimulation of IL-1, IκBα is phosphorylated, ubiquitinated and degraded. This leaves the NFκB dimers such as p50/p65, free to translocate to the nucleus. The translocation of p65 to the nucleus is therefore a hallmark of activation of NFκB. Once in the nucleus activation of NFκB-dependent genes occurs (Verstrepen, Bekaert et al. 2008). The A52 targets TRAF6 and IRAK2 are part of the signalling process upstream from IκBα phosphorylation, ubiquitination and degradation. Previously it has been shown in the lab that A52 can inhibit IL-1 induced IκBα degradation. Therefore K7 was tested to see if it could act in a similar manner.

Although the NFκB subunits are highly regulated by the IκB proteins, once liberated from these inhibitory molecules NFκB subunits must undergo posttranslational modifications to realise full transactivational potential. The most common posttranslational modification is phosphorylation. In most cases p65 phosphorylation enhances p65 transactivational potential (Viatour, Merville et al. 2005)

IL-10 is a Type II cytokine produced by macrophages, dendritic cells (DC), B cells and various subsets of CD4^+ and CD8^+ T cells. IL-10 was first identified by Mosmann et al to be an inhibitor of cytokine synthesis in Th1 cells (Couper, Blount et al. 2008). It is now known that IL-10 can inhibit the production of numerous cytokines including IL-2, IFNγ, TNFα, IL-4, IL-3, IL-1 and GM-CSF. In addition IL-10 inhibits surface molecules such as MHC class II proteins and co-stimulatory
molecules (B7.2 or CD86). Due to the immunomodulating role of IL-10 many pathogens exploit IL-10 as a mechanism of immune evasion. (Liu, Chen et al. 2006)

In most cells IL-10 is an inducible cytokine. In macrophages, LPS activates IL-10 gene expression. LPS-induced IL-10 production is dependent on p38 in PBMCs, monocytic and macrophage cells (Liu, Chen et al. 2006). As was previously mentioned Maloney et al showed that A52 was found to activate p38 and JNK MAP Kinase and potentiate LPS induced IL-10 production. A52-induced p38 activation enhanced LPS-induced IL-10 production and A52 expression lead to activation of the IL-10 promoter (Maloney, Schroder et al. 2005). The ability of A52 to drive IL-10 production serves as an immunoevasion strategy of VACV. In order to fully understand the role K7 plays in intracellular signalling, the effect K7 had on MAP kinase signalling and IL-10 production was tested.

Apart from the observation that A52 inhibits IκBα degradation, very little is known about the detailed effects of A52 on the NFκB system. In addition, the effect of K7 on NFκB was unknown. Furthermore, whether K7 would affect NFκB and IL-10 in a similar manner to A52 was a question of interest. Therefore in this chapter, I compared the effects of K7 and A52 on host signalling. In particular I examined in detail the effects of A52 and K7 on IL-1 induced NFκB signalling by measuring IκBα degradation and phosphorylation, p65 phosphorylation and binding to a consensus NFκB sequence. K7 and A52 showed inhibition in all of these assays. This was expected as the targeting of these VACV proteins to IRAK2 and TRAF6 can explain all of these effects.
Figure 3.1.: Alignment of K7 and A52 proteins from VAVC (WR strain)

The K7 and A52 protein sequences were retrieved from SWISSPROT (accession numbers P68466 and Q01220 respectively) and aligned using the CLUSTALW programme.

* = Identical amino acids

- VK07_VACCW = K7
- VA52_VACCW = A52
<table>
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<th>µg K7</th>
<th>-</th>
<th>0.5</th>
<th>1.0</th>
<th>2.3</th>
</tr>
</thead>
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16kDa $\rightarrow$  

IB: anti-K7

**Figure 3.2: Ha-K7 is expressed in a dose-dependent manner as detected with a K7 antibody**

HEK293T cells were seeded at $1 \times 10^5$ cells/ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the 'genejuice' method, increasing amounts of a plasmid encoding for K7 were transfected into the cells as indicated. Total DNA amount was kept constant by supplementation with the matching empty vector for the K7 plasmid. Cells were harvested 24 hours post transfection and lysed in SDS-containing sample buffer. The lysates were resolved using an SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with an antibody specific for K7. This data is representative of three experiments.
Figure 3.3: GFP-K7 and GFP-A52 have different cellular localizations

Coverslips were placed into 6 well plates prior to seeding of the cells. HEK293 R1 cells were seeded into 6 well plates 24 hours prior to transfection. Using the 'genejuice' method, 2300ng of plasmids encoding for GFP-K7 or GFP-A52 were transfected into cells as indicated. Cells were harvested 24 hours post transfection, fixed and permeabilized as described in Materials and Methods. The coverslips were stained with DAPI and mounted on slides. The slides were viewed by phase contrast and confocal microscopy. This data is representative of three experiments.
HEK293 T cells were seeded at 1 x 10^6 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the 'genejuice' method, the cells were co-transfected with pFR luciferase reporter plasmid (60ng), TK-Renilla (20ng), pFA-CHOP transactivator (0.2ng) and various amounts of a plasmid encoding for K7 or A52 as indicated. Total DNA was kept constant using empty vector. The cells were harvested 24 hours post transfection and reporter gene activity was measured. The data is expressed as mean fold induction ± s.d relative to control levels from an experiment performed in triplicate. This data is representative of three experiments.

P<0.02= **
P<0.05= ***
Figure 3.5: K7 and A52 activate the IL-10 promoter

HEK293 T cells were seeded at $1 \times 10^5$ cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the 'genejuice' method, the cells were co-transfected with a IL-10 pFR luciferase reporter (60ng), TK-Renilla (20ng) and various amounts of a plasmid encoding for K7 or A52 as indicated. Total DNA was kept constant using empty vector. The cells were harvested 24 hours post transfection and reporter gene activity was measured. The data is expressed as mean fold induction ± s.d relative to control levels from an experiment performed in triplicate. This data is representative of three experiments.

P<0.01=  ***
Figure 3.6: The effect of K7 on LPS-induced IL-10 production

Murine Macrophage RAW 264.7 cells (2 x 10⁴ cells per ml) were seeded into 96-well plates 24 hours prior to transfection. The cells were transfected using the 'genejuice' method with 230ng of a plasmid encoding for K7, A52 or the matched empty vector. 24 hours after transfection the cells were stimulated with 1μg/ml LPS as indicated. Supernatants were harvested 24 hours post-stimulation and assayed for IL-10 production by ELISA. Data is expressed as mean pg/ml +/- s.d. This data is a representative experiment from three separate experiment, each performed in triplicate.
Figure 3.7: Expression of K7 truncation mutants

HEK293T cells were seeded in 6 well plates 24 hours prior to transfection. Using the 'genejuice' method, 2.3μg of plasmids encoding for the various HA-tagged truncation mutants were added to the cells. Cells were harvested 24 hours post transfection and lysed in SDS-containing sample buffer. The lysates were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to Ha. This data is representative of three separate experiments.
Figure 3.8: K7 and K7 1-123 activate p38 MAP kinase

HEK293T cells were seeded into 96 well plates 24 hours prior to transfection. Using the 'genejuice' method, the cells were co-transfected with pFR luciferase reporter plasmid (60ng), TK-Renilla (20ng), pFA-CHOP transactivator (0.2ng) and various amounts of a plasmid encoding for K7 or K7 1-123 as indicated. Total DNA was kept constant using empty vector. The cells were harvested 24 hrs post transfection and reporter gene activity was measured. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of three experiments.

P<0.05= ***
Figure 3.9: K7 and K7 1-123 activate the IL-10 promoter

HEK293T cells were seeded into 96 well plates 24 hours prior to transfection. Using the 'genejuice' method, the cells were co-transfected with a IL-10-luciferase reporter plasmid (60ng), TK-Renilla (20ng) and various amounts of K7 or K7 1-123 as indicated. Total DNA was kept constant using empty vector. The cells were harvested 24 hrs post transfection and reporter gene activity was measured. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of three experiments

P<0.05= *

P<0.02= **
Figure 3.10: Like A52, K7 antagonises IL-1 induced NFκB activation

HEK293T cells were seeded into 96 well plates 24 hours prior to transfection. Using the ‘genejuice’ method, the cells were co-transfected with a κB-luciferase reporter plasmid (60ng), TK-Renilla (20ng) and various amounts of K7 or K7 1-123 as indicated. Total DNA was kept constant using empty vector. 24 hours post-transfection, cells were stimulated with 20ng/ml of IL-1 and 6 hours later cells were harvested. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of three experiments.

P< 0.05= *
P<0.02= **
P<0.05= ***
**Figure 3.11: Like A52, K7 blocks IL-1 mediated \( \text{IkB}\alpha \) degradation**

HEK293 R1 cells were seeded into 6 well plates 24 hours prior to transfection. Using the 'genejuice' method 1\( \mu \)g of an \( \text{IkB}-\text{C} \) tagged plasmid and 1.3\( \mu \)g of plasmids encoding for either K7 or A52 or the matched empty vector control were added to the cells. Cells were stimulated for 7 minutes, 24 hours post transfection with 20ng/ml IL-1 and lysed in SDS containing sample buffer. The lysates were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to \( \text{IkB}\alpha \). This data is representative of three separate experiments.
Figure 3.12: Like A52, K7 enhances IL-1 induced phosphorylated Ser\textsuperscript{32} IκBα

HEK293 R1 cells were seeded into 6 well plates 24 hours prior to transfection. Using the ‘genejuice’ method, 2.3μg of plasmids encoding for either K7 or A52 were added to the cells. Cells were stimulated for 7 minutes, 24 hours post transfection with 20ng/ml IL-1 and lysed in SDS containing sample buffer. The lysates were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to pIκBα\textsuperscript{ser32}. This data is representative of three separate experiments.
Figure 3.13: K7 blocks an IL-1 induced, negatively charged posttranslational modification of p65

HEK293R1 cells were seeded into 10cm dishes 24 hours prior to transfection. Using the 'genejuice' method, 8μg of plasmids encoding for either K7 or empty vector were added to the cells. Cells were stimulated for 7 minutes, 24 hours post transfection with 20ng/ml IL-1 and lysed in SDS containing sample buffer. The lysates were resolved by SDS-PAGE according to pI first and then according to size. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to IκBα. This data is representative of three separate experiments.
Figure 3.14: K7 inhibits IL-1 induced phosphorylation of p65 on Ser\textsuperscript{536}

HEK293 R1 cells were seeded into 6 well plates 24 hours prior to transfection. Using the 'genejuice' method, 2.3μg of plasmids encoding for either K7, A52 or empty vector were added to the cells. Cells were stimulated for 15 minutes, 24 hours post transfection with 20ng/ml IL-1 and lysed in 100μl of SDS containing sample buffer. The lysates were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to pp65\textsuperscript{ser536}. This data is representative of three separate experiments.
Figure 3.15: K7 inhibits p65 translocation to the nucleus as measured by Confocal microscopy

(a) HEK293 R1 cells were seeded into 6 well plates containing coverslips 24 hrs prior to transfection. Using the 'genejuice' method, 2.3μg of plasmids encoding for either empty vector, GFP-K7 or GFP-A52 were added to the cells. Cells were stimulated for 15 minutes, 24 hours post transfection with 20ng/ml IL-1. The coverslips were fixed and permeabilized. The coverslips were removed from the wells and stained for p65, using an antibody specific to p65. Dapi was used to stain the nucleus. The coverslips were mounted on slides and viewed under a confocal microscope. Cells in a given field of view were counted and the number of p65 stained nuclei in the given field of view was expressed as percentage of the total cells staining positive for GFP in the stimulated cells. For the p65 sample with stimulation, cells without GFP were counted. This data is representative of three separate experiments.

(b) A representative picture from one experiment was chosen to demonstrate p65 location in unstimulated cells in the presence of GFP-K7, the location of p65 upon IL-1 stimulation in the absence of K7 and upon IL-1 stimulation in the presence of GFP-K7.
Figure 3.16: K7 inhibits IL-1 induced p65 translocation to the nucleus as measured by cell fractionation

HEK293 R1 cells were seeded into 6 well plates 24 hours prior to transfection. Using the 'genejuice' method, 2.3μg of plasmids encoding for either K7 or empty vector were added to the cells. Cells were stimulated for 15 minutes, 24 hours post transfection with 20ng/ml IL-1. The cells were harvested and fractionated into cytoplasmic or nuclear pools of proteins. SDS containing sample buffer was added to each fraction. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to IκBα. This data is representative of three separate experiments.

n= nuclear fraction, c=cytoplasmic fraction
Figure 3.17: IL-1 induced association of p65 with the κB motif is inhibited in the presence of K7

HEK293 R1 cells were seeded into 6 well plates 24 hours prior to transfection. Using the 'genejuice' method, 2.3μg of plasmids encoding for either K7 or empty vector were added to the cells. 24 hours post transfection cells were stimulated as indicated with 20ng/ml IL-1. The cells were harvested and then lysed for 20 minutes. The lysates were incubated with 1μg/ml of a consensus sequence for NFκB and 20μl of strepavidin/agarose beads and rolled for 2 hours at 4 degrees. The beads were spun down and the supernatants harvested in SDS containing sample buffer and probed for β-actin. The beads were washed, resuspended in SDS containing sample buffer and boiled. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to p65. This data is representative of three separate experiments.
Figure 3.18: IL-1 induced association of p65 with the κB motif is not inhibited in the presence of recombinant K7

HEK293 R1 cells were seeded into 6 well plates. 24 hours post seeding the cells were stimulated as indicated with 20ng/ml IL-1. The cells were harvested and lysed for 20 minutes. The lysates were incubated with 1μg/ml of a consensus sequence for NFκB, 20μl of strepavidin/agarose beads and 1mg/ml of recombinant K7 or lysozyme as indicated and rolled for 2 hours at 4 degrees. The beads were spun down and the supernatants harvested in SDS containing sample buffer and probed for β-actin. The beads were washed, resuspended in SDS containing sample buffer and boiled. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to p65. This data is representative of three separate experiments.
LPS (hours)  0  0  1  1  2  2

K7  -  +  -  +  -  +

IP:p65

Figure 3.19: LPS-induced association of p65 with the κB motif is inhibited in the presence of K7

HEK293 TLR4 cells were seeded into 6 well plates 24 hours prior to transfection. Using the 'genejuice' method, 2.3μg of plasmids encoding for either K7 or empty vector were added to the cells. 24 hours post transfection cells were stimulated as indicated with 100ng/ml LPS. The cells were harvested and lysed for 20 minutes. The lysates were incubated with 1μg/ml of a consensus sequence for NFκB and 20μl of strepavidin/agarose beads and rolled for 2 hours at 4 degrees. The beads were spun down and the supernatants harvested in SDS containing sample buffer and probed for β-actin. The beads were washed, resuspended in SDS containing sample buffer and boiled. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to p65. This data is representative of three separate experiments
Figure 3.20a: Western Blot of Lentivirus K7 or GFP expression in HEK 293T cells

HEK293T cells were seeded at 2 x 10^5 cells per ml in 6 well plates (2 ml per well) 24 hours prior to infection. For infection the lentivirus was thawed and diluted as indicated to a final volume of 1 ml in full culture medium. The cell medium was removed and replaced with the virus containing medium. Polybrene was added to the cells at a final concentration of 6μl/ml. 24 hours later the virus containing medium was removed and replace with 2mls of full culture medium. Cells were harvested 24 hours post transfection and lysed in SDS-containing sample buffer. The lysates were resolved using SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with an antibody specific for K7 (a) Virus expressing K7, (b) Virus expressing GFP. This data is representative of three separate experiments.
3.2 Results

3.2.1 Expression and cellular location of K7

K7R from VACV strain WR was cloned into a mammalian expression vector containing an N-terminal HA epitope (pCMV-HA-K7R). Following transfection of increasing amounts of the K7 encoding plasmid into HEK 293T cells (Figure 3.2), a band of the predicted molecular mass of 17.5 for K7 was observed when the resultant immunoblot was probed with an antibody specific for K7. Given the sequence similarities between K7 and A52 it was of interest to see if K7 and A52 had similar intracellular locations. Plasmids encoding for either GFP-K7 or GFP-A52 were transfected into 293R1 cells (stably transfected with the IL-1 receptor) and viewed under a confocal microscope using the nuclear stain DAPI as a reference for intracellular location. Figure 3.3 shows that K7 has a predominately nuclear location compared to the cytoplasmic location of A52. This came as a surprise as I expected K7 and A52 to have comparable cellular locations. This was the first indication that K7 and A52 might not have redundant functions in the VACV genome despite their sequence similarities.

3.2.2 K7 activates p38 MAP kinase and induces IL-10

Previously it was shown that A52 could activate p38 MAP kinase in a dose-dependent manner (Maloney, Schroder et al. 2005). For this study Maloney et al used the Stratagene PathDetect™ System. This assay 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. I used the same system to see if K7 could activate p38 MAP kinase comparably to A52. Figure 3.4 shows that similar to A52, expression of K7 led to promoter induction in a dose-dependent manner. Downstream from p38, A52 activates the IL-10 promoter. Activation of the IL-10 promoter can be studied using a reporter plasmid whereby the luciferase gene is fused to the IL-10 promoter. Again I compared K7 to A52 using this system. Figure 3.5 shows that similar to A52, expression of K7 led to promoter induction in a dose-dependent manner. A52 stimulates IL-10 protein production but only in the presence of LPS (Maloney, Schroder et al. 2005). IL-10 has immune regulating properties and it was suggested that A52 switches TLR signalling from NFκB activation to IL-10 production in order to dampen down a potential immune response against VACV. Figure 3.6 shows that K7 does have a positive effect on IL-10 protein production, which was not surprising since K7 can induce p38 MAP kinase activation and the IL-10 promoter like A52. However in contrast to A52, K7 could induce IL-10 production in the absence of LPS (figure 3.6). This would suggest that A52 and K7 are not redundant with regards to IL-10 protein production. Apart from the ability to drive basal IL-10 protein production, it also seems that K7 has a more potent effect on LPS induced IL-10 production. This suggests that K7 is acting on the IL-10 promoter in a different manner to A52.

3.2.3: K7 truncation mutants activate p38 MAP kinase and the IL-10 promoter

In order to carry out a more detailed study on the effects K7 has on p38 and IL-10, various truncation mutants of K7 were tested, in the hope of mapping the effect on host signalling to particular regions within K7. Four K7-Ha tagged mutants were made lacking the N and C terminus: K7^{41-149}, K7^{1-108}, K7^{1-93} and K7^{1-123}. 
Following transfection of the plasmids encoding the K7 truncation mutants into HEK293T cells, bands of the predicted molecular mass were detected when the resultant immunoblot was probed with an antibody specific for the Ha tag (figure 3.7). All of the various mutants were expressed slightly better than wild type Ha-tagged K7. The Ha-tagged mutant K71-123 was of particular interest as it was though at the time that the region of 123-149 in K7 was of importance in interacting with an important K7 host interaction partner. This mutant was tested to see if it could still activate p38 MAP kinase. Using the Stratagene PathDetect™ System Figure 3.8 shows that K7 (1-123) activated p38 MAP kinase in a dose-dependent manner, just like wild type K7. In a similar manner to figure 3.8 it was of interest to see if K71-123 would have the same effect as wild type K7 on the IL-10 promoter. When various doses of either a plasmid encoding for K71-123 or wild type K7 were transfected into HEK293T cells both showed dose dependent activation of the IL-10-luc promoter (figure 3.9). Thus the C terminal amino acids 124-149 are not required by K7 for MAP kinase and IL-10 promoter activation.

3.2.4: Like A52, K7 antagonises IL-1 induced NFκB activation

As well as stimulating p38 and IL-10, A52 also inhibits IL-1/TLR induced NFκB. Thus A52 has the ability to promote an anti-inflammatory response (IL-10 protein) and inhibit a pro-inflammatory response (NFκB). A52 has been shown to inhibit multiple IL-1/TLR pathways to NFκB, but not affect TNF induced NFκB activation (Keating, Maloney et al. 2007)

Given the similarities between K7 and A52 in terms of p38 activation, the effects of K7 on IL-1 induced NFκB were compared to A52. Upon IL-1 stimulation, the NFκB-luciferase reporter gene showed a ten-fold activation (figure 3.10), which
was inhibited dose dependently by either A52 or K7 expression. It should be noted that K7 had a more potent inhibitory effect on IL-1 induced NFκB reporter gene activation when compared to A52 at same plasmid doses (figure 3.10). In addition, K7 had an inhibitory effect on basal, unstimulated NFκB reporter gene activation while A52 did not (figure 3.10). Given the ability of K7 to inhibit IL-1 induced NFκB reporter gene activation it was of interest to study this pathway in a more detailed manner. Seeing that IkBa degradation is a key event of NFκB activation this signalling event was studied. Previous studies in the lab showed that A52 could inhibit IL-1 induced IkBa degradation. Therefore K7 was compared to A52 in its ability to block IL-1 induced IkBa degradation. IL-1 induced IkBa degradation is a rapidly occurring event. Cells were seeded and transfected with IkBa C-Tag. This allows transfected as well as endogenous IkBa to be studied. Figure 3.11 shows that when HEK293 R1 cells were stimulated for 7 minutes with 20ng/ml of IL-1, IkBa degradation was obvious while expression of plasmids encoding for either A52 (lane 5) or K7 (lane 6) prevented IL-1 induced IkBa degradation. Phosphorylation of IkBa by the IKKs at Ser32 and Ser36 marks IkBa for degradation. Surprisingly, although A52 inhibits NFκB it has been shown to enhance ligand-induced IkBa Ser32 (personal communication, Tara Hurst). In order to examine the effects of K7 on IkBa Ser32 phosphorylation HEK293 R1 cells were stimulated for 7 minutes with 20ng/ml of IL-1. Figure 3.12 shows that like A52, K7 also enhances IL-1 induced phosphorylation of IkBa Ser32. This effect seems to be in disagreement with the ability of A52 and K7 to block IkBa degradation but may reflect a block in IkBa degradation leading to accumulation of the phosphorylated form of IkBa.
3.2.5: K7 inhibits p65 phosphorylation and nuclear translocation

Once IkBα is degraded, p65-containing dimers translocate to the nucleus. Furthermore p65 is subject to multiple phosphorylation events which regulate its, DNA binding and transactivational potential (Viatour, Merville et al. 2005)

Therefore in order to study IL-1 induced NFκB activation in greater detail the post-translation modifications of p65 induced by IL-1 stimulation were examined. This was first studied using a 2D gel method. This can reveal subtle differences in post translational modifications by detecting small changes in the pI of proteins. Figure 3.13 shows that after 7 minutes IL-1 stimulation in the absence of K7, there is the presence of a distinct smear representing a negative charged form of p65 (circled). In the presence of K7 this negative form of p65 is reduced. It is a reasonable assumption that this negative form of p65 is a hyper-phosphorylated form of p65. Thus this suggests that K7 inhibits p65 phosphorylation.

Ser536 phosphorylation of p65 is a well characterised event in the activation of NFκB. The Ser536 residue is located in the C-terminal transactivation domain of p65. This phosphorylation enhances the transactivation potential of p65 (Chew, Biswas et al. 2009). Good antibodies are available commercially to this phosphorylation site. Figure 3.13 showed that K7 can block a negative form of post translationally modified p65. Therefore using an antibody to phosphor p65 Ser536 K7 and A52 were tested to see if they had the ability to block this key event in NFκB activation. Initial experiments indentified 15 minutes as a good time point to detect phosphor. p65. The cells were stimulated for 15 minutes with IL-1 and the resultant blot was probed for phosphor. p65 Ser536. From figure 3.14 it appears that
both A52 (lane 5) and K7 (lane 6) can block phospho p65 Ser\(^{536}\). It should be noted that K7 has a slightly more potent inhibitory effect on phosphor. p65 Ser\(^{536}\). The translocation of p65 from the cytoplasm to the nucleus is a feature of NF\(\kappa\)B activation. K7 and A52 were compared in their ability to block p65 translocation to the nucleus using confocal microscopy. HEK293 R1s cells on coverslips were transfected with GFP-K7 or GFP-A52 and stimulated for 15 minutes with 20ng/ml of IL-1. The coverslips were viewed under a confocal microscope and the number of GFP positive cells staining positive for endogenous p65 in the nucleus were counted. Figure 3.15 shows that both A52 and K7 inhibited IL-1 induced p65 nuclear translocation. Again K7 had a more potent effect on this than A52. To confirm the result in figure 3.15 using an alternative approach, IL-1 induced p65 translocation was tested by cell fractionation and western blot analysis. Cytoplasmic and nuclear fractions were isolated from samples by a process of lysis and centrifugation. The samples were run on a gel and the resultant blot was probed for p65, lamin and tubulin. Lamin is used as a marker for nuclear fractions and tubulin is used as a marker for cytoplasmic fractions. Figure 3.16 shows that K7 prevented IL-1 induced appearance of p65 in the nuclear fraction (compare lane 6 and lane 8). This is in agreement with figure 3.15. Further downstream of p65 translocation after IL-1 stimulation, p65 binds to the \(\kappa\)B DNA element within the promoter region of a gene. A consensus sequence to this NF\(\kappa\)B binding site was designed with a 5' biotinylated tag in order to examine the effects of K7 on DNA binding of p65. HEK 293T cells were transfected with a plasmid encoding for K7 or a matched empty vector control. The cells were stimulated for 7 and 15 minutes with 20ng/ml of IL-1 and lysed. The lysates were incubated with 1\(\mu\)g/ml of a consensus sequence for NF\(\kappa\)B and 20\(\mu\)l of strepavidin/agarose beads and rolled for 2 hours at 4°.
samples were then run on a gel and the resultant blot was probed for p65. Figure 3.17 shows that treatment of cells for 7 minutes or 15 minutes IL-1 causes p65 binding. By comparing lanes 3 and 4 in figure 3.17 it can be seen that after 7 minutes stimulation with IL-1 K7 can block p65 binding to a consensus sequence, while the inhibition is more marked after 15 minutes (compare lanes 5 and 6). Given the ability of transfected K7 to inhibit p65 DNA binding, it was of interest to test if K7 could directly inhibit this after stimulation had occurred. Therefore recombinant K7 was added to cell lysates after stimulation and harvesting of cells. Lysozyme was used as a control protein. Figure 3.18 shows that under these conditions K7 lost its ability to inhibit p65 DNA binding. Thus K7 can inhibit IL-1 induced Ser phosphorylation of p65, p65 translocation and p65 DNA binding. It is also known that K7 is a negative regulator of TLR induced NFκB activation. In order to compare the mechanism of inhibition to that seen for IL-1, I examined the ability of K7 to prevent LPS induced p65 DNA binding. Figure 3.18 shows that p65 DNA binding is seen after 1 and 2 hours LPS stimulation. By comparing lanes 5 (absence of K7) and 6 (presence of K7) it can be seen that LPS induced p65 DNA binding is inhibited in the presence of K7.

3.2.6: Generation of K7 expressing Lentivirus

The Lentivirus system of expressing genes in any cell type is a valuable tool to study in vitro effects of Immunomodulating genes. Therefore K7 was cloned into a vector that is often used to make Lentivirus systems. This involved a process of firstly cloning K7 into a TOPO™ vector, preceded by subcloning into a pENTR™ vector (specifically pLenti4/TO/v5-DEST). A GFP gene was cloned in a similar manner as K7 to produce a control GFP virus. Figure 3.20 shows HEK293T cells
infected with increasing MOIs of the K7 expressing Lentivirus. The resultant immunoblot from this infection shows a dose-dependent expression of K7. Figure 3.21 shows the same dose range of the control GFP virus. This infection with the control GFP virus also led to a dose-dependent expression of GFP. Using these K7 and GFP expressing Lentivirus' it was of interest to see what effects K7 would have in the RAW264.7 mouse macrophage-type cell line. Previous studies in the lab showed that expression of K7 could inhibit RANTES and IL-8 protein production. Therefore I tested the K7 Lentivirus for inhibitory effects on LPS induced mRANTES and MIP-2 (a mouse IL-8 homologue). Figure 3.21 and 3.22 shows that the expression of K7 from a K7 expressing Lentivirus leads to the inhibition of LPS induced RANTES and MIP-2.

Thus K7 is a potent inhibitor of IL-1/TLR induced NFκB and can prevent expression of chemokines.
3.3 Discussion

In this chapter I compared the effects of A52 and K7 on signalling pathways and demonstrated that like A52, K7 activates p38 'MAP' kinase activation and induces IL-10 protein production. Additionally both A52 and K7 can inhibit IL-1 induced NFκB activation, reflected in multiple readouts for NFκB activation. Finally, in this chapter I showed expression of K7 from a lentivirus vector led to inhibition of LPS induced MIP-2 and RANTES in a murine macrophage cell line.

A52 and K7 are members of a poxviral protein family (PF06225) which also includes VACV proteins B14, C16/B22, A46 and N1. The structures of K7 and A52 have been resolved, as has the structure of the IKKβ inhibitor B14 (Graham, Bahar et al. 2008; Kalverda, Thompson et al. 2009) and this has revealed that these proteins share a Bcl-2-like fold. The mammalian Bcl-2 family of proteins are regulators of apoptosis (Huang, Petros et al. 2003). These proteins are characterised by the presence of a Bcl-2-homology (BH1-4) region. These regions are structurally distinct and support the functional roles of Bcl-2 domain containing proteins. (Aoyagi, Zhai et al. 2007). The structure of six Bcl-2 family members have been resolved to date, namely the anti-apoptotic proteins Bcl-xL, Bcl-2, KSHV-Bcl-2 and Bcl-and the pro-apoptotic Bax and Bid. Despite a variance in amino acid sequence they all displayed a homologous fold. This fold consists of six or seven α-helices of various lengths surrounding two core α-helices (Huang, Petros et al. 2003).

N1L was initially shown to inhibit NFκB activation through its association with the IKK complex and more recently was shown to inhibit apoptosis (DiPerna, Stack et al. 2004; Cooray, Bahar et al. 2007). A46 can inhibit activation of the TLRs through
the targeting of the TIR adaptors MyD88, MAL, TRIF and TRAM (Stack, Haga et al. 2005). A52 can block NFκB activation through an interaction with IRAK2 and induce IL-10 production through TRAF6-dependent MAPK activation (Maloney, Schroder et al. 2005; Keating, Maloney et al. 2007). As previously mentioned B14 can inhibit IKKβ and finally K7 can inhibit both TLR and non-TLR activation of the transcription factors NFκB and IRF3 (Schroder, Baran et al. 2008). Thus K7 and A52 are part of an expanding family of viral immune evasion proteins that share the Bcl-2 fold, but have distinct functions in targeting innate immunity.

K7 consists of six α-helices and connecting loops that match the topology of the Bcl-2 family of proteins (Kalverda, Thompson et al. 2009). A52 and B14 both form homodimers in crystallo and in vitro (Graham, Bahar et al. 2008). In contrast, K7 is a monomer with a closed BH3 groove (Kalverda, Thompson et al. 2009). The fact that K7 is a monomer could serve to be significant in separating the functions of A52 and K7. Essentially by existing as a monomer, K7 has an extra surface to form protein-protein interactions compared to A52. This additional surface could potentially allow K7 to bind host interaction partners that A52 cannot, and indeed a further host target has been identified (see chapter 4).

K7 has been shown to interact with the A52 host target protein TRAF6. For A52, the targeting of TRAF6 leads to the activation of p38 MAPK (Maloney, Schroder et al. 2005). Therefore the fact that K7 can also drive p38 MAPK activation (figure 3.4) might be explained by its ability to bind TRAF6. As figure 3.1 shows, K7 and A52 have sequence similarities and show regions that tightly align. Maloney et al showed that a truncated version of A52 (lacking 46 amino acids at the C-terminus) was unable to bind to TRAF6. Therefore the last 46 amino acids in A52 were
proposed to contain the TRAF6 binding site in A52 (Maloney, Schroder et al. 2005). In fact previous studies in the lab have shown that the TRAF6 binding region in A52 is the RNEKLF sequence at amino acids 148-153 (Tara Hurst, personal communication). From the alignment (figure 3.1) K7 and A52 align strongly within this area of A52 where the proposed TRAF6 binding site is. Kalverda et al structurally aligned K7 and A52 and found that there was some degree of structural similarity between these two proteins (Kalverda, Thompson et al. 2009). Therefore it is possible that K7 can bind TRAF6 in a similar manner as A52 does. Various truncation mutants of K7 were made in the hope that regions of K7 important for the effects of K7 on host cellular signalling could be mapped out. Four different mutants were made K7 41-149, K7 1-108, K7 1-93 and K7 1-123 all with a HA tag. Figure 3.8 shows that all of these mutants are expressed strongly. It was believed that K7 1-123 would have particular significance and this construct was therefore tested in its ability to drive p38 activation and the IL-10 promoter. In figure 3.8 the 1-123 truncation of K7 can still activate p38 MAPK. The levels of potentiation by K7 1-123 versus full length K7 are similar within the same dose range. Therefore residues 124-149 are not required for p38 activation. These amino acids are outside of the region where K7 aligns with the A52 TRAF6 binding region. Therefore K7 may interact with TRAF6 in a similar manner to A52 given that K7 1-123 still contains the region where K7 aligns with the A52 TRAF6 binding site. Consequently it is quite possible that K7 can drive p38 MAPK activation simply through the targeting of TRAF6.

Both K7 and A52 activated p38 MAP kinase. It should be noted that in figure 3.4, A52 is a slightly better activator of p38 when compared to K7. Given the sequence
similarities between K7 and A52 and their similar host targets, K7 could have arisen from a duplication of A52 in the VACV genome. Therefore this ability of K7 to interact with TRAF6 and activate p38 MAPK activation may merely be a relic from A52. Given that A52 activates p38 better than K7 it may be that TRAF6 is a more important target of A52 than of K7 in infected cells.

From the studies on A52 it was shown that the ability of A52 to activate p38 and induce the IL-10 promoter relied on the TRAF6 interaction (Maloney, Schroder et al. 2005) and here I show that K7 can also activate p38 and induce the IL-10 promoter. A52 can only stimulate IL-10 production in the presence of LPS (Maloney, Schroder et al. 2005), whereas K7 can induce IL-10 production in the absence of LPS stimulation. This suggests that K7 may stimulate IL-10 protein production in a different manner to A52. It is possible that K7 is utilizing TRAF6 and p38, like A52, to induce the IL-10 promoter, but that K7 has an additional mechanism to enhance IL-10 protein synthesis. This might be facilitated by K7 targeting a host protein involved in enhancing IL-10 production.

Like A52, K7 was found to inhibit IL-1-simulated IκBα degradation and NFκB reporter gene activity. The second host target of A52 is IRAK2. Using A52 as a tool the function of IRAK2 in IL-1/TLR signalling was described (Keating, Maloney et al. 2007) and the mechanism whereby A52 inhibits IL-1/TLR mediated NFκB activation can be explained by the ability of A52 to interact with IRAK2 (Keating, Maloney et al. 2007). Given the capacity of K7 to interact with IRAK2 (Schroder, Baran et al. 2008), it might be assumed that K7 inhibits IL-1/TLR mediated NFκB activation in a similar manner to A52. However it must be noted that K7 can inhibit the basal level of NFκB reporter gene activity whereas A52 does not have this
ability (figure 3.10). In addition, K7 has a more inhibitory effect on IL-1-induced NFκB reporter gene activity compared to A52, and this indicates that K7 may have an additional mechanism of inhibition of IL-1/TLR-induced NFκB reporter gene activation or indeed a completely separate mechanism to A52. As is the case for K7-induced IL-10 protein production, it is possible that K7 has additional host interaction partners that could explain these more potent effects on IL-1-induced NFκB.

Upstream from its degradation, IκBα must be phosphorylated. This tags IκBα for ubiquitination and proteasomal degradation. IκBα is phosphorylated at Ser\(^{32}\) and Ser\(^{36}\) (Verstrepen, Bekaert et al. 2008) and when the effect of A52 and K7 on IL-1 induced IκBα phosphorylation was tested, it was found that both A52 and K7 enhance IκBα Ser\(^{32}\) phosphorylation. This result was unexpected since one would assume that this phosphorylation event would then lead on to enhanced IκBα ubiquitination and degradation with the eventual outcome of improved NFκB gene activity. However, it is possible that IκBα phosphorylation is enhanced in the presence of K7 and A52, due to an accumulation of this form of IκBα because of an inhibition of IκBα ubiquitination or degradation.

The DNA binding ability of p65 is primarily controlled by its interaction with its inhibitory protein IκB. In addition, however p65 is also regulated by post-translational modification such as phosphorylation, acetylation and ubiquitination. There are numerous phosphorylations that p65 undergoes that are necessary for the full transcriptional activation of this NFκB subunit. A well characterised p65 phosphorylation event occurs at Ser\(^{536}\) of p65 (Schmitz, Mattioli et al. 2004). The
importance of Ser$^{536}$ phosphorylation of p65 is highlighted by the existence of regulatory mechanisms in the cell that can dephosphorylate p65 at this Ser residue. Recently Chew et al showed that the WIP1 phosphatase inhibited NF$\kappa$B signalling by acting as a phosphatase for Ser$^{536}$ p65 (Chew, Biswas et al. 2009). Of the possible phosphorylation sites present on p65 that enhance NF$\kappa$B activation, Ser$^{536}$ was of particular interest due to the well-characterised nature of this event and the availability of good antibodies raised against this site. From figure 3.14 it can be seen that IL-1 induces the Ser$^{536}$ form of p65. In the presence of A52 and K7 the appearance of Ser$^{536}$ p65 is inhibited. This result might be expected as again this event is downstream of the involvement of IRAK2 in IL-1 induced NF$\kappa$B activation. It should be noted that K7 has a slightly enhanced ability to block this phosphorylation event compared to A52. This is in agreement with the K7 enhanced ability to block NF$\kappa$B reporter gene activity compared to A52 and again suggests that K7 could have an additional inhibitory mechanism for NF$\kappa$B activation.

The translocation of p65 to the nucleus is a necessary event in NF$\kappa$B activation. This allows p65 to bind to its promoter and carry out transcription of p65 dependent genes. This event was measured by two independent methods, namely confocal analysis and cell fractionation (detectable via western blot) and both methods showed that A52 and K7 inhibited p65 translocation. By reducing nuclear p65 NF$\kappa$B activation cannot occur to full potential.

Although p65 translocation to the nucleus is a vital property of NF$\kappa$B activation, p65 DNA binding is the main determinant of transcription activity (Schmitz, Mattioli et al. 2004). Using a biotinylated consensus NF$\kappa$B binding sequence, p65 promoter binding was studied. Since whole cell lysates were used as the source of
p65 for this assay this does not exactly represent the true portion of nuclear p65 available to bind to the promoter *in vivo* as some of the p65 binding to the consensus sequence may still reside in the cytoplasm. None the less, this assay does indicate that K7 can inhibit the binding abilities of p65 to a NFκB sequence. This effect was further studied using recombinant K7. The recombinant K7 was added to the whole cell lysates after stimulation and harvesting. From figure 3.18 there appears to be no inhibitory activity of recombinant K7 on p65 binding to a consensus sequence. This would indicate that K7 is indirectly blocking p65 binding to a promoter and that the presence of K7 is required prior to stimulation in order for this inhibition to occur. This is consistent with an upstream role for K7. This p65 binding assay also showed LPS induced p65 binding to the κB motif was inhibited by K7. Once more this inhibitory effect of K7 may be explained by the ability of K7 to interact with IRAK2.

Finally in this chapter I used a Lentivirus system to show that expression of K7 could block LPS-induced NFκB-dependent chemokine release. This system will be of benefit for future studies of the effect of K7 on host signalling in a broad range of hard to transfect cells.

In conclusion the effects of K7 and A52 on host signalling pathways seen in this chapter can conceivably be explained by the upstream interaction of K7 or A52 with IRAK 2 and TRAF6. Thus there may be some redundancy between K7 and A52 in their roles in virus virulence. However the more potent inhibitory effects of K7 on NFκB and stimulatory effects on IL-10 production may suggest further mechanism are employed by K7 to manipulate host signalling.
IL-10 has been shown to have anti-inflammatory actions in conditions coupled to increased IFNγ, IL-1 or TNF release. Therefore IL-10 is a particularly attractive host cytokine for VACV to induce. Consistent with this, VACV replication is impaired in IL-10⁻/⁻ mice (Maloney, Schroder et al. 2005). The importance of IL-10 in immunomodulatory terms is highlighted by the observations that many viruses promote the signalling cascade that leads to its release. Furthermore, Epstein Barr Virus (EBV) encodes a viral homologue of IL-10 (Aliberti and Bafica 2005). It should be noted that VACV has no known viral homologue of IL-10. Therefore it is likely that A52 and K7 are employed for the means of which VACV promotes IL-10 signalling.

There is a lot of data on TLR recognition of VACV. Dissecting the precise role of TLRs in VACV recognition is complicated by the fact that TLRs appear to play the role of a double-edged sword for the host. TLRs provide the host with anti-poxvirus defence, but also are responsible for viral pathogenicity and tissue damage (Hutchens, Luker et al. 2008). TLR4 has recently been implicated in the host response to VACV and this role has been shown to be of one of protection, in comparison to the role that TLR3 appears to play. To date, surprisingly TLR2 has been shown to be the most important anti-viral TLR. Zhu et al and Delaloye et al have both independently shown that TLR2 is critical for a successful host response against VACV in vivo (Zhu, Martinez et al. 2007; Delaloye, Roger et al. 2009).

Continuing on from this observation, Barbalat et al recently showed that TLR2 could induce a type I interferon response upon stimulation with VACV (Barbalat, Lau et al. 2009). This response was pinpointed to a subset of cells that the authors referred to as inflammatory monocytes (IMs). In addition the authors postulated that these IMs play a critical anti-viral role, which is TLR-dependent (Barbalat, Lau et
More evidence of the importance of TLR2 in VACV responses was shown by Quigely et al. This study showed that TLR2-MyD88 signalling was important in order for an intact host adaptive response (Quigley, Martinez et al. 2009). The dogma of TLR recognition of viruses has centred around nucleic acid acting as the PAMP. Recent studies have shown that this might not be the case, especially for innate recognition of VACV by TLR2 and TLR4 (Hutchens, Luker et al. 2008; Barbalat, Lau et al. 2009). Given the importance of TLRs in responding to VACV, the observations that I have made in this chapter that K7 is capable of inhibiting TLR-induced NFκB are in keeping with the literature and the inhibition by K7 of TLR signalling may contribute to blocking the host innate and possibly adaptive immune response.

Overall the effects I have seen of A52 and K7 on host signalling are consistent with the virus promoting the completion of its life cycle through the modulation of host signalling to favour of the virus.
4.1 Chapter Introduction

Chapter 3 compared the effects that A52 and K7 had on host intracellular signalling. K7 and A52 appear to have similar effects on p38 MAP kinase (activation) and IL-1 induced NFκB (inhibition). Furthermore, both stimulate the IL-10 promoter and enhance LPS induced IL-10 protein production. Therefore at a quick glance it would seem that K7 and A52 have redundant effects on host signalling pathways. On the other hand there are subtle differences between these two VACV proteins. K7 and A52 have distinct cellular localisations, K7 alone can drive IL-10 protein production in unstimulated cells and it appears that K7 is a better inhibitor of both basal and stimulated NFκB activation. This suggests that K7 has additional effects on host signalling. Therefore these two VACV proteins are not quite as redundant as they seem to be.

The effects K7 and A52 have on IL-1-mediated activation of NFκB were explored in Chapter 3. Both K7 and A52 inhibited many steps leading to activation of NFκB by IL-1. IL-1 relies on an adaptor protein, MyD88, that contains a TIR domain to transduce signalling following ligand binding. MyD88 then signals through the IRAKs and TRAF6 (Li and Qin 2005). Following this TRAF6 activates a TAB1/2/3:TAK1 complex, which then leads to the IKK complex activation. The IKK complex then leads to the phosphorylation of IκBα, which then results in the degradation of IκBα. This leaves p65 free to translocate to the nucleus and carry out its transcriptional duties (Li and Qin 2005). Thus any inhibitory effects of K7 and A52 can partially be explained through their known targets on the IL-1/TLR signalling pathway.
TNF-α is a cytokine that potently activates NFκB. TNF signalling can occur through TNF-R1 or TNF-R2, although TNF-R1 plays a broader role in NFκB activation (Bonif, Meuwis et al. 2006). Unlike the IL-1R which utilises the TIR domain containing adaptor MyD88, TNF uses the death domain (DD) containing adaptor TRADD to transduce a signal from the receptor after ligand binding. TRADD in turn recruits TRAF2, TRAF5 and RIP-1. RIP-1 then causes the TAK1/TAB1-TAB2 complex or MEKK3 to activate the IKK kinase complex. The IKK complex phosphorylates IκBα to induce its degradation. This then allows the p65/p50 dimers to translocate to the nucleus and activate transcription (Verstrepen, Bekaert et al. 2008; Vallabhapurapu and Karin 2009). Therefore, although the eventual outcome is the same in terms of NFκB activation, TNF uses a distinct set of adaptor and signalling molecules at the level of the receptor compared to IL-1/TLRs. In contrast to IL-1/TLR signalling which uses TRAF6, TNF uses TRAF2 and TRAF5 for signal transduction. In addition, to date no IRAKs have been shown to be involved in TNF signalling to NFκB. Consistent with this, Bowie et al (2000) showed that A52 was not capable of inhibiting TNF induced NFκB activation (Bowie, Kiss-Toth et al. 2000). It was of interest therefore to test if K7 had an effect on TNF-induced NFκB activation. In fact, in this chapter I show that K7 can indeed block NFκB activation by TNFα, demonstrating that A52 and K7 are not redundant and suggesting that K7 has an additional host protein interaction partner.

Of note, Schroder et al (2008) showed that K7 does have an additional host target, namely DDX3. This RNA helicase, was pulled down using recombinant His-tagged K7 (Schroder, Baran et al. 2008). DDX3 is a 73kDa protein that is ubiquitously expressed. It belongs to the DEAD box family of ATP-dependent RNA helicases. This DEAD box family as the name suggests is characterized by an Asp-Glu-Ala-
Asp (DEAD) motif. Previous reports have shown that DDX3 and its orthologs are involved in a variety of cellular function including, transcription, regulation of splicing, nuclear export and translation (Lee, Dias et al. 2008; Schroder 2009).

The main focus of this chapter therefore was to study the effects K7 and A52 have on TNF-induced NFκB activation. Having found that K7 and not A52 inhibits TNF driven NFκB activation, I then examined the potential role of DDX3 in IL-1 and TNF induced NFκB activation.
Figure 4.1: Unlike A52, K7 antagonises TNF-induced NFκB activation

HEK293T cells were seeded into a 96 well plate 24 hours prior to transfection. Using the ‘genejuice’ method the cells were co-transfected with the κB-luciferase reporter gene, TK-Renilla and various amounts of a plasmid encoding for K7 or A52. Total DNA was kept constant using empty vector. 24 hours post-transfection, cells were stimulated with 50ng/ml of TNF and 6 hours later the cells were harvested and assayed for NFκB activity by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels and is from a single experiment performed in triplicate. This data is representative of more than three experiments.

P< 0.01= ***
Figure 4.2: Unlike A52, K7 blocks TNF-stimulated \( \text{IkB} \alpha \) degradation

HEK293T cells were seeded into 6 well plates 24 hours prior to transfection. Using the genejuice method, 2.3\( \mu \)g of plasmids encoding for either K7, A52, and \( \text{IkB} \) C-TAG were transfected into the cells. Total DNA was kept constant using empty vector. Cells were stimulated for 15 minutes, 24 hours post transfection with 50ng/ml TNF and lysed in SDS-containing sample buffer. The lysates were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to \( \text{IkB} \alpha \). This data is representative of three separate experiments.
Figure 4.3: Unlike A52, K7 blocks TNF-induced phosphorylation of p65 on Ser^{536}

HEK293T cells were seeded into 6 well plates 24 hours prior to transfection. Using the genejuice method, 2.3μg of plasmids encoding for either K7, A52 or empty vector were transfected into the cells. Cells were stimulated for 15 minutes, 24 hours post transfection with 50ng/ml TNF and lysed in 100μl of SDS containing sample buffer. The lysates were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to pp65^{Ser^{536}}. This data is representative of two separate experiments.
Figure 4.4: K7 inhibits TNF induced p65 translocation to the nucleus

HEK293T cells were seeded into 6 well plates 24 hours prior to transfection. Using the genejuice method, 2.3μg of plasmids encoding for either K7 or empty vector were transfected into the cells. Cells were stimulated for 15 minutes, 24 hours post transfection with 50ng/ml TNF. The cells were harvested and fractionated into cytoplasmic or nuclear pools of proteins. SDS containing sample buffer was added to each fraction. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to p65 or lamin. This data is representative of three separate experiments.

n= nuclear fraction, c= cytoplasmic fraction
Figure 4.5: DDX3-myc interacts with IKKβ-flag and the interaction slightly enhanced with TNF stimulation

HEK293T cells were seeded at 1 x 10^5 cells/ml in 10cm dishes (15mls per dish) 24 hours prior to transfection. Using the genejuice method, 8µg of plasmids encoding for either DDX3-myc, IKKβ-flag or empty vector were added to the cells. Cells were stimulated for 30 minutes, 24 hours post transfection, with 50ng/ml TNF. The cells were harvested and cell lysates were generated. The lysates were spun down and some sample was retained for a lysate blot. The remaining supernatants were Immunoprecipitated using 20µl of Protein A/sample. The immunoprecipitated supernatants were washed three times, resuspended in SDS-containing sample buffer and resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to myc or flag. This data is representative of three separate experiments.
HEK293T cells were seeded into 10cm dishes 24 hours prior to transfection. Cells were stimulated as indicated, 24 hours post transfection with 50ng/ml TNF. The cells were harvested and lysates were generated. The lysates were spun down and some sample was retained for a lysate blot. The remaining supernatants were Immunoprecipitated using 20μl of Protein A/sample. The Immunoprecipitated supernatants were washed three times, resuspended in SDS containing sample buffer and resolved by SDS containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or IKKβ. This data is representative of three separate experiments.
Figure 4.7: Endogenous DDX3 interacts with endogenous IKKβ in both wild type and TBK1-deficient macrophages

Cells were seeded into 10cm dishes 24 hours prior to transfection. The cells were stimulated as indicated 24 hours post seeding. The cells were harvested and lysates were generated. The lysates were spun down and some sample was retained for a lysate blot. The remaining supernatants were Immunoprecipitated using 20μl of Protein A/sample. The immunoprecipitated supernatants were washed three times, resuspended in SDS containing sample buffer and resolved by SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or IKKβ. This data is representative of three separate experiments.

Lanes 1-3 = TBK1-deficient macrophages

Lanes 4-6 = wild type macrophages
Figure 4.8: Expression of IKKβ-flag induces a higher molecular weight form of DDX3-myc

HEK293T cells were seeded into 10cm dishes 24 hours prior to transfection. Using the genejuice method, 8µg of plasmids encoding for either DDX3-myc, IKKβ-flag or empty vector were added to the cells. Cells were stimulated as indicated, 24 hours post transfection with 50ng/ml TNF. The cells were harvested and lysed for 1 hour on ice. The supernatants were resuspended in SDS containing sample buffer and resolved using SDS containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to myc or flag. This data is representative of three separate experiments.

DDX3*=higher molecular weight form of DDX3
Figure 4.9: Optimisation of DDX3 knockdown by siRNA in HEK 293T cells

HEK293T cells were seeded at 1.5 x 10^5 cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected with varying amounts of a DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of siRNA as 48 hours post seeding. 24 hours after the second hit the cells were harvested and lysed in SDS containing lysis buffer and resolved using a SDS containing gel. The membrane was probed with an antibody specific for DDX3 or β-actin as a loading control. This data is representative of three separate experiments.
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Figure 4.10: Knock down of DDX3 using siRNA has no effect IL-1 induced activation of NFκB

HEK293Ts cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipofectamine with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA 48hrs after seeding and also co-transfected with an NFκB-luciferase reporter gene reporter plasmid and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of IL-1 and the cells were harvested 6 hours post stimulation.

(a) NFκB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
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DDX3

IB
β-actin

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Figure 4.11: Knock down of DDX3 using siRNA has no effect TNF induced activation of NFκB

HEK293Ts cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipo with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA 48hrs after seeding and also co-transfected with an NFκB-luciferase reporter gene reporter plasmid and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of IL-1 and the cells were harvested 6 hours post stimulation.

(a) NFκB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
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HEK293T cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipofectamine with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA 48hrs after seeding and also co-transfected with an ifb-luc promoter and a TK Renilla construct. The cells were infected 8 hours post transfection and harvested 24 hours post infection.

(a) IFNβ activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels and each performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.

P<0.01= ***
Figure 4.13: DDX3 depletion has no effect on IL-1-induced \( \text{IκBα} \) degradation

HEK293T cells were seeded at \( 1.5 \times 10^5 \) cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA 48 hours post seeding. 24 hours after the second hit the cells were stimulated as indicated with 20ng/ml of IL-1. The cells were harvested and lysed in SDS containing lysis buffer and resolved using a SDS containing gel. The membrane was probed with an antibody specific for \( \text{IκBα} \), DDX3 or \( \beta \)-actin. This data is representative of three separate experiments.
Figure 4.14: DDX3 depletion has no effect on IL-1 induced phosphorylation of IκBα

HEK293T cells were seeded at 1.5 x 10^5 cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA 48 hours post seeding. 24 hours after the second hit the cells were stimulated as indicated with 20ng/ml of IL-1. The cells were harvested and lysed in SDS containing lysis buffer and resolved using a SDS containing gel. The membrane was probed with an antibody specific for phoso IκBα , DDX3 or β-actin. This data is representative of three separate experiments.
Figure 4.15: DDX3 depletion has no effect on TNF-induced IκBα degradation

HEK293T cells were seeded at 1.5 x 10^5 cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA 48 hours post seeding. 24 hours after the second hit the cells were stimulated as indicated with 50ng/ml of TNFα. The cells were harvested and lysed in SDS containing lysis buffer and resolved using a SDS containing gel. The membrane was probed with an antibody specific for IκBα, DDX3 or β-actin. This data is representative of three separate experiments.
Figure 4.16: DDX3 depletion does not prevent TNF-induced phosphorylation of IκBα

HEK293T cells were seeded at 1.5 x 10^5 cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA 48 hours post seeding. 24 hours after the second hit the cells were stimulated as indicated with 50ng/ml of TNFα. The cells were harvested and lysed in SDS containing lysis buffer and resolved using a SDS containing gel. The membrane was probed with an antibody specific for pIκBα^{ser32}, DDX3 or β-actin. This data is representative of three separate experiments.
4.2 Results

4.2.1 Unlike A52, K7 can inhibit TNF-induced NFκB activation

It was shown in chapter 3 that both K7 and A52 can block IL-1-induced NFκB activation. Bowie et al (2000) showed that A52 was not capable of inhibiting TNF induced NFκB activation. Therefore I tested whether K7 could block TNF-induced NFκB activation. From figure 4.1 it can be seen that upon stimulation with TNF, the NFκB reporter gene showed approximately 8-fold stimulation in HEK293T cells. This activation was inhibited in a dose-dependent manner in the presence of a plasmid encoding for K7. In contrast, there was no inhibition in the presence of a plasmid encoding for A52. In a similar manner to IL-1-induced IκBα degradation, TNF induced IκBα degradation was tested by using cells transfected with IκBα C-tag. Figure 4.2 shows that when HEK293T cells were stimulated with 50ng/ml of TNF for 15 minutes, IκBα degradation occurred. This degradation was blocked by the presence of an expression plasmid encoding for K7 (lane 6), whereas the degradation occurred as normal in the presence of an expression plasmid encoding for A52 (lane 5). TNF-induced phosphorylation of p65 on Ser536 was studied next. HEK293T cells were stimulated for 15 minutes with 50ng/ml of TNF. Figure 4.3 shows that strong phosphorylation of p65 (Ser536) was detected with an antibody specific for this phosphorylation site at this time point. This band was decreased in the presence of an expression plasmid encoding for K7 but not A52. Following on from this TNF-induced p65 translocation to the nucleus was examined by generating nuclear and cytoplasmic fractions by a process of lysis and centrifugation. From Figure 4.4 it can be seen that the resulting blot shows that the
presence of K7 prevented TNF-induced appearance of p65 in the nuclear fraction (compare lanes 6 and 8).

4.2.2 DDX3 interacts with IKKβ

Apart from the differential sensitivity of TNF signalling to A52 and K7 an additional difference between K7 and A52 is the observation that K7 can inhibit the PRR induced interferon response (Schroder, Baran et al. 2008). Schroder et al, 2008, showed that K7 but not A52 could interact with DDX3, and that DDX3 was a positive regulator in the kinase complex that activates the interferon pathway, particularly in response to Sendai Virus. DDX3 could interact with IKKe and this interaction was ligand dependent. Therefore in the case of interferon induction, K7 can target this positive regulator of this pathway and thus inhibit the host’s antiviral response to VACV. Given that the IKKs involved in TNF induced NFκB activation are structurally similar to IKKe, it was of interest whether DDX3 might be involved in NFκB activation by TNF via an interaction with IKKβ. If so this would explain how K7 inhibits TNF signalling, via DDX3 targeting.

To initially test this theory of DDX3 involvement in IKKβ activation, an over-expressed system was used in the HEK293T cells. Cell lysates were generated from HEK293T cells after transfection and stimulation. The lysates were incubated with either an antibody to flag or myc and resultant immune complex were run on a gel and immunoblotted for either flag or myc. By looking at the ‘top blot’ in figure 4.5 it can be seen that over-expressed DDX3-myc can interact IKKβ-flag (lane 3). In addition this interaction was enhanced when the cells were stimulated for 30 minutes with 50ng/ml of TNFα (lane 4), although expression of DDX3-myc was lower in the unstimulated lane compared to the simulated lane (third blot down,
lanes 3 and 4). Therefore the difference in expression of DDX3-myc could account for some of the apparent enhancement of the interaction.

In order to confirm this result, the endogenous interaction between DDX3 and IKKβ was examined. Once more HEK293T cells were used and as can be seen from the top blot in figure 4.6, DDX3 and IKKβ interact endogenously. It should be noted that this endogenous interaction is also enhanced by TNF stimulation (compare lane 1, absence of TNF with the stimulated lanes 2-5, presence of TNF). From the observation that DDX3 and IKKε could interact it was important to test the DDX3/IKKβ interaction in the absence of some of the kinases involved in the interferon response. This was particularly important given the degree of crosstalk between the interferon and NFκB responses. Due to availability, TBK1-deficient macrophages and the matched wild type control cells were tested for the DDX3/IKKβ interaction. Figure 4.7 shows that the DDX3/IKKβ interaction was intact in the absence of TBK1. [compare lanes 1-3 (TBK1 deficient macrophages) and lanes 4-6 (matched wild type cells)].

An important observation that was noted during the generation of lysate blots for the overexpression interaction studies can be seen in figure 4.8. In the presence of IKKβ-flag a higher molecular weight form of DDX3 was induced. This hints that IKKβ could have a role to play in the regulation of DDX3 through post-translational modification.

42.3 DDX3 does not contribute IL-1 or TNF induced NFκB activation.

Given that DDX3 and IKKβ interacted in a ligand dependent manner, the involvement of DDX3 in the NFκB pathway was tested. siRNA targeting DDX3
was used in order to test whether transient depletion of DDX3 in cells would lead to impaired NFκB activation. Given the importance of IKKβ in IL-1-induced NFκB activation, IL-1 induced NFκB activation was studied in addition to TNF-induced NFκB activation. Initially the siRNA (purchased from Invitrogen™) was tested in a dose response for its ability to reduce the cellular expression of DDX3. A control siRNA was also purchased from Invitrogen™ that had a matched GC content. These siRNA oligonucleotides were tested in HEK293T cells plated out into 6 well plates. The cells were seeded into six-well plates, allowed to rest for 24 hours. Following this the cells were given two separate hits of the siRNAs, 24 hours apart. Then the cells were stimulated and harvested. The resultant immunoblot (figure 4.9) shows that the DDX3-targeting oligonucleotide led to dramatically reduced DDX3 expression, whereas the control oligonucleotide did not affect expression levels of DDX3. The most effective doses of DDX3 targeting siRNA were 25-50pg/ml. Therefore these concentrations of siRNA were used figure 4.10 and 4.11 to examine the effects of DDX3 depletion on IL-1- and TNF-induced NFκB-luciferase reporter gene activation. IL-1 stimulation led to approximately 10-fold activation of the reporter gene in figure 4.10. Figure 4.10 shows that no major inhibition or potentiation occurs for IL-1 induced NFκB reporter gene activation in the presence of DDX3-targeting siRNA by comparing control siRNA to #12 (DDX3 targeting oligonucleotide) Figure 4.10b confirms that efficient knockdown of DDX3 was achieved in this experiment when the DDX3 targeting oligonucleotide was used. Figure 4.11a shows a similar result was found for TNF-induced NFκB reporter gene activation. Figure 4.11b shows the representative DDX3 protein blot for figure 4.11a. Again, DDX3 targeting siRNA leads to reduced expression of the protein levels, but no effect on NFκB activation.
Previous studies from this laboratory have shown that siRNA-mediated DDX3 depletion reduced SeV-induced IRF3 activation (Schroder, Baran et al. 2008). Therefore to fully appreciate the lack of effect seen on the NFκB-luciferase reporter gene in the presence of reduced DDX3 protein levels, Sendai Virus induced IFNβ-promoter luciferase induction was tested for effects of DDX3 knockdown. Using the same dose of control and #12 siRNA oligonucleotides and an identical protocol, figure 4.12 shows that in the presence of reduced DDX3 protein levels (figure 4.12b), SeV induced IFNβ activation is reduced (figure 4.12a).

Figures 4.10 and 4.11 showed that DDX3 depletion by siRNA had no effect on IL-1 and TNF induced NFκB reporter gene activation even though DDX3 and IKKβ interact. Therefore in order to confirm the lack of a role of DDX3 in NFκB activation, IL-1 and TNF induced IκBα phosphorylation and degradation were tested in DDX3-depleted cells. Figure 4.13 shows that IκBα degradation occurred when the cells were stimulated for 7 and 15 minutes with 20ng/ml of IL-1. There appeared to be recovery of the IκBα protein levels after 30 minutes stimulation. By comparing the control siRNA lanes with #12 (DDX3 targeting) siRNA lanes within the same time point, figure 4.13 shows that knock down of DDX3 has no effect on IL-1 induced IκBα degradation. Figure 4.14 shows that pIκBα is rapidly induced after 7 minutes IL-1 stimulation. After 30 minutes IL-1 stimulation, pIκBα levels reduced to close to basal levels. This figure shows that DDX3 depletion did not have a significant effect on IL-1 induced pIκBα induction at 7 minutes, although it does appear that after 15 minutes stimulation of IL-1, pIκBα was slightly inhibited when DDX3 levels were reduced. In a similar manner to figures 4.13 and 4.14, figures 4.15 and 4.16 show that DDX3 depletion does not affect TNF induced IκBα phosphorylation and degradation. In figure 4.15, IκBα degradation occurs after 7
minutes TNF stimulation, with maximum degradation occurring after 15 minutes stimulation. Figure 4.16 shows $p\text{IkB}\alpha$ is induced after 7 minutes TNF stimulation, is reduced after 15 minutes stimulation and is then followed by a second wave of phosphorylation after 30 minutes. It must be noted that #12 oligonucleotide (DDX3 specific) appears to induce this phosphorylation event in the absence of stimulation. For both figures 4.15 and 4.16 again by comparing control and DDX3 siRNA lanes within the same time point, it can be seen that $\text{I kB}\alpha$ degradation and phosphorylation are not affected.
Discussion Chapter 4

Chapter 3 explored the effects of K7 and A52 on the various signalling pathways that occur upon IL-1 engaging its receptor. In this chapter I showed that K7 but not A52 inhibited TNF-induced NFκB reporter gene activation. A52 is unable to block TNF-induced NFκB activation as its host target IRAK2 and TRAF6 have not been shown to be involved in the activation of this host signalling pathway to date. In this chapter I demonstrate that K7 is capable of inhibiting TNF-induced NFκB activation. Since K7 can inhibit this pathway, this demonstrates that K7 and A52 are not functionally redundant. However, K7 is not the only known VACV inhibitor of TNF-induced NFκB activation. Other VACV inhibitors of TNF induced NFκB activation include B14 (Graham, Bahar et al. 2008).

Multiple assays for TNF induced NFκB activation confirmed a potent inhibitory effect of K7 on the TNF pathway, namely NFκB reporter gene activation, IκBα degradation, p65 Ser\(^{536}\) phosphorylation and p65 translocation. The data from chapter 3 and 4 clearly show that K7 is a potent p65 antagonist. p65 Ser\(^{536}\) phosphorylation has been shown to be mediated through TRAF2/TRAF5 and TAK1 signalling for TNF stimulation (Verstrepen, Bekaert et al. 2008). The inability of A52 to block TNF stimulated NFκB activation can be explained by the fact that its host targets IRAK2 and TRAF6 are not involved in TNF induced NFκB activation. Therefore, seeing that K7 can block TNF stimulated NFκB, K7 must have additional targets when compared to A52 or be using its cellular targets in a different manner to A52. Recently Funakoshi et al. demonstrated that TRAF6 negatively regulates TNFα induced NFκB activation (Funakoshi-Tago, Kamada et
al. 2009). It is possible that K7 targets TRAF6, which in turn negatively regulates TNF induced NFκB activation. However A52 clearly targets TRAF6, but it is not capable of blocking TNF stimulated NFκB activation. Therefore although it cannot be formally excluded that K7 inhibits TNF via TRAF6, given the known existence of a K7 target that A52 does not interact with (namely DDX3) here I focused on the question of whether DDX3 had a role in NFκB activation. To date DDX3 has not been involved in NFκB activation.

Schroder et al showed that DDX3 had a stimulatory effect on IRF3 activation and that it could interact with the TBK1/IKKe complex involved in IFNb promoter activation. It was also shown that the positive effect DDX3 exerts on the ifnb promoter was independent on the ATPase activities of DDX3. Therefore K7 targeting of DDX3 inhibits the activation of IRF3 and thus blocks the induction of INFβ. By blocking IFNβ induction, K7 prevents the promotion of an anti-viral environment in the cell (Schroder, Baran et al. 2008).

Since DDX3 acted at the level of TBK1 and IKKe in the interferon pathway, I looked at the potential role of DDX3 in the related kinase complex involved in NFκB activation. Full activation of NFκB is a result of the degradation of its cytosolic inhibitor IκBα. Prior to degradation, IκBα becomes phosphorylated. This phosphorylation marks IκBα for degradation. The two kinases IKKα and IKKβ are capable of this phosphorylation. These two kinases are in a complex with IKKγ/NEMO. IL-1 and TNF activate the canonical NFκB pathway. For this pathway IKKβ is the more crucial kinase (Schmid and Birbach 2008). In order to explore the potential role of DDX3 in the NFκB pathway it was therefore of interest to see if DDX3 could interact with IKKβ. Indeed both overexpressed and
endogenous DDX3 and IKKβ were shown to interact. Similar to the interaction seen by Schroder et al for DDX3 and IKKe, the DDX3/IKKβ interaction here was stimulus-dependent. In fact previous studies have linked DDX3 to TNF signalling. Sun et al showed that DDX3 was part of an antiapoptotic complex (Sun, Song et al. 2008). It was shown that Glycogen synthase kinase-3 (GSK3), DDX3 and the inhibitor of apoptosis cIAP-1 form a complex at death receptors to inhibit apoptotic signalling. GSK3 was required for DDX3 to associate with the death receptor and cIAP-1 and inhibition of GSK-3 increased TNF-induced apoptosis. Seeing that apoptosis and NFκB are very tightly associated, the interaction of DDX3 with IKKβ could be indicative of a link or feedback loop from apoptosis to NFκB (Bonif, Meuwis et al. 2006; Sun, Song et al. 2008).

In order to test the involvement of DDX3 in the NFκB pathway it was logical to test siRNA targeting DDX3 in the NFκB reporter gene system in which the IL-1 and TNF induced blockage by K7 of activation was seen. However, although the DDX3 siRNA was effective in reducing DDX3 protein levels, it had no inhibitory effect on IL-1 or TNF-induced NFκB reporter gene activation or on IκBα phosphorylation and degradation. Schroder et al showed siRNA against DDX3 inhibited Sendai virus and polyI:C activation of an IRF-3 reporter gene assay (Schroder, Baran et al. 2008). Therefore as a positive control I tested the siRNAs used here to see if they showed similar effects as was seen by Schroder. Indeed in my hands DDX3 siRNA-treated cells, there was an inhibition of the IFNβ promoter luciferase reporter in response to Sendai Virus.
Since I comprehensively excluded a role for DDX3 in NFκB activation the question arises as to what the relevance of the DDX3/IKKβ interaction. Schroder et al showed that DDX3 targeted the TBK1/IKKe complex. There is a lot of literature linking TBK1/IKKe to IKKa/IKKβ. Therefore it was possible that the interaction with IKKβ was indirect, via the TBK1 complex. TANK is considered to be necessary in the scaffolding of the TBK1/IKKe complex that is responsible for IRF activation. Chariot et al showed that TANK could interact with NEMO/IKKγ via yeast-two-hybrid and co-immunoprecipitation studies in HEK293T cells (Chariot, Leonardi et al. 2002). NEMO/IKKγ are the scaffolds required for IKKa/IKKβ complex formation. The authors suggested that TANK could be an adaptor to mediate influence of IKKγ/NEMO on the IKK complex. Additionally the TBK1/IKKe complex was shown by Buss et al to phosphorylate p65 on residue Ser^{536} and importantly that TBK1/IKKe was not regulated by IL-1. The authors hypothesised that TBK1/IKKe were likely candidates for p65 Ser^{536} phosphorylation where p65 is not bound to IκBα or the IKK kinase complex (Buss, Dorrie et al. 2004).

TANK has been shown to associate with IKKβ and therefore possibly have a role in NFκB activation or be involved in crosstalk between the NFκB and IRF pathways. In addition TNF can induce IKKβ to phosphorylate TANK (Bonif, Meuwis et al. 2006). The literature to date remains a little confused on whether TANK plays a positive or negative role in NFκB activation. However studies using a TANK^-/- mouse recently showed that TANK was only involved in the negative regulation of NFκB. It has been demonstrated that DDX3 is not inhibitory in the NFκB pathway. Therefore the likelihood of the DDX3 interaction with IKKβ being mediated by TANK is not likely to explain this interaction. Given other literature linking the
TBK1 pathway to DDX3, possibly more weight should be assigned to TBK1/IKKe being the real interaction partner of DDX3. Therefore the IKKβ/DDX3 interaction is possibly indirect and is mediated by the presence of TANK or TBK1/IKKe. However I did find the interaction of IKKβ with DDX3 was TBK-1 independent in macrophages. Also DDX3 may be like TANK, in that it is regulated by IKKβ. Therefore, this maybe a way that IKKβ regulates IRF activation.

It should be noted from figure 4.8 that a higher molecular weight (MW) form of DDX3 is induced in the presence of IKKβ. This higher MW form of DDX3 could possibly be a phosphorylated form of DDX3. Therefore, IKKβ may be regulating DDX3 function via the ability to phosphorylate it. Given the fact that DDX3 does not have a role in IL-1 and TNF induced NFκB, it is possible that IKKβ is exerting its influence on other pathways through its regulation of DDX3. Previous studies in the literature have shown that DDX3 does indeed become phosphorylated. Sekiguchi et al showed that cyclinB can phosphorylate DDX3 at Thr\textsuperscript{204}. This demonstrates that DDX3 can be a substrate of kinases (Shih, Tsai et al. 2008).

Given the initial theory that K7 inhibits TNF induced NFκB activation through the targeting of DDX3 is now probably not the case, it must be debated how K7 can inhibit TNF signalling to NFκB. It is possible that K7 has additional host targets, apart from A52, that are involved in TNF signalling to NFκB. Additionally this effect on TNF signalling could be down to an RNA effect of the K7 encoding plasmid.

In summary, I have shown in this chapter that K7 can inhibit non-IL-1/TLR induced activation of NFκB. A52 does not have this ability and thus this inhibition can not be fully explained by the targeting of IRAK2 and TRAF6 by K7. I then moved on to
demonstrate that DDX3, a target for K7 and not A52 interacts with IKKβ in a
ligand-enhanced manner. Finally I showed using siRNA, that DDX3 depletion does
not inhibit IL-1 and TNF induced NFκB activation. Thus the mechanism whereby
K7 inhibits non IL-1/TLR-induced NFκB activation remains unclear.
5.1 Chapter Introduction

Chapter 4 explored the difference between the effects of K7 and A52 on non-TLR signalling to NFκB. I tried to implicate DDX3, the third host interaction partner of K7, in NFκB activation in the hope that this would explain the difference between A52 and K7. I saw that DDX3 could interact with IKKβ but failed to see any effect of DDX3 knockdown on the NFκB pathway. Given the novel nature of the role that DDX3 plays in virus induced IFNβ induction [chapter 4 and (Schroder, Baran et al. 2008)], in this chapter I examined the potential role of DDX3 in gene induction by other innate signalling pathways.

Recently the phenomenon of transfected DNA in the cytosol triggering IFN-β has generated a lot of interest. This response is independent of the CpG DNA recognising TLR, TLR9 (Karayel, Burckstummer et al. 2009). One well-characterised DNA motif that is used to activate this interferon response is poly(dA:dT)-(dA:dT) [or poly(dAdT) for short]. Poly(dAdT) contains random stretches of As and Ts and varies in length. The exact signalling components that are involved in IFNβ induction by poly(dA:dT) have yet to be extensively mapped. Miyahira showed that TBK1 and IRF3 have a major role in the induction of IFNβ in response to DNA. It was also demonstrated by this group that TBK1 played a more important role than IKKε (Miyahira, Shahangian et al. 2009). In addition, recently STING has been demonstrated to be critical in this response. Ishikawa et al, 2009 showed that in STING-deficient cells there was a reduced response to transfected DNA. Additionally it was demonstrated that TBK1 could localise with STING upon DNA stimulation. TBK1 then goes on to activate IRF3, which in turn causes induction of type I interferon production (Ishikawa, Ma et al. 2009)
To date the only DNA sensor implicated in the induction of IFN-β has been DAI (DNA-dependent activation of interferon regulatory factors). Despite initial excitement, this DNA-recognising receptor is only important in certain cell types such as L929 cells (Takaoka, Wang et al. 2007). Therefore the presence of other receptors must explain the response to cytosolic DNA in other cells. More recently there has been a massive interest in identifying novel DNA recognising receptors capable of mounting the interferon response. Previous studied have shown that DNA from malaria can induce this response. This response is robust in HEK293Ts and murine macrophages.

It was of interest to see if DDX3 was involved in the poly(dAdT) and malaria DNA pathway leading to IFNβ induction and if so where in the pathway DDX3 is placed. Another level of complexity in interpreting the role of DDX3 in the DNA interferon response is the observation that in HEK293Ts, poly(dAdT) can be processed into an RNA intermediate. This RNA intermediate is recognised through RIG-I, and SeV which is recognised via RIG-I induces INFβ in a DDX3-dependent manner. RIG-1 is known to signal through TBK1, therefore DDX3 can then be linked to the PolIII/RIG-I pathway through TBK1 (Chiu, Macmillan et al. 2009).

In addition to the DNA-induced interferon pathway, the IL-1/TNF-induced IL-8 pathway was explored to test for the involvement of DDX3. Schroder et al showed that K7 could inhibit LPS-induced IL-8 protein production in TLR4 expressing HEK293 cells (Schroder, Baran et al. 2008). In addition, I showed in chapter 3 that a Lentivirus expressing K7 was capable of inhibiting LPS induced MIP-2 in a murine macrophage cell line.
Figure 5.1: Knock down of DDX3 using siRNA inhibits the Interferon response to polydAdT in HEK293T cells

HEK293Ts cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipofectaimine with varying amounts of DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding and also co-transfected with an IFNβ-promoter luciferase reporter, TK Renilla construct and poly(dAdT). 24 hours later the cells were harvested.

(a) IFNβ activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels from an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.

P< 0.02=★★

P<0.01= ★★★
A

- **Control siRNA**
- **#12 (DDX3)**

---

**SiRNA 25 pmol**

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<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
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<td>-</td>
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</tr>
<tr>
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**IB**

- P-actin
- DDX3

**B**

- β-actin

---

**Control**

- 25
- 50
- 100

**#12**

- 25
- 50
- 100
Figure 5.3: Immunoprecipitation of DDX3 and STING using biotinylated Malaria DNA in HEK293T cells

HEK293T cells were seeded into 6 well plates 24 hours prior to transfection. 24 hours post seeding the cells were harvested and lysed for 20 minutes. The lysates were incubated with 1μg/ml of the indicated oligos and 30μl of strepavidin/agarose beads and rolled for 2 hours at 4 degrees. The beads were spun down, washed, resuspended in SDS-containing sample buffer and boiled. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3. The membrane was then washed and reprobed using an antibody specific to STING. This data is representative of one experiment from two separate experiments.

1 A2O 5' bio= IFN activating oligonucleotide in 293T cells that is 5' biotinylated
2 A2O unbio = IFN activating oligonucleotide in 293T cells that is not tagged
3 A2E 5' bio= control oligonucleotide in 293T cells that is 5' biotinylated
4 A2E unbio= control oligonucleotide in 293T cells that is not tagged
Figure 5.4: Immunoprecipitation of DDX3 using biotinylated Malaria DNA is independent of STING

The macrophages were seeded into 6 well plates 24 hours prior to transfection. 24 hours post seeding the cells were harvested and lysed for 20 minutes. The lysates were incubated with 1µg/ml of the indicated oligos and 30µl of strepavidin/agarose beads and rolled for 2 hours at 4 degrees. The beads were spun down, washed, resuspended in SDS containing sample buffer and boiled. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3. This data is from one experiment

A2E unbio= control oligo in macrophage that is not tagged
A2E5' bio= control oligo in macrophages that is 5' biotinylated
A5E unbio = IFN activating oligo in macrophages that is not tagged
A5E 5' bio= IFN activating oligo in macrophage that is 5' biotinylated
A5E 3' bio= IFN activating oligo in macrophages that is 3' biotinylated
**Figure 5.5: Immunoprecipitation of DDX3 using biotinylated Malaria DNA is dependent on TBK1**

The macrophages were seeded into 6 well plates 24 hours prior to transfection. 24 hours post seeding the cells were harvested and lysed for 20 minutes. The lysates were incubated with 1μg/ml of the indicated oligos and 30μl of strepavidin/agarose beads and rolled for 2 hours at 4 degrees. The beads were spun down, washed, resuspended in SDS containing sample buffer and boiled. The samples were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3. This data is from one experiment.

- **A2E unbio** = control oligo in macrophage that is not tagged
- **A2E 5' bio** = control oligo in macrophages that is 5' biotinylated
- **A5E unbio** = ifn activating oligo in macrophages that is not tagged
- **A5E 5' bio** = ifn activating oligo in macrophage that is 5' biotinylated
- **A5E 3' bio** = ifn activating oligo in macrophages that is 3' biotinylated
Figure 5.6: Knock down of DDX3 using siRNA impedes IL-1-induced IL-8 production

HEK293T cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200µl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipofectamine with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding. 24 hours later the cells were stimulated with 20ng/ml of IL-1. The supernatants were harvested 24 hours post stimulation assayed for IL-8 production by ELISA. Data is expressed as pg/ml +/- s.d The data is expressed as mean IL-8 ± s.d for a single experiment performed in triplicate. This data is representative of more than three experiments.
HEK293 cells were seeded at $1.5 \times 10^5$ cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. The cells were given 2 hits of siRNA as previously described. 24 hours after the second hit the cells were stimulated as indicated with 20ng/ml of IL-1. The cells were harvested and the RNA from each sample was isolated using a Qiagen RNeasy Mini Kit. The RNA samples were reverse transcribed into cDNA and real time PCR was carried out using primers specific for IL-8 and β-actin. The data is expressed as mean fold induction normalised to β-actin ± s.d from a single exp performed in triplicate. This data is representative of more than three experiments.
**A**

Wild type IL-8-luc promoter

- **Control**
- **siRNA**
- **#12 (DDX3)**

Fold Induction

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<td>-</td>
<td>-</td>
</tr>
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<td></td>
<td>+</td>
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**B**

- **IB DDX3**
- **IB β-actin**

- Control-25 pg/ml
- Control-50 pg/ml
- Control-100 pg/ml
- #12.25 pg/ml
- #12.50 pg/ml
- #12.100 pg/ml
Figure 5.9: Knock down of DDX3 using siRNA enhances IL-1 stimulated induction of the IL-8 promoter

HEK293Ts cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipo with varying amounts of DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding and also co-transfected with an IL-8 promoter luciferase reporter gene reporter plasmid and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of IL-1 and the cells were harvested 6 hours post stimulation.

(a) NFκB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
A

Del AP-1 IL-8-luc promoter

- Control
- siRNA
- #12 (DDX3)

Fold Induction

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<th>Pg/ml</th>
<th>siRNA</th>
<th>IL-1</th>
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<td>0</td>
<td>25</td>
<td>-</td>
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</table>

B

Control-25 pg/ml
Control-50 pg/ml
Control-100 pg/ml
#12-25 pg/ml
#12-50 pg/ml
#12-100 pg/ml
Figure 5.10: Knock down of DDX3 using siRNA enhances IL-1 stimulated induction of the IL-8 promoter lacking the AP-1 element

HEK293Ts cells were seeded at $1.5 \times 10^5$ cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipo with varying amounts of DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding and also co-transfected with an IL-8-luciferase reporter gene reporter plasmid without the AP1 binding site and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of IL-1 and the cells were harvested 6 hours post stimulation.

(a) NFκB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
A

Del c/EBP IL-8-luc promoter

Control

siRNA

#12 (DDX3)

Fold Induction

Pg/ml 25 50 100 25 50 100

siRNA

IL-1 - - - + + +

B

Control-25 pg/ml

Control-50 pg/ml

Control-100 pg/ml

#12-25 pg/ml

#12-50 pg/ml

#12-100 pg/ml
Figure 5.11: Knock down of DDX3 using siRNA enhances IL-1 stimulated induction of the IL-8 promoter lacking the C/EBP element

HEK293T cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipo with varying amounts of DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding and also co-transfected with an IL-8-luciferase reporter gene reporter plasmid without the C/EBP binding site and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of IL-1 and the cells were harvested 6 hours post stimulation.

(a) NFkB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
A

Wt IL-8-luc promoter

Control siRNA

#12 (DDX3)

Fold Induction

siRNA

TNF - - - + + +

B

Control-25 pg/ml
Control-50 pg/ml
Control-100 pg/ml
#12-25 pg/ml
#12-50 pg/ml
#12-100 pg/ml
Figure 5.12: Knock down of DDX3 using siRNA enhances TNF stimulated induction of the IL-8 promoter

HEK293Ts cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipo with varying amounts of DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding and also co-transfected with an IL-8-luciferase reporter gene reporter plasmid and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of TNF and the cells were harvested 6 hours post stimulation.

(a) NFκB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
A

Del AP-1 IL-8

Control
siRNA

#12 (DDX3)

Fold Induction

pmol
siRNA

TNF

- - - + + +

25 50 100 25 50 100

B

Control-25 pg/ml

Control-50 pg/ml

Control-100 pg/ml

#12-25 pg/ml

#12-50 pg/ml

#12-100 pg/ml
Figure 5.13: Knock down of DDX3 using siRNA enhances TNF stimulated induction of the IL-8 promoter lacking the AP-1 element

HEK293Ts cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200μl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipo with varying amounts of DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding and also co-transfected with an IL-8-luciferase reporter gene reporter plasmid without the AP1 binding site and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of TNF and the cells were harvested 6 hours post stimulation.

(a) NFκB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
Figure 5.14: Knock down of DDX3 using siRNA enhances TNF stimulated induction of the IL-8 promoter lacking the C/EBP element

HEK293Ts cells were seeded at 1.5 x 10^5 cells/ml in 96 well plates (200µl per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected using lipo with varying amounts of DDX3 targeting siRNA or a control oligonucleotide as indicated. The cells were given a second hit of the same amount of siRNA 48hrs after seeding and also co-transfected with an IL-8-luciferase reporter gene reporter plasmid without the C/EBP binding site and TK-Renilla. 24 hours later the cells were stimulated with 20ng/ml of IL-1 and the cells were harvested 6 hours post stimulation.

(a) NFκB activation was measured by reporter gene activation. The data is expressed as mean fold induction ± s.d relative to control levels for an experiment performed in triplicate. This data is representative of more than three experiments.

(b) The remainder of the lysates from the stimulated samples were transferred to an eppendorf and SDS-containing sample buffer was added to each sample, which were then resolved using SDS-containing gels. The proteins were transferred to a PVDF membrane. The membrane was probed with an antibody specific to DDX3 or β-actin.
Figure 5.15: DDX3 depletion has no effect on IL-1 induced JNK phosphorylation

HEK293T cells were seeded at 1.5 x 10^5 cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA as indicated 48 hours post seeding. 24 hours after the second hit the cells were stimulated as indicated with 50ng/ml of TNFα. The cells were harvested and lysed in SDS containing lysis buffer and resolved using a SDS containing gel. The membrane was probed with an antibody specific for pJNK, DDX3 and total JNK. This data is representative of three separate experiments.
Figure 5.16: DDX3 depletion has no effect on IL-1 induced p38 phosphorylation

HEK293T cells were seeded at $1.5 \times 10^5$ cells per ml in 6 well plates (2ml per well) 24 hours prior to transfection. Using the lipofectamine method 24 hours post seeding, the cells were transfected with varying amounts of DDX3 targeting siRNA or a control oligo as indicated. The cells were given a second hit of siRNA as indicated 48 hours post seeding. 24 hours after the second hit the cells were stimulated as indicated with 50ng/ml of TNFα. The cells were harvested and lysed in SDS containing lysis buffer and resolved using a SDS containing gel. The membrane was probed with an antibody specific for pp38, DDX3 or β-actin. This data is representative of three separate experiments.
Figure 5.18: Theory of DDX3 involvement in intracellular DNA recognition

Figure 5.3 showed that DDX3 and STING were both pulled down in an immunocomplex with an Interferon stimulatory oligonucleotide. Therefore DDX3 and STING are involved in the complex involved in DNA recognition or signal transduction leading to the Interferon response.
Figure 5.18: Second possible signalling scenario for DNA sensing receptors

Figure 5.4 showed that DDX3 is immunoprecipitated by an Interferon stimulatory oligonucleotide in a STING independent manner. Figure 5.5 showed that the presence of TBK1 was necessary for DDX3 to be immunoprecipitated by the same oligonucleotide. This therefore places DDX3 and TBK1 upstream of STING in the DNA sensing pathway leading to Interferon production.
5.2 Results

5.2.1 The role of DDX3 in DNA-induced IFNβ

In order to test the role of DDX3 in the poly(dAdT)-induced interferon response, the siRNA protocol developed in chapter 4 was used. HEK293T cells were used to carry out this investigation. Figure 5.1a shows that upon transfection of poly(dAdT) into the cells, the IFNβ-promoter luciferase plasmid shows strong induction. By comparing control siRNA to siRNA targeting DDX3 (#12) it is quite clear that the poly(dAdT)-induced interferon response is impaired in the absence of DDX3. Figure 5.1b shows the degree of DDX3 protein knockdown for figure 5.1a. It should be noted that each dose of siRNA against DDX3 results in at least a 50% inhibition of the interferon response.

Previous studies in other labs have shown that other DNA sequences apart from the commonly used poly(dAdT) and ISD DNA motifs can induce an interferon response. ISD is synthetic dsDNA that is 45 base pairs in length with a GC content of 35.6%. Another IFN stimulatory DNA motif has been found in malaria DNA (personal communication, Kate Fitzgerald, Shruti Sharma). Given the dependency of poly(dAdT) on DDX3 for the interferon response (figure 5.1,) the immunostimulating malaria DNA was tested in a similar fashion. The exact same experimental setup that was used in figure 5.1a was used for figure 5.2a. Figure 5.2a shows that there is some dependency on DDX3 for the malaria DNA interferon response. It should be noted that the dependency is not as dramatic as that found in figure 5.1a especially for the higher doses of DDX3 siRNA. The corresponding protein expression blot for figure 5.2a can be seen in figure 5.2b.
Given that the malaria DNA response showed some dependency on DDX3, it was of interest to study if DDX3 was part of a signalling complex with the malaria DNA. This was studied using a short (14bp) strand of malaria DNA that was biotinylated and strepdavidin beads as the immunoprecipitating agent. Previous studies have shown that in HEK293T cells the interferon response to the malaria DNA was dependent on the nature of the backbone of the DNA (personal communication Shruti Sharma). It has been shown previously that the malaria DNA requires a phosphorothiorate backbone to induce an interferon response in the 293T cells. Therefore the malaria DNA in a phosphodiester backbone serves as an excellent control in the HEK293T cells as malaria DNA of this nature cannot stimulate an interferon response. Unbiotinylated malaria DNA of both the phosphodiester and phosphorothiorate served as a further control as these should not be pulled down by the strepdavidin beads. Following lysis of the cells, various malaria DNAs were added to the cell lysates along with the strepdavidin beads. The samples were incubated for two hours and the beads were spun down and washed three times. The resultant immunoblot, seen in figure 5.3, showed that only the malaria DNA with the phosphorothiorate backbone (that was biotinylated at its 5’ end) pulled down a protein complex containing DDX3. Given the recent interest in STING in the DNA induced interferon response I also probed the strepdavidin pulldown blots to see if STING also came down with the DNA/DDX3 complex. In figure 5.3 STING can only be detected in the lanes where DDX3 came down. Thus STING and DDX3 likely form part of the signalling complex used by malaria DNA.

The malaria DNA that stimulates an interferon response in the HEK293T cells does not induce this response in macrophage cells. Previous studies have shown that in
order to induce an interferon response in macrophages, this malaria DNA must have slightly different sequence. In addition, this DNA needs to have a phosphodiester backbone to induce the response in macrophages. In order to examine the malaria DNA inducing interferon response in macrophages and the role DDX3 has in this response, various stimulatory and non-stimulatory DNAs were used in the macrophages. Given the result seen in figure 5.3, STING-knockout and matched wild-type macrophages were tested in their ability to pull down DDX3 with the IFN-stimulatory DNA. A similar set-up was used for this experiment as was used in figure 5.3. Various control DNAs (A2E) and stimulatory DNAs (A5E) that were biotinylated were used to pull down DDX3 using streptavidin beads. Unbiotinylated DNAs were used as additional controls. Figure 5.4 shows that in wild-type cells the DNA that most potently pulled down DDX3 was the 5′biotinylated stimulatory A5E DNA. However there was a small amount of DDX3 pulled down by the 5′ biotinylated unstimulatory A2E DNA. It should also be noted that DDX3 could only be pulled down when the A5E DNA was 5′ and not 3′ biotinylated. An identical result was obtained for STING−/− macrophages (figure 5.4). This result indicates that DDX3 can be pulled down by the malaria DNA in the macrophages independent of STING presence (experiment was only done once). Given previous studies by Schroder et al (Schroder, Baran et al. 2008) and others (Soulat, Burckstummer et al. 2008) showing that DDX3 is part of the TBK1 signalling complex it was of interest to see if DDX3 could be pulled down in the absence of this signalling molecule. Therefore DDX3 pulldown was tested in the TBK1 knockout and matched wild type macrophages. Figure 5.5 shows that in a similar manner to figure 5.4, the stimulatory A5E DNA that was 5′biotinylated pulled down DDX3 in the wild type cells. In addition there was also pulldown by the A2E unstimulatory DNA that was
5' biotinylated. It should be noted that DDX3 was not pulled down in the TBK1 knockout cells. This experiment suggests the ability of the malaria DNA to pulldown DDX3 is dependent on the presence of TBK1, although this would have to be repeated.

5.2.2 The role of DDX3 in proinflammatory cytokine stimulated IL-8 gene induction

Having shown that DDX3 is required for IFNβ induction by DNA, I next examined the potential role of DDX3 in an IRF3-independent gene pathway. Schroder et al, 2008, showed that K7 could inhibit LPS-induced IL-8 protein production. Although this could be explained by K7 inhibiting TLR induced NFκB (chapter 3) given the ability of K7 to interact with DDX3 it was possible that K7 could also inhibit IL-8 production due to novel regulatory effects of DDX3 on the IL-8 protein production pathway. Given this hypothesis I tested the DDX3-targeting oligonucleotide on IL-1 induced IL-8 protein production in the HEK 293Ts. Figure 5.6 shows that when the cells were stimulated with 20ng/ml of IL-1, this led to roughly three fold induction of IL-8 protein production after 24 hours. By comparing the control and DDX3 targeting oligonucleotide treated cells within the same dose of siRNA it could be seen that IL-1 induced IL-8 protein production was reduced by DDX3 siRNA at 24 hours post stimulation. The corresponding immunoblot from this experiment shows that DDX3 protein levels are indeed reduced (figure 5.6) This was a surprising result given that I showed in chapter 4 that DDX3 has no role in IL-1 induced NFκB activation. Therefore I hypothesised that DDX3 must be acting at another point on
the IL-1 signalling pathway leading to IL-8 induction. Therefore I next examined the effect of DDX3 depletion on IL-1 induced IL-8 mRNA. Figure 5.7 shows that upon IL-1 stimulation there is induction of IL-8 mRNA in the control lane that reaches a maximum after 8 hours. The message then reduces to near basal levels after 24 hours stimulation. In the DDX3-depleted cells it is clear that the pattern of IL-1 induced IL-8 messenger RNA has been radically affected. The absence of DDX3 led to a more potent induction of IL-8 mRNA after 1 hour when compared to control treated cells. This was followed by a more rapid decrease in the message from 4-24 hours. Therefore DDX3 affects the kinetics of IL-1 induced IL-8 mRNA induction. The supernatants from the cells used to study IL-8 mRNA were retained and IL-8 protein levels were examined. Figure 5.8 shows that similar to figure 5.6 I saw the inhibitory effect of IL-1 induced IL-8 protein production at 24 hours in the DDX3-depleted cells, while little inhibition was observed at the earlier time points of 4 and 8 hours. A portion of the cell lysates in figure 5.7 were also retained and used to check for DDX3 expression levels. The immunoblot in figure 5.8 confirms that DDX3 expression levels were reduced in cells treated with the siRNA targeting DDX3.

Therefore absence of DDX3 enhances IL-8 mRNA induction at early time points, but reduces IL-8 mRNA levels more rapidly from 4-24 hours. This translates to an overall inhibitory effect on IL-8 protein 24 hours post stimulation. Given these observations I went on to study the effects absence of DDX3 would have on the IL-8 promoter. In addition to NFκB regulatory elements, the IL-8 promoter contains AP-1 and C/EBP binding regions. Chapter 4 showed that DDX3 knock down did not effect NFκB activation. It is possible that DDX3 could have a role in these other regulatory elements involved in IL-8 promoter activation. Therefore I first examined
the effect of DDX3 knockdown on the wild type IL-8 promoter and then went on to look at IL-8 promoter elements lacking the AP-1 binding site and lacking the c/EBP binding site in the hope of mapping the effects of DDX3 depletion to a particular element on the IL-8 promoter. For each promoter luciferase reporter construct both IL-1 and TNF activation was examined. In Figure 5.9a it can be seen that when the cells were treated with IL-1 this led to 20-40 fold induction of IL-8 wild type promoter in the control siRNA-treated cells. By comparing DDX3 targeting siRNA to the control siRNA within the same dose of siRNA it is clear that DDX3 depletion (confirmed in Figure 5.9b) enhances IL-1 induced activation of the wild type IL-8 luciferase promoter. This is consistent with the early effect of DDX3 depletion on IL-8 mRNA. The IL-8 promoter luciferase lacking the AP-1 binding site was next examined. Again stimulation with IL-1 leads to 20-40 fold activation of the promoter in the control siRNA treated lanes. DDX3 depletion showed enhancement of the IL-8 promoter luciferase lacking the AP-1 binding site. It should be noted that basal activation was potentially increased in the absence of DDX3. Figure 5.10b shows the corresponding protein blots for figure 5.10a. Finally the IL-8 promoter luciferase lacking the c/EBP binding site was tested for IL-1 activation. In figure 5.11a, IL-1 stimulation resulted in 30-fold activation of the promoter in the control cells. In the DDX3 depleted cells activation was increased by at least two-fold. The matched protein blots for figure 5.11a can be seen in figure 5.11b. Next these IL-8 promoter luciferase constructs were tested for TNF activation. In figure 5.12a stimulation of the wild type IL-8 promoter luciferase resulted in approximately 20-40 fold activation of the promoter. DDX3 depletion caused increased activation of the promoter when control and DDX3 siRNA lanes area compared within the same dose of siRNA. In the case of TNF induced activation of the IL-8 luciferase
promoter lacking the AP-1 binding site, stimulation resulted in 20 fold activation in the control lanes (figure 5.13a). In the DDX3 depleted cells activation increased by at least two-fold when compared to the control activation. Finally, when the IL-8 luciferase promoter lacking the c/EBP binding site was stimulated with TNF, the promoter was activated approximately 20-60 fold over basal activation (figure 5.14a). DDX3 depletion resulted in roughly 3-fold increase in promoter activation when compared to control activated cells. Thus depletion of DDX3 enhances IL-1 and TNF stimulated induction of the IL-8 promoter in an AP-1 and C/EBP-independent manner. It was not possible to test an IL-8 promoter lacking the NFκB elements as this was not induced by IL-1 or TNF (data not shown). However chapter 4 showed no effect of DDX3 depletion on NFκB activation of IL-1 or TNF.

The MAP kinases p38 and JNK are known to have roles in cytokine promoter induction. Given the effects that were seen on the IL-8 luciferase promoter it was of interest to see if DDX3 depletion could affect JNK or p38 activation. HEK293T cells were used to study this possible effect of DDX3 upon IL-1 induced JNK and p38 activation. Figure 5.15 shows JNK activation by the appearance of a phosphorylated form of JNK after 7 minutes IL-1 stimulation. By comparing this band within time points for control and DDX3 siRNA treated cells it should be noted that there is no effect on JNK activation in DDX3 depleted cells. In a similar manner p38 activation was measured through the appearance of a phosphorylated form of p38 after 7 minutes IL-1 stimulation. Again there is no obvious difference in the intensity of this band when control and DDX3 siRNA treated cells are compared within a single time point.
Hence DDX3 depletion has complex effects on DNA, IL-1 and TNF signalling. Thus DDX3 has multiple roles in gene induction, ranging from transcription activation (IRF3) to promoter repression (IL-8 promoter) to cytokine induction (IL-8 protein release). Therefore of great interest to access the overall role of DDX3 in the immune response in vivo would be of great benefit.
5.3 Discussion

In this chapter I explored the role of DDX3 in the DNA-induced IFNβ response and the IL-1/TNF pathway leading to IL-8 protein production. It can be seen from this chapter that DDX3 has a positive role to play in the DNA IFNβ response, for both poly(dAdT) and malaria DNA. Finally in this chapter I show that DDX3 has a complex role in IL-8 induction, but overall is a positive regulator of IL-8 production.

For both poly(dAdT) and malaria DNA stimulation of the HEK293T cells, absence of DDX3 causes inhibition of the DNA-induced IFNβ response. Recently Chiu et al (2009) and Ablasser et al (2009) noted that for the poly(dAdT) pathway leading to IFNβ in HEK293 cells, treatment with siRNA against RIG-1 or IPS-1 blocked IRF3 dimerization and IFNβ induction (Ablasser, Bauernfeind et al. 2009). The IRF3 pathway induced by RIG-I is known to be TBK1-dependent. Chiu et al showed that RNA Polymerase III converts the poly(dAdT) into an RNA intermediate which is then subject to recognition by the RIG-I pathway (Chiu, Macmillan et al. 2009). Thus the dependency of the poly(dAdT) IFNβ pathway on DDX3 in HEK293T cells could be a reflection of the role TBK1 plays in this pathway. If DDX3 functions mainly downstream of RIG-I for TBK1 activation this could also indicate that the malaria DNA is being converted into an RNA intermediate and being detected via RIG-I.

It should be noted that these DNAs are not at all similar. Poly(dAdT) consist of chemically synthesised long stretches of As and Ts. It can vary in lengths of up to hundreds of base pairs. In contrast, the malaria DNA used in figure 5.2 is only 14 basepairs in length. Given the difference in nature of these two DNAs it is possible
that the Malaria DNA is being recognised by a different receptor in the HEK293T, independent of the PolIII/RIG-I pathway. If this is the case, this gives a strong indication that DDX3 could be an essential component of machinery necessary to cause IFNβ induction by novel cytosolic DNA sensor pathways. Ishikawa et al showed that STING was necessary for DNA-mediated IFNβ production and that STING co-localized with TBK1 (Ishikawa, Ma et al. 2009). It is likely that TBK1 is also required for all DNA responses leading to IRF3 activation and this might also be the case of DDX3. Therefore this may also link STING to DDX3, given that previous studies have linked DDX3 to the TBK1/IKKε complex. Figure 5.3 serves as evidence for this as when the blots from the DDX3 pulldown experiment were reprobed for STING, STING was also detected in this complex. This scenario is illustrated in figure 5.17

This could also be the case even if these two DNA types are recognised by the same novel DNA receptor.

The other scenario is that DDX3 is the actual missing receptor. Evidence for this can be seen in figures 5.3, 5.4 and 5.5. In each of these figures, DDX3 is pulled down by the tagged DNA when the DNA is added to cell lysates. Therefore DDX3 is capable of interacting with the DNA in the absence of signalling in intact cells. Therefore this binding of the tagged DNA to DDX3 is a direct interaction. If the complex is not preassembled, then DDX3 may be a sensor of this DNA. On the other hand, the missing receptor could simply be interacting with DDX3 constitutively.

The observation that DDX3 was pulled down in the STING knock-out macrophages could serve as further evidence for an upstream role for DDX3 (figure 5.4). This
result indicates that the direct binding of DDX3 to the DNA is upstream of STING, i.e. that the presence of STING is not necessary for DDX3 binding to the DNA. Even if DDX3 is not the receptor, this result still places DDX3 upstream of STING. Finally figure 5.5 in which DDX3 is only pulled down in the wild-type and not the TBK1 knockout cells would place DDX3 at the same level or upstream of STING. This in turn places TBK1 upstream of STING (figure 5.18). Again, it should be noted that this could simply reflect the role DDX3 has in the TBK1 complex, involved in the signal transduction of an unknown DNA receptor.

It must be noted that only the malaria DNA with a phosphorothioate backbone caused an IFNβ response in the HEK293Ts. This backbone is possibly more stable and less prone to degradation and could be a substrate for the Pol III/RIG-I dependent pathway that was observed by Chiu et al (2009) and Ablasser et al (2009) (Ablasser, Bauernfeind et al. 2009; Chiu, Macmillan et al. 2009). It is also a possibility that these various DNAs are signalling through both the novel DNA receptor and the PolII/RIG-I pathways. Evidence to support this theory is that previous studies have shown that the IFNβ response to the malaria DNA and polydAdT is only partially dependent on PolIII (Kate Fitzgerald, from siRNA studies, Personal communication). Overall it can be seen that DDX3 does have a role to play in IFNβ induction by DNA, although the precise role that DDX3 has in this pathway has yet to be determined.

Surprisingly, given the lack of a role for DDX3 in NFκB activation, depletion of DDX3 inhibited IL-1 induction of IL-8 protein production. The effect that DDX3
depletion has on IL-8 protein production could potentially be at any point in the pathway to induction of IL-8. Therefore the effect of DDX3 depletion on IL-8 mRNA induction and IL-8 promoter induction was tested.

This inhibition of IL-8 protein levels in the absence of DDX3 is mirrored by the inhibition of IL-8 mRNA at late time points. The effect that DDX3 knockdown can inhibit IL-1 induced IL-8 protein production can be placed at any level of IL-8 gene induction. From the literature DDX3 can exert effects on every level of gene expression. This inhibitory effect of DDX3 indicates that DDX3 is playing a positive role in IL-1 induced IL-8 protein production.

In contrast to the overall positive effect of DDX3 on IL-8 release, it was found that at early time points DDX3 plays a negative role in IL-1 induced IL-8 mRNA. From Chapter 4 it can be seen that DDX3 depletion has no effect on IL-1 or TNF induced NFκB activation. A lot of literature has demonstrated that the promoter element in the IL-8 gene that is essential and sufficient for transcriptional regulation of the gene corresponds to the NFκB element. Although the IL-8 promoter also contains activating protein (AP-1) and CAAT/enhancer-binding protein (C/EBP)-binding sites. These sites are dispensable for transcriptional activation in some cells, but necessary for the activation in other cells. Thus, NFκB is essential for induction, but in certain conditions does not induce maximal gene expression (Medin, Fitzgerald et al. 2005). There is some evidence in the literature that DDX3 can influence transcription. Soulat et al showed by ChIP analysis that DDX3 can be recruited to the IFN-β-promoter. The authors suggested that this was how DDX3 could positively regulate the IFN-β promoter (Soulat, Burckstummer et al. 2008). Figure 5.9 and figure 5.12 show that for IL-1 and TNF respectively, in the absence of
DDX3 there is an enhancement in the activation of the wild type IL-8 luciferase promoter. When the AP-1 binding site was absent from the promoter, DDX3 absence still caused increased activation of the luciferase promoter, and this was also the case for deletion of the C/EBP site. It should be noted that the IL-8 luciferase promoter lacking the NFκB binding sites did not respond to IL-1 or TNF stimulation (data not shown). Despite this lack of response, the results from chapter 4 would indicate that the NFκB binding sites in the IL-8 promoter would not contribute to any effect of DDX3 on the promoter. Therefore the negative role of DDX3 on IL-8 seems independent of AP1 (consistent with this, there was no effect on MAPK activation), C/EBP or NFκB. This does correlate with early effects on mRNA. Other possible literature on DDX3 being a positive regulator of gene induction include the findings by Chao et al. This group showed that DDX3 could transactivate the p21waf/cip1 promoter and cause promoter activation through the transcription factor Sp1 (Chao, Chen et al. 2006). Therefore DDX3 can play a specific role in transcription and perhaps is a negative regulator in the case of the IL-8 promoter. Another DEAD box helicase, DDX5 (p68) has been shown to act as both a transcriptional co-activator and repressor. The balance of negative and positive regulation is in part controlled by SUMOylation of DDX5 on residue K53 of DDX5. SUMOylation of DDX5 promotes a more repressive state in this RNA Helicase (Fuller-Pace, Jacobs et al. 2007). In chapter 4 I showed that DDX3 could be post-translationally modified by IKKβ. This post translational modification of DDX3 could possibly cause a change in the transcriptional activities of DDX3. This could also reflect the two effects seen in figure 5.7 on IL-8 mRNA in the absence of DDX3. Whereby absence of DDX3 leads to both positive and negative effects on IL-1 induced IL-8 mRNA.
Previous reports found that DDX3 knockdown decreased expression of long structured 5'-UTR reporters (Lai, Lee et al. 2008). The 5'-UTR of IL-8 RNA is not long. In fact it is only 101 bases in length, therefore it is unlikely that this accounts for the specific effects on IL-8 mRNA. DDX3 has shown to interact with the major nuclear mRNA export receptor Tip associated protein (TAP) (Lai, Lee et al. 2008). DDX3 was show to interact with TAP via mass spectotormetry and immuno-precipitation. The authors showed that DDX3 interacts with TAP mainly in the nucleus. In addition DDX3 binds to mRNA in the nucleus and cytoplasm and TAP is not needed for DDX3 to interact with mRNAs. It is possible that DDX3 functions in conjunction with TAP in the export of general or specific mRNA to the cytoplasm for initiation of translation. The authors also found that DDX3 interacts with the translation initiation factors eIF4A, eIF2α and PAB1. Although, they found no significant effect on general translation, the authors did suggest that they did not detect lower abundance proteins. All this serves to suggest that DDX3 does perhaps have a specific role in IL-8 mRNA. Finally there is some evidence suggesting DDX3 has a role in mRNA splicing (Kanai et al) and the effects that DDX3 depletion has on IL-8 mRNA could be an effect on the splicing of this mRNA to mature mRNA (Kanai, Dohmae et al. 2004).

Although in contrast, Shih et al, 2008 found that DDX3 was capable of inhibiting global protein synthesis. The authors showed that DDX3 interacts with a mRNA cap binding protein, eIF4E, that is involved in the initiation of translation. DDX3
has an eIF4E binding motif that is necessary for the translational inhibitory effect. The authors suggested that DDX3 renders eIF4E conformationally unable to bind other translation initiators, such as eIF4G, therefore blocking translation (Shih, Tsai et al. 2008).

In summary a have demonstrated that DDX3 plays a complex role in gene induction, but overall DDX3 is required for DNA induced IFNβ and proinflammatory induced IL-8 mRNA induction.
Final Discussion

The function of the immune system is to protect an individual from invading pathogens. This protection of the host is achieved both through detection of these pathogens and prevention of their establishment within the host. Finally once the immune system detects a pathogen, its elimination must occur. The ultimate goal of a pathogen is the successful completion of its life cycle and thus this ensures propagation of the next generation. Therefore, in order for this goal to be achieved, the pathogen must infect and more importantly avoid detection and destruction by the host immune system. Given the interplay between these entities it is obvious that co-evolution must occur between host and pathogen.

VACV has evolved many mechanisms to avoid detection and destruction by the host immune system. In order to make the life cycle of the virus more efficient there must be a compromise between efficient life cycle and sufficient number of tools to avoid the immune system. Therefore, a balance must be struck with the virus between the number of genes it encodes for viral immune evasion and the number of essential genes needed for completion of the life cycle. Therefore it would seem fundamental for the virus to avoid unnecessary duplication of immune evasion genes. At a glance it would seem that K7 and A52 might be an example of this unnecessary duplication. Although at a closer look, it has been demonstrated in this thesis that these VACV proteins do show some differences in immune evasion properties.

Given the similarities it is very possible that one of these genes arose from the duplication of the other. If this is the case, given the fact that both genes are still intact and expressed, the result was a more advantageous virus in terms of life cycle...
and immune avoidance. K7 and A52 do not appear to have role in viral replication (G. Smith, personal communication), therefore this improved virus had an advantage in terms of host immune evasion. Therefore, it is likely that this extra gene could ‘carry its own weight’ by making up for the extra energy it takes to transcribed and translated by not being redundant in the virus.

Both K7 and A52 belong to the group of immune evasion proteins that are structurally similar to Bcl-2 proteins. Other members of this family include N1, M11 and B14 (Graham, Bahar et al. 2008). It is likely that this fold represents a very stable conformation for a protein to take. It is quite unlikely that the viral Bcl-2 fold and the host cellular fold evolved independently of one another rather the virus may have captured a bcl-2 host gene. It should be noted in the unlikely scenario that this fold evolved independently in viruses and host proteins, the fact that these two organisms came to utilise this fold also serves as evidence of how advantageous this fold is.

The viral proteins N1 and M11 have this Bcl-2 fold and have likely retained the ability to bind BH3 helices (REF). It is possible that these proteins were founding members of this family given the fact that they are most like the host Bcl2 proteins in that they can modulate apoptotic signalling. In addition, in the evolution of the virus these founding members of the family served to show how stable this fold is and therefore other proteins such as A52 and K7 were developed. K7 and A52 probably emerged in the virus close together in time. Finally A46 may also be more Bcl2-like than TIR-like (J. Stack, personal communication)
Kalverda et al showed that K7 is a monomer, in contrast Graham et demonstrated that A52 can homodimerise *in crystallo* and *in vitro* (Kalverda, Thompson et al. 2009). The fact that K7 is a monomer serves as an explanation of why K7 and not A52 can bind to DDX3 and the fact that the regions of K7 that interact with DDX3 have been shown to be at the A52-like dimer interface (A. Khan, personal communication). One major difference between K7 and A52 is their cellular localisation. It should be noted that the transfection method used to introduce K7 into the cell is very artificial and possibly far removed for how the viral DNA is located in the host cell. The location of K7 in the cell might suggest further affects of K7 on host gene expression apart from their cytosolic signalling pathways.

Through our study of the evasion mechanisms of viruses we can learn about unknown regulatory mechanism of the immune response. There are examples of this even in the study of VACV. It was through the observations that A52 could inhibit IL-1 and TLR induced NFκB activation, in addition to the fact that A52 could interact with IRAK2, that the importance of IRAK2 in innate immune signalling was highlighted (Keating, Maloney et al. 2007). Another example is the fact that K7 could block non-TLR induced IFN induction. This then led to the demonstration of the importance of DDX3 in innate immunity (Schroder, Baran et al. 2008).

In this study I highlighted other differences between K7 and A52 and tried to implicate DDX3 in these differences. In the case of A52, TRAF6 binding results in MAPK activation which in turn causes an increase in TLR-dependent IL-10 protein production (Maloney, Schroder et al. 2005). K7 had a different effect of IL-10 protein production in that it could directly induce it. Therefore K7 possibly has a different mechanism of stimulating IL-10 production. Seeing that K7 can interact
with DDX3 it is possible that K7 can cause these additional effects on IL-10 through manipulating DDX3. The IL-10 promoter has been shown to contain sp1 sites (REF) and DDX3 can promote the activation of certain genes through activation of Sp1 (Chao, Chen et al. 2006). Therefore, K7 may be also targeting DDX3 to promote the induction of IL-10. More study must be carried out in order to test this hypothesis of DDX3 involvement in IL-10 gene induction. The use of siRNA against DDX3 could be of benefit in determining the effect DDX3 absence has on both LPS and K7-induced IL-10 production.

K7, unlike A52, could inhibit TNF stimulated NFκB activation. As has been mentioned in previous chapters, Schroder found that through the initial finding that K7 could interact with DDX3 that DDX3 is involved in the kinase complex that activates the interferon pathway. Given this finding, I looked to see if DDX3 was capable of exerting influence at a similar level in the NFκB pathway. Indeed, DDX3 was capable of interacting in both an overexpressed and endogenous manner with IKKβ. In addition, I showed that this interaction was intact in the absence of the interferon activating kinase, TBK1. In order to complete this study it would be of great interest to test the DDX3/IKKβ interaction in cells deficient for the other interferon activating kinase IKKe. Schroder et al showed a possible more potent effect of DDX3 on IKKe then TBK1 (Schroder 2009). It was shown that siRNA targeting DDX3 probably inhibited IKKe induced interferon luciferase promoter more potently than TBK1 induced activation.

The relevance of this DDX3/IKKβ interaction remains to be clarified. Chapter 4 showed that DDX3 depletion had no effects on two well-characterised pathways involving IKKβ. It may well be the case that IKKβ is regulating DDX3. It also was
demonstrated in chapter 4 that in the presence of IKKβ-flag, DDX3 undergoes a post translational modification. This modification may alter the transactivational potential of DDX3 in terms of transcription or translation. It would be interesting to determine the exact nature of this modification of DDX3 by IKKβ. Another interesting study would be to test siRNA against IKKβ to see if this had any effect on IFNβ induction.

Having clearly shown no role for DDX3 in IL-1 and TNF induced NFκB activation it would be of interest to test other host proteins and the effect K7 has on them. Given that K7 can inhibit IL-1 and TNF stimulated NFκB activation, it would be of interest to see if K7 could target any components that are common to these two pathways. MEKK3 is one such protein, as are the proteins TAK1/TAB1 and TAB2 (Verstrepen, Bekaert et al. 2008).

The K7 expressing Lentivirus system will be of great benefit to see the influence K7 has on many hard to transfect cells. In particular, human PBMCs would be very interesting in this context. Given the literature on VACV and TLR-2 signalling, studying the role K7 plays in this innate immune signalling pathway would be of benefit. A particular set of cells that would be relevant to study are the newly characterised inflammatory monocytes (Barbalat, Lau et al 2009). Given the likely hood that these cells will be hard to transfect, the K7 expressing Lentivirus will be helpful.

DDX3 had a very complex role in the induction of IL-8 upon IL-1 stimulation. DDX3 may be one way of innate immunity controlling gene expression. Given that DDX3 can interact with, regulate (in the case of IKKe) and possibly be regulated by (in the case of IKKβ) with many of the IKK family members The thought that
DDX3 may even act as a bridging molecule to transition all the events that are necessary in gene expression is also a possibility. This suggestion is somewhat promising given the fact that there is a lot of literature involving DDX3 in every level of gene induction. Future studies would include testing to see if DDX3 had a similar role in other genes.

Recently Huang et al showed that in the case of TLR4 signalling, IKKε influences promoters by causing the clearance of repressors for promoters. In addition the phosphorylation of p65 at Ser\(^{536}\) serves as a docking site on p65 for IKKε delivery to the nucleus, which then causes repressor removal from the promoter (Huang, Ghisletti et al 2009). K7 can block this p65 phosphorylation event by both IL-1 and TNF stimulus. Therefore perhaps this is a mechanism by which K7 can inhibit TNF induced NFκB activation. From the initial finding that DDX3 could interact with IKKβ, I went on to show that DDX3 siRNA did not affect IL-1 or TNF induced NFκB activation (Chapter 4). Therefore the purpose of this DDX3/IKKβ interaction remains elusive. Huang et al proposed that other similar kinases might have a similar function in the clearance of co-repressors. This is possibly the function of the DDX3/IKKβ interaction.

Chapter 4 showed that DDX3 depleted cells affect the kinetics of IL-1 induced IL-8 production. In addition, DDX3 depletion had a complex role in IL-1 induced IL-8 induction. It would be of great benefit to the overall study to look at other cytokines and see if the depletion of DDX3 had a similar effect. In addition testing the effect of DDX3 depletion on the stability of IL-8 mRNA after IL-1 and TNF treatment will be of interest.
Chapter 5 demonstrated that DDX3 is involved in the malaria DNA pathway leading to type I IFN induction. As discussed in chapter 5 it is not known whether this dependency is a reflection of DDX3 being the actual receptor for DNA recognition, simply a part of the TBK1 complex involved in receptor signalling (for both DAI or a separate missing receptor) or part of signalling complex involved in RIG-I signalling. One way to clarify this would be to extract RNA from cells treated with the malaria DNA, and test the RNAs ability to stimulate type I IFNs. An interesting experiment would be to test the ability of K7 to inhibit DNA induced interferon responses. If K7 has this ability it could be down to the ability of K7 to target DDX3. This would add to the evidence that DDX3 is important in the DNA induced IFN response.

Identifying the receptor that is responsible for the malaria DNA response is an exciting task. In order to help achieve this task it would be interesting to identify the cytokine release after stimulation with this DNA. A range of cytokines could be tested to profile the gene induction. This might hint as to the nature of the receptor. For example, if IL-1β is upregulated then this might indicate that this novel receptor might be part of the HIN family of proteins and thus this receptor might be similar to AIM-2.

Given the multiple effects of DDX3 on innate immunity, of great benefit to all of these studies would be the development of a mouse model that is deficient from the expression of DDX3. This process has been initiated. Previous work includes the purchasing of ES DNA form the GGTC (German Gene Trap Consortium) in which the DDX3 gene has been targeted by the Rosaβ-Geo vector (clone ID P134 A05).
This vector contains a splice acceptor site upstream from a promoter reporter. Upon activation of transcription, a transcript is generated that is a fusion of the upstream 'trapped' gene coding sequence and the vector (containing a reporter). To date, it has been shown that this vector targets the first intron in the DDX3 gene in these ES cells. Mice have been generated that are viable (not foetal lethal) and in addition these mice are now germ line transmissible. A wild type PRC band has been optimised that serves as a marker for wild-type mice or mice that are heterozygous with only one copy of the gene that is targeted by the vector. This band is a 2kb product of a PCR reaction that amplifies the first and second exon (in contrast a 'trapped'gene will result in a large band shift as the vector will bridge exons 1 and 2, therefore the 'trapped gene will result in a PCR product that is 10kb). The presence of the vector has been detected in DNA from the ES cells purchased and in the germ line transmitted mice using primers that are specific to the vector. Future work for these mice will include complete genotyping to identify homozygous mice for the trapped gene. When these mice have been identified numerous assays would be used to study the effects of the lack of DDX3 expression on innate immune signalling. Assays such as testing for DNA induced IFN responses, RNA induced IFN responses and testing for a variety of other stimulus induced signalling pathways and cytokine release. This should hopefully clarify the role that DDX3 has on innate immunity.
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