Characterisation and therapeutic modulation of toll-like receptor signalling in response to the intracellular pathogen *F. tularensis*

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Declaration

I hereby declare that my dissertation entitled “Characterisation and therapeutic modulation of toll-like receptor signalling in response to the intracellular pathogen *F. tularensis*” is not substantially the same as any that I have submitted for a degree or diploma or other qualification at any other university.

I further state that no part of my dissertation has already been or is being concurrently submitted for any research degree, diploma or other qualification.

This dissertation is the result of my own investigations, except where otherwise stated. Other sources are acknowledged in the text.

Signed..................................................

Richard Saint

Date
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Abstract

Characterisation and therapeutic modulation of toll-like receptor signalling in response to the intracellular pathogen *F. tularensis*

Richard Saint

The induction of an innate immune response upon infection is dependent on the detection of the invading organism and the generation of a signalling cascade leading to the production of inflammatory mediators. Toll-like receptors are expressed on multiple cell types and induce the activation of a complex network of signalling pathways containing numerous branches with multiple interactions and cross-talk between the different branches. The TLR system is integral to the generation of a protective immune response and as such is an important target for pathogen-associated modulation. Many bacterial and viral pathogens employ strategies for interrupting or modulating TLR signalling to evade the host immune response. The obligate intracellular bacterial pathogen, *F. tularensis*, successfully invades and replicates within immune and epithelial cells. However, despite significant research the exact mechanisms used by this pathogen to successfully evade the host immune response remain elusive.

To establish the exact signalling events that occur within a host upon infection with *F. tularensis*, the activation of specific signalling proteins was characterised using *in vitro* and *in vivo* models. The MAPKs, ERK and p38, were identified as critical in generating the host response. Furthermore, the temporal regulation of these signalling proteins was found to be bi-phasic with an early transient activation of both ERK and p38 followed by a sustained activation of ERK and a suppression of p38 activation at later time points. The role of ERK was investigated further using a specific inhibitor (PD0325901). Although there was no decrease in bacterial burdens *in vitro* and no increase in survival in mice treated with PD0325901, the inhibition of ERK activation reduced the secretion of TNF and IL-6 and reduced systemic bacterial proliferation *in vivo*.

The induction of immune signalling cascades requires the activation of one or more receptors. The contribution of TLR2, TLR4 and TLR9 to the immune response to *F. tularensis* infection was examined using KO cell lines and specific antagonists. TLR2 was confirmed as a receptor for *F. tularensis* and was observed to play a role in the translational regulation of TNF. A role for TLR4 was also identified and further characterisation identified a potential priming relationship with TLR9. Sub-stimulation of
TLR4 by LPS enhanced the response induced by a subsequent stimulation of TLR9 by purified *F. tularensis* DNA.

Overall, this study has provided evidence that, during infection, *F. tularensis* interacts with innate immune signalling pathways. By simultaneously suppressing p38 activation and prolonging ERK activation *F. tularensis* is able to regulate cytokine secretion and the induction of host-cell death mechanisms. Furthermore, this work has demonstrated that the activation of TLR9 by *F. tularensis* genomic DNA can be primed by a prior sub-stimulation of TLR4, although more research is required to fully understand the contribution of this interaction to the pathogenesis of *F. tularensis*.
Conference presentations


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3'UTR</td>
<td>3' untranslated region</td>
</tr>
<tr>
<td>ACDP</td>
<td>Advisory committee on dangerous pathogens</td>
</tr>
<tr>
<td>ActA</td>
<td>Actin nucleator A</td>
</tr>
<tr>
<td>AIM</td>
<td>Absent in melanoma</td>
</tr>
<tr>
<td>αα</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AP</td>
<td>Activator protein</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARE</td>
<td>adenylate/uridylate-rich element</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein</td>
</tr>
<tr>
<td>ATF</td>
<td>AP-1 transcription factor</td>
</tr>
<tr>
<td>BCGA</td>
<td>Blood cysteine glucose agar</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase-recruitment domain</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytokine bead array</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>Common domain</td>
</tr>
<tr>
<td>CL</td>
<td>Containment level</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>D-domain</td>
<td>Docking domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>ECACC</td>
<td>European collection of cell cultures</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ED</td>
<td>ERK-docking domain</td>
</tr>
<tr>
<td>EEA1</td>
<td>Effector early endosomal antigen 1</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>Elongation factor- Tu</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FCV</td>
<td><em>Francisella</em> containing vacuole</td>
</tr>
<tr>
<td>FimH</td>
<td>Fimbriae H protein</td>
</tr>
<tr>
<td>FPI</td>
<td><em>Francisella</em> pathogenicity island</td>
</tr>
<tr>
<td>GSK3B</td>
<td>Glycogen synthase kinase-3B</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HLH</td>
<td>Helix-loop-helix</td>
</tr>
<tr>
<td>HMGB-1</td>
<td>High-mobility group box-1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>I-kappa-B kinase complex</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IPS-1</td>
<td>Interferon-β promoter stimulator</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon-response factor</td>
</tr>
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</table>
IκB  Inhibitor of κB
JNK  c-Jun NH₂-terminal kinase
LAMP  lysosomal-associated membrane protein
LDH  Lactate dehydrogenase
LiCl  Lithium chloride
LPS  Lipopolysaccharide
LRR  Leucine-rich repeats
LVS  Live vaccine strain
MAC  Membrane attack complex
Mal  MyD88-adaptor-like
MALP-2  Macrophage-activating lipopeptide-2
MAPK  Mitogen-activated protein kinase
MCP-1  Monocyte chemotactic protein-1
M-CSF  Macrophage colony-stimulating factor
MDA-5  Melanoma differentiation-associated gene
MDC  Monodansylcadaverine
mDAP  D-γ-glutamyl-meso-DAP
MDP  Muramyl dipeptide
MEK  MAPK-kinase
MHC  Major histocompatibility complex
MIP  Macrophage inflammatory protein
MK-2  MAPK-activated protein kinase-2
MKP  MAPK phosphatase
mmLDL  minimally modified low density lipoprotein
MOI  Multiplicity of infection
MR  Mannose receptor
MyD88  Myeloid differentiation primary response gene-88
NBD  Nucleotide binding domain
NBD/γBD  NEMO/IKKγ-binding domain
NEMO  NFκB essential modulator
NFA  Negative feedback amplifier
NF-κB  Nuclear factor kappa B
NK cells  Natural Killer cells
NLR  Nod-like receptor
NOD  Nucleotide-binding oligomerisation domain
OD  Optical density
ODN  Oligonucleotides
PAC  Phosphatase of activated cells
PAGE  Polyacrylamide gel electrophoresis
PAMP  Pathogen associated molecular pattern
PBS  Phosphate buffered saline
PEC  Peritoneal exudate cells
PGN  Peptidoglycan
PI3P  Phosphatidylinositol-3-phosphate
PRR  Pattern recognition receptor
PVDF  Polyvinylidene fluoride
Rab-7  Ras-related in brain-7
RANTES  Regulated upon activation, normal T-cell expressed, and secreted
RHD  Rel-homology domain
RIG  retinoic acid inducible gene
RING  Really interesting gene
RIP  Receptor-interacting protein
RLR  RIG-like receptor
RNA  Ribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute medium</td>
</tr>
<tr>
<td>SAP</td>
<td>SRF-accessory protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEAP</td>
<td>Secreted embryonic alkaline phosphatase</td>
</tr>
<tr>
<td>SR-A</td>
<td>Scavenger receptor-A</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
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<tr>
<td>TA</td>
<td>Transcriptional activation</td>
</tr>
<tr>
<td>TAB</td>
<td>TAK-1-binding protein</td>
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<tr>
<td>TAK</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF family member-associated NFκB activator</td>
</tr>
<tr>
<td>TBK</td>
<td>TANK-binding kinase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R domain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-receptor-associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<td>TRIF</td>
<td>TIR-domain containing adaptor-inducing interferon-β</td>
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<td>Ubc</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>USSR</td>
<td>Union of Soviet Socialist Republics</td>
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</table>
Chapter 1: General Introduction

1.1 Innate immunity

The innate immune system has previously been considered a simple and non-specific mechanism whose function was to eliminate pathogens and present antigen to the cells of the adaptive immune response. However, with more focussed research it has been found that, far from being non-specific, the innate immune response exhibits a highly developed ability to discriminate between pathogens and respond accordingly. Unlike adaptive immunity, which has a slower response time and is mediated by T and B cells, innate immunity displays a rapid response relying on groups of pattern recognition receptors (PRRs) to initiate the response 1,2. The function of PRRs is to detect evolutionary conserved components of microbial pathogens called pathogen associated molecular patterns (PAMPs). PAMPs include a range of molecules such as lipids, proteins and nucleic acids and it is the detection of particular PAMPs by a broad range of PRRs which confer the innate immune system with much of its specificity. The PRRs involved in inducing a response can be categorised into three major families called retinoic acid inducible gene (RIG)-like receptors (RLRs), nod-like receptors (NLRs) and the toll-like receptors (TLRs) 3. In addition to the high degree of specificity and the rapid induction of the innate response, it has recently been shown that the innate immune response is actually a requirement for the induction of the adaptive immune response 1,2. It is clear, therefore, why innate immunity is now considered to represent the first line of defence against invading microbial pathogens.

The three major PRR families are categorised according to the functions they perform and their structure. In dendritic cells, TLRs appear to be responsible for virus detection 4. However, in all other cell types RLRs take the lead role 5. RIG-1 was the first RLR to be identified as functioning within the innate immune response, initiating a response to double-stranded RNA 5,6. A second RLR called melanoma differentiation-associated gene (MDA-5) was subsequently identified and also detects double-stranded RNA. Interestingly, these two receptors do not perform redundant functions as observed using knock-out mice 7,8. Both RIG-1 and MDA-5 share a similarity with NLRs in that they contain a caspase-recruitment domain (CARD) which enables the recruitment of the CARD-containing adaptor protein ‘interferon-β promoter stimulator-1’ (IPS-1) 3. Recruitment of IPS-1 leads to signalling events resulting in the activation of nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs) and interferon-response factors (IRFs) 9.
NLRs comprise the largest family of PRRs with 22 members in humans and more than 33 in mice. NLRs are intracellular receptors and detect a variety of bacterial components. The structure of NLRs is formed of three domains with a CARD domain at the N-terminus and a series of leucine-rich repeats (LRRs) at the N-terminus. These two domains are linked by a nucleotide-binding domain (NBD). NLRs share homology with both TLRs and RLRs as they contain LRRs and CARD domains, respectively. The first two NLRs to be reported were nucleotide-binding oligomerisation domain (NOD)-1 and NOD2 which detect the components of peptidoglycan (PGN), D-γ-glutamyl-meso-DAP (mDAP) and muramyl dipeptide (MDP), respectively. Although NLRs share a CARD domain with RLRs, their subsequent adaptors are different. Signalling via NLRs involves the recruitment of receptor-interacting protein (RIP-2) which leads to the activation of the IκB kinase complex (IKK). In recent years a lot of research has been done on this family of receptors leading to the discovery of numerous NLRs. As different groups simultaneously study these receptors numerous names have been used for specific proteins. Recently, a unified nomenclature was agreed on that allocates the majority of the NLRs into 5 subfamilies called NLRA, NLRB, NLRC, NLRP and NLRX based on the different effector domains.

A major step forward in innate immune research was the discovery of the inflammasome complex. The inflammasome is a multi-protein complex which can be formed after activation of one of three NLRs, NLRP1, NLRP3 and NLRC4. Upon assembly of the inflammasome, caspase proteins are cleaved from their pro-forms to an active state leading to the processing of interleukin (IL)-1β and IL-18. The most studied complex is formed of NLRP3, apoptosis-associated speck-like protein (ASC) and caspase-1 and is activated upon stimulation by numerous and varied microbes and associated ligands, in addition to some endogenous stimuli. To date, no direct binding of ligands to the NLRP3 inflammasome complex has been demonstrated and so the mechanism by which such a variety of ligands can activate this complex is still under investigation.

Although the NLRP3-inflammasome is able to respond to many ligands, one group of ligands that appear not to directly signal through this inflammasome complex is cytoplasmic bacterial, viral, mammalian or synthetic DNA. In 2009, two groups successfully identified the cytoplasmic receptor responsible for initiating a response to these ligands as absent in melanoma 2 (AIM2). AIM2 is able to directly bind to cytoplasmic DNA through its oligonucleotide/oligosaccharide binding domain and can interact with the NLR-associated protein, ASC, by means of its amino-terminal pyrin domain. Construction of the AIM2-inflammasome, similarly to NLRP3, results in the activation of caspase-1 and production of active IL-1β and IL-18.
The third major family of PRRs is the TLR family. As this family of receptors is the main focus of this work, TLRs are discussed in more detail below (Section 1.2).

## 1.2 Toll and toll-like receptors

### 1.2.1 Early milestones of TLR research

Strangely, the event that initiated research on TLR signalling actually occurred long before the identification of the first TLR. Dr Charles Janeway, in a lecture in 1989, hypothesised the process whereby receptors present on immune cells detect microbes and instigate a protective immune response. Despite being seven years previous, this seminal lecture paved the way for the identification of the first Toll gene in 1996. At this time, Lemaitre et al. observed that the *toll* gene of *Drosophila melanogaster* was important in dorso-ventral patterning and subsequently that mutations in *toll* led to the fly succumbing to overwhelming fungal infections. Characterisation of this gene identified it as a receptor now termed “Toll” whose role, in *Drosophila*, is in the detection of invading fungal pathogens. A search for Toll homologues led to the identification of the first human TLRs called TLR2 and TLR4, that are responsible for the detection of bacterial lipoproteins and lipopolysaccharide (LPS), respectively. Subsequently, TLRs have been identified across a wide range of species including the sea urchin that, with 222 TLRs (and 203 NLRs), has the most of any organism identified to date. In humans 11 TLRs have been identified which are expressed on numerous cell types including immune cells such as dendritic cells, neutrophils and alveolar macrophages as well as on non-immune cells such as endothelial cells and epithelial cells.

### 1.2.2 Structure and ligands of TLRs

TLRs are type I integral membrane glycoproteins and their cytoplasmic regions demonstrate substantial homology with another family of receptors known as IL-1 receptors (IL-1Rs). Together with these receptors TLRs form a large superfamily of receptors called the TLR/IL-1R superfamily. The cytoplasmic portion of all TLR/IL-1R family members is formed of a conserved chain of 200 amino acids, known as the Toll/IL-1R (TIR) domain. Within this domain are three regions, with high homology, that are key for homologous interactions with downstream signalling adaptors that also contain TIR domains within their structure. Despite the homology in the cytoplasmic regions, the extracellular regions of TLRs differ considerably from IL-1Rs. The extracellular region of
IL-1Rs is formed of three immunoglobulin-like domains whereas the extracellular region of TLRs consists of LRR motifs 47.

The LRR motif was first identified from an α2-glycoprotein of human serum in 1985 and was characterised as having a 24-residue repeated sequence containing multiple hydrophobic residues 48. Subsequently, in 2003 the basic framework of the TLR ectodomain was suggested to be a horseshoe-shaped solenoid formed of parallel β-sheets on its concave surface and α-helices on the convex surface 49. Typically, the β-sheets have uniform twist angles and radii throughout the entire structure, such as in TLR3 50. However, TLR4 has an unusual ectodomain as it contains sharp structural transitions that divide this protein into three domains; N-, central, and C-terminal domains 51. The largest changes from typical ectodomains are found within the central domain where the radius is 35% smaller and twist angles are three times greater than those of TLR3 51. These structural alterations can be attributed to the lack of the usual asparagine ladder from LRR9 – 12 and breaking of the phenylalanine spine at the border between the central and C-terminal domains 51. Similar to TLR4, TLR2 can be divided into three domains with its central and C-terminal domain deviating considerably from the “typical” structure. Again this is due to the absence of both the asparagine ladder and the phenylalanine spine 52. The PAMP binding region of TLR2 is also located in an unusual place. Typically, PAMP-binding occurs on the concave surface of the solenoid structure where the structure provides a binding surface with a 10-fold greater area than the binding surface of antibodies 49. In contrast, the convex surface provides the ligand-binding pocket located at the border of the central and C-terminal domains 52. The unusual location of ligand-binding is suggested to enable a more efficient mechanism of binding small-molecule ligands such as lipopeptides in contrast to the large surface area provided by the concave surface for macro-molecule binding.

In addition to structural differences specific residue insertions confer further specificity to ligand binding. For example, LRR12 and LRR20 of TLR3 contain large protruding loops that are unique to TLR3 and are highly conserved indicating a possible role in ligand binding 50,53. Furthermore, the crystal structure of the TLR1/TLR2 heterodimer bound to a synthetic ligand, Pam3CSK4, revealed multiple key regions including two acyl chains bound to a hydrophobic pocket on TLR2 and a third amide-bound acyl chain bound to a hydrophobic groove on TLR1 52. A key residue of TLR1, Gln-316, was also observed interacting via hydrogen-bond formation with a lipopeptide ligand 54.

One method of subdividing TLRs is by the type of ligand that they primarily recognise. Using this method the human TLR1, TLR2, TLR4 and TLR6 are grouped on the basis of recognising lipids.
Similarly, TLR3, TLR7, TLR8 and TLR9 all recognise nucleic acids and finally TLR5 recognises protein ligands. However, this method does not reflect the full specificity of recognition that is characterised by each individual TLR. Alternatively, TLRs can be categorised by the cellular localisation where they are predominantly expressed. In this manner TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are classed as cell surface expressed receptors whereas TLR3, TLR7, TLR8 and TLR9 are intracellularly expressed receptors located on endosomes and lysosomes. Interestingly, classification in this way displays a degree of overlap with the type of PAMP being recognised. Cell surface receptors mainly recognise bacterial products not produced by the host whereas intracellular receptors tend to recognise nucleic acids. In this case, prevention of host-derived ligand detection is supplied through physical separation rather than completely on the molecular structure of PAMPs. However, the classification of TLRs is made more complex by the fact that the receptors do not always function as a homologous dimer. For example, TLR2 is able to form dimers with both TLR1, TLR6 and, more recently identified, TLR10. In combination with TLR1, TLR2 is able to detect tri-acylated lipoproteins and in combination with TLR6 it is able to recognise di-acylated lipoproteins. The dimer formed between TLR10 and TLR2 shares some similarities with the TLR1/TLR2 dimer in that TLR10 preserves the TLR2 dimer interface and the lipopeptide-binding channel found in TLR1. Interestingly, although both TLR1/TLR2 and TLR10/TLR2 recruit MyD88, the downstream signalling cascades initiated are not the same. Where TLR1/TLR2 is able to initiate NF-κB transcription, TLR10/TLR2 is unable to indicating distinct functions of these two different TLR2 dimers.

The cooperation between certain TLRs, such as TLR2, further diversifies the already broad range of PAMPs supported by the TLR system. The most descriptive and accurate, if not most concise, way of categorising TLRs is by examining each TLRs individual set of ligands. These are summarised in Table 1-1. As mentioned previously, TLR2 is able to detect di- or tri-acylated lipoproteins when in combination with either TLR6 or TLR1, respectively. In addition, TLR2 recognises a wide variety of PAMPs including peptidoglycan, mycoplasma lipopeptides, fungal zymosan and cylindrical LPS. TLR3 recognises double stranded RNA (dsRNA) produced by many viruses during replication. TLR4, together with its accessory proteins MD-2 and CD14, recognises LPS from Gram-negative bacteria. TLR5 detects bacterial flagellin. TLR7, TLR8 and TLR9 all detect nucleic acids with TLR7 and TLR8 specifically detecting single stranded RNA (ssRNA) and TLR9 detecting unmethylated CpG motifs present in bacterial and viral genomes. TLR10 shares the ligand repertoire of TLR1 and the human TLR11 is thought to be non-functional due to a stop codon present within its sequence. This list of PAMPs is by no means exhaustive and only mentions the primary ligands of these TLRs.
<table>
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<tr>
<th>TLR #</th>
<th>Cellular location</th>
<th>Ligand group</th>
<th>Specific ligands</th>
<th>Microorganism</th>
<th>Synthetic ligands</th>
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<td>Plasma membrane</td>
<td>Lipids</td>
<td>Tri-acylated lipoproteins&lt;sup&gt;70&lt;/sup&gt;</td>
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<td>Compounds A,B,C&lt;sup&gt;54&lt;/sup&gt;</td>
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Table 1-1: Cellular location of TLRs and their respective ligands

The ligands of TLRs grouped into categories. TLRs that detect lipids are shown in orange. TLRs that recognise proteins are shown in purple. TLRs that bind to nucleic acids are shown in red and TLRs with unknown ligands are shown in green. Also shown are the cellular locations and further details of specific natural and synthetic TLR ligands.
receptors. It is also likely that many more ligands exist that have yet to be identified. The activation of TLRs is not only restricted to pathogen-associated ligands. There exists another class of ligands called danger-associated molecular patterns (DAMPs). The function of these host-derived ligands is to alert the host of damage inflicted upon its cellular structure through infection or trauma. Subsequent to non-infectious lung injury, fragmented hyaluronan builds up at the site of damage. This DAMP signals through TLR2 and TLR4 initiating the production of inflammatory mediators and ensuring the maintenance of epithelial cell integrity resulting in the recovery of injury \(^88\). Another well studied DAMP is called high-mobility group box-1 (HMGB-1). HMGB-1 is passively released when tissues are damaged in the absence of an invading organism \(^89\). In contrast to hyaluronan-induced signalling, HMGB-1 release promotes excessive inflammation and subsequent tissue damage \(^89\).

1.2.3 Expression of TLRs

In addition to certain TLRs being preferentially expressed on different cellular membranes, diverse cell types also preferentially express sets of receptors. Neutrophils and macrophages, two of the major immune cells, express all TLRs except TLR3 \(^36\). In contrast, mature dendritic cells (DCs) do express TLR3 in addition to TLR1, TLR2, TLR4 and TLR5 although they lack TLR7 and TLR9 \(^36\). Mononuclear cells, such as B-cells, have been shown to express high levels of TLR1, TLR6, TLR9 and TLR10 but low levels of TLR2, TLR4 and TLR7 \(^36\). However, it is not just antigen presenting cells (APCs) that express TLRs. Natural killer (NK) cells, a subtype of T-cell, express high levels of TLR3 and can become activated after direct detection of the synthetic TLR3 ligand poly(I: C) \(^90\). More recently, it has been found that NK cells can respond to other ligands such as CpG DNA perhaps suggesting the involvement of other TLRs in addition to TLR3 \(^91\). As NK cells are major anti-viral immune cells it is perhaps not surprising that they express TLR3, a detector of viral components, and have the capacity to respond to CpG DNA.

TLR expression is not restricted to immune cells and TLRs have been located on numerous epithelial cells isolated from the respiratory, intestinal and genitourinary tracts \(^36\). Within the intestinal tract, the epithelial cells are in constant contact with commensal bacteria and the cells need to be able to maintain the potential to respond to pathogenic bacteria without repeatedly being activated by commensal bacteria. This is achieved through two mechanisms. Firstly, intestinal epithelial cells exhibit very low levels of expression of TLR4, keeping the cells in a hypo-responsive state to LPS expressed by commensal bacteria \(^92\). The second, and possibly more tightly regulated, mechanism is through TLR5 exhibiting a strict polarised expression in these cells whereby TLR5 is only expressed
on the basolateral edge and not on the intestinal lumen (apical) side. This mechanism prevents activation by the flagella of commensal bacteria and reserves detection for pathogenic bacteria that make the journey across to the basolateral side. Unlike intestinal epithelium, TLR4 is widely expressed in pulmonary epithelial cells. In addition, TLR2 is highly expressed by type II alveolar epithelial cells. This widespread expression of TLR2 and TLR4 in the lungs could be due to the requirement to detect and eliminate invading pathogens rapidly before too much organ damage is sustained. However, the response must be strictly regulated to prevent autoimmune damage.

With the range of TLRs available, human cells are able to initiate a response to a multitude of diverse ligands. The specificity of the response is dependent upon which TLRs are activated, the location of the host cell and the type of cell being activated. All of these factors contribute to the final response generated by the innate immune system. However, there is a requirement to link the primary detection event by TLRs to the generation of a specific immune response and this is provided by the intracellular TLR signalling cascades.

1.3 TLR signalling

The signalling cascades initiated by ligands binding to TLRs can be subdivided into two major pathways. These are classified as the Myeloid differentiation primary response gene-88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF)-dependent pathways according to which of the two primary adaptor molecules (MyD88 or TRIF) are recruited to the intracellular TIR domain of the TLRs. Following adaptor binding there is a complex cascade of protein recruitment and activation involving numerous proteins and signalling branches. A schematic representation of the major proteins and branches is shown in Figure 1.1. The figure does not display all proteins and pathways known or proposed to be involved in TLR signalling but illustrates the complexity of TLR signalling generated by numerous signalling proteins, multiple interactions and cross-talk between pathways. One paper has attempted to diagrammatically describe the TLR system and nicely illustrates the complexity of the network even if the end result is not entirely comprehensible.

1.3.1 MyD88-dependent signalling

1.3.1.1 MyD88 and Mal

MyD88 is the adaptor molecule responsible for the initiation of intracellular signalling upon ligand binding to all TLRs except TLR3. This has been demonstrated through the stimulation of MyD88-deficient macrophages by a range of different TLR ligands. These macrophages were unable
Dimerisation of TLR4, induced by ligand binding, leads to the recruitment of both major adaptors, MyD88 and TRIF, through their co-adaptors Mal and TRAM, respectively. MyD88 recruits IRAK-4 (and potentially other members of the IRAK family) which in turn attracts TRAF-6 to the complex. Ubiquitination of TRAF-6 leads to the formation of a signalling complex formed of TAK-1 and its binding proteins TAB-1,-2,-3. At this point the MyD88-dependent pathway bifurcates into the MAPK and the NFκB branches. The MAPK branch consists of a cascade of phosphorylation events beginning with TAK-1 and passing through the MAP2Ks (MKK1-7) and the MAPKs (JNK, ERK and p38) leading to the activation of the dimeric transcription factor, AP-1. The NFκB branch involves the formation and activation of the IKK complex, composed of IKKα, IKKβ and IKKγ (NFκB essential modulator – NEMO). This complex phosphorylates the NFκB inhibitory protein, IκBα, leading to its degradation and the release of NFκB. NFκB then translocates to the nucleus and initiates transcription. The TRIF-dependent pathway is able to link into both the NFκB and the MAPK branches of the MyD88-dependent pathway through the signalling proteins RIP and TRAF-6 respectively. In addition, the TRIF-dependent pathway leads to the activation of TBK-1 and IKKi, possibly through TRAF-3. This in turn leads to the activation of the transcription factor, IRF-3. Figure reproduced from “TLR Signalling”, 2006, T. Kawai and S. Akira ⁹⁷.
to secrete tumour necrosis factor (TNF) and IL-6 in response to LPS and macrophage-activating lipopeptide-2 (MALP-2), although delayed activation of NFκB was still observed in response to LPS. MyD88 is a tertiary domain protein consisting of a TIR domain at the C-terminus, which is used in the homologous binding to the TIR domains of TLRs, and a death-domain (DD) at the N-terminus, which is essential for the recruitment of downstream signalling proteins. As MyD88 is essential to the majority of signalling pathways induced by TLRs, the signalling cascade downstream of MyD88 is termed the MyD88-dependent pathway. MyD88 is the primary signalling protein recruited to the intracellular portion of TLRs. However, MyD88 is not the only adaptor utilised in the MyD88-dependent pathway. Both TLR2 and TLR4 usually require a co-adaptor called MyD88-adaptor-like (Mal) to activate their signalling cascades. In fact, the same defective TNF and IL-6 secretion profile was observed in Mal-deficient macrophages stimulated with LPS and MALP-2 as was seen in MyD88-deficient macrophages indicating the essential requirement for Mal. In addition, activation of the downstream signalling proteins extracellular regulated kinase (ERK), p38 and c-Jun NH₂-terminal kinase (JNK) was only observed in the presence of Mal when the cells were stimulated with MALP-2. Interestingly, more recent research demonstrates a role for Mal in TLR2 signalling only when low concentrations of ligand are available since the requirement for Mal can be overcome with high concentrations of TLR2 ligand when only MyD88 is required. It is possible that the requirement for Mal is dependent on the concentrations of ligand and acts as a sensitizer for the system when ligand is sparse. Work performed using the bacterial pathogen Francisella tularensis extends this hypothesis by showing that increasing the retention time of the bacteria within the phagosome also negates the requirement for Mal in TLR2 signalling. It appears then that Mal is required to enhance the sensitivity of detection only when low concentrations of ligand or a transient interaction with the ligand is available. Mal displays close homology with MyD88 in terms of having a TIR domain at its C-terminus but Mal does not have a DD and its N-terminus is 75 amino acids shorter than MyD88. The current understanding is that TLR dimerisation, in all cases except TLR3, induces the recruitment of MyD88 via homotypic TIR domain interactions. In the case of TLR4, Mal is required to bridge the connection between the TLR and MyD88 TIR domains via its own TIR domain. For TLR2, Mal is only a requirement if low concentrations of ligand are present. However, this model of Mal recruitment is not true in all cases. Infection of mice with Salmonella typhimurium induces early signalling events through TLR4 that aids in controlling early bacterial growth. Interestingly, this response is unaltered in mice deficient in the expression of Mal indicating that, in this case, Mal is not required for TLR4-induced signalling cascades. This observation clearly demonstrates the flexibility of the TLR signalling system and acts as a warning against assuming that
signalling proteins and interactions downstream of one stimulus will necessarily be present in the cascade induced by another.

1.3.1.2 IRAKs

The recruitment of MyD88 and Mal to the intracellular portion of TLRs leads to the recruitment of the next family of signalling proteins termed the interleukin-1 receptor-associated kinases (IRAKs). There are 4 members of the IRAK protein family and each has a specific role to play in TLR signalling. IRAK-4 is the first family member to be recruited to the DD of MyD88 via interactions with its own DD. The essential role of IRAK-4 has been demonstrated in TLR2 signalling, as IκB degradation was prevented after stimulation with PGN in IRAK-4 deficient macrophages. Signalling downstream of TLR4 activation in IRAK-4 deficient macrophages was only delayed, as judged by NFκB activation, due to TLR4 having the capacity to signal through other pathways besides the MyD88-dependent cascade. The requirement for IRAK-4 is also observed in vivo as mice deficient in IRAK-4 are unable to secrete IL-6 and TNF in response to LPS. The function of IRAK-4 is dependent on the kinase activity, which phosphorylates subsequent signalling proteins resulting in the progression of the signalling cascade. Although the role for IRAK-4 is well defined, the substrate which it interacts with is less certain although both IRAK-1 and IRAK-2 are potential IRAK-4 substrates.

IRAK-1, similarly to MyD88, has a DD at its N-terminus that is able to mediate binding to other DD-containing proteins. Also present in its structure is a Ser/Thr kinase domain and a C-terminal domain that contains motifs for TNF-receptor-associated factor-6 (TRAF-6) binding. It has been proposed that in its inactive state, IRAK-1 is maintained in a folded form involving interactions between the DD and motifs present in the C-terminus. Both IRAK-4 and IRAK-1 have DDs although it has been observed that a homotypic binding between these domains is not required for their interaction. Despite this, a linkage between IRAK-4 activation and IRAK-1 activation and degradation has been seen. In macrophages stimulated with LPS, a degradation of IRAK-1 was observed within 15 min and this was maintained until 60 min. However, in the absence of IRAK-4, no degradation of IRAK-1 was seen. IRAK-1 contains residues that enable it to both become autophosphorylated, at Thr-209, and phosphorylated, at Thr-387, by other kinases. It is thought that a conformational change, induced by autophosphorylation, enables phosphorylation within its activation loop leading to full activation of its kinase activity. A subsequent conformational change might enable it to interact with MyD88 and recruit TRAF-6 via motifs in its C-terminus. The degradation of IRAK-1 is deemed essential for the release of the signalling complex from TLRs and might also function as a negative regulator.
feedback mechanism terminating TLR-mediated NFκB activation \(^{107}\). Recent structural studies have provided insight into the associations between MyD88 and members of the IRAK family, known as the Myddosome \(^{110,111}\). Alone, MyD88 forms a heterogeneous population of homo-oligomers via DD interactions \(^{110}\). However, when IRAK-4 is present, stable complexes are formed between these two proteins with predictable ratios \(^{110}\). Solving of the crystal structure of the Myddosome complex further refined knowledge of its configuration revealing the presence of six MyD88, four IRAK-4 and four IRAK-2 units arranged in a left-handed helix \(^{111}\). Interestingly, although this structure suggests a sequential mechanism of protein addition, the process which interrupts the addition of MyD88 protein to the complex and converts it to the addition of IRAK-4 followed by IRAK-2 is currently unknown. Assembly of the Myddosome is yet to be delineated but its composition provides valuable information of the proteins involved in the initiating stage of a signalling cascade. The Myddosome brings IRAK-4 into close proximity with IRAK-1 enabling the phosphorylation of IRAK-1 and progression of the signal \(^{112}\).

Despite evidence pointing to IRAK-1 functionality within the MyD88-dependent pathway, there are some unexplained inconsistencies. TRAF-6, the proposed effector of IRAK-1, requires autoubiquitination for its function but IRAK-1 lacks the capacity to initiate this step \(^{113}\). In addition, recent data shows that IRAK-1 is dispensable for NFκB activation for some TLRs and might even have an alternative role in IRF activation \(^{114}\). Another hypothesis for progression of signalling from IRAK-4 involves another IRAK family member, IRAK-2, that can associate with both Mal and MyD88 via homotypic DD interactions. Two putative TRAF-6 binding motifs have also been identified and mutation of these motifs inhibited IRAK-2 functions suggesting TRAF-6 as a potential effector protein \(^{109,114}\). In addition, unlike IRAK-1, IRAK-2 displays the ability to initiate TRAF-6 polyubiquitination \(^{114}\). However, like IRAK-1, IRAK-2 does not fully account for all process downstream of IRAK-4. Although NFκB DNA-binding activity was impaired 4 h after stimulation of IRAK-2 deficient macrophages, there was no impairment seen up to 40 min \(^{108}\). In addition, the activation of JNK, p38 and ERK, after MALP-2 stimulation of IRAK-2 deficient macrophages, was not impaired suggesting that IRAK-2 is dispensable for this function \(^{108}\). It is likely then that both IRAK-1 and IRAK-2 are required downstream of IRAK-4 and perform distinct functions. One model suggests that both IRAK-1 and IRAK-2 function redundantly in the initial stages of signalling ensuring a robust response to TLR stimulation. However, within 1 h of TLR stimulation IRAK-1 exhibits degradation acting as a negative feedback mechanism preventing the overproduction of inflammatory cytokines. Meanwhile the activation of IRAK-2 is sustained, up to 8 h after stimulation, ensuring a lower but maintained level of cytokine response \(^{108}\).
The final IRAK family member associated with MyD88-dependent signalling is IRAK-M which is responsible for negative regulation. Along with the other IRAK proteins, IRAK-M contains a DD and imposes negative regulation upon IRAK-1 via homotypic DD interactions.

1.3.1.3 TRAF-6

TRAF-6 belongs to a family of six TRAF proteins. TRAF-6 was identified in 1996 through the use of a yeast two-hybrid system investigating molecules crucial for CD40 signalling. TRAF-6 is composed of a highly conserved TRAF-C domain at its C-terminus that is preceded by a coiled-coil domain and a really interesting gene (RING) domain and zinc-finger motifs at its N-terminus. It is known that, although TRAF-6 and TRAF-3 are both able to bind to CD40, only TRAF-6 induces the downstream activation of NFκB, indicating functional differences between these two TRAF proteins. Since the discovery of its role in CD40 signalling, a role for TRAF-6 has also been identified in numerous physiological processes including bone metabolism, cell differentiation, dendritic cell maturation and innate immune responses. Many, if not all, of these processes rely on the location of TRAF-6 within signalling pathways, and its ability to activate downstream signalling proteins such as NFκB and the MAPKs.

Each of the domains within the structure of TRAF-6 is essential to its function. The TRAF-C domain is the most conserved region within the TRAF proteins with 35.9% homology between TRAF-6 and TRAF-2 and homology between other TRAF family members ranging from 41.7% to 66.2%. This domain forms a trimeric mushroom head-like structure with three protein-binding pockets. The TRAF-C domain is important in TRAF-6 oligomerisation and in binding to upstream signalling proteins. Interestingly, multiple single changes of highly conserved residues within the TRAF-C domain do not affect the downstream activation of NFκB indicating a compact structure of this domain which is stabilised by multiple different residues.

Located next to the TRAF-C domain is the coiled-coil domain that is considered essential in the autoubiquitination of TRAF-6. In studies where the coiled-coil domain is abolished from its structure, TRAF-6 does not induce IKK complex activation or induction of NFκB transcription. This indicates that autoubiquitination through the coiled-coil domain is crucial for the function of TRAF-6 and the progression of signalling to downstream proteins. However, despite the role of the coiled-coil domain in autoubiquitination of TRAF-6, it is not the actual site for attachment of ubiquitin. Rather it has been proposed that it is the bridge that links TRAF-6 to one of its accessory proteins.
ubiquitin-conjugating enzyme (Ubc)-13. Ubiquitination performs two functions. Lys-48-linked ubiquitination acts as a label targeting the protein for proteasomal degradation, as in the case of IRAK-1. In contrast, lys-63-linked ubiquitination acts as a scaffold leading to the formation of signalling complexes. The autoubiquitination of TRAF-6 is lys-63-linked and enables the recruitment of transforming growth factor-β (TGF-β)-activated kinase-1 (TAK-1) through its accessory proteins TAK-1-binding protein (TAB)-1, TAB-2 and TAB-3.

Finally, the RING domain comprises the core of the ubiquitin ligase catalytic domain. Studies that altered single amino acids within this domain led to the identification of a potentially necessary but insufficient role for the RING domain in Lys-63-linked polyubiquitination of TRAF-6. The domains of TRAF-6 all seem to be linked to the process of ubiquitination either directly or through the recruitment of accessory proteins. Therefore, it seems apparent that the major function of TRAF-6 is in inducing the construction of Lys-63-linked ubiquitin chains that subsequently act as scaffolds for the recruitment of downstream proteins, in particular TAK-1.

1.3.1.4 TAK-1

Upon recruitment of TAK-1 to the TRAF-6 complex, TAK-1 becomes activated through dual phosphorylation of the Thr-178 and Thr-184 residues within the kinase activation loop. The initial phosphorylation of TAK-1 has been demonstrated to be an autophosphorylation event as a kinase-defective TAK-1 mutant remains unphosphorylated. The TAK-1 accessory protein, TAB-1, is constitutively associated with TAK-1 through a 68 αα region located on the C-terminus of TAB-1. The association of TAK-1 with TAB-1 is required but not sufficient for activation indicating that another process is also required for the progression of signalling. However, in contrast, a more recent study provided evidence that TAB-1 is not required for TAK-1 activation as a result of IL-1-induced signalling. Another inconsistency between studies relates to the dimerisation of TAK-1. One study demonstrated that TAK-1 is activated in the absence of dimerisation whereas another study observed oligomerisation of TAK-1. Two key differences between these studies may explain the different conclusions. First, in one study oligomerisation was observed only in the presence of high concentrations of TAK-1. Since high concentrations of proteins often result in the formation of complexes, this may be an artefact of the experiment. Secondly, the differing requirements for TAB-1 might be due to the manner by which the cells were activated as one was induced by IL-1-stimulation and the other by TNF and IL-1 stimulation.
An additional function potentially attributed to TAB-1 is the negative feedback of TAK-1. TAB-1 becomes phosphorylated by a downstream MAPK at Ser-423 and Thr-431 and this coincides with the down-regulation of TAK-1. A mechanism for this process may involve one of the other TAK-1 accessory proteins, TAB-3 that becomes phosphorylated on residues Ser-60, Thr-404 and Ser-506 residues as a direct or indirect result of the recruitment of p38 to the TAK-1 complex by TAB-1. The link between p38 recruitment by TAB-1 and phosphorylation of TAB-3 is demonstrated by the fact that in murine embryonic fibroblasts deficient in TAB-1, phosphorylation of TAB-3 is greatly reduced. It seems then that activated TAB-1 recruits p38 which leads to the phosphorylation of TAB-3 and the down-regulation of TAK-1 activation coinciding with negative regulation of TAK-1 activity. TAB-2 and TAB-3 both interact with TAK-1 through a region located on TAK-1 between amino acids 479 and 553. TAB-2 and TAB-3 are able to bind to ubiquitin and may function to tether TAK-1 to upstream proteins such as TRAF-6 and also downstream effector proteins through ubiquitin scaffolding. Following IL-1 stimulation, TAB-2 becomes phosphorylated at Ser-372 and Ser-524 and TAB-3 becomes phosphorylated at Ser-60, Thr-404 and Ser-506.

At the point of TAK-1 recruitment and activation the MyD88-dependent pathway bifurcates into the MAPK pathway and the NFκB pathway, which are discussed separately in sections 1.3.3 and 1.3.4, respectively.

1.3.2 TRIF-dependent signalling

1.3.2.1 TRIF and TRAM

The presence of adaptor molecules other than MyD88 and Mal were identified following the finding that macrophages deficient in MyD88 still display a delayed response to stimulation despite the immediate response being impaired. This finding led to the discovery of TRIF as the second major TLR adaptor protein that, at 712 amino acids long, is much larger than the 300 amino acid MyD88. Unlike MyD88, which is involved in signalling from all TLRs except TLR3, TRIF has been linked to signalling downstream of TLR4 and TLR3 only. The unique feature of TLR4 is that it is able to induce both the MyD88-dependent and the TRIF-dependent signalling cascades. This means that MyD88-deficient macrophages stimulated by LPS retain the capacity to signal through TRIF-dependent mechanisms and, similarly, TRIF-deficient macrophages can still signal through MyD88-dependent processes. The major difference between the two pathways is the kinetics of the NFκB response. MyD88-dependent signalling leads to an early phase activation of NFκB whilst the TRIF-dependent pathway leads to a late phase of activation.
Similarly to Mal bridging the connection between MyD88 and the TIR domain of TLR4, TRIF also utilises a co-adaptor, termed TRIF-related adaptor molecule (TRAM), for this purpose. The role for TRAM in TLR4 recruitment of TRIF is shown by the finding that macrophages deficient in TRAM show normal cytokine responses (i.e. IL-6 and TNF secretion) to stimulation via TLR2, TLR7 and TLR9 but defective responses to TLR4. In comparison, the use of TRAM as a co-adaptor has not been demonstrated for TLR3-induced signalling. In fact, stimulation of TRAM-deficient macrophages via TLR3 does not induce defective responses.

Like MyD88, TRIF-dependent signalling can lead to NFκB and MAPK activation although, unlike MyD88, TRIF can also lead to activation of the IRF transcription factors, particularly IRF-3 and IRF-7. The link into the NFκB pathway is mediated through the interaction of TRIF with RIP-1 and the link into the MAPK pathway is made through TRAF-6. This occurs through the three TRAF-6 binding motifs located on TRIF. The downstream pathway from this point is the same as in the MyD88-dependent pathway. However, the activation of IRF transcription factor occurs through an entirely separate pathway starting with the recruitment of TRAF-3.

### 1.3.2.2 TRAF-3, TBK-1 and IKKi

The requirement for TRAF-3 in signalling cascades downstream of TRIF activation was shown using TRAF-3 deficient macrophages that displayed elevated levels of IL-12 and IL-16 due to defective production of IL-10, one of the end products of TRIF-dependent signalling. TRAF-3 is involved in the recruitment of two kinases, TRAF family member-associated NFκB activator (TANK) binding kinase-1 (TBK-1) and IKKi, to the signalling complex. Upon binding to the TRIF/TRAF-3 complex, TBK-1 and IKKi become activated and go on to directly phosphorylate serine residues within IRF-3. IRF-3 resides within the cytoplasm in an inactivated state but, upon phosphorylation, dimerises and translocates to the nucleus. In the absence of TBK-1 or IKKi the majority of IRF-3 and IRF-7 is located within the cytoplasm. However, when TBK-1 or IKKi is activated, approximately 35% of IRF-3 and 95% of IRF-7 translocates to the nucleus.

### 1.3.3 MAPK-induced transcription

Within the MAPK signalling cascade are several parallel pathways and the best characterised utilise the kinases known as JNK, ERK and p38. These kinases represent only one level of kinase activity within a cascade involving numerous levels. In the case of TLR signalling, one of the major kinases at the starting point of MAPK-induced transcription is TAK-1, discussed in 1.3.1.4. TAK-1 is a MAP3K protein and directly activates the group of protein kinases known as MAP2Ks. These diverse
proteins are situated directly upstream of JNK, ERK and p38 and demonstrate specificity to one or two of these proteins. JNK is situated downstream of MKK4 and MKK7, p38 is activated by MKK3 and MKK6 and ERK activation is preceded by MKK1 and MKK2.

Despite being activated by specific and different kinases, the MAPKs do demonstrate substantial homology in terms of structure. In fact, there is an overall sequence homology of >40% through X-ray crystallographic analysis and amino acid mutation studies, two key domains for determining the specificity of interactions with both upstream MAP2Ks and downstream substrates have been identified. One domain, termed the common domain (CD), is universal to all MAPKs and is important for docking to both upstream MAP2Ks and downstream substrates. CDs are characterised by negatively charged amino acid such as aspartine and are located on the opposite side to the active site. The second region of importance is termed the ERK-docking (ED) domain and specific residues within this domain are key to substrate specificity. In fact, the exchange of two amino acids present in the ED domain of ERK to the corresponding ones in p38 alters the docking specificity of ERK to that of p38. The proposed model for substrate specificity currently involves the concept of a docking groove that comprises both the CD and ED domains. Evidence supporting this model is provided by the finding that when both domains are mutated in p38 an almost complete loss in docking to its substrate is observed.

Further specificity for substrates is provided by the dual phosphorylation motif present in all the MAPKs. This is characterised by a three amino acid chain formed of Thr-Xaa-Tyr with Xaa being Glu, Gly and Pro for ERK, p38 and JNK, respectively. However, it is not just residues and domains within the MAPKs that determine their downstream specificity. Within the structure of many transcription factors, which are targets for the MAPKs, there is a docking domain (D-domain). Each individual D-domain is about 20 amino acids long and does not demonstrate a high level of sequence homology between separate transcription factors. They do, however, contain similar regions characterised by an area of basic amino acids followed by a Lys-X-Lys motif and a triplet of hydrophobic amino acids. Interestingly, a single variation of D-domain is able to target the transcription factor Elk-1 to ERK and JNK whilst also conferring serum response factor (SRF)-accessory protein-1 (SAP-1) specificity towards ERK and p38. This might be explained by the fact that the D-domains are targeted by the docking groove of MAPKs which themselves contain specificity-determining residues within the CD and ED domains. It is likely that it is the combination of the CD and ED domains located on the MAPKs and the D-domains of their substrates that enables
the high level of specificity observed and also the ability to confer cross-talk between the parallel pathways of the MAPK signalling system.

The duration of the signalling response also determines the final output of the MAPK signalling pathways. For example, sustained ERK activation causes the differentiation of pheochromocytoma cells whereas transient activation does not \(^{150,151}\). Similarly, transient activation of JNK is considered a survival signal within host cells whereas sustained activation is linked with the induction of cell death \(^{140,152,153}\). One mechanism which confers detection of signal duration has been characterised in ERK signalling leading to transcription by c-Fos. The half-life of c-Fos is relatively short (approximately 30 min) and c-Fos only accumulates to sufficient concentrations for efficient transcription activation when phosphorylated under sustained ERK activation \(^{154}\). This means that only stimuli that induce a sustained ERK activation will lead to the expression of genes targeted by c-Fos.

The production of immune mediators through the MAPK signalling pathways is regulated mainly by the transcription factor activator protein (AP)-1. AP-1 is a collective term referring to a dimeric transcription factor composed of members of the Jun, Fos or AP-1 transcription factor (ATF) subunit families that are able to bind to the common AP-1 DNA binding site. Each MAPK has a set of transcription subunits that they can activate through phosphorylation.

### 1.3.4 NFκB-induced transcription

In mammals, there are five members of the NFκB family characterised by the presence of a 300 amino acid Rel-homology domain (RHD). These are NFκB-1 (p105/p50), NFκB-2 (p100/p52), RelA (p65), RelB and c-Rel (Figure 1-2) \(^{155}\). The RHD consists of a nuclear localisation sequence which is key to many of the proteins roles including dimerisation, DNA binding and interactions with their inhibitory proteins \(^{156}\). The NFκB proteins are able to form multiple homo- and heterodimers which allows them to preferentially target precise transcription sites and act as either repressors or stimulators of transcription depending on the proteins involved in dimerisation. For example, the p50 and p52 homodimers, which lack transcriptional activation (TA) domains, function as repressors whereas dimers that contain RelA or c-Rel are transcriptional stimulators \(^{157}\). Dimerisation also enables NFκB proteins that do not contain a TA domain, such as p50 and p52, to dimerise with RelA, RelB or c-Rel thereby acquiring a transcription stimulator function \(^{158,159}\). To prevent continuous transcription by NFκB proteins, the majority of dimers are held in an inactive state in the cytoplasm.
The five members of the mammalian NFκB transcription factor family are p100, p105, RelA, RelB and c-Rel. All members contain a Rel homology domain which itself contains a nuclear localisation sequence that is key in many roles including dimerisation, DNA binding and inhibitory protein interactions. RelA, RelB and c-Rel also contain a transcriptional activation domain that enables them to be part of a dimer stimulating transcription whereas p100 and p105 homodimers are suppressors of transcription due to the lack of a TA domain. The ankyrin repeats present only in p100 and p105 enable these transcription factors to self-inhibit dimers without the requirement for an IκB family member. The self-inhibition also involves the “GGG” motif that acts as a “hinge” region enabling the ankyrin repeats, present at the C-terminus, to bind to the RHD located at the N-terminus. Figure adapted from “The two NF-κB activation pathways and their role in innate and adaptive immunity”, 2004, G. Bonizzi and M. Karin. 

Figure 1-2: Members of the NFκB transcription factor family
by a family of proteins called inhibitor of κB (IκBs). IκBs are able to bind to the RHD of NFκB proteins preventing localisation to the nucleus. This interaction occurs via a series of 5-7 ankyrin repeats which are found in all nine IκB family members (IκBα, IκBβ, BCL-3, IκBε, IκBγ, IκBζ, IκBNS, p100 and p105)\(^{160}\). The two NFκB family members p100 and p105 are also members of the IκB family as they contain ankyrin repeats within their structures and are therefore able to act as self-inhibitors of their own transcription dimers\(^{161}\).

The initiation of NFκB mediated transcription can occur via two major branches of signalling known as the canonical (classical) and non-canonical (alternative) pathways (Figure 1-3).

1.3.4.1 Canonical pathway

The canonical pathway begins downstream of TAK-1 activation with the formation of an IKK complex which is a trimeric complex composed of two kinase subunits IKKα (85 kDa), IKKβ (87 kDa) and the regulatory subunit IKKγ (52 kDa) (also known as NFκB essential modulator – NEMO)\(^{162}\). For the IKK complex to become fully functional several phosphorylation events must take place.

Autophosphorylation of the helix-loop-helix (HLH) domain of IKKβ occurs and this has been shown to require IKKγ\(^{163}\). The regulation of this phosphorylation is dependent on the phosphorylation of the (NBD/γBD) in the C-terminal portion of IKKβ and so this is deemed the major regulation event of IKK complex activation\(^{163}\).

The IKK complex is able to phosphorylate both IκBα at Ser-32 and Ser-36 and IκBβ at Ser-19 and Ser-23, located in the N-terminus of these proteins\(^{164}\). Phosphorylation of these residues enables the recognition and polyubiquitination of IκBα and IκBβ by a protein complex called B-TrCP-SCF\(^{164}\). Subsequently, the polyubiquitination labels the proteins for proteasomal degradation. Upon IκB degradation, the RHD of NFκB proteins becomes un-suppressed enabling localisation to the nucleus and the initiation of transcription\(^{163}\). Despite the structural similarities of IKKα and IKKβ, it appears that IKKβ is the dominant kinase and is therefore the crucial subunit involved in canonical NFκB transcription\(^{164}\). In fact, IKKβ demonstrates a 30-fold higher activity towards IκBα than IKKα and is required for the induction of most NFκB targeted genes in response to TNF\(^{163,165}\). However, aside from NFκB activation, multiple roles have been assigned to IKKα but these mainly do not require IκBα degradation\(^{166-169}\). In addition to the preference of IKKβ activation over IKKα activation for IκBα, both IKKα and IKKβ demonstrate a preference for IκBα over IκBβ\(^{162}\). This might explain why IκBβ displays slower degradation kinetics than IκBα. Upon release from IκB-directed suppression, NFκB dimers localise to the nucleus where they are able to bind to numerous promoter and enhancer
Figure 1-3: The canonical and non-canonical NFκB pathways

(a) Canonical NFκB pathway

(b) Non-canonical NFκB pathway

The canonical pathway (a) requires the activation of the trimeric IKK complex formed of IKKα, IKKβ and IKKγ (NEMO). This complex phosphorylates IκBα leading to its targeting for proteasomal degradation. Upon IκBα degradation, the NFκB dimer is free to translocate to the nucleus and initiate transcription. The major NFκB dimer involved in the canonical pathway is composed of p50 and RelA (p65).

The non-canonical pathway (b) does not require a trimeric IKK complex, instead utilising a dimer formed of two IKKα subunits. This complex phosphorylates two serine residues located at the C-terminus of p100 that subsequently results in the proteasomal degradation of the C-terminus end with sequences present in the hinge region preventing complete degradation. Removal of the ankyrin repeat region releases p100 from self-inhibition and allows the NFκB dimer to translocate to the nucleus and initiate transcription.
regions containing κB sites with the sequence GGGRNNYYCC (N = any base, R = purine, Y = pyrimidine) \(^{162}\).

### 1.3.4.2 Non-canonical pathway

Although IKKα does not appear to play a major role in the canonical branch of NFκB transcription, it performs a critical role in the non-canonical branch where IKKβ and IKKγ are not required \(^{162}\). The only NFκB family member that is vital to the non-canonical branch is p100 \(^{160,170}\). Phosphorylation of p100 by IKKα occurs at two serine residues located at the C-terminus. Subsequently, p100 undergoes polyubiquitination mediated by the B-TrCP-SCF E3-ligase complex \(^{171}\). Similarly to the IκB proteins, polyubiquitination labels p100 for degradation. However, p100 undergoes only partial proteolysis that is regulated by the presence of a glycine-rich region between amino acids 376 and 404 that serves as a stop signal \(^{162}\). This ensures that the N-terminal portion of p100 containing the RHD is released and, combined with its usual dimerisation partner RelB, localises to the nucleus and initiates transcription \(^{155}\).

### 1.3.4.3 Regulation of NFκB

Despite there being only 5 NFκB family members there still exists a broad range of target sites where NFκB transcription can occur. This indicates that there must be control mechanisms present that confer a degree of specificity and regulation of the sites targeted and of the duration of binding in response to specific stimuli. Indeed, it has been observed that in response to just TNF stimulation there exists two phases of NFκB activation \(^{172}\). The end result of NFκB signalling appears to also be reliant on the oscillations of its transport from an inactivated state in the cytoplasm to an activated state in the nucleus \(^{173-175}\). Sustained stimulation of signalling by TNF induced substantial oscillations in NF-κB activation \(^{174}\). Alterations in oscillation frequency, amplitude and duration can be brought about through modulation of the mode of stimulation \(^{175}\). For example, maintained expression of RANTES (regulated upon activation, normal T-cell expressed, and secreted) is induced by repeated short pulses of TNF stimulation \(^{176}\). These pulses can be modulated to generate a frequency of NF-κB activation of 100 min and 200 min, although only the 100 min frequency is able to maintain RANTES expression \(^{176}\). Similarly, expression of late but not early genes is brought about by high-dose TNF stimulation that induces NF-κB oscillations with a long duration \(^{177}\). The same late gene expression is not induced by short duration NF-κB oscillations \(^{177}\). Therefore, it is likely that the consequences of signalling may depend, not only on the activation state of NFκB, but on the number, duration and amplitude of oscillations \(^{173}\). As the response depends so heavily on precise modulation of NF-κB activation it is clear that strict regulation of NF-κB activation must be present.
The IκB family members confer one mechanism of regulation upon NFκB. There are 7 IκB proteins and studies on these proteins have reported differing regulatory functions for each one. One function is the negative feedback associated with IκBα inhibition. IκBα synthesis is tightly controlled by a highly NFκB responsive promoter generating autoregulation of NFκB signalling. Simply, upon IκBα degradation, NFκB is released inducing transcription and the synthesis of more IκBα which then binds to NFκB, ceasing transcription. This results in IκBα-controlled transcription displaying a rapid and oscillatory profile. The function of two other IκB proteins, IκBβ and IκBε, were examined in cells containing only these two inhibitors. In this case, NFκB activation displayed a slower response that did not display any subsequent repression. Combining the observed profiles of all three IκB proteins generates a regulatory mechanism whereby the slower response of IκBβ and IκBε act to dampen the long-term oscillations of IκBα. The result is that short-term stimulation of the pathway induces a robust response, reliant on IκBα degradation, and prolonged stimulation generates a response proportional to the duration of the stimulation controlled by IκBβ and IκBε.

IκBα, IκBβ and IκBε act in the cytoplasm of the cell by masking the NLS of NFκB proteins. However, the other IκB family members are located within the nucleus and interact with NFκB at this location to regulate transcription. For example, Bcl-3 functions to elongate the inhibitory role of p50 dimers within the nucleus by blocking proteasomal degradation and stabilising the p50 complex. Regulation is not just provided by the IκB proteins. Within the nucleus, post-translational modification of RelA, through phosphorylation of Ser-376, promotes interactions with the histone acetyltransferases cyclic-adenosine monophosphate (cAMP)-response element-binding (CREB)-binding protein (CBP) and p300 and aids in the displacement of histone deacetylase (HDAC) proteins, thereby promoting binding of RelA. Studies have observed that this mechanism is only important to a subset of NFκB-regulated genes such as those that are rapidly induced by TNF-stimulation, such as IL-8. Phosphorylation is not the only post-translational modification and acetylation of RelA has also been seen to provide enhancement of transcriptional activation.

1.3.5 TLRs – Cooperation, integration and priming

It is true that one source of specificity programmed into the innate TLR signalling response is generated by the range of TLRs that are expressed by cells with each one responding to a certain set of ligands. However, the individual pathways induced by TLR activation do not solely act in isolation. The range of potential responses is expanded further by the cooperation and integration of multiple
signalling pathways. This is possible when more than one TLR is activated by an invading pathogen, resulting in the initiation of at least two signalling cascades which are able to interact and produce a refined response.

Signalling cascade cross-talk may produce a synergistic or enhanced response when compared to a single cascade acting in isolation. This has been observed in murine dendritic cells simultaneously stimulated with both a TLR4 and a TLR2 ligand \(^{40}\). Stimulation with the TLR4 ligand, LPS, or the TLR2 ligand, Pam3CSK4, induced either low or no production of IL-10. However, when both ligands were used a synergistic production of the anti-inflammatory cytokine IL-10 was observed, although no synergy was seen in the secretion of the pro-inflammatory cytokines IL-12p40 and TNF \(^{40}\). This study demonstrates the precise and selective nature of signalling synergy, being able to enhance one response whilst not affecting another. In this case, the synergistic interaction was traced to the MAPK pathways involving p38 and JNK that displayed a synergistic enhancement of activation upon simultaneous ligand stimulation. Similar cooperation has also been observed between TLR2 and TLR3 in dendritic cells demonstrating that signalling crosstalk can occur, not only within one type of major pathway but, between the MyD88-dependent and TRIF-dependent pathways \(^{181}\). Interestingly, the cooperation between TLR2 and TLR3 consists of both synergy and cross-inhibition in that the production of TNF, IL-6 and IL-12p40 is enhanced but the secretion of MIG, IP-10 and IL-12p35 is reduced when both receptors are stimulated simultaneously \(^{181}\). The function of crosstalk between the two major pathways may be to generate a greater response than that which can be generated by activation of either pathway alone whilst still retaining a level of control and integration.

However, it is not just simultaneous stimulation that gives rise to synergistic or cross-inhibited signalling. The prior stimulation of cells with a certain ligand has been shown to result in enhanced or reduced responses to subsequent stimulation by the same or a different ligand. The enhancement of a response is known as priming and the reduction of a response is known as tolerance or cross-tolerance, depending on whether it is due to subsequent stimulation by the same or a different ligand, respectively \(^{182\text{-}184}\).

One signalling pathway that has been the focus of much research into priming and cross-tolerance is the TLR9-induced pathway. In 2000, TLR9 was identified as the TLR responsible for the detection of bacterial DNA through unmethylated CpG motifs \(^{67}\). The fact that this is such a ubiquitous ligand, present in bacteria and viruses, could explain why so many studies have investigated the potential for TLR9 to integrate with other TLR signalling pathways. One study of the effect of TLR2, TLR4 or
TLR9 stimulation on tolerance and cross-tolerance observed that repeated stimulation with the same ligand induced tolerance. In contrast, and unlike TLR2-directed priming that induced cross-tolerance to subsequent TLR4 stimulation, priming of macrophages through TLR9 actually enhanced TNF production upon subsequent LPS stimulation. Of note is the fact that priming appears to be time dependent as 1 – 3 h pre-treatment with CpG enhanced TNF production, in response to LPS stimulation, whilst suppression of TNF production was seen after 6 – 9 h pre-treatment. An enhanced response was also observed in dendritic cells which secreted 5 times more IL-12p40 and IL-12p70 when pre-treated (primed) with CpG prior to LPS stimulation. This response is time-dependent with an optimal delay after CpG priming of 4-6 h, which may be explained by the time taken for CpG to be taken up by the cells and to come into contact with the intracellularly expressed TLR9. Similarly to simultaneous TLR2 and TLR4 stimulation, CpG priming does not result in the up-regulation of the whole innate immune response but just a portion, as demonstrated by another study that showed a suppression of IL-6 in response to LPS stimulation after CpG pre-treatment. Therefore, CpG priming appears to enhance IL-12 and TNF production while decreasing the level of IL-6 in response to LPS stimulation. However, it should be noted that the type of integration and cross-talk between TLR signalling pathways may be highly cell-type and situation dependent. For example, in RAW264.7 macrophages stimulation with LPS induced tolerance towards a subsequent LPS stimulation. However, in alveolar macrophages tolerance is not observed and, in fact, the opposite occurs. Stimulation of alveolar macrophages with LPS resulted in enhanced secretion of IL-6 upon subsequent stimulation with LPS with an associated reduction in the secretion of cytokines.

More recently, the inverse relationship between TLR4 and TLR9 has been studied where TLR4 is the priming event and TLR9 is the subsequent stimulation. Following LPS stimulation, TLR9 gene expression is up-regulated and reached a peak level of expression 3 h after stimulation. This was thought to be due to the ERK, p38 and NFκB signalling pathways. This could provide an explanation for the observation that rather than inducing tolerance, LPS pre-treatment primes TNF and IL-6 production by murine bone marrow-derived macrophages (BMDMs) to the TLR9 agonist, CpG. Similarly to that seen in the previous study, where TLR9 gene expression was up-regulated, an enhanced ERK and p38 activation correlated with the priming effect of LPS prior to CpG stimulation. A further refinement of the increased TLR9 expression mechanism proposed previously is suggested to involve the macrophage growth factor M-CSF (macrophage colony-stimulating factor). When M-CSF binds to its cell surface receptor c-Fms, one of the results is the down regulation of TLR9 expression. It has previously been reported that LPS is able to reduce the surface expression of c-Fms at very low concentrations. It is possible then that LPS priming of
macrophages induces a down-regulation of c-Fms resulting in the removal of the suppression of TLR9 gene expression. This, in turn, leads to an increased expression of TLR9 and an enhanced response to subsequent CpG stimulation \(^{189}\). In contrast, a previous study demonstrated a suppression of CpG-induced TNF production after LPS pre-treatment \(^{184,187}\). However, a 24 h delay between LPS pre-treatment and CpG stimulation was used in the study displaying tolerance compared to a 2 h gap in the study showing priming. This is likely to be the reason for the contrasting reports of these two studies and clearly shows the sensitivity and critically time-dependent nature of TLR signalling and integration.

Further evidence for TLR4 and TLR9 cooperation was provided by a study investigating the interaction between these two receptors in a murine model of Gram-negative bacterial pneumonia caused by *Klebsiella pneumoniae* \(^{193}\). In wild-type mice, bacterial infection resulted in the rapid increase in the expression of several cytokines including IL-12, TNF, IL-17 and interferon-γ (IFNγ). In mice lacking either TLR4 or TLR9 the secretion of IL-17 was moderately reduced but in double TLR mutants IL-17 secretion was almost completely abolished \(^{193}\). In addition, TLR4 contributed significantly to the early production of IL-12 and TNF whilst later expression was more reliant on TLR9. It appears then that TLR4 and TLR9 have distinct time-dependent functions but also interactive and cooperative roles in the production of a response \(^{193}\).

### 1.3.6 Role of TLRs in innate immunity

#### 1.3.6.1 Chemokines

One process that is key to combating infection is the recruitment of immune cells to the site of infection. The recruitment process can be split broadly into two categories. Homeostatic migration involves the passive movement of cells and does not require the secretion of chemotactic molecules \(^{194}\). In contrast, inducible migration requires the secretion of chemokines to attract circulating immune cells specifically to the site of infection \(^{194}\). TLRs play an important role in the preliminary secretion of chemokines such as IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1a and RANTES, thereby attracting the immune cells by inducible migration \(^{195,196}\). However, TLR signalling is also heavily involved in the up-regulation of certain cell surface receptors called selectins on endothelial cells. These receptors bind to carbohydrate ligands on leukocytes enabling movement of these cells along the endothelial surface \(^{197}\). The increase of receptor expression is likely to enhance the capture of immune cells moving round the system via homeostatic migration. In addition, the recruited leukocytes near the site of infection encounter
TLR-induced chemokines bound to the vascular endothelium and become activated allowing adhesion and translocation across the lumen wall into infected tissues 197,198.

1.3.6.2 Cytokines

Despite many of the TLRs sharing common signalling pathways they are able to induce the secretion of specific sets of cytokines in response to different TLR agonists. Added flexibility of the response is observed as the same agonists can induce differing cytokine secretion profiles depending on the type of cell stimulated or when signalling pathway cooperation occurs 40,130,199,200. One set of cytokines known as type I interferons are mainly produced after activation of TLRs which can use TRIF as a downstream adaptor, specifically TLR3 and TLR4, as they both have the potential of inducing the range of IRF transcription factors 80,98,201. Type I IFNs are implicated in the control of viral infections although they also have the ability to induce the production of a variety of proteins through binding to their own receptors 137,202,203. If type I IFNs are the characteristic cytokines produced in response to viral infection, IL-6 and TNF are the characteristic anti-bacterial cytokines implicated in the control of many bacterial pathogens, such as Legionella pneumophila and Mycobacterium tuberculosis 204,205. IL-6 performs many and diverse roles acting as both a pro-inflammatory and anti-inflammatory cytokine depending upon cell-type and location 206-209. TNF, on the other hand, is a major pro-inflammatory agent and is an essential component of the arsenal used against bacterial pathogens. The secretion and full effects of TNF are tightly controlled by the innate immune system as, due to its highly pro-inflammatory characteristics, it has the capacity to negatively affect the host. In fact the dysregulation of TNF secretion is associated with several autoimmune diseases, rheumatoid arthritis and septic shock 210,211.

1.3.6.3 Induction of adaptive immunity

As mentioned previously, the innate immune system is responsible for the induction of the adaptive immune response and TLR signalling is part of this process. The adaptive response not only requires the presentation of antigen bound to major histocompatibility complexes (MHCs) on the surface of APCs but also the associated signals generated by these cells via the TLR detection system 1. A large proportion of APCs are formed of DCs that require a maturation stage before they are able to effectively activate naïve T cells. DCs deficient in MyD88 demonstrate severe defects in the up-regulation of several cell surface markers such as MHC class II in response to mycobacteria, indicating a lack of maturation 1. In MyD88-deficient mice a similar defect in the induction of the
adaptive immune response was observed, characterised by a lack of Th1 responses although Th2 responses were induced indicating an alternative priming mechanism for these effects.

1.4 Francisella tularensis

1.4.1 Strains and symptoms

*Francisella tularensis* is a non-motile, gram-negative, facultative intracellular bacterium. The bacterium was initially isolated from ground squirrels in Tulare County, California, just over 100 years ago and was originally classified as *Bacterium tularense* by George Walter McCoy and reported as a plague-like disease of rodents in 1912. Since then the classification of this bacterium was refined and it was renamed to *Francisella tularensis*.

*F. tularensis* causes a spectrum of diseases, depending on the route of infection, collectively termed tularaemia. Tularaemia is a zoonotic disease as it survives in the environment, primarily in mammals such as rabbits and rodents as well as in arthropods such as ticks and flies. The usual infection route for humans is through an arthropod vector although *F. tularensis* can infect humans via a number of routes including inhalation of aerosolised bacteria, ingestion of contaminated food or water or through cuts and abrasions in skin coming into contact with contaminated animal hides. The route of entry is important in determining the clinical presentation of the infection.

Ulceroglandular tularaemia is characterised by an ulcer at the site of infection and swelling of the regional lymph node. Oropharyngeal tularaemia occurs within the mouth and lymph nodes in the neck display severe swelling. The inhalation of aerosolised bacteria causes pneumonic tularaemia which has the highest mortality rate of 30%, although this can be reduced to 2% with rapid administration of antibiotics. Independent of the route of entry, *F. tularensis* proliferates within the host and migrates from the primary site of infection to the liver and spleen, the major secondary sites of infection. Simultaneously with colonisation of secondary sites, the disease develops into typhoidal tularaemia leading to septicaemia. Symptoms of tularaemia include headache, fever, chills, nausea, diarrhoea, and myalgia. The ability of *F. tularensis* to rapidly and successfully colonise the host is demonstrated by the fact that the infectious dose of *F. tularensis* can be as low as 10 organisms via the inhalational route.

There are now four subspecies of *F. tularensis* classified which share about 97% genome identity. These are subspecies *tularensis*, *holarctica*, *mediasiatica*, and *novicida*. Of these four, only *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* are thought capable of causing disease.
in humans. *F. tularensis* subsp. *tularensis*, also known as type A, is found mainly in North America and is the most virulent subspecies, causing the most severe forms of tularemia. *F. tularensis* subsp. *holarctica*, also known as type B, is found throughout the northern hemisphere in North America, parts of Europe and Asia and causes a more mild form of tularemia. More recently, it has been suggested that type A *F. tularensis* could be further subdivided into A1a, A1b and A2 categories based on differences in observed virulence. Type A1 infections are characterised by a high mortality rate whereas type A2 strains rarely lead to a fatal outcome.

### 1.4.2 Bioterrorism agent

*F. tularensis* is able to cause high morbidity and mortality, to infect humans via multiple routes, and has a very low infectious dose. These characteristics made *F. tularensis* a prime candidate for use as a biological warfare agent and in the 1950s both the USA and the USSR succeeded in weaponising *F. tularensis*. As such, the United States Centres for Disease Control have classified *F. tularensis* as a category A select agent, the same category as pathogens such as *Bacillus anthracis* (anthrax) and *Yersinia pestis* (plague). This classification for potential use as a bioterrorism agent and the current worldwide interest in biodefence has led to a massive upsurge of research with this bacterium. Despite the development of *F. tularensis* for use as a biological warfare agent in the 1950s, there is no currently licensed vaccine against this pathogen. Previously, an attenuated *F. tularensis* subsp. *holarctica* strain, denoted the live vaccine strain (LVS), has been used as a vaccine. This was generated in the USSR by serial passage through mice.

However, the causes of attenuation in this strain are not well defined as it appears to involve massive genomic rearrangement as opposed to specific mutations. As such, this strain is not licensed for use as a vaccine in the US or the UK and the development of a well-defined attenuated mutant strain or other vaccine is of interest. Despite being unsuitable for license as a vaccine, the use of *F. tularensis* LVS in a murine model of infection is a useful tool with which to study human infection as *F. tularensis* LVS retains virulence in mice when given intraperitoneally or intranasally and the course of infection shows similarities to that observed in humans. Current therapies for human tularemia involve a rigorous course of antibiotics. Antibiotics used to treat tularemia include streptomycin, gentamicin, doxycycline, and ciprofloxacin. However, due to the potential development and dissemination of antibiotic-resistant strains, and an associated mortality rate of 30% in patients with severe tularemia without rapid treatment with antibiotics, further research into novel therapeutics is required.
Due to the severity of disease, particularly associated with inhalational tularemia, and the lack of a licensed vaccine, laboratory research using the majority of *F. tularensis* strains, such as the highly virulent SchuS4 strain, is carried out under Advisory Committee on Dangerous Pathogens (ACDP) category III containment (http://www.hse.gov.uk/pubns/misc208.pdf).

### 1.4.3 Infection cycle

*F. tularensis* is a facultative intracellular pathogen and, as such, much of its life cycle occurs within host cells. This requires the bacterium to enter host cells, evade host-derived pathogen-killing mechanisms, replicate within the cells and then escape from the host cell in order to proliferate around the body.\(^{238}\) The processes and mechanisms by which *F. tularensis* accomplishes these different stages are discussed in the following sections and a diagram outlining the life cycle of *F. tularensis* is shown in Figure 1-4.

#### 1.4.3.1 Host cell entry

*Francisella tularensis* is capable of invading numerous cell types including epithelial cells, alveolar macrophages, dendritic cells, and neutrophils.\(^{212,239-242}\) To gain entry to these cells *F. tularensis* has to cross the cell membrane without initiating an immune response or killing the cell.

Two mechanisms of internalisation have been characterised in other intracellular pathogens. *Neisseria meningitidis* and *Pseudomonas aeruginosa* are internalised by conventional phagocytosis, a process where the bacterium is observed to sink into the macrophage surrounded by multiple pseudopodia.\(^{243}\) In contrast, *Legionella pneumophila* and *Serpulina perlisicoli* are internalised by coiling phagocytosis involving a single pseudopod which tightly coils around the bacterium.\(^{243,244}\) However, *F. tularensis* has been observed entering macrophages by a novel process involving engulfment of bacteria within asymmetric, spacious pseudopod loops.\(^{241}\) The pseudopod loops were apparent on the surface of macrophage-like THP-1 cells within 5 min of incubation with either virulent *F. tularensis* or avirulent LVS.

The process of phagocytosis, either conventional or coiling, involves complement and complement receptors in studies using other intracellular pathogens such as *Mycobacterium tuberculosis*.\(^{245}\) Complement was therefore examined as an initiation mechanism for *F. tularensis* internalisation. It was observed that the uptake of LVS and a virulent strain increased as the percentage of serum in the incubation medium increased. In addition, incubation of LVS with HeLa cells demonstrated a very
Figure 1-4: The life-cycle of *Francisella tularensis* within the host cell

*F. tularensis* enters the host cell through phagocytosis using a novel pseudopod looping mechanism. Upon phagocytosis, *F. tularensis* resides within a phagosome that acquires both early (EE) and late (LE) endosomal markers but does not fuse with lysosomes (Lys). *F. tularensis* rapidly escapes the phagosome through an unknown mechanism and replicates within the cytosol. After sufficient replication, *F. tularensis* causes host cell death by apoptosis releasing the bacteria to proliferate to neighbouring cells. In murine cells, an alternative pathway has been observed involving reintegration of bacteria into the endosomal pathway by the formation of a *Francisella*-containing vacuole (FCV). Whether this alternative route is host-induced or pathogen-induced is yet to be determined. Figure reproduced from “The Francisella intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation”, 2010, A. Chong and J. Celli.
low level of uptake. HeLa cells, unlike professional phagocytes, do not express mannose, Fc or complement receptors. It was hypothesised that the lack of these receptors explained the poor internalisation. This hypothesis was confirmed by other studies which observed a role for both complement receptor 3 (CR 3) and Fcγ receptors in macrophage uptake of F. novicida. The use of complement receptors to initiate phagocytosis can be termed a stealthy method of entry into cells as internalisation in this way avoids triggering an oxidative burst which can be initiated through other entry routes and is detrimental to bacterial growth. Despite evidence suggesting that complement and complement receptors are important in the uptake of F. tularensis, the target of complement fixation is currently unknown. However, the fact that formalin-killed, heat-treated or protease-treated preparations of F. tularensis are taken up by macrophages suggests that the target for complement binding is a very stable molecule, possibly LPS.

1.4.3.2 Escape from phagosomes

Once internalised by a host cell, intracellular pathogens must evade host cell defence mechanisms, the majority of which are focussed at the phagosomal stage. Three pathways are recognised that enable the pathogen to do this. The extraphagosomal pathway is characterised by rapid degradation of the phagosome membrane releasing the pathogen into the cytoplasm, as occurs in infection with Listeria monocytogenes and Shigella flexneri. The phagolysosomal pathway requires the pathogen to be resistant to acidic environments as it remains within phagosomes which have fused to lysosomes, as occurs in infection with Coxiella burnetii. Finally, the phagosomal pathway involves the pathogen remaining within a phagosome that does not fuse with lysosomes, as occurs with Legionella pneumophila. Of these, F. tularensis follows the extraphagosomal pathway. The phagosomes containing F. tularensis firstly acquire the early endosome marker, effector early endosomal antigen 1 (EEA1), followed by late endosomal markers such as lysosomal-associated membrane protein 1/2 (Lamp-1/2), Cd63 and Rab7, indicating maturation of the phagosomes along the endocytic pathway. Although lysosome fusion does not occur, the phagosomes become acidified by acquiring the vacuolar ATPase. Acidification has been shown to be important for bacterial escape as inhibition of the ATPase results in a delay of bacterial escape from the phagosome.

F. tularensis replicates within the cytoplasm of host cells and, as such, must leave the phagosome compartment. Using transmission electron microscopy, Golovliov et al. visualised LVS free in the cytoplasm 2 h after infection of three separate cell lines, including both human and mouse macrophages. Several other studies have also observed a similar rapid escape for LVS and a virulent, clinical isolate of F. tularensis. As prevention of replication represents a possible
therapeutic strategy, significant research has been focussed on the process of, and genes required for, phagosomal escape. Despite this, little is known about the exact mechanism apart from the fact that soon after internalisation the phagosomal membrane becomes disrupted and degraded, allowing *F. tularensis* access to the cytoplasm.

Other intracellular bacteria, such as *L. monocytogenes*, that have to escape the phagosome before replicating have been shown to utilise pore-forming lysteriolysin and phospholipases, for example [259]. However, *F. tularensis* does not seem to use either of these mechanisms. Although the process of phagosomal escape of *F. tularensis* is not fully characterised, experiments using mutant forms of LVS have shed a little light on the genes that are important. The genome of *F. tularensis* contains a region known as the *Francisella* pathogenicity island (FPI) which includes four genes collectively known as the intracellular growth locus operon. The transcription of these genes is regulated by the global regulator MglA and one gene in particular, *iglC*, is up-regulated during intracellular growth [260]. Infection of peritoneal exudate cells (PECs) with an LVS *iglC* mutant (Δ*iglC*) demonstrated the importance of this gene in phagosomal escape. It was observed that < 1% of the mutant bacteria were able to escape compared to 97% of wild-type bacteria [258]. The reduction in the ability to escape is also seen in the J774A.1 cell line when infected with Δ*iglC* [254]. The importance of the FPI in phagosome escape may lie in the suggestion that it encodes a type VI-like secretion system [261]. The role of a type VI secretion system is to insert bacterial effector proteins directly into the surrounding environment, in this case, the phagosome. These bacterial proteins may be cytolysins, pore-forming toxins or hydrolytic enzymes, all of which are used by other intracellular pathogens. However, none of the genes identified within the FPI of *F. tularensis* display any of the characteristics of proteins used by other pathogens indicating that the process used by *F. tularensis* to enable phagosome escape is a previously uncharacterised one. Alternatively, the effector proteins secreted through the type VI-like system are encoded elsewhere in the genome and have yet to be located and characterised.

The primary host defence mechanisms are located within the phagosome and so, upon its escape, *F. tularensis* succeeds in separating itself from the majority of the host defences. Within the relative safety of the cytosol, *F. tularensis* rapidly replicates until it induces apoptosis and proliferates throughout the host. Interestingly, although immune defence mechanisms are in short supply in the cytosol, *F. tularensis* is not free from detection and the subsequent activation of the host cell defences.
Further evidence demonstrating an active role for *F. tularensis* survival within the cytosol is provided by the fact that, to date, at least 268 genes have been identified that are important for intracellular replication\(^\text{262}\). The surprisingly high number of genes that are required for intracellular replication indicates that replication within the cytosol may not be straightforward\(^\text{262}\).

### 1.4.3.3 Autophagy

Autophagy is a process whereby cells can deliver cytoplasmic substrates for lysosomal degradation and can occur via three pathways known as chaperone-mediated, micro- and macro-autophagy\(^\text{263}\)-\(^\text{265}\). Macro-autophagy is particularly important in relation to clearance of intracellular pathogens as it is the only mechanism by which cells can dispose of cytoplasmic substrates too large for destruction through proteasomal degradation\(^\text{263}\). One major difference of macro-autophagy from chaperone- or micro-autophagy is the requirement to engulf the target in a double-membrane vesicle called an autophagosome prior to fusion with lysosomes. Engulfment of a target substrate involves the formation of an isolation membrane formed at membrane sites marked with phosphatidylinositol-3-phosphate (PI3P) and the sequential activation of two ubiquitin-like systems\(^\text{263,266,267}\). The subsequent targeting of autophagosomes by lysosomes is facilitated by Ras-related in brain-7 (Rab-7) and Beclin-1\(^\text{268,269}\). In the context of intracellular bacterial infections, macro-autophagy does not only lead to the lysosomal-degradation of the pathogen but also limits its proliferation and can reduce the possibility of pathogen-induced manipulation of cellular processes and signalling events\(^\text{263}\).

The role of autophagy in several bacterial infections has been examined. Restriction of cytoplasmic colonisation has been observed in *Salmonella enterica* and *Streptococcus pyogenes* infection\(^\text{270,271}\). However, bacteria have also developed mechanisms of autophagosome evasion. Recently, the mechanism of autophagy-activation in response to *L. monocytogenes* has been elucidated and was demonstrated to involve the activation of TLR2 and RIP2 signalling pathways and particularly ERK activation\(^\text{272}\). Despite activation of autophagic pathways *L. monocytogenes* evades efficient macro-autophagy by coating itself with the host protein actin nucleator A (ActA)\(^\text{272}\). This “disguise” prevents ubiquitin binding to its outer surface which would usually target the bacteria for engulfment into an autophagosome. In contrast to evading autophagy, some bacterial species, such as *S. aureus*, use autophagy to assist their survival using the autophagosome as a replicative niche or, as is the case for *Brucella abortus*, use autophagosomes as a means of transport from cell to cell\(^\text{273-275}\).
Although *F. tularensis* rapidly extracts itself from the phagosome in order to replicate in the cytoplasm, it is becoming apparent that at later time points during infection *F. tularensis* is able to re-enter into the endocytic pathway causing the formation of a *Francisella* containing vacuole (FCV) (Figure 1-4). FCV formation was first observed by Checroun et al. in 2006 as clusters of bacteria, 24 h post-infection, were unable to be bound by stains delivered into the cytoplasm \(^{276}\). This was the case for both LVS and SchuS4 strains and the vacuoles were identified to be of autophagic origin due to monodansylcadaverine (MDC) staining \(^{276}\). Unlike other bacteria that are targeted by autophagy, a significant delay of up to 16 h was observed before *F. tularensis* was seen within FCVs \(^{270,276,277}\). This result indicates that *F. tularensis* may be preventing progression of the autophagic process until sufficient replication has taken place. Recent, re-examination of a microarray analysis of host cell signalling pathways affected by *F. tularensis* infection provided some clues for the delay in autophagy \(^{278}\). Beclin-1, involved in the formation of an isolation membrane, and several ATG genes that are required for elongation of autophagosomes were down-regulated as a result of *F. tularensis* infection of human peripheral blood monocytes \(^{278}\). Autophagosomes usually express high levels of MHC class II which could be detrimental to the progression of infection \(^{279}\). However, microarray analysis also showed down-regulation of several MHC class II-related genes indicating that *F. tularensis* may also be modulating the efficient presentation of antigen \(^{278}\). The function of re-entry into the endocytic pathway has not yet been elucidated. It could be that the acidic environment of the autophagosome provides a cue to induce genes involved with escape from the cell or re-infection of neighbouring cells. Alternatively, as is the case for *B. abortus*, autophagosomes may enable the spread of *F. tularensis* from cell to cell \(^{275}\).

An important point to note is that, in contrast to previous studies, a recent paper failed to observe the re-entry of *F. tularensis* into the endocytic pathway at late stages of infection in human cells (HEK-293T) \(^{280}\). This indicates that the down-regulation of Beclin-1 and ATG proteins observed by microarray in human monocytes may not only delay autophagy but could actually prevent its occurrence entirely. Furthermore, studies using *F. tularensis* SchuS4 observed that only replication-deficient mutants were observed within autophagosomes in human macrophages \(^{281}\). These contrasting reports between murine and human cells clearly demonstrate the care that must be taken when comparing data derived from different species.

### 1.4.3.4 Regulation of apoptosis

Cell death can occur via several routes including pyroptosis, necrosis and apoptosis. Each of these routes activates the surrounding cells in a different way. Pyroptosis and necrosis initiate a significant
inflammatory response due to total cell lysis, resulting in an uncontrolled release of inflammatory activators. Apoptosis, or programmed cell death, is a controlled event where the cell fragments into smaller bodies that contain inflammatory mediators thereby preventing the activation of inflammatory pathways. It is not surprising then that a number of intracellular pathogens including *F. tularensis* cause cell lysis via the apoptosis route. Hrstka *et. al.* found that LVS induced apoptosis of J774.2 cells by 12 h post-infection.

Apoptosis is a well-characterised mechanism of cell death and is known to be initiated via two pathways. The extrinsic pathway involves receptor activation whereas the intrinsic pathway is mitochondria-mediated. Despite different origins, both pathways follow a similar, sequential cascade pattern in which an initiator pro-caspase is cleaved, leading to the cleaving of an executioner caspase. Apoptosis initiated by *F. tularensis* acts through the intrinsic, mitochondria-mediated route. It is known that cytochrome-c release from mitochondria is an early step in the intrinsic pathway and western blots indicated a four-fold increase in cytochrome-c levels in the cytosol of LVS-infected macrophages. In addition, western blots showed the cleaved product of the initiator caspase, pro-caspase-9 and the executioner caspase, pro-capsase-3 from 12 h after infection.

A recent study examining the p38, ERK and JNK pathways revealed another possible mechanism that could contribute to apoptosis. Hrstka *et. al.* found that interrupting the ERK pathway inhibited macrophage apoptosis. In contrast, inhibition of p38 activity induced apoptosis in uninfected cells and a reduced p38 activity was seen in LVS-infected cells. The conclusion drawn was that LVS-induced apoptosis of macrophages requires activation of the ERK pathway and reduced activation of the p38 pathway. Another MAPK that has been linked to the induction of apoptosis is JNK. Similarly to ERK, it is the sustained activation of JNK that induces apoptosis. In contrast, the transient activation of JNK is thought to be linked to cell survival strategies. These studies together indicate that there are at least three routes to apoptosis as sustained activation of both the ERK and JNK pathways and reduction of p38 activation all lead to cell death via the apoptotic pathway.

Studies using LVS deficient in *iglC* demonstrated a possible role for this gene in the induction of apoptosis. Lactate dehydrogenase (LDH) release from cells is a good indicator of cell apoptosis and *iglC* mutants caused the same amount of LDH to be released as uninfected J774A.1 cells indicating that the mutants could not induce apoptosis. This could indicate that *iglC* has a direct role in
inducing apoptosis or that an indirect role exists, for example that the escape from the phagosome is an action required for activation of the apoptosome.

Apoptosis does not occur alone in an *F. tularensis* infection and cell death through pyroptosis has also been observed. Unlike apoptosis, pyroptosis is a pro-inflammatory method of cell death. Pyroptosis is a caspase-3-independent process requiring instead the activation of the caspase-1 inflammasome. This results in the release of pro-inflammatory cytokines concurrent with the loss of plasma membrane integrity and release of highly inflammatory cytoplasmic factors. It appears then that pro-inflammatory and non-inflammatory mechanisms of cell death are induced in the course of a *F. tularensis* infection.

Irrespective of the mechanism by which apoptosis is brought about, this process completes the intracellular phase of *F. tularensis* and allows the bacteria to escape host cells with minimal activation of the inflammatory response.

### 1.4.4 *F. tularensis* and TLRs

Many studies have examined the response of the host innate immune response to infection by *F. tularensis* species. However, many gaps in knowledge remain. The primary detection of *F. tularensis* was originally thought to be mediated by TLR4, although more recent studies show that it is likely that TLR2 not TLR4 confers the ability to detect *F. tularensis*. TLR4 was ruled out as a major receptor in the detection of *F. tularensis* as TLR4-defective mice were no more susceptible to an inhalational infection with *F. tularensis* than wild-type mice and the survival rates were also similar in both mouse strains. In contrast, several reports have shown a requirement for TLR2 in the detection of *F. tularensis* following infection by multiple routes and cell types. Furthermore, a TLR2 deficiency enhanced murine susceptibility to pulmonary infection by *F. tularensis* LVS and resulted in decreased production of proinflammatory cytokines including TNF and IL-6. In addition, TLR2 controls the hepatic lymphocyte IFN-γ response to *F. tularensis* by regulating dendritic cell IL-12 production and is involved in the mediation of the murine macrophage response to LVS as shown by co-localisation of TLR2 with the adaptor MyD88 and increased levels of cytokines and chemokines. Recently, more evidence was provided to support the role of TLR2 in *F. tularensis* detection. Two *F. tularensis* lipoproteins, termed TUL4 (or LpnA) and FTT1103, were found to be responsible for the stimulation of the TLR2/TLR1 heterodimer. The same study also indicated that *F. tularensis* might have proteins that could activate the TLR2/TLR6 heterodimer.
Further evidence against the detection of *F. tularensis* through TLR4 is that the ligand usually recognised by TLR4 is LPS. LPS from bacteria is typically highly stimulatory for TLR4 and atypical LPS generally signals through TLR2. However, LPS from *F. tularensis* does not seem to activate either TLR4 or TLR2 as suggested by the fact that *F. novicida* LPS did not activate signalling pathways in a human embryonic kidney cell line (HEK293) expressing either human or murine TLR4 or TLR2. Additional controversy surrounding the role of TLR4 and *F. tularensis* LPS is shown by contrasting reports of the susceptibility of C3H/HeJ mice which lack TLR4 and are therefore deemed unresponsive to LPS. Depending on route and challenge dose, different groups have observed increased susceptibility to subcutaneous and intravenous challenge when compared to wild-type mice. Conversely, decreased susceptibility of C3H/HeJ mice has been demonstrated through the intraperitoneal and aerosol route. More recent work has observed that the apparent unstimulatory character of *F. tularensis* LPS can be reversed in the presence of alternative pro-inflammatory factors associated with *F. tularensis*. In combination with *F. tularensis*-LPS, the heat shock protein GroEL was used to activate human monocyte-derived macrophages resulting in a synergistic enhancement of IL-8 secretion. This suggests that the low activity of *F. tularensis* LPS may be enhanced during infection through the action of other pathogen-associated factors. Furthermore, treatment of mice with *F. tularensis* LPS induces an antibody response that protects wild-type mice against a lethal challenge with *F. tularensis* LVS. This is not due to signalling of LPS through TLR2 as TLR2-deficient mice still generate the antibody response but, interestingly, are no longer protected. Full protection against LVS challenge was shown to require both the LPS-induced antibody response and macrophage activation through TLR signalling, either through TLR2 in wild-type mice or through activation of alternative TLRs in TLR2-deficient mice. It would seem that, despite the apparent low potency of *F. tularensis* LPS and the controversy surrounding its potential to signal through the TLR system, there is a definite role for LPS in the generation of an immune response to *F. tularensis* infection.

Recently, significant progress has been made in characterising the immune response to infection with *F. tularensis*. Experiments monitoring the total immune response in terms of cytokine production have demonstrated that certain cytokines, such as IL-1β and TNF-α, are produced at similar levels in response to *F. tularensis* infection via all routes whereas specific cytokines (e.g. IL-6) are up-regulated only in response to certain routes of infection. A study comparing respiratory and intradermal routes of infection with *F. tularensis* strain SchuS4 in mice showed that the pro-inflammatory cytokines IFN-γ, TNF-α, IL-6, IL-1β and IL-12p70 and the chemokines KC and MCP-1 were up-regulated in both cases. However, mRNA levels of IFN-γ were higher in respiratory...
infection whereas mRNA levels of IL-6, KC and MCP-1 secretion were higher in intradermal infection. Other experiments confirmed the up-regulation of TNF-α and IFN-γ production and also demonstrated that IL-10 mRNA was greatly enhanced in the lungs after intra-peritoneal and intra-dermal infection with LVS. Up-regulation of IL-10 in the lungs only was also seen in infections involving *F. tularensis* SchuS4.

Macrophages are the primary replication site for *F. tularensis*. The bacteria are internalised by macrophages in a phagosome from which they rapidly escape and replicate within the cytoplasm. Macrophages are perfectly suited to detecting and responding to infection as they are equipped with both membrane-bound and intracellular TLRs. The response of macrophages to *F. tularensis* requires coordination of multiple signalling pathways. Although TLR2 is able to initiate transcription and secretion of a number of cytokines in response to the detection of an *iglC* mutant, including TNF-α and IL-12p35, an inconsistency was observed between IL-1β mRNA transcription and secretion of IL-1β. As the *iglC* mutant is unable to escape from the phagosome, these results suggest that phagosomal escape and initiation of a separate signalling pathway is required for secretion of active IL-1β.

Another group of immune cells responsible for controlling infection are mast cells, which have been implicated as mediators of both innate and adaptive immunity. They are able to release immune modulators, such as cytokines, and also have the ability to phagocytose micro-organisms. In one study, after an intranasal challenge of mice with *F. tularensis* LVS, numbers of all immune cells were raised in lymph nodes but only the frequency of mast cells were increased in the lung. In addition, mast cells demonstrated the ability to affect *F. tularensis* uptake and growth within macrophages. Furthermore, mast cell-deficient mice exhibited greater susceptibility to pulmonary infection (20% survival) when compared to wild-type mice (80% survival), providing supporting evidence for the important role of mast cells in host defence against *F. tularensis*. However, it is not just immune cells that are able to respond to *F. tularensis* invasion. Alveolar epithelial cells have been implicated in host defence against LVS and SchuS4 infection. Through NF-κB activation, alveolar epithelial cells have been shown to raise levels of IL-8 and MCP-1 present in the lungs. These products are potent chemoattractants for immune cells such as neutrophils so alveolar epithelial cells may play a key role in clearing the lungs of bacteria via neutrophil invasion.
1.4.5 *F. tularensis*-induced modulation

The TLR signalling system is designed to detect and initiate a response to invading pathogens resulting in the generation of an appropriate defence strategy. However, as a first line of defence, the TLR system is also a perfect target for pathogens to inhibit or modify, thereby altering the response to a more favourable end result for the pathogen. Many studies have identified pathogens that use the TLR system to their own advantage either by altering the expression of certain factors on their cell surface, evading detection by TLRs, or by actually secreting proteins that are able to inhibit or modify host proteins within the signalling pathways themselves. Viruses are particularly efficient at altering TLR signalling pathways. For example, Vaccinia virus secretes two proteins called A52R and A46R that are able to inhibit TLR signalling. A52R binds to both TRAF6 and IRAK-2, resulting in the prevention of NFkB activation and cytokine production. A46R acts upstream of A52R and binds to all four major TLR adaptors as well as TLR4 to inhibit signalling. However, TLR inhibition is not restricted to viruses. *Salmonella enterica* secretes protein A, a TIR-like domain-containing protein, which is able to impair TLR and MyD88-associated activation of NFkB, improving the ability of this bacterium to replicate within host cells.

Unlike the examples discussed above, *F. tularensis* is not known to secrete any proteins that are able to inhibit or modify TLR signalling pathways. However, at each stage of its life cycle, from initial binding and entry through to escape and proliferation throughout the host, *F. tularensis* is modulating host signalling pathways and processes to facilitate its survival. A list of currently reported mechanisms of immune modulation is shown in Table 1-2.

*F. tularensis* has been described as a stealthy pathogen and this is certainly true of its method of entry into host cells whether immune or epithelial. As described in section 1.4.3.1, *F. tularensis* enters cells through a novel looping phagocytosis. Prior to this, the bacteria are exposed to one of the first antimicrobial mechanism deployed by the host in the form of complement. Complement consists of the formation of a protein called C3b that is able to enhance phagocytosis and also generates C5b, a component of the membrane attack complex (MAC) that functions to lyse bacteria. However, *F. tularensis* is able to prevent lysis through this mechanism through binding to another complement protein, Factor H. Factor H cleaves the active C3b into the inactive iC3b which disables the formation of the MAC. Additionally, and key to the stealthy entry of *F. tularensis* into host cells, iC3b is a ligand for the complement receptor CR3. As discussed in section 1.4.3.1, phagocytosis induced through CR3 does not induce an oxidative burst and so *F. tularensis*, by producing iC3b, not only disables the generation of MAC, preventing lysis, but also enables entry into host cells without initiating an immune response.
<table>
<thead>
<tr>
<th>Stage of life cycle</th>
<th>Modulation</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell entry</td>
<td>Complement factor H binding(^{240,319-321})</td>
<td>Cleaves C3b into inactive iC3b preventing formation of MAC</td>
</tr>
<tr>
<td></td>
<td>Tetra-acylated LPS (^{322})</td>
<td>Production of iC3b enables host cell entry via CR3 which does not induce an oxidative burst</td>
</tr>
<tr>
<td>Residence within phagosome</td>
<td>Catalase and superoxide dismutase production (^{323})</td>
<td>Production of iC3b enables host cell entry via CR3 which does not induce an oxidative burst</td>
</tr>
<tr>
<td></td>
<td>Preventing the phosphorylation of p47 subunit of NADPH oxidase (^{324})</td>
<td>Much lower stimulation of TLR4 than hexa-acylated LPS preventing activation of innate immune signalling cascade</td>
</tr>
<tr>
<td></td>
<td>Rapid disruption of phagosome membrane (^{252,257,258})</td>
<td>These enzymes neutralise the products of oxidative burst</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prevents the correct assembly of the complexes required for oxidative species production</td>
</tr>
<tr>
<td>Replication within cytosol</td>
<td>Production of immune signalling modulators: MviN and protein products of FTT_0584 and FTT_0748 (^{325,326})</td>
<td>Mutations of these genes indicate they may function as down-regulators of ASC mediated cytokine production in wild type bacteria reducing the inflammatory response</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Induction of host cell death (^{284,287,288})</td>
<td>Induction of apoptosis via the release of mitochondrial cytochrome-c ensures cell death and proliferation with a very low release of pro-inflammatory mediators</td>
</tr>
</tbody>
</table>

Table 1-2: Modulation of the immune response by *F. tularensis*
In addition to the expression of complement receptors on the surface of cells, TLRs are also present which pathogens must try and evade in order to prevent the initiation of an immune response. *F. tularensis* is able to bypass detection, particularly by TLR4, through expressing low stimulatory LPS. Unlike other Gram-negative bacteria that express hexa-acylated LPS, the LPS of *F. tularensis* is tetra-acylated which is thought to reduce its immunogenicity measured by its ability to stimulate human or murine TLR4 \(^{322}\). Several alternative TLR ligands have been identified on the surface of *F. tularensis*, such as the TLR2 ligand Tul4 \(^{300}\). However, it appears that the major target cells of *F. tularensis*, such as alveolar macrophages, are unable to respond to this ligand despite expressing TLR2 \(^{318}\). One hypothesis accounting for this defect is the inability of these cells to express high levels of CD14, a co-receptor that aids TLR2-ligand detection \(^{318}\). When dendritic cells were supplemented with CD14 they were able to respond to infection by *F. tularensis* through the secretion of pro-inflammatory cytokines, a response they were unable to generate previously \(^{327}\). In the case of alveolar macrophages and dendritic cells this could be due to the failure of these cells to express the co-receptor, CD14, possibly required for activation of TLR2 \(^{318}\).

Once inside the cell, *F. tularensis* is not free from cell defence mechanisms. Indeed, within the phagosome bacteria are faced with reactive oxygen and reactive nitrogen killing mechanisms. Once again though, *F. tularensis* is able to interfere with this process in several ways. Firstly, *F. tularensis* produces enzymes, such as catalase and superoxide dismutase, which are able to neutralise products of the oxidative burst \(^{323}\). However, a mechanism by which *F. tularensis* prevents the correct assembly of complexes responsible for generating oxidative species has also been observed \(^{324}\). It is thought that this occurs by preventing the phosphorylation of the p47 subunit of NADPH oxidase. As mentioned previously, *F. tularensis* is able to rapidly escape the phagosome representing a further mechanism for evasion of oxidative death \(^{257}\). Rapid escape from the phagosome also prevents prolonged exposure to TLR2 and TLR9 that can be found on the surface of phagosomes. This was confirmed using mutants of *F. tularensis* that are incapable of phagosome escape \(^{199}\). These mutant strains demonstrate increased secretion of pro-inflammatory cytokines linked to TLR activation \(^{199}\). Despite a brief interaction with TLRs within the phagosome, secretion of TNF in a TLR2-dependent manner can still be detected. Interestingly, upon phagosome escape, a down-regulation of NFκB activity is observed and a reduction in TNF, IL-6, and IL-12 secretion is seen \(^{296,328}\).

Although residence within the cytosol removes the potential of detection through the TLRs, another family of PRRs are present in the cytosol, the NLRs. In murine macrophages, the receptor responsible for cytosolic detection of *F. tularensis* has been identified as AIM2 \(^{329}\). Upon detection, AIM2 forms a
complex with ASC and activates the caspase-1 associated inflammasome. This results in the processing of the proinflammatory cytokines IL-1\(\beta\) and IL-18 into their active forms. Once again, \textit{F. tularensis} has mechanisms to suppress the inflammatory response. Particularly, several \textit{F. tularensis} proteins are able to suppress AIM2-dependent IL-1\(\beta\) secretion. Mutations in a \textit{F. tularensis} protein called MviN and two genes, FTT_0584 and FTT_0748 all show an increase in ASC-mediated IL-1\(\beta\) production, indicating that they may function as immune modulators in wild type bacteria.

The final stage of the \textit{F. tularensis} life cycle is the induction of cell death of the host cell and proliferation throughout the host. Cells within the tissues of mice infected with type A \textit{F. tularensis} exhibited activation of the caspase-3 associated process of cell death between 6 – 12 h post-infection. This involves the release of mitochondrial cytochrome-c into the cytosol with subsequent activation of caspase-3 and caspase-9. However, this appears to be just one method of inducing apoptosis by \textit{F. tularensis} as it has been shown that \textit{F. tularensis} is also able to induce cell death through the down regulation of the MAPK p38. Interestingly, although capase-3 activation is observed at 6 – 12 h post-infection, it is not until 18 h post-infection that apoptosis occurs in human monocyte-derived macrophages. One explanation for this could be the induction of anti-apoptotic processes which prevent premature cell death until sufficient bacterial replication has taken place.

In addition to the innate immune response, \textit{F. tularensis} is able to modulate alternative immune mechanisms. It appears that, during infection, \textit{F. tularensis} is able to induce the degradation of MHC class II proteins whose role is in the presentation of antigen to cells of the adaptive immune response. This occurs through an ubiquitin-dependent mechanism potentially involving two ubiquitin proteins, the ubiquitin hydrolase USP22 and the ubiquitin ligase CDC27. Degradation of MHC class II receptors will prevent efficient presentation of antigen therefore disrupting the induction of the adaptive immune response.

Finally, \textit{F. tularensis} modulates host cytokine secretion, although the mechanism by which this occurs remains elusive. Both \textit{Mycobacterium tuberculosis} and \textit{Mycobacterium avium} inhibit IFN-\(\gamma\) responses and this is partly dependent on TLR2 engagement. \textit{F. tularensis} also suppresses IFN-\(\gamma\) induced responses and so it is possible that TLR2 is also involved in this response. Another study identified an inhibition of TNF-\(\alpha\) and IL-1\(\beta\) secretion by macrophages infected with LVS and this inhibition was prevented in an \textit{iglC} mutant strain. This suggests that escape from the phagosome is required for the inhibition to take place although the exact mechanism is unknown. Specific to TLR...
signalling proteins, an initial up-regulation of three signalling proteins (p38, IκB and c-Jun) was observed, following infection of murine macrophages with *F. tularensis* LVS, but then their production was down-regulated. Interestingly, the down-regulation corresponded with internalisation of the bacteria and was not observed in *iglC* mutant bacteria. The modulation of cytokine production is particularly clear in the murine model of *F. tularensis* infection. Mice challenged with *F. tularensis* SchuS4 via the inhalational route display active suppression of the inflammatory response associated with decreased cytokine production. No cytokine secretion is observed at the primary site of infection, the lungs, until 72 h post-infection. By this time, the bacteria have successfully colonised the lungs and have begun to disseminate to the secondary sites of infection, the liver and spleen. It is at this point that an efficient immune response is generated. However, even this is subject to modulation by *F. tularensis* and inflammation is exacerbated to the point where the immune response is detrimental to the host rather than beneficial. This bi-phasic response is characteristic of *F. tularensis* infection and suggests that therapeutic strategies focussed at re-balancing the inflammatory response may be beneficial to the outcome of infection.

It is clear that *F. tularensis* is able to modulate the host immune system at numerous points from the initial detection by TLRs all the way through to disrupting the efficient induction of the adaptive immune response. The modulatory effects of *F. tularensis* seem to involve both the up-regulation of certain signalling pathways with the down-regulation of others therefore producing a finely balanced and tuned response that is beneficial for replication and proliferation of the bacteria within the host. *F. tularensis* influences the immune response in many ways and interacts with signalling pathways at multiple points during its intracellular life-cycle. This makes therapeutic modulation of signalling pathways, aimed at optimising the host response to *F. tularensis* infection, a significant challenge.

### 1.4.6 Therapeutic modulation

Potentially the simplest method of therapeutic modulation of TLR signalling is through the direct binding of TLRs themselves using synthetic or naturally occurring ligands. Bacterial DNA containing unmethylated CpG motifs has previously been used to stimulate TLR9 and induce protection against subsequent challenge with the intracellular pathogens *F. tularensis* LVS and *Listeria monocytogenes*. This was brought about through the induction of a Th1 cytokine response and IFN-γ, TNF and IL-12 production. However, in some circumstances, co-stimulation with bacterial DNA appears to increase the likelihood of septic shock, probably through the synergistic production of TNF. In addition, further work using CpG oligonucleotides (ODNs) to protect mice challenged with the highly virulent SchuS4 strain of *F. tularensis* failed to show any protection regardless of challenge dose or route of infection. In fact, it appeared that the treatment actually adversely affected the survival
of infected mice which all exhibited dramatically elevated cytokine levels in the lungs, livers and spleens 6 days after challenge. Alternatively, stimulation of TLR3 using the synthetic dsRNA analogue poly(I:C) has been evaluated for the treatment of F. tularensis LVS or SchuS4 infection of Balb/c mice. Treatment 1 h prior to or 1 h after intranasal infection led to significant improvement in the survival of mice, correlating to increased influx of neutrophils to the lungs and earlier up-regulation of IL-6, MCP-1 and TNF. However, for full recovery, subsequent treatment with the antibiotic levofloxacin was still required.

To try to prevent the wide-ranging effects of direct TLR stimulation, treatments targeting specific proteins within the signalling cascades are now being developed. One of these targets is located downstream of TLR2 and is a Ser/Thr protein kinase called glycogen synthase kinase-3B (GSK3B). GSK3B has been implicated in the regulation of the inflammatory response as it is active in resting cells but becomes inactive upon phosphorylation of its N-terminus, affecting the activation status of both NFκB and CREB. Inhibition of GSK3B with lithium chloride (LiCl) significantly reduced the production of pro-inflammatory cytokines, such as IFN-γ, IL-12p40 and TNF, and increased survival from 60% in untreated mice to 90% in F. tularensis LVS-infected mice.

The diversity of treatments does raise a question about the optimal treatment protocol for F. tularensis infection. In some instances, the treatment is trying to promote cytokine production whereas, in other cases, the treatment is aiming to reduce the levels of cytokines induced. Which of these treatment strategies is appropriate? This question highlights an interesting characteristic of F. tularensis infection. Early in infection it appears that this bacterium suppresses the inflammatory response whilst at later time points there is an excessive stimulation of the immune system resulting in an overwhelming inflammatory response. Perhaps the ideal therapeutic strategy would be to enhance the early response followed by a suppression of the later inflammation.

### 1.4.7 Aims of this research

In order to defend against invading pathogens, a host must be able to detect and respond appropriately to the invading organism. The TLR signalling system, consisting of cell surface and intracellular receptors linked to the manufacture and secretion of cytokines by a complex signalling protein network, is perfectly placed to fulfil this role. However, due to the role of the TLR system in generating an immune response numerous pathogens express mechanisms of evading and even modulating this system for their own benefit. The intracellular bacterial pathogen F. tularensis demonstrates the characteristics of an immune-modulating pathogen. Upon infection of a host there is a suppression of the inflammatory response that is subsequently followed by an up-regulation of
immune signalling leading to overwhelming inflammation and associated organ damage. Despite the exact mechanisms behind its ability to modify the immune response not being fully understood, it is likely that *F. tularensis* interacts with the TLRs and downstream signalling pathways in order to achieve the observed bi-phasic inflammatory response.

This project aims to determine the signalling proteins that are activated upon infection by *F. tularensis* and begin to elucidate the role that the TLR signalling system plays in determining the host response to infection by this pathogen. As *F. tularensis* displays the characteristics of an immune-modulatory pathogen there is a possibility that the immune response induced is not optimal for host survival. Therefore, the potential for therapeutic immune modulation to optimise the host immune response will also be investigated.

This specific aims of this study are:

- Characterise the activation of signalling cascades in response to *F. tularensis* infection.
- Identify the contribution of specific TLRs in initiating an immune response to infection by *F. tularensis*.
- Investigate the potential interaction between separate signalling pathways.
- Investigate the potential for specific inhibition of a TLR signalling protein to optimise the immune response to *F. tularensis*. 
Chapter 2: Materials and methods

2.1 Microbiological techniques

2.1.1 Bacterial techniques

2.1.1.1 Growth and maintenance of *Francisella tularensis*

*Francisella tularensis* strains were maintained in 30% glycerol at -70°C for long-term storage. LVS or the SchuS4 strain of *Francisella tularensis* was routinely cultured for use in infection assays. *F. tularensis* was grown on blood cysteine glucose agar (BCGA) plates and incubated overnight at 37°C.

2.1.1.2 Preparation of *F. tularensis* inoculum

One loop from a glycerol stock of *F. tularensis* was streaked onto BCGA plates for growth (2.1.1.1). Subsequently, bacteria were harvested from the plates into the appropriate cell culture growth medium. Bacterial concentrations were calculated by taking optical density (OD) readings, using a spectrophotometer at a wavelength of 600 nm, and concentrations were adjusted accordingly by the addition of phosphate buffered saline (PBS).

2.1.1.3 Bacterial enumeration of *F. tularensis*

To enumerate bacteria, samples were subjected to ten-fold serial dilutions in PBS (100 µl sample into 900 µl PBS). Subsequently, 100 µl of the appropriate dilutions were plated onto BCGA plates and incubated at 37°C until colonies could be seen (approximately 3 days).

2.1.1.4 Preparation of *F. tularensis* genomic DNA

Genomic DNA was prepared using the Gentra Puregene Yeast/Bacteria Kit (Qiagen, UK), according to manufacturer’s instructions. Bacterial colonies harvested from plates were inoculated into 300 µl cell lysis solution and the sample was mixed by pipetting and incubated at 80°C for 5 min. RNA contamination was removed by adding 1.5 µl RNase A solution, mixing by inversion 25 times, and incubation at 37°C for 60 min. The samples were chilled on ice for 1 min and 100 µl protein precipitation solution was added. The samples were vortexed for 20 s and protein was pelleted by centrifugation at 10,000 rpm for 3 min. DNA was precipitated by adding the supernatant into 300 µl isopropanol, inverting 50 times and centrifuging at 10,000 rpm for 1 min. The supernatant was discarded and the pellet was air-dried and then washed with 300 µl of 70% (v/v) ethanol followed by
centrifugation at 10,000 rpm for 1 min. The supernatant was discarded and the DNA pellet was air
dried for 5 min before being rehydrated through addition of 100 µl DNA hydration buffer, vortexed
for 5 s and incubated at 65°C for 1 h. To ensure full rehydration the DNA samples were incubated
overnight at room temperature with gentle shaking. The samples were stored at -20°C. To determine
the concentration of the DNA preparations the OD was measured at 260 nm using a NanoDrop 1000
(Thermo Scientific, UK) according to manufacturer’s instructions. For assays requiring stimulation by
genomic DNA, the DNA was diluted in DNA hydration buffer (Qiagen, UK) to the required
concentration.

2.1.2 Cell culture techniques

2.1.2.1 Acquisition and maintenance of cell lines

J774A.1 (macrophage-like) and MH-S (alveolar macrophage-like) cell lines were obtained from
the European Collection of Cell Cultures (ECACC) (HPA, UK). The TLR4-knockout, TLR2-knockout and
parental BMDM cell lines were a kind gift from Dr K. Fitzgerald (University of Massachusetts Medical
School, US). The HEK-Blue™ hTLR9 and HEK-Blue™ Null1 cell lines were obtained from (InvivoGen,
France). HEK-Blue™ cell lines have been transfected with an NF-κB-inducible secreted embryonic
alkaline phosphatase (SEAP) reporter gene. This allows the degree of stimulation of the cells to be
determined by a colour change in the supernatant upon combining with a QUANTI-Blue™
(InvivoGen, France) reagent, a substrate for the SEAP enzyme. J774A.1 cells were maintained in
endotoxin-free Dulbecco’s modified Eagle’s medium (DMEM) (Gibco®, UK) supplemented with 10%
foetal calf serum (FCS) (Gibco®, UK) and 2 mM L-glutamine (Sigma-Aldrich, US). MH-S cells were
maintained in endotoxin-free Roswell Park Memorial Institute medium (RPMI) supplemented with
10% FCS and 2 mM L-glutamine. BMDM cell lines were maintained in DMEM supplemented with
10% FCS and 2mM L-glutamine. The HEK-Blue™ cell lines were resuscitated from frozen stocks by
culturing for two passages in DMEM supplemented with 10% FCS and 2 mM L-glutamine.
Subsequent passages of HEK-Blue™ hTLR9 cells included 100 µg/ml Normocin™, 10 µg/ml Blasticidin
and 100 µg/ml Zeocin™, (InvivoGen, France), in addition to FCS and L-glutamine. Subsequent
passages of HEK-Blue™ Null1 cells included 100 µg/ml Normocin™ and 100 µg/ml Zeocin™ in addition
to FCS and L-glutamine. All cells were maintained at 37°C, 5% carbon dioxide (CO₂) and 95% humidity
in vented tissue culture flasks (Corning, US).

Cell line stocks were stored in liquid nitrogen. To produce stocks, cells were grown to 90%
confluence and then scraped into 10 ml of suitable media. The cells were centrifuged at 1000 rpm
for 5 min and then re-suspended in 3 ml culture supernatant. To this, 3 ml of freezing medium (20 % Dimethyl sulfoxide (DMSO) in FCS) was added and then the cells were split into 1 ml aliquots. These were slowly frozen overnight to -70°C and then transferred into liquid nitrogen. When needed, cells were resuscitated by rapid thawing and culturing in suitable media.

2.1.2.2 LVS infection

J774A.1 or MH-S cells were seeded into 6-well plates (Corning, US) at 4 x 10^5 cells/ml and incubated overnight at 37°C to allow the cells to adhere. Supernatants were then removed and replaced with 1.5 ml of the LVS inoculum (Section 2.1.1.2). Plates were incubated at 37°C for 2 h (the uptake period) to allow for internalisation of the bacteria and then the supernatants were removed and replaced with 1.5 ml fresh media. The plates were incubated at 37°C and, at a range of timepoints, supernatants were removed and stored at -20°C for cytokine analysis (Section 2.3.1). The cells were washed once with chilled PBS and then lysed by adding 350 µl PhosphoSafe™ extraction reagent (Merck, US) and incubating on ice for 5 min. Full cell recovery was ensured by scraping wells with a rubber policeman (Corning, US). 100 µl of the lysate was used for bacterial enumeration (Section 2.1.1.3), and a further 250 µl was centrifuged at 13,000 rpm for 5 min to remove cell debris before being stored at -20°C for analysis by western blotting (Section 2.2.2).

For the treatment of infection with a MAPK inhibitor, cells were treated at either 0 h or 2 h after the uptake period. PD0325901 (InvivoGen, France), a MAPK kinase-1/2 (MEK-1/2) inhibitor, was used at a final concentration of 0.05 µM 343,344. At 0 h, the inhibitor was prepared to the correct concentration in growth media and then added to the cells instead of fresh media. At 2 h, the inhibitor was added directly to the supernatants in the wells to the required final concentration.

2.1.2.3 LVS infection and TLR-blocking

Into the wells of a 96-well plate, 20 µl of a TLR4-blocking antibody, IMG-428E (Imgenex, US) (50 µg/ml – 5 ng/ml), or a TLR9 antagonist, G-ODN (InvivoGen, France) (100 µg/ml – 10 ng/ml), or both were added. Subsequently, 20 µl LVS was added to a final multiplicity of infection (MOI) of 150 and then 140 µl MH-S cells were seeded into the wells at 60,000 cells/well. The wells were made up to 200 µl with RPMI. A summary of the plate layout is shown in Figure 2-1. The plates were incubated at 37°C in 5% CO₂ for 24 h before samples of supernatants were collected. Stimulation of signalling by LVS or inhibition of signalling by the antagonists was determined by measuring the levels of inflammatory cytokines in the supernatants (Section 2.3.1).
Figure 2-1: Assay plate layout for the inhibition of TLR4 and TLR9 signalling in response to LVS infection in MH-S cells

Layout of 96-well plate showing concentration of antagonist and neutralising antibody used (µg/ml). Row A contained IMG-428E (+ IMG or + I) only at concentrations ranging from 50 µg/ml to 5 ng/ml. Row B contained both IMG-428E and G-ODN (+ G) at concentrations ranging from 50 - 5 µg/ml (IMG-428E) and 100 - 10 µg/ml (G-ODN). Row C contained G-ODN only at concentrations ranging from 100 µg/ml to 10 ng/ml. Blue wells contained infected but untreated cells, purple wells were naive MH-S cells with G-ODN, yellow wells were naive MH-S cells with IMG-428E and green wells were untreated naive MH-S cells. Black outlined wells were test wells and dotted outlined wells were control wells.
2.1.2.4 SchuS4 infection

MH-S cells were seeded into 6-well plates at 4 x 10^5 cells/ml and incubated overnight at 37°C in 5% CO₂ to allow the cells to adhere. The supernatants were removed and 1.5 ml of SchuS4 inoculum (Section 2.1.1.2) was added to each well. Plates were incubated at 37°C, 5% CO₂ for 45 min to allow for internalisation of the bacteria and then the supernatants were removed and replaced with 1.5 ml fresh media. Plates were incubated at 37°C, 5% CO₂ then, at specific time points, supernatants were removed and stored at -20°C for cytokine analysis (Section 2.3.1). The cells were washed once with chilled PBS and then 500 µl Phosphosafe™ lysis buffer (Merck, US) was added to each well to lyse the cells. The plates were incubated on ice for 5 min and, to ensure full cell recovery, the wells were scrapped with rubber policemen. Subsequently, 100 µl of the lysate was serially diluted and used for bacterial enumeration (Section 2.1.1.3). The remaining 400 µl was filter sterilised using a 0.2 µm syringe filter (Sartorius Stedim Biotech, France) to remove any bacteria and then stored at -20°C for subsequent screening by western blotting (Section 2.2.2) to measure signalling protein activation.

2.1.2.5 Genomic DNA stimulation of HEK-Blue™ or MH-S cells

All wells were set up as shown in Figure 2-2. Genomic DNA purified (Section 2.1.1.4) from F. tularensis was ten-fold serially diluted in DNA hydration buffer (Qiagen, UK) to give all required concentrations, (10 µg/ml – 1 ng/ml). Subsequently, 20 µl genomic DNA or DNA hydration buffer was added to each well. A known TLR9-agonist, ODN-2395, (InvivoGen, France) and its non-stimulatory partner, ODN-2395 control, (InvivoGen, France) were used as positive and negative controls, respectively. For HEK stimulation, suspensions of HEK-Blue™ hTLR9 or HEK-Blue™ Null1 cells were prepared at 3.5 x 10^5 cells/ml in DMEM and then 180 µl was added to each well. For MH-S stimulation, a suspension of MH-S cells was prepared at 3.75 x 10^5 cells/ml in RPMI and 160 µl was added to each. To simulate a lack of TLR9, when using MH-S cells, 20 µl of the TLR9-antagonist G-ODN (InvivoGen, France) or DNA hydration buffer was added to each well. The cells were incubated at 37°C in 5% CO₂ for 24 h and then 50 µl supernatant was removed and stored at -20°C for cytokine analysis (Section 2.3.1) and the degree of cell stimulation was measured (Section 2.1.2.6).

2.1.2.6 Measurement of HEK-Blue™ stimulation

The degree of HEK cell stimulation was measured using an NF-κB-inducible SEAP reporter gene expressed by the cells. In a 96-well plate, 20 µl cell supernatant was added to 180 µl re-suspended
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<td>10 µg/ml</td>
<td>1 µg/ml</td>
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<td>10 µg/ml</td>
<td>1 µg/ml</td>
<td>1 µg/ml</td>
<td>0.1 µg/ml</td>
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<tr>
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<tr>
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**Figure 2-2: Assay plate layout for the genomic DNA stimulation of HEK-Blue™ or MH-S cells**

QUANTI-Blue™ (InvivoGen, France). The production of SEAP enzyme by stimulated cells enables the conversion of the QUANTI-Blue™ substrate from a pink to a blue colour. The plate was incubated at 37°C for 40 min and the SEAP levels were determined by measuring the absorbance at 620 nm using a spectrophotometer (Thermo Scientific, US).

2.1.2.7 Cytotoxicity measurements

The degree of cell lysis was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, UK) according to manufacturer’s instructions. This assay quantitatively measures the amount of LDH which is a cytosolic enzyme released upon cell lysis. Briefly, 50 µl samples of cell supernatant were combined with 50 µl reconstituted substrate mix in a 96-well plate. The plate was incubated for 30 min in the dark before the addition of 50 µl stop solution. The degree of cytotoxicity was determined by measuring the absorbance at 490 nm using a spectrophotometer (Thermo Scientific, US).

2.1.2.8 Priming of MH-S cells with pure LPS

To the wells of a 96-well plate, 20 µl LPS or PBS was added in the presence of either 20 µl of the TLR4-neutralising antibody IMG-428E or PBS. A suspension of MH-S cells was prepared at 5 x 10^5 cells/ml and 120 µl of the cell suspension was added to each well. The wells were made up to 200 µl using RPMI. The plates were incubated for 2 h at 37°C in 5% CO₂ to allow priming by ultrapure *E. coli* LPS (InvivoGen, France) to occur. After priming, the supernatants were removed and 20 µl genomic DNA, ODN2395 positive control, ODN2395 negative control or DNA hydration buffer was added to each well. To block TLR9 signalling in specific wells, 20 µl of the TLR9 antagonist G-ODN was added. The wells were made up to 200 µl using RPMI. The plates were incubated for 24 h at 37°C in 5% CO₂ and then supernatants were removed and stored at -20°C for cytokine analysis (Section 2.3.1). A schematic diagram of the assay layout is shown in Figure 2-3.

2.2 Protein techniques

2.2.1 SDS-PAGE

Samples of cell lysates were prepared for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) by mixing in a ratio of 1:1 with 2x Laemmli buffer (Sigma-Aldrich, US) before boiling for 5 min. Subsequently, 19 µl of each sample was loaded into the wells of a 4 – 20% gradient
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<td>LPS 0.1</td>
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<td>LVS 1</td>
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**Figure 2-3: Assay plate layout for the LPS-priming and TLR9 stimulation of MH-S cells**

tris-glycine polyacrylamide gel (Invitrogen, UK). Cell lysates from stimulated or unstimulated cells (New England Biolabs, UK) were used as positive and negative controls, respectively. Routinely, two protein standards were used: 4 µl SeeBlue® Plus2 pre-stained standard (Invitrogen, UK) was used to visualise the running of the gels and 0.5 µl MagicMark™ XP Western Protein Standard (Invitrogen, UK) was used to visualise bands after western blotting and chemiluminescent detection (Section 2.2.2). The standards were mixed and loaded into the end wells of each gel. Once loaded, the gels were run for 2 h at 125 V and 200 mA submerged in running buffer (900 ml dH₂O and 100 ml Novex® Tris-Glycine Running Buffer 10x (Invitrogen, UK)).

2.2.2 Western blotting

Proteins were transferred from polyacrylamide gels onto Invitrolon™ Polyvinylidene fluoride (PVDF) membranes (Invitrogen, UK) using a Novex® Semi-Dry Blotter (Invitrogen, UK) according to manufacturer’s instructions. Briefly, PVDF membranes were activated in methanol for 1 min, washed in dH₂O for 1 min, and then soaked in transfer buffer (820 ml ultrapure H₂O (Millipore, US), 100 ml methanol and 80 ml Novex® Tris-Glycine Transfer Buffer 25x (Invitrogen, UK)). The gel was carefully removed from its plastic cassette and, with 4 filter papers, was soaked in transfer buffer. The separate components were then stacked into the Semi-Dry Blotter (Figure 2-4) and the transfer was run for 45 min at 10 V and 250 mA. To detect proteins of interest, the PVDF membranes were blocked for 2 h at room temperature using Blotto (5% (w/v) skimmed milk powder and 0.1% Tween® 20 (Sigma-Aldrich, US) dissolved in PBS). They were then washed for 5 min in wash buffer (0.1% Tween® 20 in PBS) before being incubated over night at 4°C with the primary antibody at a dilution of 1:1000 in Blotto. Table 2-1 shows the primary antibodies used in this study. The membranes were then rinsed twice for 5 min in wash buffer and then incubated for 1 h at room temperature with the secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidise (HRP) (Bio-Rad, US)) at a dilution of 1:3000 in Blotto. The membranes were rinsed 4 times in wash buffer for 2 x 5 min, 1 x 15 min and 1 x 5 min. The membranes were developed using enhanced chemiluminescence (ECL) Plus detection reagents (GE Healthcare, UK) according to manufacturer’s instructions. Briefly, the membranes were placed on a plastic sheet and 3 ml of detection reagent was added. The membranes were incubated for 5 min and then the detection reagent was drawn off using absorbent paper. The membranes were placed in a film cassette and exposed to ECL hyperfilm (GE Healthcare, UK) for 5 s – 2 h.

If the membranes were to be re-probed, they were removed from the cassette and washed twice for 5 min in wash buffer. They were then stripped for 2 h at room temperature in 100 mM glycine
Figure 2-4: Stacking order of components for semi-dry transfer of proteins

Two transfer buffer-soaked filter papers are placed onto the anode plate of the semi-dry blotter. The activated PVDF membrane is placed onto the filter papers with the gel placed on top. Finally two filter papers are placed onto the gel and the cathode plate is lowered gently onto the stack and loosely secured.
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Table 2-1: Details of the primary antibodies used in this study

NEB – New England Biolabs, UK.
Sigma-Aldrich, US) pH 3 made up in dH$_2$O. After stripping, the membranes were washed twice for 5 min in wash buffer and then re-blocked in Blotto. Detection using primary and secondary antibodies followed by ECL detection was then carried out as before. Exposed hyperfilm was developed by immersion in Kodak® developer solution (Sigma-Aldrich, US) for 1 min followed by washing in dH$_2$O for 1 min, then immersion in Kodak® fixer solution (Sigma-Aldrich, US) for 1 min followed by a final wash in dH$_2$O for 1 min. The hyperfilm was allowed to air-dry for at least 2 h before further analysis by densitometry.

### 2.2.3 Densitometry

The hyperfilms were scanned using a GS-800 Imaging Densitometer (Bio-Rad, US) combined with Quantity One® analysis software v 4.2.1 (Bio-Rad, US) set to transmissive-scan gray X-ray film (Figure 2-5A). Each lane of the gel was overlaid onto the image using anchoring lines to match the contours of the gel (Figure 2-5B). Once the lanes had been mapped, the background intensity of the film could be removed so that only the intensity of the bands is measured. Using the analysis software, bands could be identified and classified as proteins of interest, loading controls or standards (Figure 2-6). The software allocates a value to each band according to the area under the band’s intensity curve (trace quantity). This value is then converted to a normalised quantity which accounts for loading differences. The normalised quantity is the trace quantity of a band expressed as a percentage of a selected band (β-actin) in the same lane (Figure 2-7A). A report containing the relevant band values was produced and this was exported into Microsoft Office® Excel® 2007 (Figure 2-7B). In Excel®, a formula was used to normalise the data collected from repeated experiments. This was performed, using the equation shown in Figure 2-8, to account for variations in the separate exposures. A flowchart displaying the individual stages of the densitometry analysis is shown in Figure 2-9.

### 2.3 Immunological techniques

#### 2.3.1 BD™ Cytometric bead array (CBA)

The secreted levels of specific cytokines released into the cell supernatants were measured using BD™ cytometric bead array (CBA) technology (BD Biosciences, US) on a BD™ FACScan flow cytometer (BD Biosciences, US) according to manufacturer’s instructions. Samples obtained from J774A.1, MS-S and BMDM cells were screened using the BD™ mouse inflammation CBA kit (BD Biosciences, US). Samples obtained from HEK cells were screened using the BD™ human flex CBA set (BD Biosciences, US). Into the wells of a 96-well plate 50 µl of either test sample or standard were added. The capture
Figure 2-5: Densitometry: Scanning and applying lanes

A. Selection of the appropriate film category (red box) applies default settings to filter type and scanning mode (blue box). B. The lanes (red lines) are manually applied using anchors (white dashed lines) fitting the lanes to the contours of the gel. The background intensity is also removed at this stage.
Figure 2-6: Densitometry: Band identification and classification

Known bands (green) are manually selected and matched bands (red) of equal molecular weight are identified automatically using the standards (blue).
Figure 2-7: Densitometry: Generation of band values

A. Values are allocated to each band depending on size and intensity. “Mol. Wt.” (purple box) is the molecular weight of the band as related to the chosen standard. “Peak Density” (red box) is the intensity of the bands peak. “Trace Qty” (blue box) is the area under the band’s intensity profile curve. “Normalized Qty” (green box) is the trace quantity of a band expressed as a percentage of a selected band in the same lane. B. A report is generated containing the selected criteria. This report is exported to Microsoft Office® Excel® 2007 for further analysis.
The values produced by densitometry for each signalling protein were normalised across the three repeat experiments performed in duplicate. This was performed using the equation shown to account for variations in the separate exposures. Equation used from Chopard et al., 2000.45

$$\text{Band's normalised value} = \frac{\text{Value of band}}{\left(\frac{\sum \text{Bands from all gels}}{6 \times \sum \text{Bands from that gel}}\right)}$$
Figure 2-9: Flowchart of densitometry process

A simple flowchart showing the individual steps in the densitometry process from the original scanning of the film to the final normalised values.
beads specific for each of the cytokines IFN-γ, IL-6, IL-10, IL-12p70, TNF and MCP-1 were combined in equal amounts and then 25 µl aliquots were added to the samples. The plate was incubated for 1 h at room temperature in the dark with gentle shaking. Subsequently, 25 µl PE detection reagent (BD Biosciences, US) was added to each well and the plate was re-incubated for 1 h. An extra 100 µl wash buffer (BD Biosciences, US) was added to the plate which was then centrifuged at 1,350 rpm for 5 min. The supernatant was removed and 150 µl fresh wash buffer was added to each well. The beads were re-suspended by gentle pipetting. The plate was then run on the FACs machine (BD Biosciences, US) and, using the standards on the same plate to generate a standard curve, the concentration of each cytokine was interpolated using GraphPad Prism® 5.01 software (GraphPad, US).

### 2.4 Animal studies

#### 2.4.1 Preparation of inoculum

LVS was grown overnight on BCGA plates incubated at 37°C (Section 2.1.1.2). The following day, bacteria were added to 10 ml PBS to an OD₆₀₀ of 0.15 (~1 x 10⁹ CFU/ml). This solution was diluted to give a final concentration of ~5 x 10⁵ CFU/ml. To confirm the CFU/ml of the final dose, dilutions of both the ~1 x 10⁹ CFU/ml and the ~5 x 10⁵ CFU/ml solutions were plated onto BCGA plates which were incubated at 37°C for subsequent bacterial enumeration (Section 2.1.1.3).

#### 2.4.2 Animals and suppliers

All animal studies were carried out in accordance with the UK Scientific Procedures Act 1986. Animal handling and infection procedures were carried out by trained staff at Dstl. Pathogen-free 6-8 week old female BALB/c mice were obtained from Charles River Laboratories, UK, and housed in high-efficiency particulate air-filtered barrier units. Mice were provided with a continuous supply of food and water and allowed to acclimatise to new surroundings for at least a week before experimental procedures were done. The mice were kept at 25°C with alternating 12 h periods of light and dark to simulate day and night. Animals that reached the humane endpoint, described in the UK Home Office project license used for this work, were culled by cervical dislocation.

#### 2.4.3 LVS infection

Thirty-six, 6-8 week old, female BALB/c mice (Charles River, UK) were infected via the intranasal
route with \( \sim 1 \times 10^4 \) CFU of LVS. Groups of 5 mice were culled at 0 h (naive), 2 h, 12 h, 24 h, 48 h, 72 h, and 96 h and the lungs, liver, spleen and blood were removed into aliquots of 2 ml PBS. The organs were homogenised through a 0.2 µm cell sieve (BD Biosciences, US). The blood and homogenates were screened for the levels of cytokines (Section 2.3.1), activation of signalling proteins (Section 2.2.2) and bacterial enumeration (Section 2.1.1.3).

### 2.4.4 PD0325901 treatment

Sixty-five, 6-8 week old, female BALB/c mice (Charles River, UK) were separated into 4 groups based on treatment type. All mice, except group IV, were treated daily with either PBS or PD0325901 by oral-gavage at a dose of 100 µl. Groups I and II were treated from 1 day pre-infection whilst group III was treated from 2 days post-infection. Mice were challenged with \( 2.5 \times 10^4 \) CFU LVS by the intranasal route. Weighing and scoring of the mice occurred twice daily throughout the study. On day 2 and day 4 post-challenge, 5 mice from groups I, II, and IV were culled and the lungs, liver and spleen were removed and placed into aliquots of 2 ml PBS. Ten mice from groups I, II and III were monitored for changes in survival. A summary of the study protocol is shown in Table 2-2. Organs were homogenised though a 0.2 µm cell sieve (BD Biosciences, US). Serial dilutions of 100 µl of the homogenates were plated onto BCGA for bacterial enumeration (Section 2.1.1.3). Cytokine analysis was carried out on 100 µl samples (Section 2.3.1) and 300 µl was mixed with 300 µl Phosphosafe™ (Merck, US) and analysed for signalling protein activation (Section 2.2.2).

### 2.5 Statistics

All transformations of data and statistical tests were performed using GraphPad Prism® 5.01 software (GraphPad, US). A p-value of <0.05 was considered significant for all studies. The specific statistical tests used are detailed in the results chapters.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment and Route</th>
<th>-1</th>
<th>Infect 0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>&gt;4</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>PD0325901 Oral-gavage</td>
<td>PD (20)</td>
<td>PD (20)</td>
<td>PD (20)</td>
<td>PD (15)</td>
<td>Cull 5</td>
<td>PD (15)</td>
<td>PD (10)</td>
</tr>
<tr>
<td>III</td>
<td>PD0325901 Oral-gavage</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>PD (15)</td>
<td>PD (15)</td>
<td>PD (10)</td>
<td>Cull 5</td>
</tr>
<tr>
<td>IV</td>
<td>Uninfected Untreated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Cull 5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2-2: PD0325901 – Inoculation and treatment schedule

Mice in group I were treated daily for 6 days with PBS from 1 day pre-challenge. Mice in group II were treated daily for 6 days with PD0325901 from 1 day pre-challenge. Mice in group III were treated daily for 3 days with PD0325901 from 2 days post-challenge. Mice in group IV remained untreated and uninfected for the duration of the study. Numbers in brackets show the number of animals treated in each group.
Chapter 3: Characterisation of TLR signalling induced by infection with *F. tularensis*

3.1 Introduction

The generation of an immune response to any pathogen relies heavily upon immune and epithelial cells being able to detect and respond appropriately to the invading organism. One of the first lines of defence for a host is the innate immune system and the “watchmen” of this system are the PRRs. One family of PRRs that, along with their associated signalling pathways, has received intense scrutiny is the TLR family of receptors. TLRs convert the ligand-binding event into an intracellular signalling cascade, involving numerous signalling proteins, resulting in the secretion of immune mediators and, ideally, clearance of the pathogen. A better understanding of how TLR signalling pathways induce a specific response to specific pathogens could enable the identification of the key signalling components that are essential to generating an optimal immune response leading to pathogen clearance. The full characterisation of a signalling cascade requires several pieces of information to give a complete picture of signalling events. The identity of proteins involved in a specific signalling response and their function within the pathway needs to be determined. This process has been demonstrated in the characterisation of the two major signalling cascades; the MyD88-dependent and TRIF-dependent signalling pathways. For example, TAK-1 is known to have a role in MyD88-dependent signalling and functions as a diverging point bifurcating into the MAPK and the NF-κB branches of signalling. Equally, knowledge of the interactions between separate signalling proteins provides valuable information about the sequence in which the proteins become involved in the pathway. This has been demonstrated through the solving of the structure of, and therefore the interactions within, the Myddosome. In this case, IRAK-4 was shown to be recruited to the complex first followed by the addition of IRAK-2, providing insight into how these two proteins regulate each other. Additional information can be provided through determining the actual time course of activation of specific proteins within a pathway. This can be carried out through the direct measurement of protein activation that occurs predominantly through phosphorylation of specific residues within their structure. This information enables the identification of signalling proteins that are important in the early response to infection and those that function later in an infection.

Previous studies have examined the net effect of TLR signalling induced by *F. tularensis* infection composed of the production of immune mediators such as cytokines and chemokines. For example, TLR2 activation by *F. tularensis* has been shown to induce the secretion of IFN-γ, IL-1β, IL-
12p40, RANTES and TNF \(^{298}\). Furthermore, the secreted cytokines can be categorised using temporal factors as the secretion of specific cytokines peaked at 3 different time points after stimulation: pre-8 h (TNF), 8-12 h (IL-12p40) or post-12 h (IFN-\(\gamma\)) \(^{298}\). Secretion of TNF and IFN-\(\gamma\) has also been observed in several other studies \(^{306,308,334}\). TLR2-deficiency in vivo resulted in lower levels of TNF but increased amounts of IFN-\(\gamma\) indicating differential regulation of the production of these two cytokines \(^{41}\). These data suggested a synergistic action of these cytokines with the microbicidal activity of IFN-\(\gamma\) being reduced in the absence of TNF \(^{41}\).

The outcome of \(F.\) tularensis infection, with regards to the cytokine output, has been closely examined but there has been less focus upon examining the time course of signalling activation after infection. An understanding of this process is particularly important for a pathogen such as \(F.\) tularensis whose infection strategy in vivo relies upon suppressing the immune response early in infection but subsequently over-activating it \(^{296}\). This pattern of immune response to infection suggests that \(F.\) tularensis is modifying signalling responses to prevent bacterial clearance and the resolution of infection.

Characterisation of signalling pathways has greatly enhanced our understanding of the pathogenesis of several other pathogens. For example, studies of two strains of Legionella pneumophila identified an up-regulation and sustained activation of p38 and JNK signalling proteins only in the virulent AA100 strain \(^{351}\). This highlights key differences between virulent and avirulent Legionella strains and similar studies with strains of \(F.\) tularensis therefore has potential to identify signalling proteins correlating with virulence. Signalling characterisation can also lead to novel therapeutic approaches. For example, during infection of HEK-293 cells, herpes simplex virus type 2 (HSV-2) induced sustained ERK activation and targeting of an up-stream signalling protein with an inhibitor reduced viral propagation \(^{352}\). Similarly, transient activation of p38 after Mycobacterium avium subspecies paratuberculosis infection of bovine monocytes was identified as being a key mechanism for bacterial survival within these cells \(^{353}\). Blockage of the p38 pathway altered the cytokine output and increased phagosome acidification resulting in improved pathogen killing by infected cells \(^{353}\). As with these examples, a more detailed understanding of the TLR signalling response to \(F.\) tularensis infection could underpin approaches aiming to improving the outcome of infection to this bacterial pathogen.
3.1.1 Aims

The aim of this chapter was to characterise the intracellular signalling cascades within host cells infected with *F. tularensis* using both *in vitro* and *in vivo* infection models. Initial studies were performed using the low virulence *F. tularensis* strain LVS before characterising the signalling response to the highly virulent SchuS4 strain.

The specific aims of this chapter were to:
- Establish and optimise an *in vitro* infection protocol to enable consistent measurement of TLR signalling cascades.
- Select signalling proteins for focussed screening of signalling in further studies.
- Compile a profile of the signalling response to *F. tularensis* LVS consisting of the activation of specific proteins during infection and the resulting cytokine output in both *in vitro* and *in vivo* infection models.
- Compile a profile of the signalling response to *F. tularensis* SchuS4 for comparison to the LVS profiles.

3.2 Results

3.2.1 *In vitro* assay optimisation

3.2.1.1 Standardisation of bacterial inoculum

In order to enable consistent preparation of bacterial inoculum for infection assays, a standard curve was produced correlating absorbance measurements to bacterial concentration. Specifically, several LVS broth samples were prepared by resuspending LVS colonies in appropriate cell culture media with absorbance readings ($A_{600}$) between 0.1 and 0.6. The preparations were then plated onto BCGA for bacterial enumeration. The calculated CFU/ml was plotted against the known absorbance readings and a best-fit line was calculated (Figure 3-1). From the graph it was possible to interpolate that an absorbance reading of 0.15 correlates with the desired starting inoculum of approximately $1 \times 10^9$ CFU/ml.

3.2.1.2 Optimisation in J774A.1 cells

An *in vitro* infection model was established to enable the characterisation of TLR signalling in response to *F. tularensis*. The macrophage-like J774A.1 cell line was initially selected for optimisation...
Figure 3-1: Correlation between spectrophotometer reading and CFU/ml of *F. tularensis*

The CFU/ml correlating to specific spectrophotometer readings was determined by diluting LVS in DMEM and then reading the absorbance at 600 nm. A best-fit line is shown which was used to determine which absorbance level correlated to approximately $1 \times 10^5$ CFU/ml.
of the infection protocol since this cell line is very well characterised and expresses all TLRs of interest to this study\textsuperscript{354-356}. In addition, J774A.1 cells support infection and replication of \textit{F. tularensis} \textsuperscript{239,246,260,261}. Therefore, the cells were considered suitable for the study of TLR signalling induced by an LVS infection. It was important to determine which multiplicity of infection (MOI) to use in the infections since high MOIs could result in high levels of host cell death and low MOIs might not create an established infection model. \textit{F. tularensis} enters host macrophages through phagocytosis, requiring CR3 alone or in cooperation with mannose receptors (MRs) and scavenger receptor-A (SR-A), and does not actively invade these cells\textsuperscript{240,241,246}. J774A.1s were infected with MOIs ranging from 10 to 150. The cells were incubated for 2 h to phagocytose the bacteria and then at several time points after uptake cells were lysed and enumeration of bacteria was performed on BCGA plates (Figure 3-2). As expected, the number of bacteria at 0 h (which corresponds to 2 h after infection) increased with an increase in MOI. Over the following 72 h, the growth pattern of LVS was the same irrespective of the starting MOI. There was an initial decrease in bacterial numbers over the first 2 h after uptake followed by a 3 x log increase in bacterial numbers at 24 h. From this time point through to the end of the time course, at 72 h, there was no increase in bacterial numbers. For subsequent infections, an MOI of 150 was chosen as this value generated an established infection within host cells without causing a high rate of host cell death.

3.2.1.3 Optimisation in MH-S cells

The J774A.1 cell line is classified as a macrophage-like cell line and readily uptakes bacteria. However, for the modelling of \textit{F. tularensis} infection, specifically via the inhalational route, a lung-associated macrophage may more fully represent the natural infection. Therefore, optimisation of \textit{F. tularensis} infection in the alveolar macrophage-like MH-S cell line was performed. To ensure that MH-S cells display similar infection characteristics to the J774A.1 cell line, which was used in the preliminary optimisation studies, a LVS growth curve in MH-S cells was generated and then compared to the growth curve from J774A.1 cells (Figure 3-3). Both cell lines were infected with \textit{F. tularensis} LVS at an MOI of 150 and incubated for 2 h to phagocytose the bacteria. After uptake the cells were lysed and bacterial enumeration was performed on BCGA plates. Both cell lines were able to phagocytose LVS to the same degree with bacterial numbers of around 5x10\textsuperscript{5} CFU/ml at 0 h (which equates to 2 h post-infection). Indeed, the initial infection and survival of LVS within the two cell types was near identical with a decrease of bacterial numbers in the first 2 h followed by an increase back to original values at 6 h. However, from this point the two growth profiles diverge. In J774A.1 cells, LVS replicated at a greater rate than in MH-S cells, reaching 5x10\textsuperscript{11} CFU/ml compared to 1x10\textsuperscript{8} CFU/ml by 24 h. Therefore, although the initial infection demonstrates large similarity
Figure 3-2: The effect of MOI on the growth pattern of *F. tularensis* LVS within J774A.1 macrophages

J774A.1 macrophages were infected with one of four MOIs and bacteria were enumerated at a range of time points after uptake. The experiment was performed in duplicate (n = 2) and each replicate was performed in duplicate. Error bars show the standard deviation.
Figure 3-3: Comparison between survival and growth of *F. tularensis* LVS within J774A.1 macrophages and MH-S macrophages

J774A.1 and MH-S cells were infected with *F. tularensis* LVS at an MOI of 150 and at specific times (0 h, 2 h, 4 h, 6 h, 24 h) after uptake bacteria were enumerated to determine intracellular bacterial numbers. This experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the standard deviation. Significance, between the two cell types at each time point, was determined by two-way ANOVA and a Bonferroni post-test (* = p<0.001)
between the cells, the later stages of infection show differences in how the host cells handle infection.

### 3.2.2 In vitro characterisation of the immune response to *F. tularensis* LVS

The MH-S model of *F. tularensis* LVS infection was selected for the measurement of signalling protein activation and cytokine secretion. Since it was likely that significant changes in signalling protein activation could occur at the earliest stages of infection the assay was further developed to include a series of new time points at 5 min, 15 min, 30 min, 45 min, 1 h, and 1.5 h post-uptake. As shown in Figure 3-4, the bacterial numbers decreased throughout the pre-2 h time points and at 2 h is significantly lower than the number of bacteria originally taken up by the cells. From 2 h, however, bacterial numbers increase and replication is maintained through to 24 h.

Signalling proteins to include in the preliminary screening panel were selected based on their known function and position within signalling pathways (IRAK-1, TAK-1, JNK-1/2/3, p38, ERK-1/2, MAPK phosphatase-1 (MKP-1), IKK-α, IKK-β, IKK-γ, IκBα, IκBβ). Importantly, these proteins are known to be intimately involved in signalling downstream of TLR2 and TLR4 activation. *F. tularensis* expresses several factors that are possible ligands for these two receptors suggesting that they are likely detectors of a *F. tularensis* infection. As such, the signalling proteins located within their downstream pathways have the most potential for activation and modulation by this pathogen and warrant examination. In total, the preliminary screen contained 14 proteins and their respective phosphorylated states (Table 3-1). The progression of signalling in response to infection was examined by immunoblotting lysates collected from LVS-infected MH-S cells. Briefly, MH-S cells were incubated for 2 h in the presence of LVS and then at multiple time points supernatants were collected and cells were lysed. Lysates were used for bacterial enumeration and also screened by western blotting for the levels of un-phosphorylated and phosphorylated proteins. Semi-quantitative values were allocated to the levels of expressed and activated signalling proteins by densitometer analysis of the images created by western blotting.

The preliminary screen identified five proteins (IRAK-1, TAK-1, IKK-α, IKK-β, IKK-γ) which were not easily visualised by western blotting due to high levels of non-specific binding or those whose activation did not appear to be affected by LVS infection (black text in Table 3-1). Nine proteins (JNK-1/2/3, p38, ERK-1/2, MKP-1, IκBα, IκBβ) were measured in further studies (red text in Table 3-1).
Figure 3-4: The growth and replication of *F. tularensis* LVS within MH-S cells

The bacterial burden of MH-S cells was determined at several time points (0 h, 0.08 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 24 h) after the 2 h uptake period. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the standard error of the mean. Significance, compared to 0 h, was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight (kDa)</th>
<th>Phosphorylated residues</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRAK-1</td>
<td>80</td>
<td>-</td>
<td>Recruited to the membrane-bound receptor complex. Subsequent recruitment of TRAF-6 leads to its degradation and release of the signalling complex into the cytoplasm</td>
</tr>
<tr>
<td>P-IRAK-1</td>
<td>77</td>
<td>Thr209</td>
<td></td>
</tr>
<tr>
<td>TAK-1</td>
<td>80</td>
<td>-</td>
<td>A MAP3K which forms a complex with TAB2 and TAB3 leading to a divergence in the pathway down both the NF-κB and MAPK branches, through activation of the IKK complex and MAPKs respectively</td>
</tr>
<tr>
<td>P-TAK-1</td>
<td>82</td>
<td>Thr184</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>82</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thr184 ; Thr187</td>
<td></td>
</tr>
<tr>
<td>JNK-1 / 2,3</td>
<td>46 / 54</td>
<td>-</td>
<td>MAPK phosphorylated by MKK4 and MKK7, upstream of AP-1 transcription</td>
</tr>
<tr>
<td>P-JNK-1 / 2,3</td>
<td>46 / 54</td>
<td>Thr183 ; Tyr185</td>
<td></td>
</tr>
<tr>
<td>p38</td>
<td>43</td>
<td>-</td>
<td>MAPK phosphorylated by MKK3 and MKK6, upstream of AP-1 transcription</td>
</tr>
<tr>
<td>P-p38</td>
<td>43</td>
<td>Thr180 ; Tyr182</td>
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</tr>
<tr>
<td>ERK-1</td>
<td>44</td>
<td>-</td>
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</tr>
<tr>
<td>ERK-2</td>
<td>42</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P-ERK-1 / 2</td>
<td>44 / 42</td>
<td>Thr202 ; Tyr204 , Thr185 ; Tyr187</td>
<td></td>
</tr>
<tr>
<td>MKP-1</td>
<td>40</td>
<td>Ser359</td>
<td>Negative regulator of MAPKs, particularly p38</td>
</tr>
<tr>
<td>P-MKP-1</td>
<td>40</td>
<td>Ser359</td>
<td></td>
</tr>
<tr>
<td>IKK-α</td>
<td>85</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IKK-β</td>
<td>87</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P-IKK-α / β</td>
<td>85 / 87</td>
<td>Ser176</td>
<td>Together form the IKK complex responsible for inducing the phosphorylation and degradation of IκB proteins</td>
</tr>
<tr>
<td></td>
<td>85 / 87</td>
<td>Ser180</td>
<td></td>
</tr>
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</tr>
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<td>-</td>
<td>Negative regulator of NF-κB</td>
</tr>
<tr>
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<td>36</td>
<td>Ser32 ; Ser36</td>
<td></td>
</tr>
<tr>
<td>IκBβ</td>
<td>48</td>
<td>-</td>
<td>Negative regulator of NF-κB</td>
</tr>
</tbody>
</table>

**Table 3-1: Signalling proteins measured in infected cells**

Red text denotes proteins that were down-selected due to occupying key positions in TLR signalling pathways or displaying significant alterations in activation in preliminary screens. Black text denotes proteins that were removed from the panel due to unclear western blot images or because their activation was not altered during infection.
### 3.2.2.1 MAPK proteins

A class of proteins which, as a group, showed the greatest change in activation during *F. tularensis* LVS infection of MH-S cells were the MAPKs. JNK, p38, and ERK are key proteins located on the MAPK-branch of the MyD88-dependent pathway and are primarily responsible for activating transcription by the transcription factor called AP-1 which is a heterodimer formed of a Fos domain and a Jun domain \(^{359}\). Although the MAPKs major link is to transcription via AP-1, links to NF-κB have also been observed \(^{360-362}\).

Figure 3-5 shows the expression and activation profiles of the three JNK proteins over the course of infection. No alteration in the levels of activated protein for any of the JNK proteins was detected, apart from at 24 h where a decrease in the level of activated JNK-2/3 from 0.75 h and 6 h was detected (Figure 3-5D). The expression of JNK-2/3 also remained constant but there was a slight decrease in the expression of JNK-1 from 6 h through to 24 h post-uptake (Figure 3-5A).

The expression and activation profiles of p38 and one of its regulators MKP-1 over the course of infection are shown in Figure 3-6 and Figure 3-7, respectively. MKP-1 is a negative regulator of p38 and is therefore responsible for the inactivation of this protein. Similar to the JNK proteins, the expression of p38 remained constant during the infection. However, there was a significant change in the activation profile. A rapid increase in activation was seen immediately after the uptake of LVS. This was a transient response and the levels of activated protein returned to naïve levels by 0.5 h. After the rapid and transient activation of p38 there was no re-activation throughout the rest of the infection. Similarly, there was no significant change in the expression of MKP-1 although significant changes in the level of activated protein did occur. Simultaneously with p38, MKP-1 became activated but MKP-1 displayed an extended period of activation, compared to p38, which lasted until 0.75 h, just after the activation of p38 had returned to naïve levels.

The final MAPKs to be screened were the two ERK proteins, the expression and activation of which are shown in Figure 3-8. As with the other MAPKs, the expression of both ERK-1 and ERK-2 did not alter at any time point during the infection. The activation profiles of these two proteins, however, did display some interesting features. There appeared to be two phases of protein activation, particularly for ERK-2. In the first hour after uptake a transient activation pattern was observed. However, from 1.5 h there was a sustained activation of the two ERK proteins which was maintained through to 6 h.
Figure 3-5: The expression and the levels of activated JNK-1 and JNK-2/3 in MH-S cells throughout the course of a *F. tularensis* LVS infection

The expression of JNK-1 (A) and JNK-2/3 (C) and the levels of activated JNK-1 (B) and JNK-2/3 (D) were determined at several time points after the 2 h uptake period. Representative western blots are shown below the corresponding graphs. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the 95% confidence interval. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
Figure 3-6: The expression and the levels of activated p38 in MH-S cells throughout the course of a *F. tularensis* LVS infection

The expression of p38 (A) and the levels of activated p38 (B) were determined at several time points after the 2 h uptake period. Representative western blots are shown below the corresponding graphs. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the 95% confidence interval. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
Figure 3-7: The expression and the levels of activated MKP-1 in MH-S cells throughout the course of a F. tularensis LVS infection

The expression of MKP1 (A) and the levels of activated MKP-1 (B) were determined at several time points after the 2 h uptake period. Representative western blots are shown below the corresponding graphs. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the 95% confidence interval. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
Figure 3-8: The expression and levels of activated ERK-1/2 in MH-S cells throughout the course of a *F. tularensis* LVS infection

The expression of ERK-1 (A) and ERK-2 (C) and the levels of activated ERK-1 (B) and ERK-2 (D) were determined at several time points after the 2 h uptake period. Representative western blots are shown below the corresponding graphs. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the 95% confidence interval. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
3.2.2.2 IκB proteins

The second branch of the MyD88-dependent pathway results in the activation of the transcription factor NF-κB. The two major members of the NF-κB family, RelA and p65, reside in an inactivated form in the cytoplasm generally bound to one of a family of IκB proteins. When the IκB protein is phosphorylated it degrades releasing NF-κB which can then be phosphorylated and translocates to the nucleus initiating transcription. Therefore, one method of observing the activation of the NF-κB branch of signalling is in measuring the level of IκB expression. If levels decrease, degradation of this protein likely indicates activation and initiation of NF-κB-controlled transcription. Two members of the IκB family were included in the screening panel and these were IκBα and IκBβ (Figure 3-9).

The expression of IκBα does show a trend towards decreased expression from naïve levels between 0 h and 0.75 h followed by an increase above naïve levels at 2 – 4 h, although this trend was not statistically significant. No statistically significant change was seen in the expression profile of IκBβ although large variability was seen in the repeated screens for this protein.

3.2.2.3 Cytokines

An end result of the signalling pathways examined is the production of immune mediators such as cytokines and chemokines. The secretion of IFN-γ, IL-6, IL-10, IL-12p70, TNF and MCP-1 was investigated in the supernatants collected from LVS-infected MH-S cells using flow cytometry. Of the six cytokines screened, only 3 (MCP-1, TNF and IL-6) were detected (Figure 3-10). MCP-1 was rapidly secreted during the early stages of infection and reached maximum levels of detection (2500 pg/ml) by 1.5 h. TNF was secreted at a slower rate than MCP-1 and by 6 h after uptake of LVS by MH-S cells was detected at 1000 pg/ml compared to 2500 pg/ml for MCP-1. IL-6 was not secreted immediately after uptake and was only detected at a level significantly above naïve levels at 6 h and 24 h.

3.2.1 In vivo characterisation of the immune response to F. tularensis LVS

After gaining insight into the activation of specific proteins located within the MyD88 signalling pathway in an in vitro model, samples from an in vivo infection with F. tularensis LVS were screened for comparison. The samples obtained for screening were taken from an experiment performed by colleagues, Riccardo D’Elia, Tom Laws and Dominic Jenner, involved with a different study. As such, the group sizes and time points at which samples were taken were limited by the study protocol.
Figure 3-9: The expression of IkBα and IkBβ in MH-S cells throughout the course of a F. tularensis LVS infection

The expression of IkBα (A) and IkBβ (B) was determined at several time points after the 2 h uptake period. Representative western blots are shown below the corresponding graphs. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the 95% confidence interval. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
The secretion of cytokines into the supernatant was measured at several time points after the 2 h uptake period. IL-1β, IFN-γ, IL-10, and IL-12p70 (not shown) were undetectable. The experiment was performed in triplicate (n=3). Error bars show the standard deviation. Significant difference from the secretion level at 0 h was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05).
Briefly, groups of 5 mice were challenged with ~ $1 \times 10^4$ CFU of LVS via the intranasal route. At specific time points (2 h, 12 h, 24 h, 48 h, 72 h, 96 h) after infection mice were culled and the blood, lungs, spleen and livers were removed. Bacterial enumeration was performed on all organs and the lungs were homogenised and screened for the expression and activation of the MyD88-dependent signalling proteins. The survival and replication of LVS within distinct organs of the mouse is shown in Figure 3-11. *F. tularensis* LVS was first detected in the lungs at 2 h, then the liver at 24 h and finally the blood and spleen at 48 h. LVS replicated well in the lungs and at 72 h there was nearly a $3 \times \log$ increase over the 2 h count. Interestingly, at 96 h there was a drop in bacterial numbers detected in the lungs. LVS was also found in increasing numbers in all other organs during the course of the infection and at 96 h there was the same number of bacteria in the lung, liver and spleen.

The lung homogenates were screened using the same western blotting approach as for the *in vitro* samples for the presence of activated signalling proteins. However, it was found that, with the experimental protocol used, there was large variation in the results obtained between samples taken from different mice in the same group. Despite carrying out five replicate screens, and the observation of trends in the phosphorylation profile of ERK (Figure 3-12), no significant change in activation levels could be detected for any of the proteins of interest (Figure 3-13, Figure 3-14). Therefore, it was decided to continue with subsequent investigations in the *in vitro* model of LVS infection in MH-S cells.

Cytokine secretion was also measured in the lung homogenates by flow cytometry screening for MCP-1, TNF, IL-6, IFN-γ, IL-10, IL-12p70, MIP-1α, MIG, IL-17, IL-13, GM-CSF and RANTES (Figure 3-15). For all cytokines screened, except MIG, significant levels of secretion could not be detected until 72 h after the initial infection. In all cases, once secretion began, the levels continued increasing through to the final time point of 96 h.

### 3.2.1 *In vitro* characterisation of immune response to *F. tularensis* SchuS4 infection

*F. tularensis* LVS may be used as a model for the more virulent type A strains, such as *F. tularensis* SchuS4, which is able to cause severe disease in humans. The severity of disease combined with the lack of a sufficiently characterised vaccine means that this strain is only handled in ACDP level 3 containment. Using LVS enables research to be done without the restrictions that working in containment can have. However, it is appropriate to validate work done using LVS through an *in
Groups of 5 mice (n = 5) were challenged via the intranasal route with 13,700 CFU of *F. tularensis* LVS. At 2 h, 12 h, 24 h, 48 h, 72 h, and 96 h post-infection the lungs, liver, spleen and blood were removed and the bacterial burdens were determined. The black lines show the position of the mean for each group.
Figure 3-12: The expression and the levels of activated ERK-1 and ERK-2 in the lungs of Balb/c mice infected with *F. tularensis* LVS

The expression of ERK-1 (A) and ERK-2 (B) and the levels of activated ERK-1 (C) and ERK-2 (D) were determined at several time points after infection, via the intranasal route, with 13,700 CFU of *F. tularensis* LVS. Representative western blots are shown below the corresponding graphs. Samples were taken from 5 mice at each time point (n = 5). Error bars show the standard error of the mean. Western blot image: L – MagicMark™ ladder; N – naive; +ve – positive control.
Figure 3-13: The expression and the levels of activated JNK-1 and JNK-2/3 in the lungs of Balb/c mice infected with *F. tularensis* LVS

The expression of JNK-1 (A) and JNK-2/3 (B) and the levels of activated JNK-1 (C) were determined at several time points after infection, via the intranasal route, with 13,700 CFU of *F. tularensis* LVS. Representative western blots are shown below the corresponding graphs. Samples were taken from 5 mice at each time point (n = 5). Error bars show the standard error of the mean. Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
Figure 3-14: The expression and the levels of activated p38 and MKP-1 in the lungs of Balb/c mice infected with *F. tularensis* LVS

The expression of p38 (A) and MKP-1 (B) and the levels of activated p38 (C) and MKP-1 (D) were determined at several time points after infection, via the intranasal route, with 13,700 CFU of *F. tularensis* LVS. Representative western blots are shown below the corresponding graphs. Samples were taken from 5 mice at each time point (n = 5). Error bars show the standard error of the mean. Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
The secretion of MCP-1 (A), TNF-α (B), IFN-γ (C), IL-6 (D), GM-CSF (E), MIP-1α (F) and MIG (G) in the lungs of mice infected via the intranasal route with 13,700 CFU of *F. tularensis* LVS was measured by flow cytometry. Samples were taken from 5 mice at each time point (n = 5). Error bars show the 95% confidence interval. Significant difference from 0 h was determined by a one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05).

Figure 3-15: Cytokine secretion in lungs of mice infected with LVS
vitro infection of MH-S cells using a F. tularensis strain such as SchuS4. As for the LVS infection assays, samples of supernatants and lysates were collected at several time points following uptake of bacteria into the cells. Cytokine levels were measured in the supernatants, signalling protein activation was measured in the lysates and bacterial enumeration was also performed.

In the first 6 h of infection, there was very little increase in bacterial numbers (Figure 3-16). Similarly to LVS, there was a slight decrease in bacterial numbers in the first 2 h of infection and then a plateau until the 6 h time point. Samples were taken for bacterial enumeration at 24 h post-uptake but viable bacteria could not be detected possibly due to high levels of cell death as a result of overwhelming bacterial numbers. Due to the requirement for this infection assay to be performed at ACDP level 3 there were no opportunities for the experiment to be repeated further for this study.

The activation of signalling proteins was also measured using the cell lysate samples. From the western blots for JNK-1 and JNK-2/3, no significant alterations in the levels of activated protein could be detected throughout the infection (Figure 3-17). There appeared to be some down-regulation of activated protein when compared to the naïve cells but, due to the high level of error associated with the naïve samples, the down-regulation was only significant at a few of the time points measured. This data has similarity with the signalling profiles generated by F. tularensis LVS infection that also did not identify any alteration in JNK activation during infection (Figure 3-5). The activation profile for p38 is similar to that of JNK-1 and JNK-2/3. No significant alterations in the activation state of this protein was detected throughout the course of the infection (Figure 3-18) and, as for the JNK proteins, there was some indication of down-regulation in the infected cells compared to the naïve levels, which was only significant only at a few time points due to the large variability observed in the naïve cells. A lack of p38 activation by F. tularensis SchuS4 is in contrast to what was seen in MH-S cells infected with F. tularensis LVS. With LVS, although no late activation of p38 was observed, there was a transient activation of this protein very soon after infection (Figure 3-6). The issue of large variability in the naïve cell levels of proteins also meant that any alteration in the expression of IκBα and IκBβ could not be significantly differentiated from the levels of these proteins in uninfected cells (Figure 3-19).

In contrast to the JNK proteins and p38, the screening of the ERK proteins during F. tularensis SchuS4 infection shows some interesting alterations in their activation profiles (Figure 3-20). At 0 h post-uptake there is a significant increase in activation of ERK-1 and ERK-2 compared to naïve cells. However, the activation is transient and decreases to the level observed in uninfected cell levels by 5
Figure 3-16. The survival of *F. tularensis* SchuS4 within MH-S cells

MH-S cells were infected with *F. tularensis* SchuS4 at an MOI of 10. At 0 h, 0.08 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h, 2 h, 4 h, and 6 h after the 30 min uptake period cells were lysed and bacteria were enumerated. Bacterial enumeration at 24 h (not shown) was not detectable. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the standard error of the mean.
The levels of activated JNK-1 (A) and JNK-2/3 (B) were determined at several time points after the 30 min uptake period. A representative western blot is shown below the corresponding graphs. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. AU = arbitrary units. Error bars show the standard deviation. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: lane 1 – MagicMark™ ladder; lane 2 – naïve; lane 3 – 0 h; lane 4 – 0.08 h; lane 5 – 0.25 h; lane 6 – 0.5 h; lane 7 – 0.75 h, lane 8 – 1 h; lane 9 – 1.5 h; lane 10 – 2 h; lane 11 – 4 h; lane 12 – 6 h; lane 13 – 24 h; lane 14 – +ve; lane 15 – MagicMark™ ladder.

Figure 3-17. The levels of activated JNK-1 and JNK-2/3 in MH-S cells throughout the course of a F. tularensis SchuS4 infection.
Figure 3-18. The levels of activated p38 in MH-S cells throughout the course of a *F. tularensis* Schu4 infection

The level of activated p38 was determined at several time points after the 30 min uptake period. A representative western blot is shown below the corresponding graph. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. AU = arbitrary units. Error bars show the standard deviation. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: lane 1 – MagicMark™ ladder; lane 2 – naïve; lane 3 – 0 h; lane 4 – 0.08 h; lane 5 – 0.25 h; lane 6 – 0.5 h; lane 7 – 0.75 h; lane 8 – 1 h; lane 9 – 1.5 h; lane 10 – 2 h; lane 11 – 4 h; lane 12 – 6 h; lane 13 – 24 h; lane 14 – +ve; lane 15 – MagicMark™ ladder.
Figure 3-19. The expression of IκBα and IκBβ in MH-S cells throughout the course of a F. tularensis Schu4 infection

The expression of IκBα (A) and IκBβ (B) was determined at several time points after the 30 min uptake period. Representative western blots are shown below the corresponding graphs. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. AU = arbitrary units. Error bars show the standard deviation. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: lane 1 – MagicMark™ ladder; lane 2 – naïve; lane 3 – 0 h; lane 4 – 0.08 h; lane 5 – 0.25 h; lane 6 – 0.5 h; lane 7 – 0.75 h; lane 8 – 1 h; lane 9 – 1.5 h; lane 10 – 2 h; lane 11 – 4 h; lane 12 – 6 h; lane 13 – 24 h; lane 14 – +ve; lane 15 – MagicMark™ ladder.
Figure 3-20. The levels of activated of ERK-1 and ERK-2 in MH-S cells throughout the course of a *F. tularensis* SchuS4 infection

The levels of activated of ERK-1 (A) and ERK-2 (B) were determined at several time points after the 30 min uptake period. A representative western blot is shown below the corresponding graphs. The experiment was performed in triplicate (*n* = 3) and each replicate was performed in duplicate. AU = arbitrary units. Error bars show the standard deviation. Significant difference from naïve levels was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Western blot image: lane 1 – MagicMark™ ladder; lane 2 – naïve; lane 3 – 0 h; lane 4 – 0.08 h; lane 5 – 0.25 h; lane 6 – 0.5 h; lane 7 – 0.75 h; lane 8 – 1 h; lane 9 – 1.5 h; lane 10 – 2 h; lane 11 – 4 h; lane 12 – 6 h; lane 13 – 24 h; lane 14 – +ve; lane 15 – MagicMark™ ladder.
min post-uptake. The second feature of ERK activation in response to SchuS4 infection begins at approximately 0.25 h post-uptake and is characterised by a sustained activation which lasts through to 6 h post-uptake. The activation profile of ERK in response to *F. tularensis* SchuS4 infection closely matches the profile obtained from a *F. tularensis* LVS infection (Figure 3-8). In both cases a rapid yet transient early activation is followed by a late sustained activation of the ERK proteins. One difference in profiles observed between the two strains is the time at which the sustained activation begins. For *F. tularensis* SchuS4 the sustained activation of ERK occurs rapidly after infection (0.25 h) whereas for *F. tularensis* LVS the same pattern of sustained activation is not seen until later (1.5 h).

### 3.3 Discussion

The optimisation of *in vitro* *F. tularensis* LVS infection models involved establishing an inoculum preparation protocol and the determination of the appropriate MOI to use. This work was initially carried out in the J774A.1 macrophage-like cell line although optimisation was subsequently performed using the alveolar macrophage-like cell line, MH-S cells. A range of MOIs (10 – 150) were tested as previous studies have used various MOIs depending upon the aspect of infection being studied and the cell line being used. One study observed that to obtain high cytokine secretion, MOIs of above 100 were required, although this was in hepatic lymphocytes. In this study, an MOI of 150 established a strong infection with a high level of intracellular replication without inducing a significant amount of cell death in the time frame being examined and so a MOI of 150 was used for subsequent studies. Although the MOI selected may seem high, a previous study observed that murine macrophages required a MOI ten times higher than human macrophages to induce the same level of infection. After preliminary optimisation in J774A.1s, the infection model was altered to use MH-S cells. These cells are considered a more appropriate cell line to model a *F. tularensis* infection via the inhalational route as alveolar macrophages are one of the first cells to come in contact with bacteria when the lung is the primary site of infection. Comparison of the growth curves of LVS within J774A.1s to those obtained from MH-S cells showed that uptake of the bacteria was similar in the two cell lines. Interestingly, the alveolar macrophages exhibited a superior resistance to intracellular growth (Figure 3-3) demonstrating nearly a 4 x log lower intracellular bacterial burden at 24 h post-uptake when compared to J774A.1s. This suggests that MH-S cells are more resistant to infection than J774A.1 and are likely a better model for *in vivo* lung macrophages whose function is to deal with pulmonary infection.
A more detailed examination of the bacterial burdens after infection showed that there is a significant decrease in bacterial numbers by 2 h after-uptake of LVS (Figure 3-4). This indicates that, at the early time points, the host cell is able to prevent replication of the bacteria and actually reduces the bacterial population by about 0.5 x log. Interestingly, a similar decrease in bacterial numbers is also observed with the more virulent SchuS4 strain suggesting that, at the early stages of infection at least, host cells are able to deal with even highly virulent *F. tularensis*. These early time points could correlate to the period of time the bacteria spend within the phagosome, exposed to bacterial killing mechanisms. However, upon escape from the phagosome *F. tularensis* is free to replicate and this is clearly observed in the growth profile of LVS which shows a 1.5 x log increase in bacterial numbers from 2 h to 24 h. A similar growth profile was observed in a study that infected J774A.1 cells with *F. tularensis* LVS where rapid bacterial replication was only present between 12 and 24 h. In contrast, bacterial numbers of *F. tularensis* SchuS4 remain low until 6 h and no counts could be obtained from 24 h. One possibility is that the MH-S cells were able to limit the replication of *F. tularensis* SchuS4 and therefore clear the infection. This is doubtful as the cells were unable to prevent *F. tularensis* LVS infection and are therefore unlikely to be able to control infection by a more virulent strain. The more likely reason is that *F. tularensis* SchuS4 induced rapid cell death and was therefore lost into the supernatant giving a falsely low bacterial enumeration value.

The rapid induction of cell death by SchuS4 may be due to the activation of the cytoplasmic receptor, AIM-2, that was recently identified as responsible for the detection of *F. tularensis* DNA. Importantly, subsequent to AIM-2 activation is the construction of the caspase-1 inflammasome complex which is integral to the secretion of IL-1β, IL-18 and the induction of cell death. Therefore, through the stimulation of AIM-2 signalling cascades, *F. tularensis* would be able to induce host cell death. Interestingly, in AIM-2-deficient mice, bacterial loads after *F. novicida* infection were greatly increased compared to wild-type mice indicating that AIM-2 is essential for generating an effective innate immune response. It would seem that the activation of AIM-2 is a fine balance between stimulating the cells to secrete pro-inflammatory cytokines and the induction of cell death. In this study, the ability of *F. tularensis* SchuS4 to induce cell death more rapidly than *F. tularensis* LVS could be due to a greater stimulation of AIM-2. This could be attributed to faster cytoplasmic replication or possibly a higher efficacy of *F. tularensis* SchuS4 DNA for AIM-2 compared to that of *F. tularensis* LVS DNA.

In addition to the typical caspase-3-induction of cell death, signalling via the MAPK pathways has been linked to apoptosis. Hrstka *et. al.* observed that a reduction in p38 activation and an increase
in ERK activation, after LVS infection, correlated with increased apoptosis. The in vitro signalling profiles generated in this study confirm these findings. Both F. tularensis LVS and SchuS4 infections displayed a suppression of p38 activation. LVS retained an early and transient activation of p38 but lacked any subsequent re-activation and SchuS4 demonstrated a sustained inactivation throughout the infection. The observed activation of p38 is in agreement with previous studies which showed that, in response to LVS infection, p38 is rapidly activated and demonstrated reduced activation by 2 h. In contrast to p38, both strains of F. tularensis induced a sustained level of ERK activation although SchuS4 induced this earlier in infection. It is possible that F. tularensis induces apoptosis through the MAPK pathway enabling it to proliferate throughout the host. Although sustained activation of JNK has previously been correlated to cell death, this study detected no significant alteration in the activation state of JNK indicating that cell death induced by F. tularensis may only require the p38 and ERK pathways.

The profiles observed in this study demonstrate a crucial aspect of signalling pathways; that it is not only whether a protein is activated or not that is important but also the temporal dynamics of the activation. Previous work has demonstrated differing roles for transient compared to sustained activation of both JNK and ERK proteins. In some cases, transient activation leads to cell survival whereas prolonged activation leads to cell apoptosis. By screening the activation of signalling proteins at multiple time points throughout infection, this study is able to discriminate between the two phases of signalling observed after LVS infection. The first phase, characterised by an early transient response of both p38 and ERK, and the second phase, characterised by repressed p38 activation and sustained ERK activation. In addition, a correlation can be made between the signalling phases and the intracellular life-cycle and replication of F. tularensis LVS. At 2 h after uptake the second phase of signalling begins and it is at this point that the bacterial numbers stop decreasing and begin increasing. It is possible that this switch correlates with the transition from the phagosome into the cytoplasm enabling the bacteria to replicate freely. At the same time, the change in intracellular compartment might induce the transition of signalling activation from the first phase to the second phase beginning the process of inducing apoptosis. The coordination of signalling and replication would enable F. tularensis to replicate to sufficient levels prior to being released from the cell via induction of apoptosis.

To confirm that cytokines were being produced as a result of the infection, the levels of several pro-inflammatory cytokines were measured in the supernatants collected from the F. tularensis LVS infected cells. This measurement showed that three cytokines were secreted at detectable levels.
Interestingly, each cytokine detected was secreted with a different temporal profile (Figure 3-10) an observation that has been seen in a previous study. MCP-1 was secreted rapidly after infection, TNF showed a more gradual secretion profile and IL-6 exhibited late stage secretion. The late secretion of IL-6 could be due to an induction mechanism involving TNF signalling via NF-κB. In LVS-infected MH-S cells, IL-6 only reached detectable levels in the supernatants late in infection. In contrast, TNF secretion began early in infection but at a relatively slow rate. Therefore, TNF may only have reached threshold levels for IL-6 induction at the later stages of infection explaining the delay until 6 h seen for IL-6 secretion. The rapid secretion of MCP-1 is perhaps not surprising as MCP-1 is a chemokine involved in attracting neutrophils to the site of infection. Neutrophils are one of the cell types that are heavily involved in the clearing of infection and therefore would be recruited soon after the detection of an invading organism. However, the recruitment of neutrophils could also be advantageous to the bacteria as *F. tularensis* has been shown to invade and replicate within this immune cell type. TNF was also secreted to a high level but this was a more gradual production exhibiting at least two “spikes” of secretion early after infection. TNF, IFN-γ and IL-12p70 have all been implicated in the resolution of *F. tularensis* infection and their secretion and function appear to be tightly linked through the mechanism of induction and synergy. Although no significant secretion of IL-12p70 or IFN-γ was detected from MH-S cells, it could be that this cell type is not responsible for the large amount seen in studies using *in vivo* models. Another study observed that although both macrophages and DCs could produce IL-12p40, only DCs could secrete IL-12p70. One reason for this may be that, in order to prevent an exacerbated immune response, different cell types are required to be present at the site of infection. If multiple cell types are recruited to the site of infection, the necessary signals to induce cytokine secretion will occur and the synergy between these cytokines produces the required immune response. If conditions to recruit multiple cell types are not met, only a mild response will be generated preventing damage through exacerbated inflammation. The response to LVS infection in this study is in agreement with an earlier study which observed secretion of MCP-1, IL-6 and TNF but not IL-12p70 or IFN-γ in LVS-infected peritoneal macrophages.

It is important to ascertain whether *in vitro* observations correspond to those observed *in vivo*. The cytokine analysis of the *in vivo* *F. tularensis* LVS infection verify the *in vitro* results as TNF, MCP-1 and IL-6 secretion was observed in the lungs of infected mice. Confirming earlier studies, IFN-γ was also detected. Interestingly, and in agreement with other studies, the secretion of all detected cytokines was delayed until 72 h after LVS infection. The reason for the delayed response is not fully understood as there is no evidence that *F. tularensis* secretes any immune-modulatory factors.
although it does seem that it has a number of “passive” strategies for evading immune detection. Despite the massive secretion of cytokines late in infection, F. tularensis is not cleared from the host and the mice succumb to infection. This suggests that the level of cytokines produced is actually detrimental to the host as has been suggested by previous studies \(^{200,370}\). In moribund mice, high levels of IL-6 and MCP-1 were detected in the lungs and spleen at day 7 post-challenge. In comparison, the levels of these two cytokines were very low in surviving animals \(^{370}\). Similarly, elevated levels of IL-6 were found in mice infected with F. novicida when they were near death \(^{200}\). These observations indicate that an immune response involving excessive secretion of MCP-1 and IL-6 is actually detrimental and suggests that a less severe response may be beneficial to the host.

As seen in the in vitro infection, it could be that F. tularensis modulates specific signalling proteins such as p38 and ERK, and the result of this is the delayed secretion of cytokines. However, it is difficult to tell from this study whether the in vitro activation of signalling proteins is apparent in an in vivo model. This is due to large variability in the levels of activation seen between lung homogenates and thus the ability to distinguish significant changes from uninfected controls is very difficult. To account for the variation between mice, another study could have been conducted using larger groups of mice at each time point. However, as the in vitro model is well defined and the cytokine profiles correlated well with those observed from the in vivo model, further studies into the signalling response generated by F. tularensis infection were continued using in vitro models.

In summary:

- MH-S cells initially demonstrate successful control and killing of LVS. However, from 2 h after uptake the numbers of intracellular bacteria increase through to 24 h.
- In response to in vitro infection several signalling proteins, particularly p38 and ERK, display changing activation levels and the secretion of MCP-1, TNF and IL-6 is induced.
- LVS successfully infects the lungs of BALB/c mice and replicates within this organ before proliferating into the liver, spleen and blood.
- The cytokine response to in vivo infection is delayed until 72 h post-infection.
- Increased virulence of F. tularensis SchuS4 compared to LVS could be in part due to an elongated and earlier period of sustained ERK activation inducing greater cell death.
Chapter 4: Targeted immune modulation

4.1 Introduction

Numerous pathogens, including bacteria and viruses, have developed mechanisms of evading or altering the immune response to their advantage. For most pathogens it involves the secretion of immuno-modulatory proteins that are able to interact with host signalling proteins and either block or enhance signalling. Vaccinia virus secretes two proteins named A52R and A46R that prevent signalling at two stages in the TLR-signalling cascade. A46R is able to bind to all four major TLR adaptors and prevent their recruitment to the TIR domain region of TLRs. A52R acts downstream and inhibits the formation of the TRAF-6 / IRAK-2 signalling complex, preventing the progression of signalling to NF-κB. Several bacterial species also use secreted proteins to interfere with host signalling. Salmonella enterica secretes protein A that impairs the TLR and MyD88-associated activation of NF-κB. These modulation strategies involve the suppression of signalling. However, in some cases an up-regulation of the immune response is required for successful proliferation of the pathogen. For example, Yersinia sp. secrete a virulence factor called LcrV that signals through TLR2 inducing the secretion of IL-10, a potent immune-suppressive cytokine. The intracellular pathogen Listeria monocytogenes, also uses signalling activation to enhance its pathogenicity. Similarly to F. tularensis, L. monocytogenes, requires host cells to replicate within. By up-regulating NF-κB activation, L. monocytogenes increases the expression of adhesins and the secretion of chemokines at the site of infection subsequently attracting phagocytes thereby increasing its replicative niche.

F. tularensis also modulates innate immune signalling albeit with a more subtle approach than most other pathogens, resulting in it being designated, by some, as a stealth pathogen. Whilst situated outside a host cell, F. tularensis prevents its detection by expressing tetra-acylated LPS that exhibits a lower stimulatory action upon TLR4 than other LPS structures. This pathogen is also able to utilise complement, usually a host defence mechanism, to gain access to host cells without generating an immune response. Within host cells, F. tularensis continues to modulate the immune response. Inside the phagosome, F. tularensis prevents the correct assembly of oxidative species-producing complexes and within the cytosol it inhibits signalling induced by the NLR family of receptors. Clearly, at every stage of its intracellular lifecycle, from initial host cell entry through to the induction of apoptosis and subsequent re-infection, F. tularensis is interacting with immune signalling to create a more hospitable niche for its survival. The result is a complex immune...
response characterised primarily by a suppression of cytokine secretion followed, after sufficient replication, by delayed and overwhelming inflammation. As a greater understanding of the variety of strategies used by pathogens to modulate the immune system has been generated, research has begun to examine the possibility of using the pathogens’ strategies as a template for therapeutic intervention. Initial research examined the use of TLR agonists and antagonists to stimulate or block particular pathways. Despite the ease with which modulation can be brought about with direct receptor stimulation, this approach to therapeutic intervention does have disadvantages. As the target is the first step in the pathway the modulation of signalling has a broad range of downstream effects. For example, by blocking TLR4 activation, both the MyD88-dependent and TRIF-dependent pathways would be suppressed leading to widespread inhibition of transcription from IRFs, NF-κB and AP-1 transcription factors. Direct stimulation of PRRs as a therapeutic strategy has a high chance of activating numerous downstream signalling pathways leading to extensive cytokine production and, potentially, overwhelming inflammation. Stimulation of TLR9 with purified bacterial DNA in mice has induced protection from subsequent challenge with Listeria monocytogenes. In contrast though, treatment with CpG motifs adversely affected the survival of mice infected with F. tularensis SchuS4. CpG stimulation had a beneficial outcome in another bacterial infection and protected mice challenged with B. pseudomallei. These data indicate that the success of therapeutic modulation is highly pathogen specific.

The “next generation” of therapeutic modulation revolves around a more refined approach of targeting specific proteins located within the signalling pathways downstream of the PRRs. Signalling pathways respond to multiple and diverse stimuli therefore therapeutics designed to modify signal transduction have been investigated for many diseases as well as infection. Research has been carried out across numerous medical disciplines including Parkinson’s disease, hepatocellular carcinoma, rhabdomyosarcoma, septic shock and rheumatoid arthritis. Drugs specifically targeting the MAPK pathway have been used to treat both viral and bacterial infections. Aerosol administration of the MEK inhibitor, U0126, successfully protected mice against a 100x lethal viral challenge with Influenza A subtype H1N1. The entry of Yersinia into HeLa cells was prevented using wortmannin, a specific PI3-K inhibitor. Targeting signal transduction pathways, however, has rarely been completely successful. Mice pre-treated with the p38 inhibitor, SB203580, and subsequently challenged with intratracheal E. coli exhibited increased lung injury and decreased survival. The failure of targeting the signal transduction pathways has probably been
due to the lack of selectivity of inhibitors for a specific kinase, but as drug selectivity improves this problem is likely to diminish.

Treatment of *F. tularensis* infection with immune-modulatory drugs has received less attention. One study has identified the signalling protein GSK3B as being a potential therapeutic target in *F. tularensis* infection\(^{209}\). Inhibition of GSK3B, using lithium, reduced the inflammatory cytokine response and increased survival of mice challenged with *F. tularensis* LVS\(^ {209}\). As *F. tularensis* interacts with TLRs and has been shown to subvert innate immunity, immune signalling pathways are a potential target for drug therapeutics in the future.

### 4.2 Aims and Objectives

The aim of this chapter was to investigate whether inhibition of specific signalling proteins could alter the outcome of infection with *F. tularensis*.

The specific aims of this chapter were to:

- Determine whether ERK inhibition affects the survival of LVS within MH-S cells.
- Determine the effect of ERK inhibition on the secretion of cytokines by MH-S cells in response to LVS infection.
- Determine whether ERK inhibition improves the outcome of *F. tularensis* LVS infection in mice.

### 4.3 Results

#### 4.3.1 Targeting ERK activation *in vitro*

My work and published data shows that infection of cells by *F. tularensis* induces a signalling response, involving the activation of multiple proteins, partly induced by TLRs (Section 3.2)\(^ {300,305,328,363,369}\). Characterisation of the signalling cascade identified the signalling protein ERK as being important in the response to *F. tularensis* LVS. The stimulation of ERK, by *F. tularensis* LVS infection *in vitro*, resulted in a very clear bi-phasic profile, with early transient activation and late sustained activation (Figure 3-8). Previous studies have observed a detrimental effect of sustained MAPK activation on host cells and, in this study, sustained ERK activation coincided with bacterial replication (Figure 3-4)\(^ {140,282,366,385}\). Inhibition of ERK, during the late stages of infection, could re-
balance the response preventing *F. tularensis* LVS replication and possibly aiding the resolution of infection.

Several ERK inhibitors are commercially available. PD0325901 can be given by oral gavage and has demonstrated a high degree of specificity and good efficacy in previous *in vivo* studies. In a study treating cancerous cells in the flanks of mice, PD0325901 was given by oral gavage and systemic inhibition of ERK was shown even 24 h after treatment. Importantly, PD0325901 is not a direct ERK inhibitor and interacts with the upstream protein MEK-1/2. However, the MEK proteins have very specific substrates and so it is likely that inhibition of MEK will only prevent the activation of ERK and not the other major MAPKs, JNK and p38.

The potential for PD0325901 to inhibit ERK activation was first examined *in vitro* using the murine alveolar macrophage-like MH-S cell line infected with *F. tularensis* LVS. The cells were infected with *F. tularensis* LVS (t = -2 h) and the cells were allowed to take up the bacteria for 2 h. Media was removed and free bacteria were washed off the cells with PBS and fresh media added (t = 0 h). As a control, some cells were left uninfected (naïve). PD0325901 was added to the cells at either 0 h or 2 h after the removal of free bacteria. At multiple time points after infection, supernatants and cell lysates were collected for cytokine analysis and screening for signalling protein activation and bacterial enumeration was also carried out.

Consistent with published data, PD0325901 specifically inhibited ERK activation (Figure 4-1 A, B). When the cells were treated at the point of removing free bacteria (0 h) there was no measurable activation of ERK and this was maintained through to 24 h. When addition of PD0325901 was delayed until 2 h post-infection, the level of ERK activation at 0 h was no different than in cells without PD0325901. However, as expected, from 4 h the activation of ERK was entirely suppressed and this was maintained through to 24 h. To ensure PD0325901 was specifically inhibiting the activation of ERK and not other, structurally similar, MAPKs the activation of p38 was also measured. No significant inhibition was observed in either of the two PD0325901 groups when compared to untreated controls (Figure 4-2). The effect of ERK inhibition on the production of immune mediators was determined by measuring the level of inflammatory cytokine secretion. The inhibition of ERK activation correlated with a decrease in cytokine secretion in response to LVS infection (Figure 4-3 A,B). Secretion of IL-6 showed a significant decrease in both PD0325901 groups at 24 h compared to the levels observed in untreated controls (Figure 4-3 A). When PD0325901 was added at 0 h, the secretion of TNF showed a reduced level from 1 h post-uptake through to 24 h.
Figure 4-1: The levels of activated ERK-1 and ERK-2 in PD0325901-administered MH-S cells throughout the course of a *F. tularensis* LVS infection

The levels of activated ERK-1 (A), ERK-2 (B) were determined at several time points after the uptake of LVS. Representative western blots are shown on the opposite page. The experiment was performed in triplicate (n=3) and each replicate was performed in duplicate. Error bars show the min and max. Western blot images: L – Ladder; N – Naive.
Figure 4-2: The levels of activated p38 in PD0325901-treated MH-S cells throughout the course of a *F. tularensis* LVS infection

The levels of activated p38 were determined at several time points after the uptake of LVS. Representative western blots are shown above the corresponding graph. The experiment was performed in triplicate (n=3) and each replicate was performed in duplicate. Error bars show the min and max. Western blot image: L – Ladder; N – Naïve.
Figure 4-3: The secretion of cytokines and bacterial burdens of MH-S cells treated with PD0325901 (0.05 µM) and infected with *F. tularensis* LVS

The secretion of IL-6 (A) and TNF (B) was determined at several time points after the uptake of LVS. Enumeration of bacteria was also performed (C). The experiment was performed in triplicate (n=3) and each replicate was performed in duplicate. Error bars show the min and max. Significant difference from untreated levels was determined by a one-tailed, unpaired t-test (* = p<0.05).
However, when PD0325901 was added at 2 h a reduced level of TNF secretion, compared to untreated, was only observed from 4 h (Figure 4-3 B). This indicates that the reduction in the levels of cytokine secretion is specifically due to ERK inhibition by PD0325901 as a reduction of TNF secretion is only observed after addition in each case. Finally, bacterial enumeration was carried out to see if ERK inhibition resulted in a lower bacterial burden within the host cells.

Despite the differences observed in ERK activation and cytokine secretion, no alteration in intracellular bacterial numbers was seen in either PD0325901 group compared to untreated cells (Figure 4-3 C). The growth profiles from all groups displayed the usual pattern of limited replication in the first 4 h followed by an increase in bacterial numbers up to 24 h.

### 4.3.1 Cytotoxicity of PD0325901

The activation of ERK has been shown to closely correlate with the balance between cell survival and apoptosis. Whether activation is prolonged or transient seems to determine the outcome of the signalling pathway. As *F. tularensis* infection causes apoptosis of host cells, the effect of ERK inhibition on cell death is an important factor to take into account when considering signalling modulation.

To determine the effect upon cell death of PD0325901 in infected and uninfected cells, PD0325901 was added to MH-S cells at a range of concentrations (0.005 µM – 5 µM) for 24 h. The level of cytotoxicity induced by LVS and PD0325901 addition was examined using an LDH assay (Figure 4-4). In addition, supernatants were collected to allow analysis of the impact of PD0325901 concentration on cytokine secretion (Figure 4-5). At all concentrations tested, except 0.005 µM, and in both the presence and absence of LVS, PD0325901 induced significantly higher cell death than naïve cells. However, LVS also induced greater cytotoxicity than naïve cells and only PD0325901 given at 0.5 µM and 5 µM at 0 h in the presence of LVS induced significantly greater cell death than LVS only. This indicates that PD0325901, at low concentrations, does not enhance the cytotoxicity already induced by LVS. From the data collected from LVS-infected cells, it can be seen that for the three highest concentrations there is a significant decrease in cytotoxicity observed if addition of PD0325901 is delayed for 2 h when compared to the same concentration given at 0 h (Figure 4-4 B). The difference when PD0325901 is added at 0 h and 2 h is only seen when LVS is present and is absent from the data collected from uninfected cells (Figure 4-4C).
Figure 4-4: The cytotoxicity induced by *F. tularensis* LVS infection and PD0325901 (µM) treatment of MH-S cells

The % cytotoxicity was determined, using a LDH assay, 24 h after infection of MH-S cells with *F. tularensis* LVS. (A) Full range of treatment groups. (B) Focussed on LVS infected cells. (C) Focussed on uninfected cells. The experiment was performed in triplicate (n=3) and each replicate was performed in duplicate. Error bars show the min and max. Significant difference from either untreated or LVS only was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.05). Significant difference between treatment times was determined by a one-tailed, unpaired t-test (* = p<0.05).
Figure 4-5: The secretion of cytokines by MH-S cells infected with *F. tularensis* LVS and treated with PD0325901 (µM)

The secretion of IL-6 (A) and TNF (B) was measured 24 h after infection of MH-S cells with *F. tularensis* LVS. The experiment was performed in triplicate (n=3) and each replicate was performed in duplicate. Error bars show 95% confidence interval. Significant difference from LVS only was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (* = p<0.005). Significant difference between treatment groups was determined by a one-tailed, unpaired t-test (* = p<0.05)
From the same experiment, supernatants were collected to observe the effect of PD0325901 concentration on the secretion of IL-6 and TNF (Figure 4-5). Infection of cells with LVS induced a strong secretion of both IL-6 and TNF which was not observed in naïve cells. Secretion of IL-6 and TNF exhibited a clear dose-dependent inhibition independent of whether PD0325901 was added to the cells at 0 h or 2 h post-uptake. All concentrations of PD0325901, except 0.005 µM, irrespective of the time it was added, significantly reduced the level of IL-6 secretion compared to the level seen in LVS only cells (Figure 4-5 A). Addition of PD0325901 at each concentration at 0 h, except 0.005 µM, reduced IL-6 secretion to lower levels than seen in the 2 h groups given the same concentration. For TNF, only PD0325901 at 0.05, 0.5 and 5 µM given at 0 h significantly reduced secretion below that seen in LVS only cells (Figure 4-5 B). Every concentration of PD0325901, given at 0 h, significantly reduced TNF secretion below the level observed in the corresponding 2 h groups.

### 4.3.2 Targeting ERK activation in vivo

The potential for ERK inhibition, through PD0325901 addition, to improve the outcome of infection in vivo was determined using the murine model of infection. Groups of Balb/c mice were infected via the intranasal route with a lethal dose of LVS ($2 \times 10^4$ CFU). The mice were either left untreated, treated daily from 24 h pre-infection or daily from 48 h post-infection. Groups of mice were culled at 48 h and 96 h and the lungs, liver and spleen were removed and analysed for cytokine secretion and bacterial counts. The level of activated ERK was also determined in the lungs.

The level of ERK activation in the lung was determined by western blot analysis of the homogenates using antibodies specific for the phosphorylated states of ERK-1 and ERK-2. In naïve mice, no elevation in the level of ERK activation was observed across the 96 h indicating that it is infection by *F. tularensis* LVS that is the cause of ERK activation (Figure 4-6 A, B). In mice infected with LVS and treated with PBS there was a substantial increase in the level of ERK activation from 48 h to 96 h. Daily treatment with PD0325901 significantly reduced the level of both ERK-1 and ERK-2 activation in the lungs of mice compared to PBS treated mice at 96 h (Figure 4-6 A, B). This was the case for both the treatment groups irrespective of whether the treatment began at 24 h pre-infection or 48 h post-infection. Interestingly, at 48 h PD0325901-treated mice demonstrated an increase in ERK activation compared to PBS-treated mice. This is in contrast to the expected result as PD0325901 is an ERK inhibitor but in this case it seems to be enhancing ERK activation.
Figure 4-6: The levels of activated ERK-1 and ERK-2 in the lungs of *F. tularensis* LVS infected Balb/c mice treated with either PBS or PD0325901 (0.05 µM)

Groups of 5 mice (n = 5) were challenged via the intranasal route with 20,000 CFU of *F. tularensis* LVS. On day 2 and 4 post-challenge, the lungs were removed, homogenised and screened for the levels of activated ERK-1 (A) and ERK-2 (B). Naïve = uninfected, PBS = infected and treated daily with PBS from 24 h pre-challenge, PD (-1) = infected and treated daily with PD0325901 from 24 h pre-challenge, PD (+2) = infected and treated daily with PD0325901 from 48 h post-challenge. Representative western blots are shown on the opposite page. Significant difference from PBS-treated group was determined by a one-tailed, unpaired t-test (* = p<0.05). Error bars show the min and max. Western blot image: L – Ladder; N – Naïve; -ve – negative control; +ve – Positive control.
Similarly to the treatment of *in vitro* LVS infection, the effect of *in vivo* ERK inhibition on the inflammatory response was examined by measuring the secretion of downstream inflammatory cytokines in the lungs, liver and spleen of naïve, PBS treated or PD0325901 treated Balb/c mice 96 h after infection. Despite the observed inhibition of ERK activation in the lungs of mice at 96 h, no reduction in the secretion of any of the measured cytokines (IFN-γ, MCP-1, IL-6 and TNF) was seen at the primary site of infection, the lungs (Figure 4-7 A-D). In addition, there was no observed decrease in the secretion of any cytokine in the liver of pre-treated compared to PBS treated mice. In mice treated 48 h post-infection with PD0325901 there was a significant increase in IFN-γ and MCP-1 in the liver compared to PBS treated mice. However, the greatest effect of ERK inhibition was observed in the spleen. No change in cytokine secretion was observed in mice treated post-infection but, for all cytokines screened, a significant reduction in secretion was observed in the spleen of pre-treated mice compared to PBS treated mice.

To examine the effect of ERK inhibition on bacterial loads in the organs of mice, bacterial enumeration was carried out on samples taken from the lungs, liver and spleen 96 h post-infection. In agreement with the *in vitro* data, where ERK inhibition had no effect on bacterial burdens (Figure 4-3), there was no significant difference in the numbers of bacteria present in the lungs of either PD0325901 treated groups compared to PBS treated mice (Figure 4-8 A). In contrast, although no changes were seen in mice treated post-infection, bacterial burdens in both the liver and spleen were reduced in the pre-treated groups compared to PBS treated mice (Figure 4-8 A). However, despite the successful inhibition of ERK in the lungs and the reduced bacterial burdens in the spleen and liver, there was no alteration in survival of any of the PD0325901 treated groups compared to the PBS treatment groups as all the mice, except one from the PBS group, were culled on either day 8 or day 9 after infection (Figure 4-8 B).

**4.4 Discussion**

The characterisation of TLR signalling induced by *F. tularensis* infection suggested ERK was a potential target for therapeutic immune-modulation. PD0325901 was selected from a range of commercial inhibitors and its suitability for use as an immune-modulator was assessed firstly *in vitro* and subsequently *in vivo*. PD0325901 functions by binding to a non-adenosine triphosphate binding site on MEK-1/2 inducing a conformational change that prevents the subsequent binding and activation of ERK. PD0325901 has previously been used in the treatment of cancer in several murine models and was demonstrated to be specific and efficacious in its ability to inhibit ERK
Figure 4-7: Cytokine secretion in the organs of Balb/c mice infected with *F. tularensis* LVS and treated with PBS or PD0325901 (0.05 µM)

Groups of 5 mice (n = 5) were challenged via the intranasal route with 20,000 CFU of *F. tularensis* LVS. On day 4 post-challenge the organs or infected mice were removed, homogenised and the levels of IFN-γ (A), MCP-1 (B), IL-6 (C) and TNF (D) were determined. Naïve = uninfected, PBS = infected and treated daily with PBS from 1 day pre-challenge, PD (-1) = infected and treated daily with PD0325901 from 24 h pre-challenge, PD (+2) = infected and treated daily with PD0325901 from 48 h post-challenge. Significant difference from PBS-treated group was determined using a one-tailed, unpaired t-test (* = p<0.05). Error bars show the min and max.
Figure 4-8: Bacterial burdens of organs and survival of Balb/c mice infected with *F. tularensis* LVS and treated with PBS or PD0325901 (0.05 µM)

Groups of 5 mice (*n* = 5) were challenged via the intranasal route with 20,000 CFU of *F. tularensis* LVS. On day 4 post-challenge the organs or infected mice were removed, homogenised and bacterial enumeration was performed (A). PBS = infected and treated daily with PBS from 24 h pre-challenge, PD (-1) = infected and treated daily with PD0325901 from 24 h pre-challenge, PD (+2) = infected and treated daily with PD0325901 from 48 h post-challenge. Significant difference from PBS-treated group was determined using a one-tailed, unpaired t-test (* = p<0.05). Error bars show the min and max. (B) 10 mice in each group were used for a survival study (*n*=10). Balb/c mice were infected via the intranasal route with 20,000 CFU *F. tularensis* LVS and treated daily with either PBS or PD0325901. The animals were scored daily and were culled upon reaching the humane endpoint. No significant difference was observed in survival for any treatment group when compared to PBS-treated animals.
activation. After dosing by oral-gavage, the systemic activation of ERK is inhibited for up to 24 h in both the flanks and prostates of mice. As in previous studies, administration of PD0325901 in vitro initially demonstrated good specificity and efficacy as it suppressed the activation of ERK for at least 24 h whilst not affecting the activation of another MAPK, p38 (Figure 4-1). In addition, targeted inhibition of ERK strongly correlated with the suppression of IL-6 and TNF secretion (Figure 4-3 A and B).

Interestingly, even when ERK was inhibited from 0 h there was an initial secretion of TNF observable at 1 h post-uptake. This may be explained by the role that ERK activation plays in the secretory pathway of TNF. TNF secretion involves several stages including transcription, transport of mRNA from the nucleus to the cytoplasm and then translation. ERK activation is required for the transport of TNF mRNA into the cytoplasm. Therefore, in the presence of PD0325901, the transport of newly transcribed mRNA cannot take place and only mRNA already present in the cytoplasm can be translated resulting in a plateau of TNF secretion at 500 pg/ml. The importance of ERK activation for efficient secretion of TNF is consistent with previous studies. One group examining the role of a signalling protein upstream of ERK observed that TNF secretion by peritoneal macrophages and BMDMs, in response to LPS stimulation, was completely abrogated in the absence of ERK activation. Similarly, inhibition of ERK activation using U0126 prevented TNF secretion by monocytes stimulated with a biological response modifier, OK-432. For the 2 h treated group, the plateau of TNF levels occurs at a slightly higher concentration (750 pg/ml) than the 0 h treated group possibly due to a greater amount of nucleocytoplasmic transport of TNF mRNA before the inhibitor is added. However, once the effects of ERK inhibition are imposed mRNA transport ceases and TNF levels plateau. The levels of IL-6 in both treated groups were significantly lower than in untreated at 24 h. However, for all groups, IL-6 levels are a lot higher than the levels of TNF produced by the same cells. The greater levels of IL-6 compared to TNF could be explained by the differences in their mRNA stability. The half-life of IL-6 mRNA, in stimulated macrophages, is more than 20 h whereas for TNF mRNA is nearer 75 min. Therefore, the majority of the IL-6 mRNA present in the cytoplasm prior to ERK inhibition would still be present for translation throughout the time course of the experiment. In contrast, the TNF mRNA rapidly degrades and would require constant replenishment from the nucleus to obtain similar secretion levels to IL-6. The correlation of mRNA stability with secretion levels has been demonstrated in a separate study which observed a 70% decrease in IL-6 protein biosynthesis when the half-life of IL-6 mRNA was reduced from 20 h to 2 h. Similarly, the secretion of TNF was enhanced by stimulation of RAW264.7 macrophages with LPS and this correlated with an increase in half-life from 30 min to 80 min.
However, despite successful inhibition of both protein activation and cytokine secretion there was no observable alteration in bacterial numbers (Figure 4-3 C). Irrespective of treatment group, LVS successfully replicated within MH-S cells throughout the course of the infection. This is in agreement with another study that exhibited an unaltered increase in bacterial numbers despite successful inhibition of ERK signalling inhibition, albeit with a different inhibitor to the one used in my work 282.

One explanation for the observed result could be the close link between ERK activation and the regulation of cell death 385,396. From my work the induction of MCP-1 secretion in MH-S cells was a result of F. tularensis infection and the production of this chemokine could lead to the recruitment of immune cells such as neutrophils. Other studies have also observed an influx of immune cells following F. tularensis infection 397,398. As an intracellular pathogen F. tularensis requires host cells to replicate and it could be that the recruitment of immune cells is a pathogenic strategy to enlarge its replicative niche. Other intracellular pathogens use the recruitment of immune cells to ensure efficient replication and proliferation. L. monocytogenes secretes listeriolysin O that activates the IKK complex inducing transcription by NFκB resulting in the up-regulation of adhesion molecules on endothelial cells 372,375. These adhesins “trap” immune cells, such as neutrophils, providing a replicative niche for the bacteria. F. tularensis LVS was recently shown to not only survive and replicate within neutrophils but also inhibit the induction of apoptosis 399, a survival mechanism used by other pathogens such as N. gonorrhoeae and C. pneumoniae 396,400,401. The treatment of MH-S cells with PD0325901, inhibiting ERK activation and thereby delaying the induction of cell death, potentially increases the number of cells available for LVS to invade and survive within. This hypothesis is consistent with a previous study that demonstrated that the inhibition of ERK, using the indirect MEK-inhibitor PD098059, prevents macrophage apoptosis and necrosis after LVS infection 282. To determine if a reduction in ERK-mediated apoptosis was causing the lack of difference between treated and untreated bacterial counts, a LDH assay was done to measure the level of cell death. In contrast to the previous study, which observed a decrease in cell death correlating to ERK inhibition 282, treatment of MH-S cells with PD0325901 did not reduce the level of cell death (Figure 4-4). In fact, at high concentrations of PD0325901, there was a slight increase in the level of cell death. A possible reason for the discrepancy between these studies is the use of different cell lines as the infection model. The previous study used the macrophage-like J774.2 cell line whereas this study used the alveolar macrophage-like MH-S cell line. From the preliminary experiments carried out at the start of this study comparing the growth of LVS in two separate cell lines, it is clear that there are major differences between cell types (Figure 3-3). In J774 cells, LVS is able to replicate to a much larger degree than in MH-S cells indicating different abilities of these cell
types to respond to infection. The altered growth rate, and corresponding bacterial burden at 24 h after infection, could explain the different effects of ERK inhibition observed between cell lines. In J774A.1 cells the high bacterial burden induces cell death mechanisms which can be inhibited by PD0325901. However, the reduced growth rate seen in MH-S cells may not generate the required bacterial burden threshold to induce cell death and so there is no signalling activation for PD0325901 to inhibit. As such, no significant difference in cell death can be observed between untreated and PD0325901-treated MH-S cells. The possibility of slower growth preventing apoptosis is confirmed by other studies that demonstrate a reduction of cell death when bacterial replication is prevented. One study observed that rapid intracellular growth of *F. tularensis* in J774A.1 cells resulted in pronounced apoptosis. However, if intracellular bacteria were prevented from replicating host cell cytopathogenicity and apoptosis was prevented. Another difference between the two studies is the inhibitor administered to inhibit ERK signalling. The previous study used PD098059 and this study used PD0325901. Although both are commercially available and are described as MEK-specific inhibitors, it is possible that the specificity of each of these inhibitors differs leading to a variation in their effect on signalling pathways. It seems then, at least *in vitro*, a link between ERK inhibition and the prevention of cell death does not explain the lack of a reduction in *F. tularensis* LVS numbers.

Although, there are limitations in characterising the potential benefit of an ERK inhibitor in an *in vitro* model, the data does provide essential information on the specificity and efficacy of PD0325901. For example, in a natural infection different cell types work in synchrony in order to produce a full and effective immune response. This has been demonstrated in the hepatic response to *F. tularensis* infection where natural killer cells, T cells and DCs were required to generate the full cytokine response. In contrast, the *in vitro* model has only one cell type present preventing the usual cell-cell interaction and cooperation found in a natural infection. Therefore, an *in vivo* study was carried out to enable the analysis of a more complex immune response generated by *F. tularensis* LVS and modulated by treatment with PD0325901.

The ERK inhibitor, PD0325901, has previously been administered in an *in vivo* setting examining its possible use as an anti-tumour treatment. From these previous studies, information about the route of dosing and the pharmacokinetics of the drug was available. It was decided to dose Balb/c mice daily via oral-gavage. One alteration to the dosing schedule used in other studies is the concentration of drug used. The preliminary investigations carried out in this study showed that PD0325901 demonstrated good efficacy at lower concentrations (0.05 µM) than the supplier’s
suggested working concentration (0.5 µM). In addition, high concentrations display cytotoxic effects (Figure 4-4) and so the lower concentration (0.05 µM) was used.

Treatment with PD0325901, via oral-gavage, suppressed the systemic level of activated ERK protein in the lungs of treated mice at 96 h (Figure 4-6)\textsuperscript{344,388}. Surprisingly, at 48 h, mice pre-treated with PD0325901 displayed an increase in ERK activation in the lungs compared to PBS-treated mice. One possible explanation for this is related to the way that PD0325901 prevents ERK activation. PD0325901 functions as a competitive inhibitor by binding to a non-adenosine triphosphate binding site on MEK-1/2 inducing a conformational change that prevents the binding of ERK (Figure 4-9A)\textsuperscript{387}. It is possible that \textit{F. tularensis} may secrete a protein that is able to prevent ERK activation in a manner similar to PD0325901. Although no effector proteins have been confirmed in \textit{F. tularensis}, this pathogen does express a type VI secretion system\textsuperscript{261}. Type VI secretion systems are used by numerous pathogens including \textit{Burkholderia mallei}, \textit{Vibrio cholera} and \textit{P. aeruginosa} to secrete effector proteins into target cells\textsuperscript{404-408}. It is highly likely that un-identified effectors exist that are secreted through the \textit{F. tularensis} type VI secretion apparatus. The delay in cytokine secretion in response to \textit{F. tularensis} infection \textit{in vivo} further supports the theory of secreted effector proteins modulating immune signalling. If either PD0325901 or the putative \textit{F. tularensis} protein is present alone they would bind to MEK-1/2 and interrupt ERK activation (Figure 4-9B). However, in my work, when PD0325901 and \textit{F. tularensis} are present in the lungs at the same moment there is actually an increase in ERK activation. One mechanism whereby the combination of two inhibitors could induce an increase in ERK activation is if there is an interaction between the inhibitors themselves that prevents their correct binding to their target, MEK-1/2 (Figure 4-9C). This would enable MEK-1/2 to activate ERK in the usual manner leading to the increase in ERK activation observed at 48 h in PD0325901-treated mice challenged with \textit{F. tularensis} LVS. Further studies would be required to identify potential \textit{F. tularensis} effector proteins and determine if an interaction with PD0325901 is possible. This is not the only potential explanation for the observed result as the regulation of ERK activation is highly complex involving at least two negative regulators MKP-3 and phosphatase of activated cells (PAC)-1\textsuperscript{409,410}. The activity of MEK-1/2 is also regulated by phosphorylation of specific serine residues\textsuperscript{411}. Phosphorylation of Ser298 and Ser212 can enhance or decrease MEK-1/2 activity, respectively\textsuperscript{412,413}. Clearly, there are multiple pathways by which \textit{F. tularensis} could modulate ERK activation. Indeed several bacterial pathogens interact with and modulate ERK activation in different ways. \textit{Staphylococcus aureus} secretes haemolysins that modulate ERK activation whereas \textit{L. monocytogenes} activates ERK via the TLR2 and RIP-2 pathways inducing autophagy\textsuperscript{272,414}. In contrast to 48 h, ERK activation at 96 h is dramatically increased in PBS-
Figure 4-9: Theoretical models of ERK inhibition and activation involving interactions between PD0325901, MEK-1/2 and an unidentified *F. tularensis* effector protein

The usual activation of ERK (A) involves the binding of ERK to MEK-1/2 inducing the phosphorylation of specific residues. PD0325901 inhibits the activation of ERK by binding to a non-adenosine triphosphate binding site on MEK-1/2 causing a conformational change that prevents the binding of ERK (B). It is possible that an unidentified *F. tularensis* protein (Ft #1) also indirectly inhibits the activation of ERK through MEK-1/2 binding. Therefore, when PD0325901 or the unknown protein is present MEK-1/2 is unable to activate ERK (B). In the case where both PD0325901 and the *F. tularensis* protein are present an interaction between these two inhibitors prevents them binding to MEK-1/2 (C). Therefore, MEK-1/2 does not undergo a conformational change and ERK can be activated in the usual manner.
treated mice indicating that the mechanism of ERK inhibition has been released. In PD0325901-treated mice, although the *F. tularensis*-associated inhibition has been released, the inhibition due to PD0325901 is still present and so the level of ERK activation is decreased compared to PBS-treated mice.

Interestingly, although ERK activation is significantly reduced in the lungs of infected mice at 96 h, there was no alteration in bacterial burden or cytokine secretion in this organ, the primary site of infection. One explanation for the lack of a decrease in cytokine expression in the lungs, despite effective ERK inhibition, could be the requirement for a controlled, rapid and robust immune response in order to resolve infection. Due to their location and function as an air-fluid interface, the lungs are particularly susceptible to infection by multiple pathogens and the potential outcomes of respiratory infection are severe. As such, it may be that the signalling pathways activated in pulmonary immune and epithelial cells contain a high level of redundancy or complexity to ensure that a proportional yet robust immune response is generated irrespective of the pathways involved. If this is the case, inhibition of ERK alone would not be sufficient to affect cytokine secretion and inhibition of multiple MAPKs or members of other signalling protein families may be required to dampen the cytokine response. For example, using a human lung slice model of *B. anthracis* infection a combination of three inhibitors targeting p38, JNK and ERK were required to prevent cytokine secretion.

In contrast to the data from the lungs, a reduced bacterial burden was observed in the liver and spleen indicating that the treatment was decreasing the systemic spread of *F. tularensis* LVS to secondary sites of infection (Figure 4-8). The reason behind reduced systemic spread could be down to the link between ERK activation and host cell death. In several other bacterial infections, an association between ERK and the induction of apoptosis has been observed, although the exact correlation differs depending on the infecting organism. It seems that the anti-apoptotic properties of *Chlamydia* are due to the activation of the Raf/MEK/ERK pathway whereas *Helicobacter pylori* induces ERK-dependent AP-1 transcription to bring about apoptosis in macrophages. Although *in vitro* data from this study indicates that ERK inhibition does not affect cell death, it is possible that in an *in vivo* setting this may not be the case. The induction of cell death by apoptosis is a strategy of *F. tularensis* to promote its survival and proliferation as it releases the bacteria allowing systemic spread. In this study, by reducing the level of ERK activation in the lung, the infected immune cells at the primary site of infection might exhibit reduced cell death. This would trap the bacteria within alveolar macrophages and neutrophils reducing the...
number that are able to escape the lung and disseminate to other organs. This hypothesis fits the bacterial counts observed in this study, as there is no alteration in numbers of LVS present in the lung, where bacteria are potentially trapped within immune cells, but a decrease in both the liver and spleen is observed compared to PBS-treated controls. The mechanism of “trapping” bacteria within lung macrophages, and thereby restricting bacterial dissemination from the lungs to other organs as a therapeutic approach, has not been widely examined. However, one field of research that has received much interest is that of sepsis. Bacterial sepsis develops when the bacteria overwhelm the local containment and enter the general circulation causing excessive systemic inflammation that may eventually lead to multi-organ failure and death. As such, the possibility of restricting the dissemination of bacteria from the local containment has been studied as a therapeutic approach. One study observed that blocking scavenger receptors prevented bacterial proliferation from local containment and increased survival of C57BL/6 mice after cecal ligation and puncture-induced sepsis. Outside sepsis research, it has been observed that intratracheal instillation of heparin in A/J mice improved survival after infection by the intracellular pathogen Legionella pneumophila. This result correlated with a prevention of bacterial dissemination from the lung to other organs such as the spleen and liver and an improvement in bacterial clearance from the lungs. These data suggests that restriction of F. tularensis to the lung, through inhibition of apoptosis, demonstrates potential as a therapeutic approach.

No alteration in cytokine secretion was observed in the lungs whereas a significant reduction in secretion was seen in the spleens of pre-treated mice. It is possible that the reduced secretion in the spleen is due to the lower bacterial colonisation of this organ compared to the lungs. This explanation is in line with the bacterial burdens observed in these two organs as no decrease in bacterial colonisation is seen in the lungs compared to the significant reduction seen in the spleen. However, it could be that pre-treatment with PD0325901 does not inhibit ERK activation only in the lungs but also targets this protein systemically. This has been observed in other studies that showed oral gavage administration of PD0325901 inhibiting ERK activation in the flanks of mice as well as other organs. In this case, it could be that PD0325901 is inhibiting, not only ERK activation in the lungs but also in the spleen and liver. This could interrupt the signalling required to induce cytokine secretion to the levels seen in PBS-treated mice. This theory cannot be confirmed in this study as ERK activation was not determined from the liver and spleen. However, the systemic bioavailability of PD0325901, after oral gavage, has been demonstrated in other studies. Further studies could examine the systemic inhibition of ERK and determine if the reduction of cytokine secretion in
liver and spleens of infected mice is due to decreased bacterial numbers or a decrease in levels of activated ERK.

Despite reduced bacterial burdens in the liver and spleen no improvement in survival was observed in any mice treated pre- or post-infection with PD0325901. This suggests that the inhibition of ERK activation alone is not an appropriate therapeutic strategy. However, the principle of specifically targeting a signalling protein has been demonstrated. In the in vitro model of infection, ERK has been specifically inhibited without significantly affecting the activation of other MAPKs, particularly p38. As a result of this inhibition, cytokine secretion was reduced in both in vitro and in vivo models of F. tularensis infection. Additionally, bacterial burdens in the spleen and liver of infected mice were shown to be reduced in PD0325901 treated groups.

Two approaches could be investigated to enhance the action of PD0325901. Firstly, an increased concentration of PD0325901 may further reduce bacterial burdens and result in an increased survival of infected mice. Alternatively, administering an immune modulator, such as PD0325901, in combination with a traditional therapeutic such as an antibiotic might show improved survival rates. The combined administration of two forms of therapeutic has been used previously. Mice challenged via the intranasal route with F. tularensis LVS or SchuS4 displayed a 0% survival rate, at day 15, even when treated with the antibiotic levofloxacin. However, if the mice were given the synthetic TLR3 agonist poly(I:C) 1 h after challenge and then given levofloxacin on day 5, 100% survival was observed. Successful combination therapy has been demonstrated with other intracellular pathogens such as Leishmaniasis. In this case, TLR4 and TLR9 agonists combined with a poly-protein vaccine candidate reduced the parasite burden in combination-treated mice when compared to single therapy mice. Similarly, although PD0325901 did not reduce bacterial burdens in the lungs of treated mice and did not improve survival, the reduction of bacterial spread from the lungs could enhance the effect of alternative therapies, such as antibiotics. This has been seen previously where the blocking of scavenger receptors combined with antibiotic treatment prevented bacterial dissemination after the induction of sepsis resulting in decreased systemic inflammation and an increased survival rate of C57BL/6 mice from 6% to 27%.

In summary:

- PD0325901 treatment inhibits the activation of ERK by F. tularensis infection in vitro resulting in the suppression of IL-6 and TNF secretion but without decreasing bacterial numbers.
• PD0325901 treatment of *F. tularensis* infection *in vitro* does not increase the level of cell death compared with untreated cells.

• In a murine model of *F. tularensis* infection, PD0325901 treatment decreases the level of ERK activation in the lungs and reduces the proliferation of bacteria from local containment to the spleen and liver.

• No significant increase in survival is seen in PD0325901-treated mice compared to untreated groups.
Chapter 5: TLR2 and TLR4 initiate signalling

5.1 Introduction

The binding of a ligand to the LRR domain of a TLR is the initial step in the induction of an immune response. The role of the TLR is not just to convert the binding event into a signalling cascade but to also confer a degree of specificity to the system as individual TLRs are able to detect specific sets of ligands. Each TLR feeds into distinct signalling pathways, for example TLR2 and TLR3 link to the MyD88-dependent and TRIF-dependent pathways, respectively, whereas TLR4 is able to induce signalling along both of these major pathways. The downstream signalling events and subsequent immune response depend heavily upon the identity of the TLR being activated. As a result, the determination of the receptors responsible for the detection of a specific pathogen can provide valuable insight into the signalling induced and possible immunological outcomes of infection. This has been demonstrated previously in studies examining the receptors involved in the detection and response to S. enterica and Chlamydia trachomatis. TLR4 was identified as being important in controlling the early growth of S. enterica but not for complete bacterial clearance. For C. trachomatis infection, TLR2 was seen to migrate to intracellular inclusions potentially identifying it, and its adaptor MyD88, as targets for therapeutic intervention.

The ability of TLRs to detect PAMPs is conferred by the LRR domain and the macro-structure of this domain across the family of TLRs is highly conserved. Despite the structural homology, the unique ligand binding repertoire is maintained by alterations in key residues within the LRR structure and also minor structural changes such as loops and grooves. Both of these are found in TLR3 whose LRRs express conserved phenylalanine and asparagine residues and LRR12 and LRR30 contain large protruding loops that are unique to TLR3 indicating a role in conferring ligand specificity. Similarly, the TLR2/TLR1 heterodimer was found to contain three hydrophobic pockets and a key glutamine at position 316 that interacted with its tri-acylated lipopetide ligand. It is not just the structure of TLRs that determine their ligand repertoire but also their location within the cell as this limits, by physical separation, the type of ligand that is able to associate with their binding region. TLRs are mainly located within either the cell surface membrane, such as TLR4 and TLR5, or the membranes surrounding endosomal compartments, for example TLR7 and TLR9, and are therefore able to detect extracellular or intracellular PAMPs, respectively.

Much research has been focussed on identifying the ligands of TLRs and, as such, predictions of which TLRs are likely to be activated in the presence of certain pathogens can be theorised.
However, assumptions should not be made without experimental evidence as a pathogen can express a known TLR ligand without it actually activating the corresponding receptor. Transfection of TLRs with an appropriate reporter construct into HEKs, either stably or transiently, provides an excellent way of determining which PRRs are activated by specific pathogen-associated ligands. Care must also be taken when using purified pathogen components to test for their ability to stimulate TLRs. A purified component may be able to activate a receptor but this does not always correlate with the same effect when integrated as part of a whole organism. Contamination has also been a significant problem in the field of TLR ligand research. For example, in 1998 a group identified TLR2 as the receptor mediating LPS-induced cellular signalling. Subsequently, repurification of the stimulatory LPS, by several groups, was found to eliminate the observed TLR-induced signalling and then two lipoprotein contaminants were identified as responsible for the stimulatory effect of unpurified E. coli LPS upon TLR2. More recently, another study demonstrated how peptidoglycan contaminants of LPS contribute to effects of LPS stimulation previously attributed to LPA alone. It is not only contamination of LPS that has been an issue. In fact contamination by LPS is just as big a problem and, during the initial stages of TLR research, many compounds that were “shown” to be TLR4 agonists turned out to be contaminated with LPS, the highly stimulatory agonist of TLR4. Contamination has been such a barrier to the correct identification of TLR ligands that a paper was published suggesting strategies to avoid mis-identification of TLR PAMPs.

With regards to F. tularensis, predicting which TLRs this pathogen would most likely activate from the bacterial ligands it possesses suggests TLR4, TLR2/(1 or 6) and TLR9 are likely candidates. This is due to the fact that, as a Gram negative bacterium, it expresses LPS, lipopeptides and contains CpG DNA motifs. Similarly, as a non-motile organism, it is unlikely to stimulate TLR5 due to a lack of flagella. As discussed earlier though, assuming these are correct, without experimental evidence, would not be sensible and much research has been carried out examining which TLRs are responsible for initiating an immune response to F. tularensis. Initial work on the interaction of F. tularensis with TLRs led to the identification of TLR4 as the receptor involved in bacterial detection as TLR4-deficient C3H/HeJ mice displayed higher bacterial burdens and decreased NO₂ production compared to C3H/HeN mice which express a fully functional TLR4 gene. However, a subsequent report ruled out TLR4 as playing an important role in generating an immune response as CSH/HeJ mice were no more susceptible to inhalational infection with F. tularensis than wild-type mice. Similarly, TLR4-KO BMDMs were still able to respond to F. tularensis LVS infection through the secretion of pro-inflammatory cytokines and the LPS of F. tularensis is now thought to be non-stimulatory for TLR4. Recent work suggests that F. tularensis LPS does play a role in the host...
response to *F. tularensis* although it is unlikely to be through TLR4. It appears that the low potency of *F. tularensis* LPS can be enhanced in the presence of other *F. tularensis* factors. For example, the heat-shock protein GroEL synergistically enhanced the secretion of IL-8 by human monocyte-derived macrophages stimulated with LPS \(^{304}\). In addition, the antibody response that contributes to the protection of wild-type mice from lethal challenge is partially dependent on the presence of *F. tularensis* LPS \(^{305}\). Important to note though is that typical LPS is not the only agonist to display a stimulatory activity upon TLR4. Diverse ligands such as minimally modified low density lipoprotein (mmLDL) and Fimбриa H protein (FimH) have both been shown to activate TLR4 \(^{355,430}\). More recently, a *Francisella* cytoplasmic protein called elongation factor Tu (Ef-Tu) has been identified as a novel TLR4 agonist \(^{431}\). This protein was only found in the proteomic analyses of virulent *F. tularensis* strains and not in an attenuated *F. novicida* strain suggesting that this protein may be a virulence factor as well as a TLR4 agonist \(^{431}\). It would certainly seem that the TLR4-*F. tularensis* story continues to be as enigmatic as ever.

*F. tularensis* also expresses membrane bound lipopeptides and so TLR2 has received a high degree of scrutiny. The role of TLR2 seems to be much clearer than that of TLR4 and TLR2 quickly replaced TLR4 as the likely candidate for *F. tularensis* detection and response initiation. Murine macrophages deficient in TLR2 failed to secrete pro-inflammatory cytokines to the level observed in wild-type macrophages \(^{212,298}\). Furthermore, mice deficient in TLR2 exhibit an enhanced susceptibility to pulmonary infection by *F. tularensis* LVS and a reduced secretion of pro-inflammatory cytokines such as TNF and IL-6 \(^{41}\). More recently, two *F. tularensis* lipoproteins have been identified, TUL4 and FTT1103, which stimulate the TLR2/1 heterodimer inducing chemokine production in murine dendritic cells \(^{300}\). TLR9 also has the potential to be a *F. tularensis* detector and this will be discussed in the subsequent chapter.

### 5.2 Aims and Objectives

The aim of this chapter was to determine the contribution of TLR2 and TLR4 to the initiation of signalling cascades after stimulation by *F. tularensis* LVS by using macrophage cell lines, with TLR2 or TLR4 knocked out, and measuring the activation profiles of downstream signalling proteins in comparison to wild-type cells. If no alteration in signalling is observed in TLR-KO cells it is likely that the specific TLR is not responsible for initiating a response to *F. tularensis* LVS.
The specific aims of this chapter were to:

- Measure the activation of signalling proteins and secretion of cytokines in response to *F. tularensis* LVS infection of wild-type and TLR-KO cell lines.
- Compare the signalling profiles and cytokine profiles generated from the different cell lines and identify where there are differences.

### 5.3 Results

#### 5.3.1 Signalling proteins

From the initial characterisation of LVS-induced signalling in MH-S cells, the three groups of MAPKs (JNK, p38, and ERK) and the two IκB proteins (α and β) were selected to characterise the roles of TLR2 and TLR4. The activation of these proteins was measured and analysed using western blotting and densitometry, generating activation profiles for each signalling protein. Similarly to the LVS infection of MH-S cells, samples of cell lysates and supernatants were collected at 12 time points throughout the course of a 24 h infection.

Throughout the course of infection no significant change in activation was seen with either JNK-1 or JNK-2 in any of the cell lines infected (Figure 5-1). This is also the case for the two IκB proteins (Figure 5-2). The activation of both ERK-1 and ERK-2, however, do show alterations in signalling caused by the absence of either TLR2 or TLR4 (Figure 5-3). In wild-type cells, the activation of both ERK-1 and ERK-2 is elevated, compared to uninfected cells, until 1.5 h after uptake of the bacteria. However, in TLR4-KO and TLR2-KO cells the early elevation in the activation level of ERK-1 is not sustained, as in wild-type cells, and is significantly decreased at 0.5 h after bacterial uptake. The same pattern is observed for ERK-2 activation in TLR2-KO cells although there is a slight difference for TLR4-KO cells which display no elevation of ERK-2 activation at the early time points. As the activation of ERK is altered in both TLR2-KO and TLR4-KO cells it is likely that stimulation of both these receptors contribute to the regulation of ERK activation in response to *F. tularensis*.

The biggest changes in signalling due to TLR-KO was seen in the activation profiles of p38 (Figure 5-4). Similarly to ERK activation, p38 activation is affected, albeit in different ways, by the absence of either TLR2 or TLR4. The activation profile for p38 observed in wild-type BMDMs closely follows that of MH-S cells. This is characterised by an early activation of p38 followed by a maintained suppression of activation throughout the remaining time points. In TLR2-KO cells, the resting level of p38 activation is greatly increased and, upon infection by LVS, rises significantly above levels seen in
A: Western blots

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<th>TLR4-KO</th>
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<td>- P-JNK-1</td>
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<td>40</td>
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Figure 5-1: The levels of activated JNK-1 and JNK-2/3 in WT, TLR2-KO and TLR4-KO BMDMs throughout the course of a *F. tularensis* LVS infection

The levels of activated JNK-1 (B) and JNK-2/3 (C) were determined at several time points after the uptake of LVS. Representative western blots (A) are shown on the opposite page. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show 95% confidence interval. Significant difference from wild-type levels was determined by two-way ANOVA and a Bonferroni’s post-test (* = p<0.05). (WT) – Wild-type BMDMs; (2) – TLR2-KO BMDMs; (4) – TLR4-KO BMDMs.

Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
A: Western blots

WT

TLR2-KO

TLR4-KO

kDa

L  N  0 h  5 min  15 min  30 min  45 min  1 h  1.5 h  2 h  4 h  6 h  24 h  +ve  L

- IκBα

- β-actin

- IκBα

- β-actin

- IκBα

- β-actin

kDa

WT

TLR2-KO

TLR4-KO

- IκBβ

- β-actin

- IκBβ

- β-actin

- IκBβ

- β-actin
Figure 5-2: The expression of IκBα or IκBβ in WT, TLR2-KO and TLR4-KO BMDMs throughout the course of a *F. tularensis* LVS infection

The expression of IκBα (B) and IκBβ (C) was determined at several time points after the uptake of LVS. Representative western blots (A) are shown on the opposite page. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show 95% confidence interval. Significant difference from wild-type levels was determined by two-way ANOVA and a Bonferroni’s post-test (* = p<0.05). (WT) – Wild-type BMDMs; (2) – TLR2-KO BMDMs; (4) – TLR4-KO BMDMs.

Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
A: Western blots

[Image showing western blots for WT, TLR2-KO, and TLR4-KO with P-ERK-1, P-ERK-2, and β-actin bands at different time points (L, N, 0 h, 5 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 24 h, ×ve, L).]
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<td>24 h</td>
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**Figure 5-3:** The levels of activated ERK-1 and ERK-2 in WT, TLR2-KO and TLR4-KO BMDMs throughout the course of a *F. tularensis* LVS infection

The levels of activated ERK-1 (B) and ERK-2 (C) were determined at several time points after the uptake of LVS. Representative western blots (A) are shown on the opposite page. The experiment was performed in triplicate (*n* = 3) and each replicate was performed in duplicate. Error bars show 95% confidence interval. Significant difference from wild-type levels was determined by two-way ANOVA and a Bonferroni’s post-test (*p* < 0.05).

(WT) – Wild-type BMDMs; (2) – TLR2-KO BMDMs; (4) – TLR4-KO BMDMs.

Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
Figure 5-4: The levels of activated p38 in WT, TLR2-KO and TLR4-KO BMDMS throughout the course of a *F. tularensis* LVS infection

The levels of activated p38 were determined at several time points after the uptake of LVS. Representative western blots are shown above. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show 95% confidence interval. Significant difference from wild-type levels was determined by two-way ANOVA and a Bonferroni’s post-test (* = p<0.05). (B) – Wild-type BMDMs; (2) – TLR2-KO BMDMs; (4) – TLR4-KO BMDMs. Western blot image: L – MagicMark™ ladder; N – naïve; +ve – positive control.
wild-type cells between 0 h and 0.25 h. Conversely, the resting level of p38 activation in TLR4-KO cells is identical to wild-type cells, but when the TLR4-KO cells are infected, p38 fails to become activated and remains significantly lower than the level seen in wild-type cells until 1.5 h. This data shows that p38 activation, like ERK activation, is dependent on the presence and activation of both TLR2 and TLR4.

5.3.1 Cytokines

To observe whether the absence of either TLR2 or TLR4 could affect the downstream result of signalling pathways the secretion of cytokines was measured in the supernatants sampled from the cells at each time point. The supernatants were screened for the production of IFN-γ, IL-6, IL-10, IL-12p70, TNF and MCP-1. Of the 6 cytokines measured, only MCP-1 and TNF could be detected from the supernatants (Figure 5-5). MCP-1 is secreted rapidly in response to LVS infection in all three cell lines and does not show a greatly increased or decreased rate of secretion in either TLR2-KO or TLR4-KO cells when compared to wild-type. However, the secretion of TNF does show significant differences between the cell-lines tested. In wild-type cells, in the first 2 h after uptake, TNF is secreted rapidly and then at 6 h reaches maximum levels at about 3500 pg/ml. In TLR2-KO cells the rapid secretion of TNF is completely abrogated and TNF levels are significantly lower than in wild-type cells from 0.75 h through to 24 h after uptake, barely reaching 900 pg/ml. In TLR4-KO cells, TNF is initially secreted at a similar rate to wild-type cells. However, at 1 h post-uptake, the levels of TNF plateau at lower values than for wild-type cells and the maximum value reached is about 1500 pg/ml, significantly lower than in wild-type cells.

5.4 Discussion

The involvement of TLR2 in initiating an immune response to F. tularensis infection is widely accepted. TLR2-KO macrophages fail to secrete pro-inflammatory cytokines in response to F. tularensis infection. TLR2-KO mice display an increase in bacterial burden in the lungs, liver and spleen after infection compared to wild-type mice. Important to note is that all the evidence pointing to TLR2’s involvement in the response to F. tularensis infection does not negate a requirement for other receptors, as a recent study demonstrated. A mutant strain of F. tularensis LVS was highly attenuated in a murine model and was able to induce a far more rapid and robust inflammation response than wild-type F. tularensis but this did not involve changes in TLR2 mediated signalling suggesting a contribution by other receptors. The examination of signalling proteins and cytokine secretion in my work confirms previous reports of TLR2’s role in the detection and response
Figure 5-5: Secretion of MCP-1 and TNF in WT, TLR2-KO and TLR4-KO BMDMs throughout the course of a F. tularensis LVS infection

The secretion of MCP-1 (A) and TNF (B) into the supernatant was measured at several time points after the uptake of LVS. The experiment performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the standard error of the mean. Significant difference from wild-type levels was determined by two-way ANOVA and a Bonferroni’s post-test. Blue asterisk – significant difference to TLR2-KO. Green asterisk – significant difference to TLR4-KO. (* = p<0.05). (WT) – Wild-type BMDMs; (2) – TLR2-KO BMDMs; (4) – TLR4-KO BMDMs.
to F. tularensis as, although TLR2-KO did not significantly impact upon the activation of JNK or the expression of IκBα/β, significant changes were observed in both p38 and ERK activation. This result is in agreement with previous studies identifying a link between TLR2 stimulation and the activation of signalling proteins, in particular MAPKs \(^{393,433-435}\). It is perhaps no surprise that JNK, and IκBα/β were not altered as their expression and activation were not greatly affected by F. tularensis infection in MH-S cells. In addition, JNK activation has been observed to be suppressed by F. tularensis infection in another study \(^{436}\). In addition, this data shows that although ERK and p38 are both located within the MAPK branch of the signalling cascade and share a high degree of structural homology \(^{143,146,437}\), their activation by TLRs is different. In the absence of TLR2, the typically sustained activation of ERK is lacking and only an early and transient activation is seen. Therefore, it seems that TLR2 stimulation, in wild-type macrophages, is responsible for inducing the signal to sustain the early activation. In another study stimulation of neutrophils with LPS induced a transient activation of ERK whilst P3CSK4-stimulation induced a sustained activation of ERK lasting for 1 h after stimulation \(^{438}\). Although this study used a different cell line and pure ligand instead of bacterial-stimulation it does demonstrate the sustained activation of ERK brought about through TLR2 stimulation similar to that seen in my work. In contrast to ERK, p38 activation is significantly enhanced in TLR2-deficient cells suggesting that, in this case, TLR2 may be required to generate a suppressive signal, although this has not been observed previously. TLR2 appears to be able to simultaneously enhance the activation of one protein (ERK) whilst simultaneously suppressing the activation of another (p38) in response to F. tularensis detection. The ability of TLR2 stimulation to differentially regulate specific MAPKs has been observed previously. Stimulation of RAW264.7 macrophages with staphylococcal PGN, a TLR2 agonist \(^{439}\), induced a strong activation of ERK, moderately activated JNK and only weakly activated p38 \(^{440}\).

Interestingly, TLR2 stimulation appears to not only regulate p38 activation in response to infection but also in naïve cells. This is demonstrated by a significantly higher level of p38 in uninfected TLR2-KO compared to wild-type BMDMs and suggests that these cells are left in a highly stimulated state even when not subjected to exogenous stimuli. One possibility is that in wild-type cells a low level of TLR2 activation maintains the cells in a resting state preventing them from reacting excessively to non-pathogenic stimuli. When the steady-state of stimulation is removed, through knockout of TLR2, signalling induced through the stimulation of other PRRs is able to induce enhanced p38 activation. Low-level constitutive stimulation as a mechanism for maintaining cells in a homeostatic state has been investigated previously in the intestinal tract. In this context, commensal bacteria are continuously detected by TLRs expressed on epithelial cells \(^{441}\). The constant stimulation does not
initiate an inflammatory response but instead maintains the cells in a steady-state ready to become activated by pathogenic stimuli. It is possible that, in BMDMs, low-level stimulation of TLR2 prevents the cells from responding excessively to non-pathogenic stimuli and so when the cells lack this receptor they are unable to restrain this disproportionate response.

Another explanation for the heightened level of p38 activation in resting cells could be related to the expression of other TLRs in the TLR2-KO cell-line. In 1999, it was demonstrated that an increased expression of TLR4 on myocytes increased the activation of NF-κB suggesting that it is not only the stimulation of a TLR that determines the outcome but also the level of expression. Additionally, a cell’s expression of TLRs is strongly interlinked meaning that an alteration in the expression of one TLR can have significant effects on the expression of another. For example, in TLR2-KO mice infected with P. aeruginosa an up-regulation of TLR4 expression was observed that was not present in wild-type C57BL/6 mice. Therefore, in the TLR2-KO BMDMs used in this study, there may be a similar up-regulation in TLR4, or other TLRs, which could explain the enhanced p38 response. To verify this possibility, expression of receptors could be measured using cell staining techniques and compared between the cell lines used.

The alterations in signalling, brought about by the knock-out of specific TLRs, are also visible in the secretion profile of TNF. TNF is a pleiotropic cytokine, transducing diverse signals including cell activation, death and proliferation. With such a broad range of functions it is not surprising that the secretion of this cytokine is tightly regulated by a complex mechanism with two major stages of control. The transcription of DNA into mRNA is initiated and regulated by the transcription factor NF-κB whereas the translation of TNF mRNA into protein is regulated by the MAPK signalling pathway. In resting cells there is a basal level of TNF transcription and translation although both these process are extremely inefficient. Upon stimulation, transcription is accelerated and translational derepression occurs resulting in a far more efficient message utilisation and rapid secretion of TNF.

In TLR4-KO cells, the translation of TNF mRNA is still able to take place as the initial secretion of TNF, in the first 1.5 h, matches that observed in wild-type cells. However, TNF levels quickly plateau at 2 h reaching a maximum of 1500 pg/ml. This indicates that, although the TNF mRNA already present in the cell can be translated there is no de novo transcription occurring to continue TNF secretion as observed in wild-type cells. This indicates that TLR4 stimulation, and the signalling cascades initiated are required for the release of transcription repression. In resting cells there is a basal level of TNF...
transcription which has been shown to rely on binding of only the p50 subunit of NF-κB to at least one of the four kB motifs present in the gene. Upon cell stimulation, an increase in nuclear NF-κB content is observed and it was proposed that binding of both the p50 and p65 subunits of NF-κB promote accelerated transcription. The result observed in this study, where a lack of TLR4 results in a plateau of TNF secretion, indicates a role for TLR4 stimulation in the acceleration of transcription involving NF-κB. Conversely, as the translation of TNF mRNA was unaffected in TLR4-KO cells, as shown by the unaltered initial rate of TNF secretion, TLR4 appears not to be critical in the regulation of this stage in the TNF secretory process. A role for TLR4 in the induction of TNF secretion has previously been demonstrated in alveolar macrophages that secreted TNF, in addition to IL-8 and MCP-1 when stimulated with LPS.

In TLR2-KO cells, the initial rapid secretion of TNF seen in both wild-type and TLR4-KO cells is absent indicating that derepression of TNF translation has not occurred and the conversion of mRNA into secreted protein remains inefficient. This is likely to be due to altered MAPK signalling as the control of TNF translation has been studied in depth by several groups and is characterised to be under the regulation of the MAPK signalling pathway involving all three of the major MAPKs, p38, JNK and ERK in non-redundant roles. The role of p38 in TNF regulation involves the activation of its downstream substrate, MAPK-activated protein kinase-2 (MK-2) and the interaction between this signalling protein and the adenylate/uridylate-rich element (ARE) located in the 3’ untranslated region (3’UTR) of TNF mRNA. The importance of these proteins and elements has been comprehensively demonstrated by numerous groups utilising MK-2 deficient cells, p38 inhibitors and ARE-deleted TNF genes. Although, these elements were identified as critical some years ago, the exact mechanism of regulation has only recently begun to be elucidated. Initial studies observed that MK-2-deficiency did not alter the half-life of TNF suggesting that the p38 pathway is not involved in the stability of TNF mRNA. In contrast, however, more recent work has linked the p38 pathway and MK-2 activation to the regulation of TNF mRNA stability, possibly through the regulation of tristetrapolin.

Despite the correlation reported in the literature between p38 activation and the increase of TNF secretion, my work appears, at first glance to disagree. In TLR2-KO cells the secretion of TNF was almost completely abrogated but this was associated with enhanced and not reduced p38 activation. This result may be explained by the activation profile of another signalling protein, ERK, in the TLR2-KO cell-line. Unlike p38 and JNK, the ERK pathway is responsible for the transport of TNF mRNA from the nucleus into the cytoplasm. In TLR2-KO cells the activation of ERK is significantly reduced
compared to wild-type BMDMs and this could result in inhibition of the nucleocytoplasmic transport of TNF mRNA. In this case, although p38 activation is enhanced, usually correlating to enhanced TNF secretion, there is no cytoplasmic mRNA for it to positively regulate as it is all contained within the nucleus and therefore TNF secretion remains suppressed. In combination, the data presented here support a hypothesis whereby both TLR2 and TLR4 contribute, in distinct ways, to efficient secretion of TNF. TLR4 stimulation appears to be involved with the initiation of TNF transcription but not translation whereas TLR2 stimulation, via the MAPK cascade involving p38 and ERK, is critical in the regulation of TNF translation. Only with coordinated stimulation of both these TLRs is the whole mechanism required for efficient TNF secretion fully functional. In agreement with these results, coordinated signalling between TLR2 and TLR4 in the secretion of TNF has been observed previously. Simultaneous stimulation of fibroblasts with pure TLR2, TLR4 and TLR6 ligands synergistically increased the secretion of TNF. Similarly, dual blockade of TLR2 and TLR4 was required to inhibit TNF secretion from peripheral blood mononuclear cells upon infection with *S. enterica*. It also appears that synergy between TLR2 and TLR4 is not restricted to cytokine secretion and, in the case of *M. tuberculosis* infection of macrophages, cooperation between these two signalling pathways plays a critical role in regulating cell death. However, not all studies observe the same level of synergy with regards to TNF secretion. In dendritic cells, stimulated with ultra-pure LPS and Pam3CSK4 a synergistic enhancement of IL-10 was seen but not TNF. The differences between studies could be explained by the use of different cell-types but also whether the stimulation of the cells was brought about by infection or through the use of pure ligands.

The observed response to *F. tularensis* infection in wild-type BMDMs cannot be fully explained by the contribution of one TLR alone. Although this study demonstrates that it is generated by the combination of at least TLR2 and TLR4, cooperation between TLR4 and other TLRs has been scrutinised previously. Pure TLR ligands were used to stimulate the cells and the synergistic cytokine response, when TLRs were stimulated simultaneously, was determined to be generated at the level of the MAPKs. This is in agreement with my work where either the absence of TLR2 or TLR4 affected the activation of MAPKs indicating that both receptors are required for the wild-type cell response. In addition, TLR cooperation has been observed in a mouse model of bacterial pneumonia indicating that it is not just an artefact of *in vitro* models. The results presented here also do not rule out the possibility of other PRRs contributing to the response, in fact the opposite is indicated. MCP-1 secretion was unaffected by either TLR2-KO or TLR4-KO supporting the hypothesis that additional PRRs are able to react to *F. tularensis* infection. Alternatively, it could be that either TLR2 or TLR4 stimulation can induce MCP-1 secretion, in a redundant fashion, meaning that in the
absence of one TLR the other can replace it. The influx of immune cells, stimulated in part by secretion of MCP-1, is a critical step in the resolution of infection. It is likely then that multiple TLRs can induce MCP-1 secretion to ensure that, whatever ligand the pathogen expresses, immune cell influx is initiated. In fact, direct stimulation of TLR2, TLR3, TLR4, TLR7/8 and TLR9 with their corresponding agonists all result in the secretion of MCP-1. Additional receptors have also recently been identified such as the cytoplasmic receptor, AIM-2, that was characterised as a detector of DNA present in the cytoplasm in 2009 by two separate groups. Subsequently, AIM-2 was identified as an additional receptor important in the macrophage response to *F. tularensis* triggering inflammasome assembly and cleavage of caspase-1 resulting in the secretion of IL-1β and IL-18. Further evidence for receptors other than TLR2 and TLR4 being involved in the response to *F. tularensis* is provided by a study using a galU mutant of *F. tularensis LVS*. In a murine pulmonary model of tularemia, the galU mutant demonstrated reduced virulence correlating with a more rapid and robust inflammatory response. Importantly, the enhanced inflammation was brought about in the absence of any alteration in TLR2 or TLR4 signalling indicating the involvement of other receptors and signalling pathways. Almost certainly, there are other *F. tularensis*-responsive receptors yet to be identified.

In summary:

- TLR2 is able to simultaneously enhance the activation of one protein (ERK) whilst suppressing the activation of another (p38) in response to *F. tularensis* detection.
- TLR2 stimulation, via the MAPK cascade involving p38 and ERK, is critical in the regulation of TNF translation in response to *F. tularensis* infection.
- TLR4 stimulation and the signalling cascades initiated are required to release the repression of TNF transcription in response to *F. tularensis* infection.
Chapter 6: Priming of TLR9 response

6.1 Introduction

The sea urchin has a repertoire of 222 TLRs and, with this arsenal available, it is easy to see how this organism can tailor a specific immune response to a wide variety of stimuli. In contrast, human cells have a much more restricted set of TLRs with which to react to an equally varied set of stimuli. Despite this, human cells are still able to generate specific immune responses. This leads us to the intriguing question: How does a limited set of receptors generate such a broad range of responses?

One mechanism that can begin to explain this phenomenon is TLR cooperation or coordination. The three major mechanisms of cooperation are cross-talk, priming and tolerance. Cross-talk occurs when signalling proteins from one pathway modulate the activation of signalling proteins on another branch. For example, activated ERK is able to bind to MKP-3 enhancing its activity and subsequently applying a positive feedback loop upon p38. Priming is characterised by a preliminary stimulation event enhancing the response to a subsequent stimulation event. In contrast, tolerance is defined as the limiting of a response after pre-stimulation. This can occur either when the two stimulation events are by the same ligand or when the primary stimulation event is by a different ligand to the subsequent event; in this instance it is known as cross-tolerance. Because macrophages may encounter multiple TLR ligands during an infection, signalling cross-talk between TLR pathways is likely to be important for the tailoring of inflammatory responses to pathogens.

Priming and cross-tolerance have been studied in depth in relation to the integration of multiple TLRs with TLR9 receiving the most attention with regards to its interaction with other TLRs, particularly TLR4. However, as usual in the field of TLR research, there are contrasting reports of the integration of TLR4 and TLR9 signalling. A recent study observed that pre-treatment of macrophages with LPS primed the cells for subsequent stimulation by CpG DNA correlating with enhanced MAPK activation. This priming effect is only apparent when subsequent stimulation is by a different ligand. A separate study that examined repeated stimulation with either LPS or CpG observed generation of tolerance to these ligands. In contrast to these studies, an earlier study observed the induction of hyporesponsiveness in RAW264.7 cells pre-treated with LPS upon subsequent re-stimulation with CpG. The hyporesponsive state correlated with the suppression of TNF and IL-12 production albeit with the potentiation of IL-6 production. This study suggests that hyporesponsiveness of RAW264.7 cells is due to a complicated re-programming of the cells and not just a simple exhaustion of cellular components. The order of stimulation events is also critical to...
determining the outcome of signalling as the same stimulation in a different order results in significantly different outcomes. For example, priming of cells via CpG-induced TLR9 activation followed by LPS stimulation results in an augmented IL-12 production whereas the same stimuli but in the reverse order has no affect on the production of IL-12 by murine DCs \(^{186}\). In an \textit{ex vivo} experiment, purified human alveolar macrophages were removed from lungs of volunteers that had been instilled with LPS and they were re-stimulated 6 hours later with either TLR4 or TLR2 ligands \(^{182}\). In both cases an elevated level of IL-1 and IL-6 secretion was observed and this correlated with a sustained activation of p38, although, due to ethical reasons p38 inhibition could not be carried out to confirm direct correlation \(^{182}\).

Although priming and tolerance have been observed in numerous cell types involving a variety of TLRs, the mechanisms that underlie these two phenomena are less well characterised. One mechanism suggested to contribute to priming effects is the cross-regulation of TLR expression. Within 1 h of LPS stimulation the expression of TLR9 was up-regulated in RAW264.7 cells and this correlated with the activation of NF-κB and MAPKs \(^{188}\). However, this effect is cell and TLR specific as LPS stimulation of human AMs primed the cells for IL-1 and IL-6 secretion after TLR2 stimulation but this did not correlate with an increase in TLR4 or TLR2 expression \(^{182}\).

An added layer of complexity to the mechanisms of priming and tolerance is that TLRs can modulate the response of another TLR without becoming fully activated themselves. Sub-stimulatory or sub-threshold concentrations of LPS have been used to prime murine peritoneal macrophages prior to re-stimulation with LPS. Although no measurable stimulation occurs after the priming step, an alteration in the cytokine response to the secondary stimulation is observed when compared to un-primed cells \(^{455,456}\). In addition, priming of cells does not universally up-regulate or down-regulate the response. LPS-primed macrophages demonstrated a suppression of NO production whilst simultaneously enhancing the secretion of TNF \(^{456}\). The ability of cells to be selectively primed by sub-stimulatory concentrations of ligand indicates a complex mechanism of cellular reprogramming.

As described previously, \textit{F. tularensis} has the potential to activate several receptors including TLR2, TLR4 and TLR9 and my work has confirmed that both TLR2 and TLR4 certainly play a part in generating the response to this pathogen. However, through the mechanisms of TLR cooperation, these receptors, in addition to acting in isolation, may also integrate their signals generating additional unique immune responses.
6.2 Aims and Objectives

The role of TLR2 in generating a response to *F. tularensis* is well characterised and my work has confirmed this. However, TLR4’s contribution remains the subject of much debate as it seems to play a role but the mechanism has not been elucidated. Previous research using pure ligands of TLR4 and TLR9 has demonstrated that TLR4 sub-stimulation is able to prime TLR9, enhancing the immune response.189 The cooperation between these two receptors may represent the mechanism by which TLR4 plays a role in the host response to *F. tularensis* and could explain the potentially detrimental immune response seen at later time points during infection. The aim of this chapter was to investigate whether sub-stimulation of TLR4 is able to prime TLR9 for an exacerbated response downstream of *F. tularensis* genomic DNA detection. The proposed mechanism behind priming of TLR9 by TLR4 is quite complex so it was decided to investigate the process in a stepwise manner. In order to examine each step individually, within this complex process, a range of cell lines, ligands and receptor antagonists were used to isolate the function of each individual receptor.

The specific aims of this chapter were to:

- Determine whether TLR9 is able to detect genomic DNA purified from several *Francisella* sub-species in HEK-Blue™ and MH-S cells.
- Investigate the integration of TLR4 and TLR9 signalling in response to *F. tularensis*.
- Establish whether LPS, at very low concentrations, is able to prime the TLR9 response.

6.3 Results

6.3.1 HEK-TLR9 Blue cells

Preliminary studies into the potential of *Francisella* genomic DNA to stimulate TLR9 were carried out in the HEK-Blue™ hTLR9 cell line. These cells express TLR9 with NF-κB and AP-1 controlled transcription linked to the production of SEAP. Levels of SEAP can be determined using the QUANTI-Blue™ medium which turns blue in the presence of SEAP. Therefore, the degree of colour change is correlated to the level of TLR9 stimulation. As these cells also express endogenous TLR3, TLR5 and NOD1, the parental cell line HEK-Blue™ Null1 was used as a control. In addition to determining SEAP production, the secretion of cytokines was also measured.

The level of SEAP production was measured by reading the absorbance at 620 nm on a spectrophotometer. Genomic DNA from all strains of *Francisella* was able to stimulate signalling through TLR9 (Figure 6-1). Interestingly, genomic DNA from the highly virulent SchuS4 strain
Figure 6-1: The detection of *F. tularensis* genomic DNA by HEK-Blue™ cells

Induction of signalling through TLR9 in HEK-Blue™ cells was determined by measuring the OD₄₉₂ of the media (A-E) and secretion of MCP-1 (F-J) after 24 h of stimulation by genomic DNA used at varying concentrations (10 µg/ml (A,F), 1 µg/ml (B,G), 0.1 µg/ml (C,H), 0.01 µg/ml (D,I), 0.001 µg/ml (E,J)). The experiments were performed in triplicate (n = 3) and each replicate was performed in duplicate. Error bars show the min and max. Significant difference between TLR9 and Null1 cells was determined by a one-way ANOVA and individual one-tailed, unpaired t-tests (* = p<0.05)
stimulated these cells significantly more than DNA from lower virulence strains at 0.1 and 1 µg/ml and both SchuS4 and LVS were significantly more stimulatory at 10 µg/ml when compared to U112 and FSC200 strains. The stimulatory properties of genomic DNA were also observed by measuring the secretion of MCP-1 24 h after stimulation. Similarly to the absorbance readings, SchuS4 DNA was able to induce significantly more secretion of MCP-1 than all other strains at both 1 and 10 µg/ml (Figure 4-1B). In contrast to absorbance readings, LVS DNA did not induce secretion of MCP-1 to a greater degree than other strains at any concentration used.

6.3.2 MH-S cells

6.3.2.1 TLR4 and TLR9 stimulation

As it was demonstrated that genomic DNA could stimulate signalling through TLR9 in HEK-Blue™ cells the assay was transferred to the MH-S cell line because these cells are a better model for pulmonary immune cells. Transcription in these cells is not linked to the production of SEAP and therefore signalling could only be determined through the detection of cytokine secretion. MH-S cells were stimulated for 24 h with a range of concentrations of genomic DNA purified from the same four *F. tularensis* strains as used for the HEK-Blue™ stimulation assay. After 24 h incubation, supernatants were removed and screened for the secretion of cytokines. To account for signalling induced by any receptor other than TLR9 expressed by these cells, the stimulation was also performed in the presence of a TLR9 antagonist, G-ODN. DNA from all four strains was able to stimulate MH-S cells to secrete IL-6 (Figure 6-2). In each case, this was dependent on TLR9 as IL-6 secretion was significantly reduced in the presence of the TLR9 antagonist, G-ODN. Differences between strains were also observed as genomic DNA from the highly virulent SchuS4 strain induced significantly greater IL-6 secretion than the less virulent LVS and U112 strains as well as the +ve control.

6.3.2.2 Stimulation by whole LVS

Although purified *F. tularensis* genomic DNA demonstrated the ability to induce a response via TLR9, the ability of a natural bacterial infection to do so remains unproven. For TLR9 stimulation to occur the DNA would need to be released from the bacteria, either through active secretion by bacteria or through lysis of the bacteria by the cell. To investigate this, MH-S cells were infected with live LVS in the presence of the TLR9-antagonist G-ODN ([Invitrogen](https://www.invitrogen.com)), the TLR4-blocking antibody IMG-428E ([Imgenex](https://www.imgenex.com)), or both. Untreated MH-S cells were used as a control. Supernatants were collected 24 h
Figure 6-2: Secretion of IL-6 by MH-S cells in response to TLR9 stimulation by *F. tularensis* genomic DNA (10 \( \mu \text{g/ml} \))

Secretion of IL-6 was determined 24 h after stimulation of MH-S cells by *F. tularensis* genomic DNA. The experiments were performed in triplicate \((n = 3)\) and each replicate was performed in duplicate. Significant difference from antagonised cells was determined by one-way ANOVA and individual one-tailed, unpaired t-tests \((* = p<0.05)\). Error bars show the min and max.
after infection and screened for the presence of cytokines. Figure 6-3 shows the secretion of TNF and IL-6 in response to LVS infection in the presence of a range of concentrations of TLR antagonists alongside LVS alone. A high level of IL-6 was secreted and TNF was also released in response to LVS infection. When TLR9 signalling was blocked by G-ODN, the level of TNF secretion remained similar to that produced by infection with LVS alone for all concentrations except 100 µg/ml where there was a significant increase in secretion (A). In contrast, IL-6 secretion was significantly reduced in the presence of 100 µg/ml G-ODN (B). When TLR4 signalling was blocked by IMG-428E, a similar effect on secretion was observed, with TNF levels increasing at 50 µg/ml (A) and IL-6 levels decreasing at 50 µg/ml (B) when compared to LVS alone. This data confirms the previous observations that both TLR9 and TLR4 play a role in the orchestration of a response to *F. tularensis*.

### 6.3.2.3 LPS priming of TLR9 response

This study has demonstrated the ability of purified *F. tularensis* genomic DNA to activate TLR9 and this has also been shown to occur in a live bacterial infection model. In addition, using a TLR4-blocking antibody, the role of TLR4 in generating an immune response to *F. tularensis* has been confirmed. The next stage of the investigation was to determine whether alternative-activation of TLR4, by very low concentrations of LPS, is able to prime TLR9 for an enhanced response to subsequent activation by *F. tularensis* genomic DNA.

Initially, a range of concentrations of *E. coli* LPS (0.01 – 10 ng/ml) were used to stimulate MH-S cells through TLR4 in order to determine the level that could be used as a “sub-stimulatory” concentration. The secretion of IL-6 and TNF by MH-S cells was used to measure the degree of TLR4 activation (Figure 6-4). The secretion of IL-6 was negligible at 0.01, 0.1 and 1 ng/ml and only reached 20 pg/ml when the cells were stimulated with 10 ng/ml LPS. TNF was secreted to a slightly higher level but the maximum concentration obtained was still only 150 pg/ml when the cells were stimulated with 10 ng/ml. Using these results, LPS at a concentration of 0.1 ng/ml was selected for use in subsequent experiments as the priming concentration.

Subsequently, LPS (0.1 ng/ml) was used to prime MH-S cells for 2 h before stimulation with *F. tularensis* LVS, Schu S4 genomic DNA or either +ve or –ve control ODNs. As a measure of signalling activation, IL-6 secretion was determined (Figure 6-5). The +ve control ODN was able to stimulate the secretion of IL-6 and this was enhanced when the cells were primed with sub-stimulatory LPS. When TLR9 was blocked by G-ODN the level of IL-6 was dramatically reduced. Significant secretion of IL-6 was not observed when the –ve control ODN was used to stimulate MH-S cells, even when the
Figure 6-3: Secretion of TNF and IL-6 in the presence of TLR4 and TLR9 inhibitors after infection with F. tularensis LVS

Secretion of TNF (A) and IL-6 (B) by MH-S cells was determined 24 h after infection by F. tularensis LVS in the presence of inhibitors of TLR4 or TLR9. The experiment was performed in triplicate (n = 3) and each replicate was performed in duplicate. Significant difference from LVS only was determined by one-way ANOVA and a Dunnett’s multiple comparison post-test (blue * = p<0.05). Significant difference between individual inhibitors and when used in combination was determined by one-tailed, unpaired t-test (black * = p<0.05). Error bars show the min and max.
Figure 6-4: Secretion of IL-6 and TNF by MH-S cells stimulated with low concentrations of ultrapure *E. coli* LPS

Secretion of IL-6 (A) and TNF (B) by MH-S cells was determined 24 h after stimulation with ultrapure *E. coli* LPS at a range of low concentrations (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml). The experiment was performed in duplicate. Error bars show the standard deviation.
Figure 6-5: Secretion of IL-6, in response to DNA stimulation, by LPS-primed and un-primed MH-S cells

MH-S cells were primed for 2 h with ultrapure *E. coli* LPS (0.1 ng/ml) or left untreated before being stimulated with *F. tularensis* genomic DNA or control ODNs. Secretion of IL-6 was determined 24 h after stimulation. (A) Stimulated with +ve ODN control (10 µg/ml). (B) Stimulated with -ve ODN control (10 µg/ml). (C) Stimulated with *F. tularensis* LVS genomic DNA (10 µg/ml). (D) Stimulated with *F. tularensis* SchuS4 genomic DNA (4 µg/ml). +G = TLR9 antagonist present. (P) = primed with *E. coli* LPS (0.1 ng/ml). The experiment was performed in duplicate. Error bars show the standard deviation. Significant difference was determined by one-way ANOVA and a Tukey’s multiple comparison post-test (* = p<0.05).
cells were primed with LPS. The most dramatic enhancement was seen with genomic DNA from *F. tularensis* LVS. Alone, *F. tularensis* LVS DNA did not induce significant IL-6 secretion (< 200 pg/ml). However, when the cells were primed with LPS, secretion was significantly greater reaching > 1500 pg/ml in 24 h. Unfortunately, no significant enhancement of IL-6 secretion was observed after *F. tularensis* SchuS4 DNA stimulation, although with a greater number of repeats the data may have obtained significance.

6.4 Discussion

The ability of TLRs to coordinate their signalling significantly enlarges the range and type of responses that can be generated by a defined and limited set of receptors. The data collected in this chapter demonstrate that the immune response to *F. tularensis* uses integration of TLR4 and TLR9 signalling to dramatically enhance the secretion of cytokines after infection. The order of stimulatory events used in this study, where sub-stimulation of TLR4 by LPS occurs first followed by stimulation of TLR9 by genomic DNA, was designed to closely match the situation in a natural infection. *F. tularensis* first comes into contact with the immune cell membrane where the majority of TLR4 is located. Subsequently, the bacteria enter the phagosomal compartment where TLR9 is found. Therefore the infective cycle of *F. tularensis* is consistent with the hypothesis that the TLR9 response is primed by prior (sub)-stimulation of TLR4.

The data obtained in chapter 5 showed that TLR4 is involved in the detection of *F. tularensis* infection as BMDMs with TLR4 knocked out displayed an altered signalling and cytokine response to infection. The data from this chapter add to the findings that, in addition to TLR2 and TLR4, TLR9 is also able to respond to *F. tularensis* infection of MH-S cells. DNA purified from four strains of *F. tularensis* was able to stimulate MH-S cells inducing the secretion of cytokines and this response was abrogated when a TLR9 antagonist was present. To negate the requirement for an antagonist to confirm stimulation via TLR9 a TLR9-KO cell line could have been used. However, often cell lines that contain receptor-KO display altered expression of other receptors. The aim of this work was to investigate possible coordination between TLR4 and TLR9 signalling and a forced over-expression of receptors due to TLR9-KO may not provide accurate results. As such, work was carried out in MH-S cells using a TLR9 antagonist. The role of TLR9 in the detection of CpG DNA was first identified by a study in 2000 that showed that TLR9-deficient mice were unable to respond to CpG DNA as demonstrated by an abrogation of inflammatory cytokine production. My work confirms these findings as cells were unable to respond to bacterial DNA stimulation in the presence of a TLR9
antagonist. Interestingly, differences were seen in the potency of the DNA from the four strains. *F. tularensis* SchuS4 and FSC200 induced significantly greater IL-6 secretion than the LVS and U112 strains. This is particularly interesting as SchuS4 and FSC200 are considered to be highly virulent strains of *F. tularensis* whereas LVS and U112 are considered low virulence strains. Could it be that the higher potency of SchuS4 and FSC200 DNA to TLR9 confers these strains with their virulence? In agreement with this data is a study which observed that DNA preparations from 15 different bacteria demonstrated varying stimulatory activities on HEK293 cells stably transfected with TLR9. The stimulatory activity was associated with the GC dinucleotide content of the DNA as the two species that displayed the highest IL-8 secretion also had the greatest frequency of GC dinucleotides. It could be that, although the GC content of the different *F. tularensis* strains are very similar (~32%), the frequency of GC dinucleotides differs between the strains resulting in enhanced stimulatory DNA in the more virulent strains. However, stimulatory DNA is unlikely to account entirely for increased virulence. A paper examining the beneficial effects of *Bifidobacterium* observed that immunostimulatory DNA, due to CpG-rich sequences, were partly responsible for these effects. *Bifidobacteria* are commensals of the human intestinal tract and can account for up to 90% of the total gut microbiota. Not only are they non-pathogenic, but they have been shown to have beneficial effects against numerous diseases. It would seem then that having immunostimulatory DNA does not confer enhanced virulence on its own and other factors, in addition to stimulatory DNA, must account for the increased virulence of *F. tularensis* strains such as SchuS4 and FSC200. It could be the ability of *F. tularensis* to stimulate TLR9 in combination with other TLRs, such as TLR4 and TLR2, which confers increased virulence, in contrast to *Bifidobacterium* species.

An explanation for increased virulence frequently observed in other bacterial species is the expression of virulence factors such as listeriolysin-O, a pore-forming exotoxin, that is secreted by *L. monocytogenes* aiding its escape from the phagosome. However, one of the enigmatic features of virulent *F. tularensis* strains is that they do not appear to have any classical virulence factors. There is a secretion system encoded on the FPI with similarities to the type VI secretion systems of *V. cholerae* and *P. aeruginosa* and the potential for this to be involved in pathogenesis has been scrutinised in depth. Although one protein, VgrG, has been shown to be secreted, the FPI is found in all the subspecies of *F. tularensis* indicating that the presence of this system does not account for the increased virulence of some strains. One significant difference between the strains can be found at the level of their DNA. Genomic alignments show that there is massive re-arrangement across different subspecies and within Type A strains. However, further work
needs to be done to confirm the role of genomic re-arrangement as the mechanism behind increased virulence. Previously, it has been identified that bacterial DNA was a more potent stimulant of TLR9 than synthetic ODNs and the reason for this was elucidated by a paper observing the differences in macrophage activation by these two stimulants. It was found that the major contribution to potency was the length of the stimulatory DNA and synthetic ODNs of more than 44 nucleotides in length were as immunostimulatory as bacterial DNA. The contribution of DNA length correlated with the presence of a length-dependent uptake process in macrophages. This is important as the quality of the DNA preparations used in these studies then becomes important. As research involving the two highly virulent strains (SchuS4 and FSC200) is carried out at containment level (CL) III the DNA preparations may be of different quality to those made at CL II (LVS and U112) leading to the differing efficiencies of TLR9 stimulation seen between these strains and not due to genomic re-arrangement.

An intriguing question regards whether the integration of TLR4 with TLR9 signalling is advantageous to the host or the pathogen. The answer, as ever in the field of immunology, is not a simple one. The sequential activation and integration of TLRs may function as a control mechanism to limit the immune response from becoming excessive by requiring the activation of multiple TLRs. The initial contact with a primary TLR would prime the immune system but not initiate a full response. If, in time, no further activation of other TLRs occurs the system resets itself preventing an un-needed immune response from being generated. However, if a secondary TLR becomes stimulated it is likely that a pathogen is present and the system, due to priming, is prepared to launch a full and appropriate immune response. A well studied example of this is the secretion of IL-1 after *F. tularensis* infection which involves integration between two families of PRRs. Firstly, the activation of specific TLRs initiates NF-κB mediated transcription of the pro-IL-1β message. Subsequently, NLR stimulation leads to the activation of the caspase-1 inflammasome and the cleavage of the pro-form into active IL-1β. A recent review article theorised that the requirement for both TLR and NLR activation for the secretion of IL-1β was due to the need to have such a potentially dangerous pro-inflammatory cytokine under tight control. The complex regulation, requiring at least two different stimuli, ensures secretion only occurs when absolutely needed. In the case of TLR4 and TLR9 integration from the *F. tularensis* infection model used in this study, TLR4 acts as the priming receptor. Importantly, the priming function seems to occur only when low levels of ligand are present as high concentrations of TLR4 ligand, such as LPS, have frequently been shown to induce an aggressive immune response without the requirement for subsequent TLR activation as in the case of sepsis. The role of secondary receptor for *F. tularensis* detection is played by TLR9 and several
characteristics make this receptor suitable for this role. TLR9 is mainly found on the membranes of intracellular compartments such as phagosomes and the ligand that it detects, CpG DNA motifs, is bacteria-specific. This means that the stimulation of TLR9 provides positive verification of the presence of an intracellular pathogen and a full immune response can be generated. The cooperation of TLR4 with TLR9 resulting in an aggressive immune response has been shown recently in a study investigating the therapeutic use of an inhibitor in the treatment of sepsis. This study found that, whereas single inhibitors targeting either LPS or CpG DNA are inefficient at combating sepsis, a dual inhibitor of these ligands, kukoamine B, protected mice against challenge with heat-killed E. coli.

Conversely, the coordination of the priming receptor with the secondary receptor may confer advantages to the pathogen instead of the host. The immune response to F. tularensis infection in vivo is well characterised by the absence of cytokine secretion in the first 72 h followed by an overwhelming and harmful inflammatory response. The delayed immune response is almost certainly due to the stealthy characteristics of F. tularensis, expressing non-stimulatory LPS and binding to and entering cells through non-inflammatory receptors. This enables the bacteria to colonise and replicate within cells undetected. However, during this time it could be that there is sub-stimulation of receptors occurring that begins to prime other TLRs, such as TLR9. When replication has taken place, a threshold may be reached where sufficient stimulation of TLR9 by CpG DNA occurs and a response is initiated that is enhanced by the previous sub-stimulation of TLR4. The mechanism for this rapid and severe secretion of proinflammatory cytokine may have similarity to the secretion of IL-1β. Activation of TLRs induces a build up of intracellular stores of pro-IL-1β which means that when secondary stimulation occurs secretion of large amounts of active IL-1β can occur extremely rapidly. Alternatively, priming may cause an up-regulation of MAPKs, particularly ERK, leading to an increased level of cytokine mRNA in the cytoplasm due to enhanced transport from the nucleus. Again this would mean that, upon secondary stimulation, the inflammatory response is rapid and exacerbated. To investigate and confirm this hypothesis more research into the properties of F. tularensis LPS is required. Similar studies to those carried out in this chapter would need to be done initially using ultra-pure F. tularensis LPS to prime the cells prior to secondary stimulation by genomic DNA. The mechanism behind the priming process could be examined using staining techniques to track the movement and accumulation of proinflammatory cytokine mRNA in primed cells.
The role of *F. tularensis* LPS in the generation of an immune response is a highly debated topic. *F. tularensis* LPS is considered non-stimulatory for TLR4 as demonstrated by a lack of cytokine secretion after exposure of TLR4 to this ligand\(^{212,301}\). However, LPS does seem to play a role in immune stimulation although the exact mechanism has not yet been elucidated\(^{304,305}\). It is possible that *F. tularensis* LPS is involved with the priming of the TLR9 response through sub-stimulatory binding to TLR4. Previous work identified that very low “sub-stimulatory” levels of *E. coli* LPS were unable to induce a cytokine response through TLR4 but could enhance the response of subsequent TLR stimulation\(^{455,456}\). The data collected in this chapter confirm and extend these findings as sub-stimulatory concentrations of *E. coli* LPS were able to enhance the response, not only to synthetic ODNs but also, to DNA purified from *F. tularensis* LVS. Due to the limitations associated with working with reagents requiring preparation at CLIII, sufficient repeats could not be carried out to determine significance for *F. tularensis* SchuS4 DNA stimulated cells. Despite this, the initial data looks promising. Although the priming studies used purified genomic DNA and not whole bacteria, it is highly likely that the same response enhancement would be observed with whole bacteria as TLR9-dependent cytokine secretion was seen in earlier studies using whole *F. tularensis* (Section 6.3.2.2). Now that the TLR4-induced priming of the TLR9-dependent response to *F. tularensis* DNA has been observed the next step would be to determine whether the non-stimulatory LPS of *F. tularensis* is able to function as the priming ligand. *F. tularensis* LPS is known to not stimulate TLR4 in the usual manner, with no observable cytokine secretion response associated with LPS binding\(^{301}\). However, the potential for sub-stimulatory binding has not been examined previously.

In summary:

- *F. tularensis* genomic DNA stimulates MH-S cells to secrete TNF and IL-6 in a TLR9-dependent manner.
- Sub-stimulation of TLR4 by LPS primes MH-S cells for an enhanced cytokine response upon secondary stimulation of TLR9 by *F. tularensis* DNA.
Chapter 7: General Discussion

7.1 Activation of ERK and p38 induced by F. tularensis infection

Detection of an invading organism by PRRs is a vital step in the generation of a rapid and specific innate immune response. Activation of PRRs initiates a downstream cascade involving the activation of multiple signalling proteins. Identification of the specific signalling proteins involved in the response to an infection can provide important information concerning the pathogenesis, the mechanisms of survival and immune evasion strategies used by the pathogen. This study identified that F. tularensis infection of MH-S cells was associated with significant alterations in the activation state of the MAPKs, p38 and ERK. As readings were taken at numerous time points throughout the course of infection this study was able to investigate the temporal dynamics of activation, which is as important in determining the subsequent response as activation itself, and identified a bi-phasic immune response to F. tularensis infection. This response was characterised by a rapid and transient activation of p38 followed by the complete suppression of p38 activation (Figure 3-6) and an early transient activation of ERK followed by a sustained activation (Figure 3-8).

The temporal dynamics, particularly of ERK, provide important clues to the pathogenesis of F. tularensis. Transient activation of ERK has been demonstrated to correspond to host cell survival strategies whereas sustained activation is associated with cell death mechanisms. The data from this study, demonstrated by the transient activation of p38 and ERK, indicate that during the early stages of infection the host cells are adopting cell survival strategies. However, during the late stages of infection, the host cells are in the process of cell death, as evidenced by the lack of p38 activation and the maintained level of ERK activation. This pattern of signalling activation points towards two possible hypotheses. The first hypothesis is that the host cell is initiating the conversion from survival to cell death in an effort to control the infection as has been seen for M. tuberculosis. Similar to M. tuberculosis, F. tularensis is an intracellular pathogen and, as such, requires host cells to replicate within. Therefore, cell death by apoptosis may reduce the replicative niche available for bacterial replication. The importance of apoptosis in host defence is highlighted by the fact that many viral pathogens encode apoptosis-inhibitors that are vital to their successful colonisation of a host. However, the second, and possibly more likely, explanation is that F. tularensis is regulating this process as a mechanism of increasing its proliferation. The timing of the switch from transient to sustained activation correlates with the transition of F. tularensis from the phagosomal compartment into the cytoplasm. This would allow sufficient bacterial replication prior to being released from the cell by apoptosis in order to successfully proliferate to...
neighbouring cells. Modulation of apoptotic pathways by bacterial pathogens has been observed previously, strongly supporting the theory that *F. tularensis* is interacting with these pathways through modulation of ERK. Chlamydiae species prevent the induction of apoptosis through interaction with the ERK signalling pathway enabling the bacteria to complete their obligate intracellular growth cycle. *Helicobacter pylori* infection induces the formation of an apoptosis complex that is again dependent on ERK signalling. It is possible that *F. tularensis* is similar to these two pathogens, using ERK signalling to alter the survival outcome of host cells. Supporting evidence for this has recently been provided by a study that demonstrated *F. tularensis* LVS inhibiting apoptosis of neutrophils via both the intrinsic and extrinsic pathways.

In addition to influencing the outcome of infected cells, activation of these two MAPKs was associated with the secretion of the immune mediators MCP-1 and TNF. In *F. tularensis*-infected MH-S cells, MCP-1 was rapidly secreted and TNF exhibited a more gradual increase throughout the infection (Figure 3-10). In agreement with other studies, these two cytokines were also detected in the lungs of mice 72 h after *F. tularensis* infection. The secretion of pro-inflammatory cytokines is complex and tightly regulated. TNF requires the release of two levels of control at both the transcriptional and the translational level regulated by NF-κB and the MAPK pathways, respectively. The activation of ERK is required for the transport of TNF mRNA from the nucleus into the cytoplasm and activation of p38, via its downstream substrate MK-2, is linked to increased TNF mRNA stability.

The importance of ERK in coordinating the immune response to *F. tularensis* infection was analysed using the specific inhibitor, PD0325901. As expected, inhibition of ERK significantly reduced the secretion of TNF induced by *F. tularensis* infection (Figure 4-3), presumably through reduced nucleocytoplasmic transport. In addition, ERK inhibition reduced the systemic spread of *F. tularensis* in a murine model of infection. In comparison, there was no decrease in bacterial numbers *in vitro* and no increase in survival was observed *in vivo* (Figure 4-3 and Figure 4-8). However, it is perhaps not surprising that the administration of a single inhibitor did not sufficiently optimise the immune response for complete bacterial clearance. Signalling pathways are amazingly complex comprising numerous interactions and both positive and negative feedback mechanisms. It is likely then that the system is able to effectively respond to inhibition, possibly through functional redundancy. For example, ERK is activated by both MEK-1 and MEK-2 indicating that there is a level of redundancy incorporated into the system. Conversely, distinct functions have also been observed for these two signalling proteins. It is possible that the MEK proteins, and other signalling proteins, do not
exhibit complete redundancy but instead share a degree of overlapping functions whilst maintaining some level of specificity.

A recent study examined how complex networks are controlled and hypothesised about what the best strategy for effective modulation might be. Networks can be described as a series of nodes that can be connected by critical (vital for the progression of signalling), redundant (two pathways leading to activation of the same substrate) or ordinary links. Nodes that need to be regulated to control the system are called driver nodes. Applying these terms to signalling pathways suggests that individual signalling proteins are nodes and the interaction between them, enabling progression of a signalling cascade, are links. Some links (critical) are essential for the progression of signalling, for example the recruitment of adaptors to the TIR domains of TLRs. In contrast, others may be ordinary or redundant links, such as the two MEK proteins upstream of each major MAPK. In order to optimally control the system, targeting of the driver nodes is required and it was found that sparse or heterogeneous networks, such as cell signalling pathways, require the highest number of driver nodes in order to control the system. If this theory is correct, it is likely that a single inhibitor would be unable to sufficiently control cell signalling in order to optimise the immune response and that multiple inhibitors, or activators, would be required. The success of using a combination of immune modulators has been demonstrated previously in the context of cutaneous leishmaniasis. Dual administration of a TLR4 and a TLR9 agonist in combination with a poly-protein vaccine candidate resulted in a reduced parasite burden whereas a single agonist alone did not. It is possible that by identifying the critical links and driver nodes downstream of F. tularensis infection an immuno-modulatory combination therapy may be identified.

In the course of examining the importance of ERK activation in generating an optimum immune response in vivo an interesting result was observed. In this work and in published studies PD0325901 has been shown to inhibit ERK activation. Similarly, this work has shown that F. tularensis can inhibit the activation of ERK at 48 h in a murine model of infection whilst inducing activation at 96 h (Figure 4-6). However, PD0325901 administration actually causes an increase in ERK activation in the lungs of F. tularensis-infected mice which is not present in mice given PBS (Figure 4-6). This result may shed some light on the mechanism by which F. tularensis suppresses the secretion of proinflammatory cytokines until late in infection. PD0325901 inhibits ERK activation by binding to its precursor, MEK-1/2, and inducing a conformational change. One mechanism by which the presence of F. tularensis could prevent this effect would be if a protein was secreted or expressed by F. tularensis that is able to block PD0325901 from interacting properly with its target substrate. It is
possible that such a protein would usually, in the absence of PD0325901, function in a similar manner to this inhibitor and be used by *F. tularensis* to inhibit ERK activation, leading to a delay in cytokine production. When both “inhibitors” are present an interaction between them could prevent the correct inhibition of MEK-1/2 and the signalling pathway is able to advance leading to ERK activation. A secretion system with some homologous components to the type-VI secretion system of *V. cholerae* is encoded by the FPI of *F. tularensis* and, although no effector proteins have been identified within the FPI, there could be effectors encoded elsewhere in the genome.  

7.2 Integration of TLR signalling enhances the immune response to *F. tularensis*

The binding of a ligand to the LRR domain of a TLR initiates the signalling cascades required to induce an appropriate immune response. As such, the downstream signalling events and subsequent response rely heavily upon the identity of the TLR being activated. In addition, signalling pathways activated by distinct TLRs are known to integrate with each other providing further specificity to the system. An improved knowledge of the TLRs responsible for initiating a response to *F. tularensis*, and the potential for signalling integration, can provide essential information about how this stealth pathogen interacts with the host response. TLR2 has previously been shown to become activated by *F. tularensis* infection and there is strong debate around the role of TLR4. This work has demonstrated that both TLR2 and TLR4 contribute to the immune response to *F. tularensis*. Furthermore, I have shown that not only can TLR9 respond to the genomic DNA of *F. tularensis* but that this response can be primed and enhanced by the sub-stimulation of TLR4 using low concentrations of LPS.

Infection by *F. tularensis* induces the activation of p38 and ERK resulting in the secretion of MCP-1 and TNF. Using BMDMs with either TLR2 or TLR4 knocked out this work has shown that both of these receptors contribute to the immune response to *F. tularensis*. It was shown that TLR2 is able to simultaneously enhance ERK activation while suppressing p38 activation and that stimulation of TLR2 is critical in the regulation of TNF translation (Figure 5-5). However, TLR2 does not act alone and this study observed that TLR4 stimulation is required to release the transcriptional regulation imposed on TNF secretion (Figure 5-5). These data indicate that coordinated activation of both TLR2 and TLR4 is required to obtain efficient secretion of TNF. As TNF is a highly pro-inflammatory cytokine, it is likely that the coordination of multiple TLR pathways is used to ensure that secretion occurs only when the initial detection of a pathogen is confirmed by a secondary detection event.
This is the case for another proinflammatory cytokine, IL-1β. The initial detection of a pathogen induces the production of the inactive pro-form of the cytokine \(^3,^{309}\). However, to induce the secretion of fully active IL-1β a secondary detection event involving the activation of the caspase-1 inflammasome is required \(^{29,30}\). The requirement for confirmation prevents the unnecessary, energy-wasting and potentially damaging effects of inappropriate cytokine secretion. The observed dual regulation mechanism for both IL-1β and TNF has not been identified for other cytokines. This could be due to the potential for IL-1β and TNF to cause significant damage to the host if uncontrolled secretion occurs. In contrast, other cytokines that do not have such a capacity to cause damage to the host do not require such complex regulation mechanisms.

Upon invasion of a host, pathogens do not solely activate a single class or type of receptor. Pathogens contain multiple ligands that are able to bind to and stimulate multiple receptors. Therefore it is through integration of several signalling pathways that the immune response to a pathogen is generated. This had been demonstrated in studies using two intracellular bacterial pathogens, *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* \(^{193,451,473}\). One of the major TLR ligands associated with Gram-negative pathogens is LPS that is usually able to stimulate TLR4. However, the role for LPS in *F. tularensis* infection is not as clear cut as it does not appear to stimulate TLR4 in the usual manner but is still required in the generation of an antibody response to this pathogen \(^{212,301,305}\). A previous study has shown that very low concentrations of *E. coli* LPS were able to enhance cytokine secretion in response to subsequent TLR9 stimulation by synthetic ODNs \(^{455,456}\). My work has extended this finding in the context of *F. tularensis*, and identified a potential function for TLR4, by demonstrating that sub-stimulation of TLR4 is able to prime and enhance subsequent TLR9 stimulation by *F. tularensis* genomic DNA. The importance of TLR4 sub-stimulation can be seen by the requirement for multiple TLR-stimulation events to induce the secretion of pro-inflammatory cytokine such as IL-1β and TNF, as discussed earlier. TLR4 sub-stimulation alone does not have the capacity to induce optimal cytokine secretion and neither does TLR9 stimulation. However, when each individual signalling event is integrated the optimal secretion of inflammatory cytokines occurs.

**7.3 Future work**

This work has mainly focussed on infections using the less virulent *F. tularensis* LVS. However, some data was produced using the highly virulent SchuS4 strain and this enabled the identification of some characteristics that may contribute to enhanced virulence. Firstly, *F. tularensis* LVS infection of MH-S cells produced a rapid and transient p38 activation which is absent when the cells are infected
with *F. tularensis* SchuS4. Furthermore, although both strains induced sustained ERK activation during infection, this occurred sooner in infection with *F. tularensis* SchuS4 (Figure 3-8 and Figure 3-20). It was also observed that *F. tularensis* SchuS4 induced a faster and greater degree of cell death and this might be associated with faster bacterial replication. Finally, the TLR9 stimulation assays identified that *F. tularensis* SchuS4 DNA demonstrated a higher stimulatory activity than *F. tularensis* LVS or *F. tularensis* U112 (Figure 6-1 and Figure 6-2). Due to the limited success in identifying secreted virulence factors that are associated with highly virulent strains, the potential for differences in genetic makeup to confer increased virulence is an interesting theory. Highly stimulatory DNA may enhance immune signalling via TLR9 that results in the overwhelming inflammation seen during the late stages of virulent *F. tularensis* infection. Although the identified ligands of TLR9 are CpG motifs present in DNA, the presence of these motifs alone does not relate to the stimulatory potency of DNA and, therefore, a genome with a high GC content does not necessarily equate to stimulatory DNA. The GC content of most of the *F. tularensis* strains, for which genomes have been sequenced, are very similar (~32%) However, it is the GC dinucleotide content that is important to the stimulatory activity imposed on TLR9. It is possible that the more virulent *F. tularensis* strains, due to genomic rearrangement, contain a greater percentage of GC dinucleotides conferring greater TLR9 potency. The advantage for *F. tularensis* SchuS4 to stimulate TLR9 signalling can only be hypothesised at present but it may involve the recruitment of immune cells thereby increasing the replicative niche for bacterial replication and proliferation. Alternatively, stimulation of TLR9 could lead to the generation of the overwhelming inflammatory response that is characteristic of the late stages of *F. tularensis* infection. In this work, *F. tularensis* SchuS4 induced a faster sustained activation of ERK, associated with the induction of cell death, compared to the less virulent *F. tularensis* LVS. A greater ability to stimulate TLR9 may lead to the more rapid activation of signalling factors enabling the escape from immune cells and proliferation throughout the host. Further characterisation of the stimulatory activity of *F. tularensis* DNA could confirm the contribution of TLR9 stimulation to increased virulence.

The use of the specific ERK inhibitor, PD0325901, has raised some interesting questions relating to the association between ERK activation and systemic spread of *F. tularensis*. When PD0325901 was administered to *F. tularensis* LVS infected mice, reduced bacterial colonisation of secondary organs (spleen and liver) was observed despite no reduction at the primary site of infection, the lungs. One mechanism that could account for this observation is that ERK inhibition reduces apoptosis of infected cells in the lungs, “trapping” *F. tularensis* in this organ and preventing systemic proliferation of bacteria. Blocking the adhesion of *L. monocytogenes* to the lung epithelium by intra-tracheal
administration of heparin prevented the systemic spread of bacteria to secondary sites such as the liver, spleen and kidneys\textsuperscript{419}. The containment of bacteria within the lung was associated with improved mouse survival\textsuperscript{419}. The effect of restricting bacterial dissemination has recently been shown in a murine model where the blockade of scavenger receptors reduced the damage to systemic organs after induction of sepsis by the cecal ligation puncture method\textsuperscript{418}. This hypothesis could be investigated further by using fluorescently labelled bacteria to allow the tracking of bacteria through the host. In this study, no improvement in survival was seen despite the reduction in systemic spread, possible due to the high bacterial numbers still present in the lungs. Previous studies investigating immune modulation as a therapeutic strategy have used a combination of therapeutics such as antibiotics\textsuperscript{340}. It is possible that using an ERK inhibitor combined with an antibiotic, to aid the killing of bacteria “trapped” within lung immune cells, might improve the rate of survival.

An unexpected finding in this study was the observation that when PD0325901 was administered to \textit{F. tularensis}-infected MH-S cells an increase in ERK activation occurred, although inhibition was expected. It seems that the combination of an ERK inhibitor with an ERK-suppressing bacterium induces activation of this signalling protein. The mechanism behind this surprising result definitely warrants further investigation as it is possible that it points towards the presence of a \textit{F. tularensis}-associated protein that, by interacting with PD0325901, prevents the modulatory action of both. First, it would be important to determine the critical binding interactions that occur between PD0325901 and its target protein MEK-1/2. The administration of other ERK inhibitors during \textit{F. tularensis} infection, which use alternative binding sites to PD0325901, would establish whether the effect is inhibitor specific. Bioinformatic approaches could also be used to identify \textit{F. tularensis} proteins that share homology to MEK-1/2 or contain similar binding sites to that targeted by PD0325901. At present there is limited knowledge of proteins that are secreted or expressed by \textit{F. tularensis} that interact with immune signalling and it is thought that much of the ability of \textit{F. tularensis} to evade immune responses and successfully colonise a host is related to indirect or passive strategies such as phagosomal escape and cytoplasmic replication. However, the observed suppression of inflammatory responses in the first 72 h after infection suggests a more direct interaction with immune signalling indicating the presence of secreted effector proteins.

This is the first study to my knowledge that demonstrates the detection of \textit{F. tularensis} genomic DNA by TLR9 being primed by non-stimulatory concentrations of LPS. Importantly, this might represent a mechanism by which the non-stimulatory LPS of \textit{F. tularensis} can contribute to the generation of
inflammation. However, more work is required to provide evidence for this hypothesis. In this study
*E. coli* LPS was used to demonstrate the “proof-of-principle” and, as such, the next logical step would
be to use purified *F. tularensis* LPS as the priming ligand. Elucidation of the mechanism behind the
priming of TLR9 also requires further investigation as it may be explained by the accumulation of
activated signalling proteins or cytokine mRNA in the cytoplasm or due to an increase in TLR9
expression both of which could be identified by using suitable cell staining techniques or
transcriptional profiling. A recent study examining the priming of alveolar macrophages with LPS
observed no up-regulation of TLR expression after priming but did identify an accumulation of
activated p38 \(^{182}\). Similarly, a study investigating the sequential activation of TLR4 and TLR9 did not
see any alteration in TLR9 expression after TLR4 stimulation by LPS and detect enhanced signalling
factor activation particularly of ERK and p38 \(^{189}\). However, after priming of murine macrophages with
IFN-γ an up-regulation of NOD-2 expression was seen associated with enhanced nitric oxide
production indicating that receptor up-regulation can play a part in the priming of immune cells \(^{426}\).

Even with the massive advances in available technology, such as next-generation sequencers,
attempting to elucidate and fully understand super-complex networks such as immune signalling
pathways can seem to be a futile task if solely using molecular and immunological techniques.
However, other scientific and non-scientific disciplines such as mathematics, electronics and physics,
also work with complex networks and have developed tools and models, novel to the microbiology
field, to analyse them. It could be that the incorporation of these tools with standard microbiological
techniques could greatly enhance our ability to visualise and understand biological networks such as
immune signalling pathways. Several groups have already begun using mathematical and electronic
techniques to better describe biological functions ranging from cell invasion and replication to the
regulation of ERK activation. Similar to cell signalling pathways, electronic circuits are used by
engineers to coordinate multiple input signals and generate specific outputs. One example is a
commonly used circuit called a negative feedback amplifier (NFA). In engineering, the NFA is used to
regulate dynamic processes by removing fluctuations in the input signal and smoothing the output
response \(^{474}\). Experimental modelling showed that the ERK signalling pathway functions very
similarly to a NFA, and converts an on/off response to a graded response \(^{474}\). This finding closely
matches the known characteristics of ERK activation where it is not just whether ERK is activated or
not that is important but also the temporal dynamics of the activation. Mathematical modelling of
macrophage invasion by *S. typhimurium* has also dramatically improved the knowledge of the
pathogenesis of this organism \(^{475}\). In combination with real-time video microscopy, it was found that
macrophages can be repeatedly infected by *S. typhimurium* but that re-infection events occur at a lower rate than the primary infection 475.

The interaction of *F. tularensis* with immune cells, such as macrophages, would seem to be a perfect mechanism to study using a similar combination of experimental and mathematical modelling that has been used with *Salmonella*. Similarly, our understanding of the activation and progression of numerous signalling pathways through the stimulation of TLRs and NLRs by *F. tularensis* could be greatly enhanced through drawing comparisons with other disciplines such as electronic circuits.

### 7.4 Final comment: Interaction of *F. tularensis* with immune signalling

*F. tularensis* has been described as a stealth pathogen due to its ability to invade the host without inducing a detectable immune response at early time points 317. Moreover, for some time it appeared to orchestrate immune evasion without expressing immune-modulators or virulence factors such as toxins or cytolysins. However, as a greater understanding of immune signalling pathways has developed, elucidation of several mechanisms have recently begun to appear. One of the key advances was the characterisation of the FPI the genes of which were critical for phagosomal escape and intracellular replication 254,258,260,328. More recently a secretion system with similarities to the type-VI secretion system of *V. cholerae* was demonstrated to be essential for delivery or surface-expression of VgrG and IglI 261. My work provides further evidence of the interaction between *F. tularensis* and the immune signalling network. Clearly, it now appears that *F. tularensis* is a much more proactive immune-modulator than originally thought, interacting with immune processes and signalling pathways at multiple levels.

The characteristic property of *F. tularensis* infection is the delay in the inflammatory response followed by overwhelming inflammation and the potential for multi-organ failure 200. The bacterium is able to modulate the immune response enabling bacterial replication unhindered by immune processes followed by systemic proliferation aided by the induction of inflammation and cell death. Previous studies have demonstrated how *F. tularensis* enters cells in a non-stimulatory manner and is able to prevent the activation of microbicidal complexes 240,319,321. These processes go some way to explain the lack of an immediate immune response. However, my work shows that *F. tularensis* may also be directly inhibiting the activation of ERK by MEK-1/2 preventing the efficient secretion of TNF,
a highly potent inflammatory cytokine. Furthermore, during the non-responsive phase of the immune response to *F. tularensis*, this stealth pathogen may be priming the host cells. Priming ensures that, once sufficient replication has taken place, the secondary induction of the immune response causes enhanced cytokine secretion and exacerbated inflammation which is both too strong and too late to protect the host.

Significant work will be required to reveal and fully characterise all the interactions that *F. tularensis* exploits during infection. However, this research is crucial to identify mechanisms by which the immune response can be rebalanced in favour of the host.
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