Immunological correlates of illness severity and course in acute Q fever

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Immunological correlates of illness severity and course in acute Q fever

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Abstract

Acute Q fever is a disease manifestation of *Coxiella burnetii* infection. This obligate intracellular bacterium is phagocytosed by innate immune cells, where it replicates within the usually bactericidal environment of the phagolysosome. As the immune response is activated, the resultant pro-inflammatory cytokines aid in pathogen clearance but also trigger an acute sickness response in the host.

This thesis describes the natural history of acute Q fever in a prospective cohort – the Dubbo Infection Outcomes Study (DIOS). In these subjects, the acute febrile illness was characterised by severe headache, drenching sweats and fatigue. In approximately 10% of subjects, symptomatic illness marked by fatigue remained present for months, or occasionally years, after the acute illness. Subjects with more severe acute illness were more likely to develop this post Q fever fatigue syndrome (QFS). The aim of this thesis was to determine whether ongoing infection or aberrant immune activation drive the prolonged symptoms of QFS.

Sensitive real time PCR detection of Coxiella DNA revealed a significant minority of subjects had very low copy numbers in circulating monocytes, with an increased prevalence in those with QFS. However, the detection was not consistently found within individual subjects and the copy number was at the threshold of reliable detection.

C. burnetii was shown here to stimulate cytokine production in monocytic cells via

interaction with Toll-like receptor (TLR)-2 and not TLR-4. Functional polymorphisms in these TLRs were identified in subjects with Q fever, but were not associated with Q fever susceptibility, severity or duration.

Phase I-specific responses are believed to be critical in the generation of protective immunity to *C. burnetii*, yet the phase II-specific responses of innate and adaptive immune components were consistently of higher magnitude. Whole *C. burnetii* organisms induced antigen-non-specific T cell activation, presumably via the indirect activation of monocytes by *C. burnetii* LPS.

No significant differences were found in the magnitude or kinetics of the host response to infection, or in the carriage of genetic polymorphisms, when comparing subjects who developed QFS with subjects who had promptly resolving illness. It remains unclear what factors mediate the progression of acute Q fever to QFS.

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Table of contents

Part I: Introduction

1. Microbiology of *Coxiella burnetii*

2. Manifestations of *C. burnetii* infection

3. Host-pathogen interaction

Part II: Research Studies

4. Natural history of Q fever: DIOS cohort

5. Innate immune responses to *C. burnetii*

List of tables

List of figures

Publications

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Abbreviations

APC	Antigen presenting cell
BDQ	Brief Disability Questionnaire
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Copy DNA
CEF	Cytomegalovirus, Epstein-Barr virus and Influenza virus
CFS	Chronic fatigue syndrome
CHO	Chinese hamster ovary
CIIR	Centre for Infection and Inflammation Research
CME	Chloroform-methanol extract
CMR	Chloroform-methanol residue
Cp	Crossing point
CR	Complement receptor
Ct	Cycle threshold
DC	Dendritic cell
DIOS	Dubbo Infection Outcomes Study
DTH	Delayed type hypersensitivity
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorption assay
ELISpot	Enzyme-linked immunospot assay
FACS	Fluorescent-activated cell sorting

FcR	Fc receptor
HHV	Human herpesvirus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
LCV	Large cell variant
LLR	Leucine-rich repeat
LPB	LPS binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MHC	Major histocompatibility complex
MIF	Macrophage inhibition factor
mRNA	Message RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NMI	Nine Mile strain phase I
NOS2	Nitric oxide synthase 2

ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Physical containment
PCA	Principal components analysis
PCR	Polymerase chain reaction
PPD	Purified protein derivative
PSC	Physical Symptoms Checklist
PV	Parasitophorous vacuole
QF	Q fever
QFS	Q fever fatigue syndrome
qPCR	Qualitative PCR
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal RNA
RRV	Ross River virus
RT	Reverse transcription
SCV	Small cell variant
SD	Standard deviation
SE	Standard error
SNP	Single nucleotide polymorphism
SOMA	Subscale of SPHERE
SPHERE	Somatic and Psychological Health Report

TAP	Transporter associated with antigen processing
TCA	Trichloroacetic acid
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TipDC	TNF and iNOS-producing DC
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor

Part I: Introduction

1. Microbiology of *Coxiella burnetii*

1.1 Identification and early characterisation of *C. burnetii*

Acute Q fever was first described in Australian meat workers by Derrick in 1937 (Derrick, 1937), however no pathogen was identified to explain the symptoms he observed. Almost simultaneously, researchers in the USA described a rickettsia-like, filterable organism isolated from ticks which caused disease in guinea pigs, but had unknown effects on humans (Davis and Cox, 1938). Both teams were working on the same organism, which was eventually to become known as "*Coxiella burnetii*". In acknowledgement of the assistance obtained from Sir Frank Macfarlane Burnet in identification and classification of the Q fever pathogen, Derrick named the organism *Rickettsia burnetii* (Derrick, 1939). This classification was revised nearly ten years later when it became clear that while filterable, the organism causing Q fever did not share significant homology with the rickettsiae, and thus was reclassified into its own genus, *Coxiella* (Philip, 1948a). *C. burnetii* remains the only known species in this genus.

C. burnetii is an extremely small bacterium. With an approximate width of 0.2 to 0.4μm, and length of 0.4 to 1μm, it is not visible via light microscopy. Early studies of *C. burnetii* using electron scanning microscopy have visualised cell division via binary fission (Anacker *et al.*, 1964) and confirmed a cell structure similar to that found in Gram-negative bacteria, including a cytoplasmic membrane, periplasmic space, and outer cell membrane (Burton *et al.*, 1975). Gram-negative bacteria typically contain lipopolysaccharide (LPS) in the outer cell membrane. Chemical analysis of *C. burnetii* LPS has found the structure to

be similar, but not identical, to that found in most other Gram negative bacteria (Amano and Williams, 1984), and of significantly reduced toxicity (via induction of host innate immune responses) in comparison to LPS from other Gram-negative organisms (Hackstadt *et al.*, 1985).

As an obligate intracellular pathogen, the study of *C. burnetii* is hindered by the complex culture techniques that require use of embryonated chick eggs or guinea pigs. Furthermore, the study of live organism requires a physical containment level 3 (PC3) laboratory unless appropriate steps have been taken to clone an avirulent strain (Hackstadt, 1996). Accordingly, there is much about the pathogenicity of *C. burnetii* and the host response to Q fever infection that remains uncharacterised.

1.2 Life cycle

Coxiella burnetii has two distinct natural reservoirs, native fauna and livestock. The spread of *C. burnetii* in the wildlife reservoir is considered to be mediated by blood-sucking ectoparasites such as ticks. It has been identified to occur naturally within a variety of tick species including *Rhipicephalus sanguineus* (Parker and Sussman, 1949), *Ixodes holocyclus* (Carley and Pope, 1953), *Dermacentor occidentalis* and *Ornithodoros coriaceus* (Enright *et al.*, 1971). In domestic animals not commonly exposed to tick bite, it is supposed that transmission occurs primarily via the inhalation of aerosol products from contaminated animal waste products. Similarly, Q fever in humans is almost invariably attributed to aerosol contact with infected animals or their waste products (Raoult *et al.*, 2000). It is

estimated that a single organism is capable of causing infection in humans (Moos and Hackstadt, 1987).

C. burnetii is extremely resistant to chemical disinfection by agents known to be effective against *Bacillus* spores, including sodium hypochlorite and formalin (Malloch and Stoker, 1952; Scott and Williams, 1990). It is also resistant to dessication, with reported revival of a reference strain more than 20 years after it was first dessicated (Giroud *et al.*, 1964). The ability of *C.burnetii* to withstand environmental degradation is possibly due to the production of a spore-like small cell variant (SCV), first identified by its differential density in comparison to the large cell variant (LCV) (Wachter *et al.*, 1975), and which has been demonstrated to be more resistant to disruption by sonication, osmotic stress and pressure than the LCV (Amano *et al.*, 1984).

The temporal appearance of each size variant of *C. burnetii* has been linked to its replicative cycle. SCV infection of Vero cells results in a predominance of the SCV for the *C. burnetii* lag phase (approximately two days), followed by increasing prevalence of the LCV during the exponential phase of growth, and reappearance of the SCV in the stationary phase (approximately day six) (Coleman *et al.*, 2004). The SCV is associated with increased expression of *scvA* (Coleman *et al.*, 2004), a gene thought to encode a protein involved in chromatin condensation, further suggesting it is a non-replicative form. Conversely, the LCV are visibly seen to divide during the logarithmic growth phase and are clearly involved in replication (Coleman *et al.*, 2004).

1.3 Phase variation

Being a Gram-negative organism, LPS predominates in the outer cell wall of *C. burnetii*. LPS from *C. burnetii* is relatively non-toxic to chick embryos at concentrations of over 80μg per embryo, in contrast with *Salmonella typhimurium* LPS which is toxic in nanogram amounts (Hackstadt *et al.*, 1985). There are two serologically distinct phases of *C. burnetii*, termed phase I and phase II, which are somewhat analogous to the smooth and rough phase variants of *E. coli* respectively (Hackstadt *et al.*, 1985). Phase II organisms contain one-tenth the quantity of LPS present in phase I organisms (Baca *et al.*, 1980). The sugars present in phase II LPS are qualitatively similar to those present in phase I LPS, but differ in relative quantities (Baca *et al.*, 1980). It is unclear whether phase variation is linked to cell size variation, however, it is clear they are not synonymous as cell wall fractions from both small and large cell variants were shown to react with sera specific for both phase I and phase II (Amano *et al.*, 1984).

Passaging of phase I organisms in cell culture results in rapid changes in LPS structure and the generation of predominantly phase II organism (Burton *et al.*, 1978; Hotta *et al.*, 2002). Using groups of monoclonal antibodies reactive to O-polysaccharide chains or outer core oligosaccharides, it was evident that with increasing passage number, higher molecular weight antibody targets were lost from the organism (Hotta *et al.*, 2002). This indicates that there is gradual phase conversion through intermediate LPS types, where phase I and phase II are two extremes in a continuum of potential LPS structures. Consistent with this notion is the finding that the lipid A moieties of phase I and phase II LPS are identical (Zamboni *et al.*, 2004).

In guinea pigs, infection by phase I organisms results in seroconversion and fever. Viable organisms are recoverable after 30 days of infection (Moos and Hackstadt, 1987). Conversely, infection by phase II organisms only results in seroconversion and fever if a very large inoculum is used, and viable organisms are not recoverable after 30 days of infection (Moos and Hackstadt, 1987). Accordingly, phase I organisms are considered the virulent strain, while phase II organisms are considered avirulent. Strains demonstrating LPS structure intermediate between phase I and phase II have also been found to have intermediate phenotype and virulence. The LPS intermediate "Crazy" strain causes infection in guinea pigs that results in fever and seroconversion (as with phase I infection) but no recoverable organisms were present after 30 days of infection (as with phase II infection) (Moos and Hackstadt, 1987).

Early reports suggested phase II organisms could revert to phase I when passaged back in guinea pigs. It seems clear that this "reversion" was due to a small number of phase I organisms remaining in the phase II culture which were able to replicate when infected into an animal host (Hackstadt, 1996). In fact, the difference in LPS structure present in phase II strains has been attributed to a significant chromosomal DNA deletion, encompassing putative LPS biosynthetic genes such as epimerases, dehydratases and glycosyl transferases of nucleotide sugars (Hoover *et al.*, 2002). Unexpectedly, the "Crazy" strain, which is intermediate to phase I and phase II LPS structures, has been found to carry a chromosomal DNA deletion including, and extending beyond, the deletion found in phase II strains (Hoover *et al.*, 2002), perhaps suggesting the mutation responsible for the phase II LPS

phenotype occurs in another gene, early in the LPS biosynthetic pathway (Voth and Heinzen, 2007).

There is evidence to suggest that phase I LPS shields immunogenic motifs close to the cell surface, which are exposed in phase II organisms. For instance, phase II-specific antibodies are able to bind to solubilised phase I cell proteins (Hackstadt, 1988). Furthermore, chemical removal of phase I LPS exposes proteins that are immunoreactive with anti-phase II antibodies (Hackstadt, 1988). Phase II organisms are readily killed by the complement membrane attack complex (MAC), while phase I organisms are resistant (Vishwanath and Hackstadt, 1988). Dendritic cells recognise phase II organisms, but not phase I organisms, by host cell Toll-like receptor engagement (Shannon *et al.*, 2005b). Phase I LPS may also interfere with pathogen interaction with complement receptor 3 (CR3), preventing efficient uptake into host cells (Capo *et al.*, 1999), and induction of interferon-gamma (IFN-γ) production in response to phase I organisms is significantly decreased in comparison to the response to phase II organisms (Izzo and Marmion, 1993). Thus, it appears that steric hindrance of the full length phase I LPS plays a significant role in the differential pathogenicity of phase I and phase II organisms.

In primary mouse macrophages, phase II organisms display impaired growth in comparison to phase I organisms (Zamboni *et al.*, 2002; Brennan *et al.*, 2004; Zamboni, 2004). This phenomenon is perhaps specific to mice, as the growth kinetics of phase I and phase II organisms are comparable in primary guinea pig macrophages (Kishimoto and Walker, 1976) and human monocyte-derived macrophages (Shannon and Heinzen, 2007). It is

possible that phase II organisms activate host immune factors in mice that are effective at clearing infection while phase I organisms are poor activators of murine macrophage immune responses. Consistent with this notion is the finding that primary macrophages from TLR-2 knockout mice are more permissible to growth of phase II organisms (Zamboni *et al.*, 2004). There is also evidence that phase I and phase II organisms differentially induce host immune responses in human immune cells including dendritic cells, neutrophils and monocytes (Fumarulo *et al.*, 1989; Izzo and Marmion, 1993; Shannon *et al.*, 2005a), suggesting that differential immune activation may not explain the varied growth kinetics of phase I and phase II organisms in murine, but not human, primary macrophages.

1.4 Replication within the phagolysosome

C. burnetti is an obligate intracellular organism, which is typically found in phagosome-derived vacuoles in macrophages. Unlike many macrophage-tropic pathogens, *C. burnetii* survives and replicates within the acidic environment of the phagolysosome. This pathogen is well adapted to survival within this harsh environment, possessing a number of mechanisms for protection against antimicrobial host defences as outlined below.

Similar rates of uptake for dead and live *C. burnetii* into the host macrophage indicate that internalisation of the bacterium is a passive process (Tujulin *et al.*, 1998) that is thought to involve both endocytic and pinocytic processes (Baca *et al.*, 1993a; Tujulin *et al.*, 1998). The ability to take up phase I *C. burnetii* varies widely, even amongst macrophage cell

lines derived from the same mouse strain (Baca *et al.*, 1981), with phase II organisms more readily phagocytosed than phase I organisms. In the human myelomonocytic cell line, THP-1, phagocytosis of *C. burnetii* involves the leucocyte response integrins, $\alpha_v\beta_3$ and complement receptor 3 (CR3, or $\alpha_M\beta_2$) (Capo *et al.*, 1999). Phase I organisms induce actin rearrangement and the formation of membrane protrusions (Meconi *et al.*, 1998) from which CR3 is absent (Capo *et al.*, 2003), perhaps accounting for the reduced rate of phase I uptake in comparison to phase II organisms. There are differential uptake rates for phase I and phase II organisms noted for other cell types, including epithelial cells (Moos and Hackstadt, 1987)and fibroblasts (Baca *et al.*, 1993a; Miller *et al.*, 2004). The differences in uptake cannot be accounted for by CR3 localisation as these cell types do not express CR3. Therefore, it seems plausible that other receptors can play a role in uptake of the organism. *C. burnetii* phase II attaches more readily to fibroblasts than do phase I organisms, and binding of phase II organisms is impaired in the presence of phase I LPS (Baca *et al.*, 1993a), therefore differential binding to the host cell remains the likely reason for slower uptake of phase I, in comparison to phase II, organisms.

Once internalised, *C. burnetii* remains within a membrane bound compartment that initially displays phagosome characteristics. The *C. burnetii*-containing phagosomes fuse with each other, with other phagosomes, and with components of the endosomal system (Heinzen *et al.*, 1996). These fusion events in a host macrophage with multiple coxiella-carrying phagosomes give rise to one large vacuole termed the parasitophorous vacuole (PV) (Baca *et al.*, 1981). In a number of inbred mouse strains, macrophage resistance to *C. burnetii* phase II infection is associated with prevention of PV formation (Zamboni, 2004). *C.*

burnetii protein production is essential for formation and maintenance of a spacious PV, however phagosome maturation occurs independently of coxiella replication or protein synthesis. Similarly, survival of the organism within the phagolysosome does not require protein replication (in contrast with *Legionella* sp. and *Chlamydia* sp.) (Howe *et al.*, 2003). The *C. burnetii*-infected vacuole is cholesterol rich, and the organism is known to upregulate host cholesterol biogenesis and cholesterol uptake to supply the vacuole membrane with sufficient cholesterol (Howe and Heinzen, 2006).

The *C. burnetii* PV has been shown to sequentially acquire Rab 5 and Rab 7, which are GTPases associated with early and late endosomes respectively (Beron *et al.*, 2002; Romano *et al.*, 2007), indicating the organism undergoes maturation through the endocytic pathway. The *C. burnetii* PV also acquires lysosomal characteristics. Antibodies specific for lysosomal membrane proteins are able to label the *C. burnetii*-infected vacuole (Heinzen *et al.*, 1996). The lysosomal enzyme, acid phosphatase, is present, and the vacuole is acidic (Akporiaye *et al.*, 1983). Furthermore, thorium dioxide, a dye that is specific for secondary lysosomes, becomes sequestered to the *C. burnetii*-infected vacuole when added to persistently infected murine macrophages (Akporiaye *et al.*, 1983).

The typical maturation time frame for phagosomes containing latex beads is approximately 15 minutes (Oh and Swanson, 1996). In *C. burnetii*-infected phagosomes there is a slight delay in acquiring lysosomal characteristics, which take approximately 2 hours to mature (Howe and Mallavia, 2000). This delay in maturation has been proposed to aid transition of *C. burnetii* from SCV to LCV, with the SCV-specific protein ScvA being more

effectively downregulated at the slightly less acidic pH afforded by the delay in phagosome maturation (Howe and Mallavia, 2000). The brief delay in phagosome maturation may therefore represent another factor contributing to the survival of *C. burnetii* within the harsh environment of the phagolysosome.

Recent studies suggest that *C. burnetii* facilitates the pause in phagosome maturation by involvement of the autophagy system (Romano *et al.*, 2007). The protein LC3, a marker of autophagosomes, is present in the *C. burnetii* PV (Beron *et al.*, 2002). *C. burnetii* actively engages with the autophagic system early after infection (Romano *et al.*, 2007). Pharmacological agents known to block the autophagic process also block formation of the *C. burnetii* PV, while induction of the autosomal pathway enhances *C. burnetii* replication (Beron *et al.*, 2002). The role of autophagy in the fate of intracellular pathogens is varied. For *Mycobacterium tuberculosis*, induction of autophagy drives pathogen clearance, however, bacteria such as *C. burnetii* and *Legionella pneumophila* make use of the autophagic system, perhaps for delivery of nutrients to the replicative vacuole (Colombo, 2007).

The *C. burnetii*-infected macrophage is able to divide, regardless of bacterial load (Roman *et al.*, 1986). When the *C. burnetii*-infected cell divides, the PV segregates into only one daughter cell, such that the other daughter cell remains uninfected (Roman *et al.*, 1986). Thus, it is possible for *C. burnetii* to establish persistent infection in cell culture models without the need to replenish the culture with uninfected host cells. It is also apparent that *C. burnetii* infection causes minimal deleterious effects on the host cell.

1.5 Metabolism in acidic conditions

C. burnetii replicates with a relatively slow doubling time (12-20h) (Zamboni *et al.*, 2002). A number of substrates have been reported to be utilised by *C. burnetii*, including α-ketoglutarate, succinate, fumarate, malate, oxoloacetate, pyruvate, glutamate and serine (Ormsbee and Peacock, 1964), indicating use of the Krebs cycle for carbohydrate metabolism. The oxygen requirement for metabolism of each substrate is substantially lower than that needed by other bacterial and fungal organisms, suggesting relatively low metabolism at neutral pH (Ormsbee and Peacock, 1964).

At low pH, as found in the *C. burnetii* PV, glutamate is a main source of energy for the bacterium and is actively transported into the organism making use of the proton motive force (Hackstadt and Williams, 1983). Whole genome sequencing has identified a putative glutamate uptake transporter and two proton-driven uptake systems for the sugars, xylose and glucose (Seshadri *et al.*, 2003). Both the Embden-Meyerhof and pentose phosphate pathways operate in *C. burnetii* at low pH, with glutamate metabolised at a rate 26 times greater than the rate at which glucose is metabolised (Hackstadt and Williams, 1981). Transport and metabolism of glutamate are maximal at low pH (approximately pH 2.7-4.0 and pH 4.5-5.0 respectively), but become negligible at neutral pH (Hackstadt and Williams, 1983). Increasing the pH of the phagolysosome prevents multiplication of *Coxiella burnetii* (Hackstadt and Williams, 1981). This is consistent with the notion of resting, inactive organisms being taken up into the phagosome, where fusion to form a phagolysosome and the resultant drop in pH activates the organisms' metabolic pathways.

Mouse macrophage cell lines infected by strains associated with either acute or persistent Q fever illnesses did not result in significantly varied pH of the phagolysosome either between strains or over time (Maurin *et al.*, 1992). Thus, it seems that while the pH of the *C. burnetii* PV seems to be critical for the functioning of metabolic pathways and replication of the bacteria, pH is independent of the infecting strain of *C. burnetii*, and does not correlate with the course of infection.

A number of putative sodium ion/proton exchangers have been identified in the *C. burnetii* genome, and may play a role in resistance to the acidic pH present in phagolysosomes (Seshadri *et al.*, 2003). As another strategy for surviving acidic conditions, many of the proteins identified in *C. burnetii* have basic isoelectric points, potentially allowing them to act as proton sinks (Seshadri *et al.*, 2003). Thus, in addition to optimal metabolism in acidic conditions, *C. burnetii* may have a variety of mechanisms for enhanced survival in the harsh phagolysosomal environment.

1.6 Genomic and plasmid DNA

The genomic sequence for *C. burnetii* Nine Mile strain phase I (NMI) has been determined relatively recently (Seshadri *et al.*, 2003). In this strain, genetic components include both chromosomal and plasmid (QpH1) DNA. The *C. burnetii* chromosome is circular, consisting of 1,999,725 base pairs (bp), with a high C+G content of 42.6% (Seshadri *et al.*, 2003). Codon usage in *C. burnetii* is considerably different to that seen in *E. coli*, with many codons being preferentially used in one organism but not the other (Lin *et al.*, 1997).

Analysis of 16S ribosomal RNA (rRNA) sequences has confirmed *C. burnetii* to be a member of the γ-proteobacteria along with bacterial species such as *Legionella pneumophila*, and distant from the rickettsia which are α-proteobacteria (Stein *et al.*, 1993; Seshadri *et al.*, 2003), confirming its earlier reclassification from *Rickettsia burnetii* to *Coxiella burnetii* (Philip, 1948a). Approximately one third of the predicted 2134 coding sequences contained in the chromosome and plasmid are hypothetical and do not match sequences of proteins with known function in other organisms. A comparison with other intracellular pathogens such as *Rickettsia*, *Chlamydia* and *Mycobacteria* has indicated that the genome of *C. burnetii* is significantly different with respect to the presence of multiple insertion sequences, the small degree of genome reduction, and its transporter and metabolic capabilities, suggesting that *C. burnetii* may have only recently become an obligate intracellular organism (Seshadri *et al.*, 2003). With the report of an insertion sequence (IS1111a) occurring at copy numbers of approximately 20 per genome in NMI, the use of this sequence as a target for sensitive PCR assays has since been validated for use in clinical samples for *C. burnetii* detection (Klee *et al.*, 2006).

Analysis of 16S ribsosomal RNA sequences confirmed that different *C. burnetii* strains are highly related and consist of a single species (Stein *et al.*, 1993). Comparative genome hybridization (CBH) has been used to assess in greater detail the degree of genomic similarity amongst *C. burnetii* isolates from geographically diverse areas against the NMI reference sequence, and confirms that the majority of open reading frames are highly conserved (Beare *et al.*, 2006). Comparison of ORF polymorphisms confirms the whole

genome typing of clinical isolates into groups that had previously been proposed by analysis of restriction endonuclease-digested genomic DNA (Hendrix *et al.*, 1991; Beare *et al.*, 2006).

Coxiella burnetii strains can differ in the presence or absence of one or more autonomously replicating plasmids, called QpH1, QpRS, QpDV and QpDG. Strains not carrying plasmids have been shown to have plasmid-homologous sequences incorporated into their chromosomal DNA (Willems *et al.*, 1997). The plasmids each contain a common "core" sequence and only a small number of ORFs are unique to particular plasmids (Mallavia, 1991; Thiele *et al.*, 1994; Willems *et al.*, 1997; Lautenschlager *et al.*, 2000). It has been proposed that the type of plasmid (Samuel *et al.*, 1985; Hendrix *et al.*, 1991), or the genes encoded on each plasmid (Minnick *et al.*, 1991), determine whether a particular strain of *C. burnetii* is likely to cause acute or chronic disease, however this theory has been disputed (Thiele and Willems, 1994).

The QpH1 plasmid was originally isolated from the Nine Mile phase I reference strain, however sequences from this plasmid have been shown to also be present in the chromosome of phase II strains (Samuel *et al.*, 1983). QpRS and QpDG were each identified in cases of endocarditis (Samuel *et al.*, 1985; Hendrix *et al.*, 1991), however subsequent sequencing has suggested that QpDG is highly similar if not identical, to the QpH1 plasmid (Jager *et al.*, 2002). The QpDV plasmid has been associated with both acute isolates and with isolates that cause chronic, localised Q fever (Valkova and Kazár, 1995).

The possible role of these plasmids as determinants of *C. burnetii* virulence therefore remains unclear.

1.7 Related microorganisms

The taxonomy of *Coxiella burnetii* is superkingdom Bacteria, phylum Proteobacteria, class γ-Proteobacteria, order Legionellales, family Coxiellaceae. The Proteobacteria are a large and diverse group of Gram-negative bacteria including purple phototrophic, nitrifying, sulfur and iron oxidising, hydrogen oxidising, sulfate and sulfur reducing, methanotrophic, nitrogen fixing, bioluminescent and enteric bacterial species. The γ-proteobacteria include many pathogenic bacterial species. While originally classified as a subgenus of the *Rickettsia* genus, *Coxiella* was later reclassified as its own genus due to its distinct properties, for example, *Rickettsia* replicate in the cytoplasm of host cells following escape from the phagosome, while *Coxiella* replicate from within the phagolysosome (Weiss and Moulder, 1984). Studies of 16S rRNA have indicated that *C. burnetii* is placed in the gamma subdivision of the proteobacteria, which includes species such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Legionella pneumophila*.

Until recently there was no known bacterial species that is closely related to *C. burnetii* (Weisburg *et al.*, 1989). New evidence suggests other species belong to the *Coxiella* genus, including a crayfish pathogen found in Australia with the provisional name *Coxiella cheraxi sp. nov.* (Tan and Owens, 2000; Cooper *et al.*, 2007), and unnamed *Coxiella* spp. isolated from *Haemaphysalis longicornis* ticks in Korea (Lee *et al.*, 2004), from

Amblyomma americanum ticks in USA (Jasinskas *et al.*, 2007) and from laboratory maintained *Ornithodoros moubata*, *Rhipicephalus sanguineus*, and *Haemaphysalis longicornis* ticks (Noda *et al.*, 1997). It has been suggested that *Coxiella* sp. and ticks are symbiotic, as a reduction in the *Coxiella* sp. population by the use of antibiotics is associated with reduced reproductive fitness of *Amblyomma americanum* ticks (Zhong *et al.*, 2007).

Some microorganisms share phenotypic similarity with *C. burnetii*, such as an ability to replicate within the phagosome (*Salmonella*, *Yersinia*, *Mycobacteria*), or obligate parasitism (*Wolbachia*, *Rickettsia*, *Chlamydia*, *Leishmania*) (Weiss *et al.*, 1991). The mechanisms for survival within the host macrophage vary considerably amongst different organisms. Like *C. burnetii*, the amastigote stage of *Leishmania donovani* demonstrates optimal metabolic rates at acidic pH (Mukkada *et al.*, 1985), facilitating survival and replication within the acidic environment of the phagolysosome. However, *C. burnetii* and *L. donovani* are relatively unique in their ability to replicate within the phagolysosome of host macrophages.

Many of the other organisms able to infect and replicate within macrophages do so by avoiding the phagolysosome. *Salmonella typhimurium* survives within phagocytic vacuoles by preventing their fusion with lysosomes, in a process that requires the *phoP* regulon and early bacterial protein synthesis (Garvis *et al.*, 2001). *Yersinia pestis* also makes use of PhoP regulated genes to enhance survival within the host macrophage (Oyston *et al.*, 2000), yet resides in phagosomes that rapidly acquire lysosomal markers

(Grabenstein *et al.*, 2006). *Legionella pneumophila* avoids phagosome-lysosome fusion (Horwitz, 1983b), and replicates within an unusual type of phagosome studded by ribosomes, that forms as the vacuole fuses with vesicles from the endoplasmic reticulum (Horwitz, 1983a). Inhibition of protein production by *Legionella pneumophila* does not affect its ability to inhibit phagosome-lysosome fusion, nor alter its ability to form the ribosome-studded replicative vacuoles (Horwitz, 1983a). *Listeria monocytogenes* escapes the phagosome by dissolution of the phagosome membrane, where it utilises the host cell actin to form a cytoskeleton (Tilney and Portnoy, 1989). Furthermore, it makes use of actin-dependent motility to direct intracellular trafficking into other host macrophages without an extracellular phase (Tilney and Portnoy, 1989).

There is somewhat conflicting evidence to suggest that mycobacteria can either prevent phagosome maturation, or escape from the phagosome into the cytosol. *Mycobacterium tuberculosis* has been shown to subvert phagosome-lysosome fusion, however only when the pathogen is viable (Armstrong and Hart, 1971), and demonstrates inhibited growth in acidified phagosomes (Gomes *et al.*, 1999). In contrast, other studies have demonstrated escape from the phagosome into the cytosol by both *Mycobacterium tuberculosis* and *Mycobacterium leprae* (van der Wel *et al.*, 2007). *Mycobacterium avium* has been shown to reside within non-acidic vesicles within the host macrophage (Crowle *et al.*, 1991), however in contrast to *M. tuberculosis*, it shows unimpaired growth in acidic conditions (Gomes *et al.*, 1999). The intracellular survival of *Mycobacterium lepraemurium* is comparable with that of *C. burnetii*; unlike other mycobacteria, it thrives in the acidic environment of the phagolysosome (Hart *et al.*, 1972).

C. burnetii was originally classified as a *Rickettsia*, and while there are some differences between the *Coxiella* genus and *Rickettsia* genus, these bacteria are similar in many respects. Both are able to infect ticks and to transmit themselves to vertebrate hosts via tick bite (Parola and Raoult, 2001). In the human host, they differ in their host cell trophism and survival mechniasms. Most *Rickettsiae* infect and replicate primarily within the cytosol of epithelial cells, and to a lesser extent hepatocytes and macrophages, while *C. burnetii* is distinctly monocyte/macrophage-tropic. Unlike *C. burnetii*, there is evidence that *Rickettsia* escape the phagosome of the host macrophage by expression of enzymes which dissolve the phagosome membrane (Whitworth *et al.*, 2005). Macrophages, in the absence of activation (via addition of cytokines), seem unable to clear *Rickettsia mooseri*, even with the assistance of bacteriostatic agents (Gambrill and Wisseman, 1973), while *C. burnetii* infection is controlled in macrophages from immunocompetent hosts (Zamboni, 2004). However activated hepatocytes, monocytes, and in some cases endothelial cells, are able to kill *Rickettsia* via one or more of the following mechanisms: nitric oxide production, hydrogen peroxide production, or by limiting tryptophan availability to the *Rickettsiae* via indoleamine-2,3-dioxygenase-mediated degradation (Feng and Walker, 2000).

Bacteria commonly have type IV secretion systems for transfer of macromolecules (for example proteins, single-stranded DNA) across kingdom barriers, to other bacteria or into the environment, however, *Coxiella burnetii* is one of only two known organisms which contain a type IVB secretion system. This system involves the *icm* (intracellular multiplication) and *dot* (defective organelle trafficking) genes. Considerable homology and

partial, but not complete, functional complementation is observed between these systems in *C. burnetii* and *Legionella pneumophila* (Zamboni *et al.*, 2003).

In *L. pneumophila*, the *icm* and/or *dot* genes are important for phagocytosis of the organism, creation and insertion of a pore in the host cell membrane resulting in high multiplicity of infection and cytotoxicity, thinning of the phagosome membrane to enable attachment with host vesicles, inhibition of phagosome-lysosome fusion, host cell apoptosis and exit of the organism from the phagosome (reviewed in Segal *et al.*, 2005). The role of these genes in *C. burnetii* is yet to be elucidated, however it seems likely that modulation of the host cell machinery, such as for production of the cholesterol needed for PV formation, or altering the trafficking of vesicles in the autophagic system, may be mediated by effector molecules secreted by the type IV secretion system (Voth and Heinzen, 2007).

2. Manifestations of *C. burnetii* infection

2.1 Coxiellosis

Coxiellosis is the name given to *C. burnetii* infection in animal hosts. The organism has a widespread reservoir in wild and domestic animals. Seropositivity has been demonstrated in a variety of wild animals including deer, moose, coyote, raccoon, opossum, badger, feral pig, fox, jackrabbit, hare, skunk, rat, shrew, bandicoot, parrot, crow, pidgeon, bat and python (Enright *et al.*, 1971; Randhawa *et al.*, 1977; Yadav *et al.*, 1979; Yadav and Sethi, 1980; Hubalek *et al.*, 1993; Marrie *et al.*, 1993; Chomel *et al.*, 1994). *C. burnetii* has also been identified in a variety of domestic animals including sheep, cattle, goats, horses, cats, dogs, and poultry (George and Marrie, 1987; Daoust and Perry, 1989; Bloch and Diallo, 1991; Lang *et al.*, 1991; Muramatsu *et al.*, 2006; Mazyad and Hafez, 2007; Torina *et al.*, 2007). The large and diverse natural reservoir for *C. burnetii* undoubtedly contributes to the worldwide prevalence of Q fever in humans.

Detection of *C. burnetii* in domestic reservoirs is usually extrapolated from serological evidence of infection in the animals. Seroprevalence can be high in domestic animals, with rates reported to be in the range of 15-25% for domestic herds of cattle, sheep and buffalo in India (Yadav and Sethi, 1979), approximately 15% of cattle in Nigerian herds (Bloch and Diallo, 1991), and approximately 13-22% of sheep, goats and camels surveyed in Egypt (Mazyad and Hafez, 2007). In other locations seropositivity has been reported to be low. As few as 1.5% of sheep tested in Canada were seropositive for *C. burnetii* (Lang *et al.*, 1991), and a survey of animals in Turkey found seroprevalence of 0.5% and 1% for cattle

and sheep respectively (Cetinkaya *et al.*, 2000). It is unknown why some animals within a contaminated herd can remain uninfected despite a high risk of exposure.

Coxiellosis is generally an asymptomatic condition, and thus attracts little attention from farmers except for its association with spontaneous abortion and stillbirth. Detection of *C. burnetii* infection via serological testing of the pregnant animal, or by detection of organism by PCR in the placenta or foetus, has been strongly associated with reproductive disorders such as infertility, mastitis and emdometritis in cattle (van Moll *et al.*, 1993; To *et al.*, 1998b), with abortion and stillbirths in goats (Berri *et al.*, 2007), and with abortion in sheep (van Moll *et al.*, 1993; Masala *et al.*, 2004). Some have suggested that occurrence of *C. burnetii* in aborted sheep and goat foetuses is actually lower than might be expected, based on seroprevalence analysis of the herd (Masala *et al.*, 2004), suggesting that *C. burnetii* infection may not necessarily trigger abortion, but increase the relative risk of reproductive disorders in animals.

The spread of *C. burnetii* in animal herds is mediated by exposure to the contaminated secretions of infected animals. Recovery of *C. burnetii* from dust-laden air was found to be possible in the yards and barns of farms known to house animals which possessed *C. burnetii*-specific antibodies, some of which were shedding *C. burnetii* in their milk, and in which human Q fever cases had been documented in employees. These organisms were capable of causing disease when injected into guinea pig hosts (De *et al.*, 1950), indicating that airborne *C. burnetii* remains infectious despite exposure to environmental hazards such as ultraviolet light and dessication. It is possible that transmission of *C. burnetii* in animals

is also mediated by arthropod vectors. Experimental infection of guinea pigs has been confirmed to be possible via the bite of an infected tick (eg *Dermacentor andersoni*), but not via house fly or mosquito (Philip, 1948b).

2.2 Q fever epidemiology

Q fever is the disease manifestation of *C. burnetii* infection in humans, and is present worldwide. In some countries, incidence rates are low, and it is only during outbreaks that Q fever gains attention. Furthermore, Q fever is a notifiable disease in some countries but not all, so accurate data regarding incidence rates worldwide are not available. Countries with high notification rates or notable outbreaks of Q fever include the United Kingdom (UK), France, Spain, Canada, USA, Slovakia and Australia, however Q fever has been identified in at least 59 countries (Norlander, 2000). Outbreaks are commonly able to be linked to contact with contaminated livestock and weather conditions, including seasonal winds (Embil *et al.*, 1990; Smith *et al.*, 1993; Manfredi Selvaggi *et al.*, 1996; Hawker *et al.*, 1998; Carrieri *et al.*, 2002; van Woerden *et al.*, 2004). Q fever is believed to be absent in New Zealand with widespread serological testing of livestock demonstrating no positive cases (Hilbink *et al.*, 1993).

In Australia, Q fever is a notifiable disease and therefore reported rates accurately represent diagnoses. In 2005, there were 355 reported Q fever cases (approximate rate of 1.8 per 100,000 population), however this is likely an under-representation of the total cases as Q fever typically presents with non-specific influenza-like symptoms, which combined with a

prevalent lack of awareness of Q fever among general practitioners, is likely to lead to common misdiagnosis. It has also been proposed that resistance to seeking treatment among farmers who become unwell may further contribute to under-estimation of incidence rates (Owen *et al.*, 2007). The annual incidence of Q fever in Australia has been declining with the implementation of a national vaccination strategy targeting workers of the meat and livestock industries who are most at risk of exposure (Owen *et al.*, 2007), although it remains to be seen whether recent discontinuation of the program will see an increase in Q fever notifications.

Cases of Q fever are usually the result of contamination from infected livestock via inhalation, with low infective doses (<10 organisms) reported (Tigertt *et al.*, 1961; Moos and Hackstadt, 1987). Excretion of infectious particles in animal fluids results in formation of infectious dust-like particles (Stoker and Marmion, 1955), which when airborne can cause infection in humans via inhalation, in the absence of direct contact with infected animals (Manfredi Selvaggi *et al.*, 1996). Accordingly, wind strength and direction has been demonstrated to contribute to infection rates in Q fever epidemics, particularly when associated with hot and dry conditions (Tissot-Dupont *et al.*, 2004). Infection rates are particularly high among those who work in the meat processing and agricultural industries, presumably due to the high risk of occupational exposure (Garner *et al.*, 1997).

Other less common transmission methods have been identified. Consumption of raw milk products has been linked to transmission of *C. burnetii*, with viable organisms being isolated from the raw milk of cattle (Ho *et al.*, 1995). Q fever has also been proposed to be

transmitted sexually from husband to wife, however such reports are extremely rare and other transmission methods were not conclusively ruled out (Kruszewska *et al.*, 1996; Milazzo *et al.*, 2001). Nevertheless, in these cases, other family members remained uninfected, no other possible exposure mechanisms were identified, and *C. burnetii* was recovered in the semen of the infected men. In general, human-to-human transmission is believed to be extremely rare.

Cases of Q fever occur predominantly in men (Domingo *et al.*, 1999; Raoult *et al.*, 2000; Alarcon *et al.*, 2003). The male predominance in infection rates is often attributed to gender bias in occupations with high risk of *C. burnetii* exposure in the meat and agricultural industries, rather than significant differences in susceptibility between men and women. However, some propose that men are more likely to suffer symptomatic illness than women in areas where seropositivity rates between men and women are comparable and it has been suggested that oestrogen may be important in control of *C. burnetii* infection (Leone *et al.*, 2004).

2.3 Acute Q fever

Human cases of *C. burnetii* infection demonstrate a wide spectrum of illness manifestations. Asymptomatic infection appears to be common, although infection rates are hard to assess in individuals who do not suffer clinical disease and therefore do not seek medical care. Cross-sectional seroprevalence studies have indicated the presence of Q fever specific antibodies in a significant minority of individuals, most of whom did not suffer apparent

clinical illness (Skerget *et al.*, 2003; Reid and Malone, 2004). Furthermore, asymptomatic infection has been demonstrated to occur in at risk individuals during a Q fever outbreak in Germany, where repeated serological testing demonstrated temporal changes in antibody titres that were consistent with acute infection in the absence of clinical disease (Skerget *et al.*, 2003). Similarly, longitudinal assessment of seronegative individuals in Australian abattoirs indicated a seroconversion rate of approximately 24% in 15 months amongst individuals who did not suffer clinical illness (Shapiro *et al.*, 1990). Asymptomatic seroconversion has therefore been estimated to occur in approximately 60% of exposed individuals (Raoult *et al.*, 2005).

When symptomatic, acute Q fever is often a severe debilitating disease with headache, myalgia, sweats and fever (Raoult *et al.*, 2000). The incubation period between infection and presentation of symptoms is approximately three weeks (Porten *et al.*, 2006). While symptom prevalence and severity varies widely between individuals, the vast majority of subjects with symptomatic illness suffer from fevers, sweats and weight loss, with severe retro-orbital headache, often considered a classical symptom of Q fever, being present in a high proportion of patients (Smith *et al.*, 1993). The non-specific nature of the acute illness may make it difficult to differentiate Q fever from several other febrile diseases that manifest with influenza-like symptoms unless serological testing is conducted (Suttinont *et al.*, 2006). However, early diagnosis may be critical in managing the complications that may arise from acute Q fever (Lin *et al.*, 2008).

The illness also commonly presents with pneumonia or hepatitis (Hofmann and Heaton, 1982; Caron *et al.*, 1998). Predisposition to pneumonia and/or hepatitis varies geographically: in France (Tissot Dupont *et al.*, 1992; Raoult *et al.*, 2000), the southern regions of Spain (Alarcon *et al.*, 2003), Israel (Ergas *et al.*, 2006), Taiwan (Chang *et al.*, 2004), and the Canary Islands (Pascual Velasco *et al.*, 1996) hepatitis is more common; while in Ireland (Connolly *et al.*, 1990), England and Wales (Pebody *et al.*, 1996), and the Basque region of Spain (Montes *et al.*, 2006) pneumonia has been reported to be a more frequent manifestation. The occurrence of both hepatitis and pneumonia was reported as the most common manifestation in a Croatian study (Luksic *et al.*, 2006). Other manifestations of acute Q fever such as upper respiratory tract symptoms, meningitis, myocarditis, and kidney disease, including haemolytic-uremic syndrome and glomerulonephritis, have been reported (Korman *et al.*, 1998; Kofteridis *et al.*, 2004; Maltezou *et al.*, 2004; Pappas *et al.*, 2004; Vogiatzis *et al.*, 2008).

The febrile phase of acute Q fever generally lasts 1-4 weeks, however cases of prolonged febrile illness do occur (Alarcon *et al.*, 2003). Antibiotic therapy, commonly with doxycycline, is believed to be effective if administered within two weeks of symptom onset, and may reduce the duration of the febrile phase (Domingo *et al.*, 1999; Alarcon *et al.*, 2003), however the use of antibiotic therapy to treat acute Q fever is unlikely to offer substantive health improvements given that the illness manifestations are believed to predominantly result from immunopathology.

Acute Q fever is reported to lead to hospitalization in 2-5% of cases (Raoult *et al.*, 2005), with the risk of infection requiring hospitalisation being increased in men (Dupont *et al.*, 1992; Luksic *et al.*, 2006). Approximately 1% of hospitalised acute Q fever cases succumb to their illness (Raoult *et al.*, 2005). Mortality may occur even when appropriate treatment is given (Ralph *et al.*, 2007).

2.4 Chronic Q fever

In a subset of patients, acute Q fever may develop into a chronic, localised infection. Even patients with asymptomatic acute infection may develop chronic complications. Chronic, localised Q fever most commonly manifests as endocarditis (Turck *et al.*, 1976), but may also affect the kidneys, joints, liver and bone marrow (Brouqui *et al.*, 1993; Karakousis *et al.*, 2006), causing localised granulomatous inflammatory lesions. Chronic, localised Q fever may evolve over months, years or even decades after acute infection (Wilson *et al.*, 1976), with case reports suggesting the slow evolution of the disease, combined with an asymptomatic phase, may account for the lag between acute infection and diagnosis of chronic Q fever (Healy *et al.*, 2006).

In pregnant women, placentitis is also a possible manifestation of chronic Q fever (Bental *et al.*, 1995). *C. burnetii* has been reported to be involved in cases of premature birth, abortion and stillbirth, particularly when the woman is infected in the first trimester of pregnancy (Raoult *et al.*, 2002). Treatment with co-trimoxazole until delivery appears to reduce the risk of abortion (Raoult *et al.*, 2002).

In cases of chronic Q fever endocarditis, *C. burnetii* is often able to be isolated from both the peripheral blood, and infected heart valves, or is detectable by immunohistochemistry or PCR of the tissue. Histologically, sites of chronic valvular infection are characterised by fibrosis and calcification, with some chronic inflammation and neo-vascularisation (Lepidi *et al.*, 2003). As many as 30-50% of patients with pre-existing valve lesions (for example, congenital bicuspid aortic valves, or rheumatic valvular lesions) develop endocarditis after acute Q fever (Fenollar *et al.*, 2001), and almost all cases of endocarditis and chronic vascular infection occur in individuals with such pre-existing lesions. Neutrophils and *C. burnetii*-infected macrophages are recruited to these pre-existing sites of abnormality, driving ongoing inflammation and immune-mediated valvular injury (Brouqui *et al.*, 1994). Treatment for chronic, localised Q fever typically requires long courses of combination antibiotic therapy, and sometimes treatment is maintained for life to prevent recurrences (Maurin and Raoult, 1999). Detection of *C. burnetii* in the heart valves of patients with endocarditis decreases significantly only after a year or more of combination antibiotic therapy (Lepidi *et al.*, 2003).

C. burnetii has been shown to be a relatively common cause of blood culture-negative infective endocarditis (Houpikian and Raoult, 2005). Diagnosis of Q fever endocarditis usually follows exclusion of other pathogens that are easily identifiable by standard blood cultures, in which *C. burnetii* is typically not detected due to fastidious culture requirements and slow growth rate. In general, the prognosis for Q fever endocarditis is guarded as it requires prolonged antibiotic therapy and often leads to valve replacement

(Turck *et al.*, 1976). With growing awareness of Q fever as a cause of endocarditis as well as faster diagnosis and therefore earlier implementation of appropriate treatment, the mortality associated with chronic, localised Q fever has improved from approximately 70% in the 1970s to 3% in the 2000s (Ferguson *et al.*, 1962; Turck *et al.*, 1976; Brouqui *et al.*, 1993; Raoult *et al.*, 2000; Houpikian and Raoult, 2005).

2.5 Post Q fever fatigue syndrome

A significant proportion of patients with acute Q fever remain symptomatic for long periods of time, reporting disabling fatigue for months or years after onset of the acute illness (Marmion *et al.*, 1990; Ayres *et al.*, 1998). This illness has been termed post Q fever fatigue syndrome (QFS) (Ayres *et al.*, 1998). Patients with prior acute Q fever illness have a significantly higher rate of self-reported fatigue (67%) than control subjects (35%) with no prior exposure to *C. burnetii*, even ten years after their initial illness (Wildman *et al.*, 2002). After medical and psychiatric evaluation a diagnosis of CFS was made in 19% of cases and 4% of controls (Wildman *et al.*, 2002).

A diagnosis of CFS requires clinically unexplained fatigue for six or more continuous months, that is not the result of ongoing exertion, is not alleviated by rest, and results in a significant impact on the daily activities of the individual. In addition, at least four of the following symptoms must be present for six or more continuous months: symptoms that are unusually severe following exercise, un-refreshing sleep, impairment of memory or concentration, headache, muscle pain, joint pain, sore throat and swollen lymph nodes.

Empirical criteria, relating to severity of these factors, have recently been applied to the definition of CFS (Reeves *et al.*, 2005). Studies of the pathophysiology ofCFS have been hampered by conflicting results, which have been proposed to arise due to heterogeneity within the subjects that are diagnosed with CFS. To overcome this limitation, prospective studies of post-infective fatigue syndrome (PIFS) have been established as a paradigm for the pathophysiological research into CFS (White *et al.*, 1995; Hickie *et al.*, 2006).

Q fever is one infection that appears to act as a trigger for CFS (or more specifically, PIFS) in a significant proportion of subjects who are infected. Several viral infection have also been proposed to trigger CFS or to play a role in perpetuation of the illness, including Epstein-Barr virus (EBV), Ross River virus (RRV), cytomegalovirus (CMV), and human herpesvirus-6 (HHV-6) (Holmes *et al.*, 1987; Patnaik *et al.*, 1995; Hickie *et al.*, 2006). It is unknown why some subjects with acute infectious illnesses such as Q fever go on to develop chronic symptoms of fatigue. Given that cases of PIFS have been recognised following a range of different infections (Hickie *et al.*, 2006), it is likely that such fatigue syndromes are not pathogen specific, but relate primarily to host response factors.

In the Dubbo Infection Outcomes Study, which is a prospective cohort study of individuals followed from the onset of acute Q fever, Epstein-Barr virus or Ross River virus infections, approximately 10% of subjects have been documented to experience a persistent and disabling illness (without a symptom-free interval) which meets the criteria of CFS when applied after medical and psychiatric assessments as well as laboratory investigations to exclude alternative diagnoses (Hickie *et al.*, 2006). Demographic and psychological factors

did not predict PIFS, whereas the severity of the acute illness was predictive (Hickie *et al.*, 2006). The acute phase symptoms of such infections are a manifestation of the acute sickness response (ASR), which is a stereotyped cluster of symptoms that is initiated by the host immune system in response to a variety of pathogens. In this context it is believed that the immune and central nervous systems are in communication; the immune system initiates a response via cytokine production that drives processes in the brain to mediate the illness manifestations of acute infection (Watkins and Maier, 1999; Vollmer-Conna, 2001). Consistent with this notion is the finding in DIOS that those individuals with high levels of pro-inflammatory cytokine production suffered more severe symptomatic acute illness (Vollmer-Conna *et al.*, 2004).

Some studies suggest that persistence of the infecting organism may contribute to the ongoing presence of fatigue. It has been reported that peripheral blood mononuclear cell (PBMC) samples from subjects with QFS produce aberrant cytokine responses to *C. burnetii* antigens, suggesting that ongoing presence of *C. burnetii* or its antigens may drive a cytokine response that plays a role in propagating symptoms of fatigue (Penttila *et al.*, 1998). Detection of *C. burnetii* genomes in peripheral blood samples taken 5-12 years after initial infection was more common in subjects with QFS than in subjects with uncomplicated acute Q fever (Marmion *et al.*, 2005), although it is noteworthy that detection of *C. burnetii* within bone marrow aspirates was common, regardless of clinical outcome. Some patients with post Q fever fatigue may respond to antibiotic therapy (Arashima *et al.*, 2004; Ledina *et al.*, 2007), which is consistent with the notion that persistence of *C. burnetii* drives the symptoms of PIFS, although these reports should be

interpreted with considerable caution as they were derived from uncontrolled case series only (rather than randomised, double blind, placebo-controlled trials).

2.6 Q fever diagnosis

The diagnosis of acute and chronic, localised Q fever is by generally made by serological testing. Commonly, an IgM test conducted on a single serum sample is utilised for a diagnosis of acute Q fever. However, high false positive rates for Q fever IgM assays mean that repeat testing is often necessary to reliably document acute infection. Typically, IgG and IgM directed against phase II organisms appear early in infection, and these antibodies are maintained for several months following acute Q fever (Guigno *et al.*, 1992; Dupont *et al.*, 1994). By contrast, high titres of IgA and IgG directed against phase I organisms are considered diagnostic of chronic, localised Q fever (Soriano *et al.*, 1993), although some dispute the relevance of phase I IgA measurement in the determination of chronic Q fever (Dupont *et al.*, 1994). Combinations of serological measures have also been reported to better characterise the disease state of the individual. For example, individuals in one study who had both high IgA directed against phase I organisms and high complement fixation antibody titres were also all patients with endocarditis (Soriano *et al.*, 1993).

A minority of subjects have atypical serological responses. For instance, in a small proportion of acute Q fever subjects, IgA directed against phase II organisms was present as the only significant antibody response (Fournier and Raoult, 1999). Serology is additionally complicated by interference of high titre IgG antibodies causing falsely high

IgM and/or IgA determinations (Soriano *et al.*, 1993). On the other hand, one study reported that 45 of 113 sera from Q fever seronegative individuals exerted an inhibitory effect on Q fever antigen-antibody detection in complement fixation assays, suggesting that components of sera may interfere with serological testing and result in false negative determinations (Schmidt and Harding, 1956). Reported cross-reactivity between *Coxiella burnetii* and *Legionella*, *Bartonella*, *Rickettsia* and *Ehrlichia* species further complicates diagnosis by serology (Musso and Raoult, 1997; Graham *et al.*, 2000). Definitive diagnosis of acute Q fever therefore requires testing of acute and convalescent sera to detect changes in antibody production indicative of seroconversion (Dupont *et al.*, 1994), or a fourfold change in IgG and/or IgA antibody titres, which are indicative of acute Q fever (Devine *et al.*, 1997).

Attempts to culture the organism from whole blood may confirm the presence of ongoing infection, however *C. burnetii* is a fastidious and slow growing pathogen so this is rarely the primary means of diagnosis. Detection of *C. burnetii* genomes by polymerase chain reaction (PCR) can similarly indicate possible infection, and provides a supplementary means of diagnosis, although this assay has not yet become routine clinical practice, and with sensitive techniques persistent coxiella DNA may prove to be universal (or at least prevalent) amongst those with previous Q fever (Marmion *et al.*, 2005). A number of conserved gene targets have been utilised for detection of *C. burnetii* in clinical samples, including amplification of regions of the *com1* gene (Zhang *et al.*, 1998), *icd* (isocitrate dehydrogenase) gene (Klee *et al.*, 2006), or regions of a repetitive, transposon-like insertion sequence (IS1111) (Vaidya *et al.*, 2008) that provide the potential for greater detection

sensitivity due to the occurrence of these sequences at multiple copy number within each *C. burnetii* genome (Klee *et al.*, 2006). While PCR methods are sensitive, detection of *C. burnetii* by PCR does not provide information about whether the infection is acute or chronic, or whether any detected genomes are derived from viable organisms. Some studies even suggest that *C. burnetii* genomes may remain detectable for decades in patients with no clinical signs of illness (Marmion *et al.*, 2005). Due to limitations associated with culture and PCR testing, serological screening remains the predominant diagnostic tool when Q fever is suspected.

2.7 Vaccination

Early attempts at vaccine design were plagued by variable efficacy of preparations, even when derived from the same strain of *C. burnetii*. Antigenic phase variation was then found to be of critical importance in Q fever vaccine design. Phase I specific antibody responses were somewhat protective against *C. burnetii* infection in mice and guinea pigs (Abinanti and Marmion, 1957). Furthermore, phase I vaccination preparations were a hundred fold more effective at preventing infection in guinea pigs than comparable doses of phase II vaccine (Ormsbee *et al.*, 1964). It seems likely that early vaccine efforts resulted in variable efficacy due to variations in the proportion of phase I and phase II organisms that were present in the mixed phase preparations. Subsequent vaccines made use of early passage organisms to ensure the preparation contained only phase I cells.

Early studies of a Henzerling phase I vaccine involved direct *C. burnetii* challenge of vaccinated individuals and unvaccinated control subjects. High vaccine efficacy was evident: five of six unvaccinated control subjects developed clinical illness while only two of 32 vaccinated subjects became unwell (Ormsbee and Marmion, 1990). This vaccine likely offered greater protection against natural infection than the study suggests, given that the *C. burnetii* challenge induced clinical illness in such a high proportion of antigen naïve individuals while natural environmental exposure often results in asymptomatic seroconversion (Shapiro *et al.*, 1990). Vaccine efficacy was dose dependent, with higher doses of vaccine offering protection from illness in a greater proportion of subjects (Ormsbee and Marmion, 1990). Other attempts to use a live attenuated vaccine focused on the use of late passage organisms that had undergone phase variation in culture to become non-infectious phase II cells, including the M-44 line of the Grita strain and the EP88 line of the Nine Mile strain (Genig, 1965; Genig *et al.*, 1965; Johnson *et al.*, 1976). These phase II vaccines induced only phase II antibody responses, and were poorly protective except at high doses.

Australia is the only country worldwide with a commercially available, licensed Q fever vaccine, QVax^{TM}, which is produced by the Commonwealth Serum Laboratories (CSL Limited). QVax^{TM} was trialled in Australian abattoir workers (Marmion *et al.*, 1990) prior to becoming commercially available in 1989 (Garner *et al.*, 1997) – a single subcutaneous injection of whole, inactivated Henzerling strain phase I vaccine is given to subjects who have no evidence of prior exposure to Q fever.

Side effects such as local granulomatous reactions are common in individuals who have pre-existing infection, or vaccine-induced cellular immunity. Vaccination, while providing virtually absolute protection, occurs with seroconversion and skin test positive rates of only about 60% (Marmion *et al.*, 1984; Marmion *et al.*, 1990; Shapiro *et al.*, 1990). Serological screening is similarly insufficient for detecting prior *C. burnetii* infection whereas specific cellular responses may remain for decades after initial exposure (Jerrells *et al.*, 1975). In addition, seroreversion commonly occurs (Marrie and Pollak, 1995). Ideally, antigen-specific cellular proliferation assays should be conducted to confirm an individual is antigen-naïve and that vaccination will be safe (Izzo *et al.*, 1991), however such assays are typically time consuming and relatively expensive and may lack specificity. Therefore, delayed type hypersensitivity (DTH) skin testing with a diluted preparation of the vaccine is recommended prior to vaccination in conjunction with serological screening. The recommended timing of assessing DTH skin test reactivity is 7-9 days after application of the antigen; although the majority of pre-exposed individuals will show a positive skin test result at 2 days, better reliability is provided via delayed skin test reactivity (Luoto *et al.*, 1963).

The efficacy of the vaccine has been reported to be in the range of 83-100% (Chiu and Durrheim, 2007). Studies in Australia depended on large numbers of subjects working in high risk occupations, where protection was demonstrated by a higher Q fever incidence in the unvaccinated control group. QVax^{TM} therefore appears to offer long lasting, almost absolute protection from disease, provided it is given prior to exposure to *C. burnetii* (Marmion *et al.*, 1990).

Vaccination causes seroconversion in approximately 80% of individuals, however only 60% of vaccinees demonstrated detectable antibody two years after vaccination. In contrast, lymphoproliferative responses remained in 85-95% of vaccinees for five years or more, suggesting that cellular immune responses may play a critical role in immunity to Q fever (Marmion *et al.*, 1990). Despite variations in LPS antigenicity being reported for different strains of *C. burnetii* (Hackstadt *et al.*, 1985), the proliferative responses of peripheral blood mononuclear cells from QVax™ recipients were demonstrated to be of equal magnitude to responses to antigens from different strains (including Priscilla, Nine Mile and Henzerling) (Izzo *et al.*, 1991), suggesting that QVax™ offers cross-reactive immunity against a range of *C. burnetii* strains.

2.8 Biological warfare

C. burnetii has recently become of particular interest as a potential agent for biological warfare (Kagawa *et al.*, 2003; Madariaga *et al.*, 2003), and is classified by the Centers for Disease Control and Prevention (CDC) as a risk category B bioterrorism agent (Pappas *et al.*, 2006). Although the disease manifestations of illness are rarely fatal, acute illness is debilitating and may be prolonged. Furthermore, the bacterium may be produced in large quantities and has been documented to survive dessicated for decades, presumably due to production of the spore-like form (Giroud *et al.*, 1964). These features mean that the pathogen would not only be stable during transportation and release, but would also remain viable in the environment for decades.

There have been no documented cases of bioterrorism using *C. burnetii*, although there were frequent outbreaks of Q fever among soldiers during World War II, presumably of natural origin (Madariaga *et al.*, 2003). While the effects of *C. burnetii* may be less dramatic than bioterrorist agents with the potential to cause massive fatalities, such as anthrax, ebola or smallpox, the high infectivity of *C. burnetii* would contribute significantly to its impact. The hypothetical release of 50kg of *C. burnetii* along a 2km line, upwind of a population of half a million people, has been estimated to have a downwind hazard range of up to 20km, and the capacity to induce acute illness in 125,000 people, chronic illness in 9,000 people, and approximately 150 deaths (Bellamy and Freedman, 2001).

3. Host-pathogen interaction

3.1 Overview of the immune response to *C. burnetii* infection

Coxiella burnetii is usually transmitted via inhalation of a contaminated aerosol into the lung, where it establishes infection (Burton *et al.*, 1971; Burton *et al.*, 1978). Phagocytes of the innate immune system, including macrophages, initiate the host immune response by taking up the pathogen. The pathogenesis of *C. burnetii* is primarily based on its ability to survive and replicate within the usually bactericidal/bacteriostatic environment of the macrophage phagolysosome (Voth and Heinzen, 2007). Trafficking of infected macrophages from the site of infection to the lymph nodes may then facilitate dissemination of the pathogen to other tissues and organs in the host.

The importance of the immune response in controlling *C. burnetii* is especially highlighted by rare reports of reactivation of Q fever illness in subjects with immunosuppression, such as during pregnancy (Heard *et al.*, 1985; Kanfer *et al.*, 1988; Raoult *et al.*, 1992; Raoult *et al.*, 2002). The host response to any infection requires the coordinated involvement of innate and adaptive immune components. Effector cells of the innate immune system, including dendritic cells, macrophages, monocytes and neutrophils, directly phagocytose and kill *C. burnetii*, recruit inflammatory cells via the secretion of chemokines, and present antigen to drive adaptive immune responses. Activated B cells secrete pathogen-specific antibodies which opsonise bacteria and target them for phagocytosis. However, the relative importance of this humoral response in protective immunity against *C. burnetii* is unclear.

By contrast, cellular immunity is critical in clearing *C. burnetii*-infection, and the generation of memory T cells is a requirement of vaccine-induced protection.

A comparison of the immune response to *C. burnetii* with that seen in response to other types of intracellular pathogens reveals many features shared in common, including a requirement for a robust Th1-type cellular response in protection against infection and/or pathogen clearance (Neild and Roy, 2004; Pamer, 2004; Reece and Kaufmann, 2008). In particular, macrophage-tropic pathogens (including species of the *Mycobacterium*, *Legionella*, *Listeria* and *Leishmania* genera) tend to utilise similar mechanisms for evading immune microbicidal effects and require broadly comparable immune responses for pathogen clearance. The role of each component of the immune response in fighting *C. burnetii* infection, and the mechanisms by which *C. burnetii* evades host defences, are discussed below in more detail, and in comparison with other intracellular pathogens.

3.2 Phagocytic cells: macrophages and monocytes

Monocytes and macrophages are phagocytic cells of the innate immune system. Circulating monocytes provide peripheral tissues with the precursor cell for both macrophages and dendritic cells, and in the absence of infection monocytes play a key role in maintaining macrophage homeostasis. Monocytes may also traffic rapidly to sites of infection or inflammation, to provide a source of renewal of tissue macrophages, or to directly participate in anti-microbial immune responses (reviewed by (Serbina *et al.*, 2008)). Monocytes and macrophages share many of the same immune functions, including

phagocytosis and cytokine production, and thus, their roles in controlling infection are often considered together.

When *C. burnetii* exposure occurs via inhalation, the first immune defense faced by the bacterium is likely to be the alveolar macrophage. Typically, bacterial pathogens interact with a family of pathogen recognition molecules, the Toll-like receptors, on the surface of macrophages, triggering chemokine and pro-inflammatory cytokine induction, causing activation of macrophage microbicidal competence and recruitment of other immune effector cells including other monocyte-derived macrophages, dendritic cells and neutrophils (Takeda and Akira, 2001; Thoma-Uszynski *et al.*, 2001; Takeda and Akira, 2004).

As described in section 1.4, the pathogenesis of Q fever is heavily dependent on the ability of *C. burnetii* to infect and replicate within the phagolysosome of host phagocytic cells (Maurin *et al.*, 1992). This section will describe the host-pathogen interaction from the perspective of the host. A number of pathogens are able to replicate within phagocytic cells, but the way in which they are able to survive within the host cell varies greatly. *Listeria monocytogenes* escapes the phagosome into the cytosol where it is able to rapidly replicate (Tilney and Portnoy, 1989), but in some cases may also replicate within non-acidic, non-degradative vacuoles derived from the autophagy system (Birmingham *et al.*, 2008). *Mycobacterium tuberculosis* resides in phagosomes, where a drop in pH triggers bacterial gene expression to stall phagosome maturation (Rohde *et al.*, 2007). *C. burnetii* adopts a strategy shared by *Legionella pneumophila* (Cianciotto, 2001) in that it does not appear to

prevent phagosome maturation, nor does it escape into the cytosol, however it has developed a variety of strategies to overcome the usually bactericidal environment of the phagolysosome and establish persistent infection in host monocytes and macrophages (Ghigo *et al.*, 2002; Zamboni, 2004). It has been proposed that evolution to chronic, localised Q fever infection is associated with macrophage polarisation to a non-bactericidal phenotype (Benoit *et al.*, 2008c), highlighting the importance of the host macrophage (or its precursor, the monocyte) in controlling and clearing *C. burnetii* infection.

Uptake of *C. burnetii* by host macrophages occurs primarily via phagocytosis (Tujulin *et al.*, 1998). The process is passive, and killed bacteria are phagocytosed as efficiently as live organisms (Tujulin *et al.*, 1998). This is interesting, given that the gene products of the icm/dot loci that exist in both *Legionella pneumophila* and *C. burnetii*, have been demonstrated to enhance phagocytosis of *L. pneumophila* (Hilbi *et al.*, 2001), while heat-killed *C. burnetii*, which have no capacity to express the icm/dot genes, do not show reduced rates of uptake into macrophages. Phase variation affects phagocytosis efficiency; phase I organisms are more resistant to phagocytosis than phase II organisms (Kishimoto *et al.*, 1977).

Phase I organisms are phagocytosed without involvement of the host cell complement receptor 3 (CR3) (Capo *et al.*, 1999). CR3 mediates type II phagocytosis of complement-opsonised pathogens, while type I phagocytosis of antibody-coated targets occurs via FcR engagement (Allen and Aderem, 1996; Caron and Hall, 1998). Other macrophage tropic bacteria may be phagocytosed via CR3 engagement, including *Mycobacterium tuberculosis*

and *M. leprae* (Schlesinger *et al.*, 1990; Schlesinger and Horwitz, 1990). Phagocytosis via CR3 is associated with poor induction of microbicidal activity in macrophages, and engagement via CR3 does not trigger the release of reactive oxygen species (Wright and Silverstein, 1983), although in the case of *M. tuberculosis*, the specific involvement of CR3 does not affect bacterial survival or replication (Zimmerli *et al.*, 1996). Phagocytosis of phase II *C. burnetii* leads to rapid bacterial clearance *in vitro* (Capo *et al.*, 1999), however it is unclear whether CR3 involvement contributes to the rapid elimination of these avirulent bacteria. The inability of host macrophages to clear phase I organisms is presumably dependent on both the ability of *C. burnetii* to withstand host defenses, and on its ability to modulate the activity of infected macrophages.

The host macrophage phagolysosome utilises acidic pH and hydrolytic enzymes to destroy phagocytosed pathogens. Some pathogens, such as *Mycobacterium tuberculosis*, survive within phagosomes by blocking their maturation, and therefore do not encounter the extremely acidic pH that is present in phagolysosomes and is bactericidal for many species (Huynh and Grinstein, 2007). Phagosome maturation is actively stalled by *M. tuberculosis* and results in inhibition of phagosome fusion with lysosomes. Mechanisms known to effect this stall in maturation include production of the complex glycolipid, trehalose dimycolate, that blocks phagosome maturation (Axelrod *et al.*, 2008), and secretion of a protein kinase that is necessary for preventing phagosome delivery to lysosomes (Walburger *et al.*, 2004). *Legionella pneumophila* inhabits phagosomes that remain completely distinct from the endocytic pathway, possibly because they do not express Rab 5, a protein involved in regulating membrane trafficking of phagosomes and endosomes

(Clemens *et al.*, 2000). *Listeria monocytogenes* escapes the phagosome into the host cell cytosol, where it will not encounter acidic pH (Tilney and Portnoy, 1989).

Unlike these pathogens, *C. burnetii* replicates within the environmentally harsh phagolysosome, a strategy that is quite unique amongst macrophage tropic bacteria. Increased survival of virulent organisms is independent of the acidity of the phagosome (Ghigo *et al.*, 2002) and acidic pH is maintained in the phagolysosome during persistent infection (Maurin *et al.*, 1992). In fact, the organism requires an acidic pH for optimal metabolism (Hackstadt and Williams, 1981). The ability of *C. burnetii* to survive and replicate within the phagolysosome is not due to deficiencies in lysosomal enzyme activity, given evidence that lysosomal hydrolases have similar enzyme activities in infected and uninfected phagolysosomes (Akporiaye *et al.*, 1983). It is unknown why *C. burnetii* is resistant to the activity of lysosomal enzymes. Resistance may be due to the structure of the cell wall being poorly accessible for lysosomal enzymes, or due to the cell wall containing particular proteins and lipids that are not efficiently broken down by lysosomal enzymes.

Phagocytes such as macrophages and neutrophils produce reactive nitrogen and oxygen species to damage the genomic DNA of the phagocytosed pathogen, or activate other microbicidal effector functions. In the case of *Mycobacterium tuberculosis*, production of nitric oxide is associated with phagosome maturation and pathogen clearance (Axelrod *et al.*, 2008). Control of *C. burnetii* infection requires production of reactive oxygen species such as hydrogen peroxide by host macrophage NADPH oxidase activity, and also requires production of reactive nitrogen species such as nitric oxide by macrophage inducible nitric

oxide synthase (iNOS, or nitric oxide synthase 2 (NOS2)) (Brennan *et al.*, 2004). At low levels, nitric oxide acts as a signalling molecule, drawing other immune cells to sites of infection. High levels of nitric oxide are cytotoxic and can inhibit replication of many intracellular pathogens, including *C. burnetii* (Howe *et al.*, 2002). In fact, high nitric oxide levels are bacteriostatic due to inhibition of the large parasitophorous vacuoles necessary for *C. burnetii* replication (Howe *et al.*, 2002), and macrophages that phagocytose apoptotic cells demonstrate increased susceptibility to *C. burnetii* infection due to down regulation of nitric oxide production (Zamboni and Rabinovitch, 2004).

Virulent phase I *C. burnetii* is able to avoid the deleterious effects of nitric oxide by poorly stimulating its production by phagocytes (Zamboni and Rabinovitch, 2003). *C. burnetti* is also able to withstand typical oxygen-dependent defence mechanisms available to mononuclear phagocytes, including production of oxygen radicals by induction of catalase and superoxide dismutase (Akporiaye and Baca, 1983). Effective control of *C. burnetii* infection requires both nitric oxide and hydrogen peroxide production (Brennan *et al.*, 2004), therefore inhibiting their induction or avoiding their effects represent key mechanisms of *C. burnetii*'s virulence. *C. burnetii* also possesses a number of DNA repair mechanisms termed the bacterial "SOS response", including genes involved in base excision, nucleotide excision, methyl mismatch and recombinational repair systems, comparable to those found in *E. coli* and *L. pneumophila* (Mertens *et al.*, 2008). Thus, any damage caused by nitric oxide or hydrogen peroxide can be readily repaired by *C. burnetii*, making it well equipped to withstand typical macrophage bactericidal activity.

Survival of virulent phase I organisms within the phagosome may be mediated by subtle alterations in phagosomal maturation (Ghigo *et al.*, 2002; Zamboni, 2004), and it appears that impairment of macrophage maturation may also be of clinical relevance in cases of chronic Q fever (Ghigo *et al.*, 2004). Consistent with this notion is the finding that *C. burnetii*-derived proteins are essential for the formation of large, replicative vacuoles, suggesting that the infecting pathogen is able to subvert usual phagosome maturation and create an environment in which it is able to replicate (Howe *et al.*, 2003). It is believed that the organism induces a pause in the phagosome maturation process, allowing appropriate gene expression for survival and replication within the phagolysosome (Swanson and Fernandez-Moreira, 2002). The presence of infected vacuoles has no immediate deleterious effects on the host cell and *C. burnetii* is able to enhance host cell viability by inhibition of the apoptotic macrophage cell cycle (Luhrmann and Roy, 2007; Voth *et al.*, 2007; Voth and Heinzen, 2008). This provides *C. burnetii* with a prolonged period of time for replication within a stable environment.

Autophagy is a catabolic process involving the lysosomal system that allows the degradation of a cell's own components so that nutrients may be recycled within the cell. Autophagy plays a role in cellular homeostasis, and specifically in the balance of synthesis and degradation. Interestingly, involvement of the autophagy system can be beneficial for some intracellular pathogens yet detrimental for others. The large replicative parasitophorous vacuole found in *C. burnetii*-infected macrophages displays autophagic markers, and blocking the autophagic system can prevent the formation of this replicative niche (Romano *et al.*, 2007). In the case of *Mycobacterium tuberculosis* (or the vaccine

strain, *Mycobacterium bovis* bacillus Calmette-Guérin [BCG]) induction of the autophagic system represents an important host defense mechanism to enhance bacterial clearance (Gutierrez *et al.*, 2004). Presumably this relates to the differing strategies employed by these bacteria in surviving within the phagosome: survival of *M. tuberculosis* represses phagosome maturation by targeting a protein known to be upregulated by the autophagy system (Gutierrez *et al.*, 2004), while *C. burnetii* possesses an ability to survive in mature phagosomes. Given that autophagy can be stimulated by Toll-like receptor (TLR) ligands (Delgado *et al.*, 2008), *C. burnetii* may be able to activate autophagy via interation with TLR-2 and/or TLR-4, thereby inducing a host defense mechanism that actually aids *C. burnetii* replication.

Many studies of the role of macrophages in the host immune response to *C. burnetii* make use of tumour macrophage cell lines. Phase II strains are able to establish infection in a range of cell lines, while phase I strains have restricted ability to infect (Baca *et al.*, 1981). Persistent infections are able to be established by either phase I or phase II organisms, however in macrophage tumour cell line cultures that are initially infected with *C. burnetii* phase I, the infection converts to a mix of both phase I and phase II organisms (Baca *et al.*, 1981). Although some studies suggest that primary macrophages perform better at controlling infection than macrophage cell lines (Zamboni *et al.*, 2002), the ease of establishing permanently infected macrophage cell lines indicates that in the absence of other immune responses, macrophages are unable to clear *C. burnetii* infection. Resistance to acidic pH and lysosomal enzymes, the poor induction of nitrogen and oxygen reactive species and resistance to their DNA damaging effects, as well as the ability to modulate the

kinetics of phagolysosome maturation, are all likely reasons why *C. burnetii* is able to resist this important host defense mechanism.

Although *C. burnetii* can withstand or prevent microbicidal activity in monocytes and macrophages, there is a dynamic interaction between host immune responses and bacterial pathogenesis. It is known that serum containing *C. burnetii*-specific antibody is unable to prevent *C. burnetii* infection of host macrophages and bacterial replication, while supernatants from lymphocyte cultures can induce macrophage resistance to infection (Hinrichs and Jerrells, 1976). Activation of monocytes with IFN-γ restores phagosome maturation and results in alkalinisation of vacuoles, both of which may result in *C. burnetii* killing (Ghigo *et al.*, 2002), highlighting the importance of a robust T cell response in driving clearance of *C. burnetii* from infected macrophages. The importance of macrophage activation is also clear in the example of *M. tuberculosis* infection, where inherited deficits of the IL-12, IFN-γ or TNF-α signalling pathways result in clinical disease and chronic infection of macrophages (Jouanguy *et al.*, 1999; Stenger, 2005). Furthermore, TNF-α is known to initiate an apoptotic pathway that is avoided by virulent strains of *M. tuberculosis* (Keane *et al.*, 2002), and apoptosis of infected cells likely represents a key host defense mechanism against intracellular bacteria, as it does in viral infections (Barber, 2001). TNF-α, a major cytokine product of monocytes or macrophages that have been exposed to *C. burnetii* antigens, can act to trigger apoptotic pathways in infected macrophages leading to destruction of the bacterium's replicative niche. The ability of *C. burnetii* to inhibit apoptosis in infected cells therefore represents an important virulence factor (Luhrmann and Roy, 2007). It is clear that the survival of *C. burnetii* within macrophages is a dynamic

process that results only when there is balance between host immune factors and the bacterium's pathogenicity.

In addition to controlling infection via phagocytosis and pathogen destruction, a major function of macrophages is the induction of cytokines. Cytokine production is an important component of the host response to *C. burnetii* infection, and provides a mechanism by which the innate and adaptive immune responses can be coordinated. An adaptive immune response may be broadly considered Th1 or Th2 in type, depending on the types of cytokines that are induced. In fact, these designations are extremes on a continuum of possible cytokine profiles, and appear to be more distinct in mice than in humans. In general, a Th1 immune response is critical in clearing intracellular pathogens, including viruses and intracellular bacteria, and involves the induction of cytokines such as IL-12, IL-2 and IFN-γ. These cytokines favour the generation of cell mediated immunity, while Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 favour antibody production (Mosmann and Coffman, 1989). Sometimes, pro-inflammatory cytokines such as IL-1, TNF-α and IFN-γ can drive immune mediated pathology, while anti-inflammatory cytokines such as IL-10 can drive resolution of aberrant immune responses (DiPiro, 1997).

Maintenance of an appropriate inflammatory cytokine profile is critical in controlling and clearing infection by intracellular pathogens. TNF-α blockade or the use of knockout mice for the p55 TNFR increased mycobacterial proliferation and inhibited nitric oxide production and granuloma formation in mice infected with *Mycobacterium bovis* bacillus Calmette-Guérin or *M. tuberculosis*, demonstrating that TNF-α is essential for protection

against mycobacteria (Kindler *et al.*, 1989; Kindler and Sappino, 1991). TNF-α induces apoptosis of infected macrophages, allowing clearance of intracellular pathogens (Rojas *et al.*, 1999). By contrast, IL-10 inhibits the induction of apoptotic pathways (Rojas *et al.*, 1999). Furthermore, IL-10 has been shown to inhibit antigen presentation (Koppelman *et al.*, 1997). Mice with T cells that overexpress IL-10 are able to generate T cell responses including pro-inflammatory cytokine secretion, yet have high bacterial burdens and can not clear infection when challenged with *Mycobacterium bovis* bacillus Calmette-Guérin (Murray *et al.*, 1997). IL-10 can also suppress microbicidal activity of macrophages against intracellular parasites such as *Toxoplasma gondii* (Gazzinelli *et al.*, 1992). Thus, it is apparent that cytokines can drive or regulate macrophage-mediated microbicidal activity, and therefore play a key role in shaping the host immune response to intracellular pathogens.

In the context of *C. burnetii* infection, the importance of the type-II IFN, IFN-γ, has been suggested by numerous studies. Mice lacking IFN-γ are unable to control infection by avirulent phase II strains of *C. burnetii*, and IFN-$\gamma^{-/-}$ macrophages produce the cytokines IL-6, IL-10 and IL-12, while lacking the typical TNF-α production seen in macrophages from wild-type animals (Ochoa-Reparaz *et al.*, 2007). Decreased TNF-α and IL-12 production have been observed in association with macrophages of mice strains that are highly permissive of *C. burnetii* replication (Zamboni *et al.*, 2004). These studies support the notion that macrophage function is cytokine dependent, and that TNF-α, IL-12 and IFN-γ are all critical in the host response to *C. burnetii* infection.

While pro-inflammatory cytokines are critical for bacterial clearance, anti-inflammatory cytokines are necessary for prevention of immune-mediated pathology. Cytokines such as IL-10 may prevent immune-mediated damage to host tissues, however in murine models, macrophage overexpression of IL-10 results in chronic infection and *in vitro* these macrophages are unable to kill *C. burnetii* (Meghari *et al.*, 2008). The over-expression of IL-10 in these macrophages results in poor expression of inducible nitric oxide synthase and poor induction of inflammatory cytokines in response to *C. burnetii* stimulation (Meghari *et al.*, 2008). Addition of IL-10 to monocytes from convalescent patients results in defective phagosome maturation and inefficient *C. burnetii* killing, while neutralising anti-IL-10 antibody reverses the effect (Ghigo *et al.*, 2004). Thus, it is apparent that excessive IL-10 prevents macrophage clearance of *C. burnetii* and is associated with generation of chronic infection, highlighting the importance of regulating the expression of IL-10 during the host response to infection.

Just as Th1 and Th2 describe cytokine profiles that support differing immune responses, macrophages have varied functional phenotypes that can be described as M1 or M2 polarised. The M2 polarised macrophage is generally considered immunomodulatory or regulatory, and poorly microbicidal, while M1 polarised macrophages are inflammatory and microbicidal (reviewed by (Martinez *et al.*, 2008). These two types of macrophages represent extremes on a continuum of potential macrophage phenotypes, and polarisation in either direction has been documented to be reversible (Porcheray *et al.*, 2005).

C. burnetii induces transcriptional activity within macrophages that results in their polarisation to an atypical M2 profile (Benoit *et al.*, 2008a), while monocytes (macrophage precursors) display a typical M1 polarisation (Benoit *et al.*, 2008b). The response of macrophages to *C. burnetii* includes secretion of cytokines or chemokines associated with an M1 profile, including IL-6 and CXCL8, and also of those associated with an M2 profile, including IL-10, TGF-β and CCL18 (Benoit *et al.*, 2008a). Importantly, monocyte-derived macrophages infected by *C. burnetii* downregulate the expression of M1 molecules such as TNF-α and IL-12, and produce little nitric oxide (Benoit *et al.*, 2008a). An increased risk of developing chronic Q fever in individuals with cardiac valve lesions has been associated with elevated levels of circulating apoptotic leucocytes. This may relate to the fact that ingestion of apoptotic cells by monocytes or macrophages induces gene expression associated with M2 polarisation and allows *C. burnetii* replication (Benoit *et al.*, 2008c). The binding of macrophages to apoptotic cells has been implicated in enhancing the growth of other pathogens, including *Trypanosoma cruzi* and *Leishmania major* (Freire-de-Lima *et al.*, 2000; van Zandbergen *et al.*, 2004), and also of avirulent *C. burnetii* phase II organisms (Zamboni and Rabinovitch, 2004). The finding that IFN-γ can restore microbicidal competence in *C. burnetii*-infected macrophages indicates that the M2 phenotype only occurs in the absence of typical M1 mediators such as pro-inflammatory cytokines or bacterial stimuli (Benoit *et al.*, 2008c). Therefore it seems likely that in the absence of a robust T cell response, *C. burnetii*-infected macrophages are M2 polarised and are unable to prevent bacterial replication, however as T cells become active and secrete IFN-γ, macrophages are able to revert to an M1 phenotype and clear intracellular bacteria.

Monocytes are important components of the innate immune response due to their role in macrophage and dendritic cell homeostasis, but they also directly participate in fighting infection. Monocytes can be divided into two subsets with differing functions, the "classical" subset that highly expresses CD14 (CD14++) and the "inflammatory" subset that express CD14 and CD16 (CD14+ CD16+) (Ziegler-Heitbrock *et al.*, 1988). The CD14+ CD16+ monocytes, which comprise approximately 13% of monocytes in healthy human donors, display altered cell surface marker expression in comparison with classical CD14++ monocytes, including increased HLA-DR expression (Passlick *et al.*, 1989), suggesting that when CD14+ CD16+ monocytes differentiate, they may generate into more efficient antigen presenting cells (APC). In fact, CD14+ CD16+ monocytes are superior APC in comparison to CD14++ monocytes, eliciting significantly greater T cell responses when presenting purified protein derivative (PPD) and influenza antigens (Grage-Griebenow *et al.*, 2001).

Tissue macrophages tend to express a cell surface marker phenotype that is comparable with that found on CD14+ CD16+ monocytes, including decreased expression of CD11b and CD33 in comparison to CD14++ monocytes (Passlick *et al.*, 1989; Ziegler-Heitbrock *et al.*, 1993). Classical CD14++ monocytes have been shown to be capable of differentiating into CD14+ CD16+ macrophages, suggesting the CD14+ CD16+ monocyte subset could be the result of classical monocyte maturation (Ziegler-Heitbrock *et al.*, 1993). This is supported by the finding that during bacterial infection, CD14 antigen density is significantly decreased on the classical CD14++ monocyte population (Fingerle *et al.*, 1993).

Acute bacterial infection causes a selective increase in the CD14+ CD16+ monocyte population (Nockher and Scherberich, 1998), suggesting they play an important role in the host immune response to infection. These "inflammatory" monocytes have been shown to be high producers of TNF-α and low producers of IL-10 in response to TLR ligands such as LPS and Pam3Cys, in comparison to the cytokine responses of "classical" monocytes (Frankenberger *et al.*, 1996; Belge *et al.*, 2002). When stimulated with tumour cells, the CD14+ CD16+ subset are also superior producers of iNOS and NO, but poor producers of reactive oxygen species, in comparison with CD14++ monocytes (Szaflarska *et al.*, 2004). Furthermore, this inflammatory subset demonstrate increased ability to phagocytose bacteria (Nockher and Scherberich, 1998), suggesting they may play an important role in controlling infection.

The "inflammatory" monocyte subset can give rise to a dendritic cell population that regardless of the maturation stimulus, drive Th2 CD4+ T cell responses (Sanchez-Torres *et al.*, 2001; Rivas-Carvalho *et al.*, 2004), suggesting that monocyte subsets may differentially contribute to the Th1-Th2 immune phenotype that is initiated in response to infection. Very little is known about the activity of CD14++ or CD14+ CD16+ monocytes in response to *C. burnetii*, although given the importance of a robust Th1 immune response in clearing *C. burnetii* infection, the function and activity of these monocytes subsets may well be critical.

3.3 Phagocytic cells: neutrophils

Another early immune defence encountered by bacterial pathogens such as *C. burnetii* is the neutrophil. These innate immune cells constitute approximately 70% of all white blood cells in the circulation (Zacharski *et al.*, 1971) and are recruited to sites of infection by chemokines (for example, by IL-8) (Leonard and Yoshimura, 1990). As with any professional phagocyte, the immunological role of these cells depends on many functional aspects including chemotaxis to sites of infection, phagocytosis of pathogens, and induction of bactericidal enzymes.

While much research has focused on the role of macrophages in the host defense against *C. burnetii*, very little is known about the role played by neutrophils, despite them having similar phagocytic roles in fighting many bacterial infections. This is probably because neutrophils, unlike macrophages, do not make good host cells for bacterial replication because of their relatively short lifespan, with studies suggesting an average half life of 7-10 hours once these cells reach the circulation (Cartwright *et al.*, 1964; Price *et al.*, 1996), although once recruited to the site of infection (and with exposure to LPS) this may be extended for several days (Badolato *et al.*, 1994). The role of neutrophils in the host immune response to *C. burnetii* infection can be inferred by the finding of neutrophils in the tissues of infected animals. Neutrophilic infiltrates can be found at sites of local *C. burnetii* infection, including in liver and bone marrow (Srigley *et al.*, 1985). Neutrophils are also prominent in histological sections of placentas from naturally infected animals (Bildfell *et al.*, 2000), and in cardiac valves affected by endocarditis (Atzpodien *et al.*, 1994). Immunohistochemical studies suggest the presence of *C. burnetii* antigen in the

neutrophils of naturally infected animals, indicating that neutrophils do play a role in *C. burnetii* phagocytosis (van Moll *et al.*, 1993). The importance of neutrophil involvement in disease control is uncertain, however neutrophils were notably absent in the lungs of one subject with fatal Q fever pneumonia (Urso, 1975), suggesting their importance in controlling *C. burnetii* infection. By contrast, there is no evidence for an increase in susceptibility to Q fever in patients with neutropenia (Papadaki *et al.*, 2001).

C. burnetii is able to modulate the neutrophil response to infection. In guinea pigs, *C. burnetii* has been reported to induce reduced lysosomal enzyme levels in circulating neutrophils, however this impairment was transient and was significantly decreased with subsequent exposure to the pathogen (Kuzina *et al.*, 1992). It has been demonstrated that phase I *C. burnetii* fails to stimulate respiratory burst when phagocytosed by human neutrophils (Akporiaye *et al.*, 1990). This deficiency is phase I-specific, as phase II LPS does not display the same inability to prime human neutrophils (Fumarulo *et al.*, 1989). The mechanism of this phase-specific ability to inhibit neutrophil activity is unknown, but perhaps relates to the phenomenon observed in dendritic cell responses where immuno-stimulatory epitopes are shielded by the longer, branched carbohydrate O antigen present in phase I LPS (Shannon *et al.*, 2005b). *C. burnetii* also actively inhibits neutrophil activity by production of an acid phosphatase, which has been shown to inhibit respiratory burst in stimulated human neutrophils (Baca *et al.*, 1993b; Li *et al.*, 1996).

Acute bacterial infection has been shown to rapidly induce a five-fold increase in alkaline phosphatase activity in host neutrophils in comparison to healthy controls (Karlsson *et al.*,

1995). Alkaline phosphatase acts via dephosphorylation of bacterial endotoxin lipid A (Poelstra *et al.*, 1997), making it significantly less toxic (Bentala *et al.*, 2002), presumably due to the comparatively poor induction of nitric oxide and IL-6 by dephosphorylated lipid A (Aybay and Imir, 1998). In animal models of septic shock, chemical blocking of alkaline phosphatase activity significantly increased mortality (Poelstra *et al.*, 1997; Verweij *et al.*, 2004), while administration of alkaline phosphatase was protective (Bentala *et al.*, 2002). In human experimental *C. burnetii* infection there was an increase in neutrophil alkaline phosphatase activity, in terms of both number of neutrophils expressing the enzyme and individual cell enzyme activity levels, but this increase in activity was delayed until after the peak fever response in each subject (Beisel, 1967). These kinetics of alkaline phosphatase activity are significantly delayed in comparison to other bacterial infections, where activity can be detected within hours of infection (Karlsson *et al.*, 1995). This suggests that *C. burnetii* is able to inhibit alkaline phosphatase activity in neutrophils, and that neutrophil function is only restored after bacterial control is underway via other components of the immune system.

3.4 Dendritic cells

The dendritic cell (DC) is an important bridge between the innate and adaptive immune system (reviewed by Banchereau and Steinman (Banchereau and Steinman, 1998)). On encountering pathogen, immature DCs capture antigen via phagocytosis, pinocytosis or endocytosis (Inaba *et al.*, 1993; Sallusto *et al.*, 1995). These pathogen-exposed DCs typically undergo maturation resulting in increased expression of MHC class II and T cell

costimulatory molecules (Cella *et al.*, 1997) and activation of lysosomal activity (Trombetta *et al.*, 2003), allowing efficient processing and presentation of pathogen-derived peptide antigens. Mature DCs migrate to lymphoid tissues (Moll *et al.*, 1993), where they are highly efficient APCs capable of stimulating naïve T cells (Ingulli *et al.*, 1997). Mature DCs can also determine the Th1/Th2 functional phenotype of the immune response, and drive an appropriate Th1 immune response against intracellular pathogens via production of high levels of IL-12 (Manetti *et al.*, 1993; Cella *et al.*, 1996). DCs may also be involved in T-independent antigen presentation to B cells and induction of antigen-specific antibody in the absence of helper T cell activity (Balazs *et al.*, 2002).

Both phase I and phase II organisms are able to infect immature DCs. Phase II infection induces DC maturation and high IL-12 production (Shannon *et al.*, 2005a). In contrast, DCs produce very low levels of IL-12 and do not display increased expression levels of maturation markers CD80, CD83, CD40 or HLA-DR when infected by phase I organisms (Shannon *et al.*, 2005a). This phenotype is observed across different phase I strains. Differences in DC activation by phase I and phase II variants are not due to different efficiencies of infection, or differences of growth kinetics – in fact, phase I and phase II follow almost identical timing of lag and exponential growth phases, and amplify to levels comparable to those seen in other cell types (Shannon *et al.*, 2005b). It has been reported that signalling via TLR-4 results in the high IL-12 phenotype required for intracellular pathogen clearance (Re and Strominger, 2001). It is therefore surprising that phase II stimulation of DCs occurs in a TLR-4 independent manner (Shannon *et al.*, 2005b). Removal of LPS from phase I variants restores significant ability to stimulate DCs,

suggesting that the long, branched terminal LPS sugars may shield other ligands for DC signalling via other TLR (Shannon *et al.*, 2005b).

A diverse phenotype has been reported for DCs, with specialised subsets being preferentially induced in response to microbial infections. A subset of DCs that make up the majority of the DC population in the blood is known to produce large quantities of TNF-α in response to bacterial, but not viral, ligands, and to be potent inducers of T cell responses (Schakel *et al.*, 2002). Another subset of TNF-α and iNOS-producing DCs (TipDCs) have been identified in the spleen of *L. monocytogenes* infected mice (Serbina *et al.*, 2003). TipDCs produce iNOS despite not being infected themselves; presumably the production of iNOS enhances microbicidal activity of neighbouring infected cells. TipDCs are the major producers of TNF-α and iNOS in the early stages of infection, and have been shown to be critical in controlling and clearing *L. monocytogenes* (Serbina *et al.*, 2003). While no studies have examined the role of specific DC subsets in the host response to *C. burnetii*, it seems likely that these specific DC subsets may aid clearance via activation of infected macrophages and by priming a robust T cell response.

3.5 Toll-like receptors

Infection by *C. burnetii* exposes the host to large quantities of lipopolysaccharide (LPS), a glycolipid endotoxin present in the cell wall of all Gram-negative organisms. Studies in humans indicate that many of the symptoms associated with acute infectious illness, including fever, headache, nausea and fatigue, may be induced by direct injection of LPS

from a range of bacteria including *Salmonella*, *Escherichia coli*, and *Pseudomonas* (Burrell, 1994).

LPS is a type of pathogen-associated molecular pattern (PAMP). Cells of the innate immune system are able to identify classes of related pathogens via interaction with the PAMPs that are present in each pathogen. LPS from a variety of Gram-negative bacterial species induce host cell responses via interaction with the cell surface receptor, Toll-like receptor (TLR)-4. When encountered in the circulation, LPS is bound by LPS-binding protein (LBP), forming high affinity complexes (Tobias *et al.*, 1986; Tobias *et al.*, 1989) that mediate adhesion of macrophages to bacteria and induce synthesis of cytokines such as TNF-α (Schumann *et al.*, 1990). Complexes of LPB-LPS are recognised by the receptor CD14, a monocyte and macrophage glycoprotein marker (Wright *et al.*, 1990), which interacts with TLR-4 to activate signalling pathways for pro-inflammatory cytokine induction. The MD-2 molecule has more recently been identified as being critical for LPS responsiveness and in localisation of TLR-4 from the Golgi apparatus to the cell surface (Nagai *et al.*, 2002).

TLR-4 belongs to a family of Toll-like receptors that are so named due to their homology to the Toll protein first identified in *Drosophila* spp. Structurally, TLRs have cytoplasmic domains that share similarity with the IL-1 receptor cytoplasmic domain, and hence are termed Toll/IL-1 receptor (TIR) domains (Medzhitov *et al.*, 1997). In addition to the common cytoplasmic TIR domain, each TLR has an extracellular leucine-rich repeat (LLR) domain. LLR domains have been identified in a range of proteins involved in innate

immunity and in embryonic development across different species, including flies, mice and humans (Dolan *et al.*, 2007). So far ten Toll-like receptors have been identified in humans (TLR-1 – TLR-10). TLRs form homodimers (eg TLR-4) or heterodimers (eg TLR-2/TLR-1) that interact with specific and different PAMPs. Interaction with appropriate ligands triggers intracellular signalling pathways, leading to transcription factor activation (eg NF-κB) and gene expression (reviewed in Takeda and Akira, 2004).

TLRs have been identified on many innate immune cells such as macrophages, monocytes, dendritic cells, mast cells and neutrophils (Brightbill *et al.*, 1999; Muzio *et al.*, 2000; Kadowaki *et al.*, 2001). TLR expression is influenced by a number of factors including exposure to cytokines (Miettinen *et al.*, 2001) and may be regulated by interaction of TLRs with their ligands. For instance, LPS has been shown to induce increased TLR-2 expression, but decreased TLR-4 expression, by macrophages (Matsuguchi *et al.*, 2000; Nomura *et al.*, 2000). TLR expression may also be modulated as cells undergo maturation processes (Visintin *et al.*, 2001).

TLRs signal via a common pathway, termed the MyD88-dependent pathway, which results in activation of the NF-κB transcription factor (Zhang *et al.*, 1999). Each TLR also makes use of a unique alternative pathway. TLR-4 signalling may occur using a MyD88-independent pathway, which involves TIRAP/Mal and subsequent activation of the IRF-3 transcription factor (Kawai *et al.*, 2001), while TLR-2 signalling requires a Rac1-dependent pathway that results in NF-κB activation (Arbibe *et al.*, 2000). By utilising common and unique signalling pathways, responses to pathogens can involve common response patterns

as well as those that are specific to the type of pathogen. For instance, LPS stimulation induces pro-inflammatory cytokines via NF-κB-directed gene transcription (MyD88-dependent pathway), but also induces IFN-β expression via IRF-3 directed gene transcription (MyD88-independent pathway). LPS stimulated IFN-β then acts via the IFN-α/β receptor, triggering a signalling pathway that activates the Stat1 transcription factor (reviewed in Theofilopoulos *et al.*, 2005). Stat1 drives expression of genes such as inducible nitric oxide synthase (iNOS) (Ohmori and Hamilton, 2001) such that bacterial pathogens may be destroyed by exposure to reactive nitrogen species. Thus, the existence of two pathways allows for the generation of both generic anti-pathogen defenses (cytokines) and more specific anti-bacterial defenses (iNOS).

TLR signalling can also influence the type of adaptive immune response mounted to a particular pathogen. Dendritic cells play a key role in skewing CD4+ T helper cells to a Th1 or Th2 biased response. Stimulation of DCs via TLR-4 results in IL-12 and skews T helper cells to a Th1 phenotype, while stimulation via TLR-2 results in little IL-12 secretion and subsequently T helper cells are skewed towards a Th2 phenotype (Pulendran *et al.*, 2001; Re and Strominger, 2001). The presence of ligands for TLR-2 or TLR-4 may therefore critically affect the host's ability to eliminate specific bacterial pathogens.

Although LPS is traditionally thought to trigger immune responses via TLR-4, there are some data to suggest that TLR-2 is a key receptor for detection of *C. burnetii*. Signalling via TLR-2 occurs upon interaction of ligands such as peptidoglycan or lipoteichoic acid (found in Gram positive bacteria) with a heterodimer of TLR-2 in combination with TLR-1

or TLR-6 (Kirschning and Schumann, 2002). Interaction of TLR-2 with its ligands may involve or even require many of the same molecules as for TLR-4 signalling, including LBP, MD-2 and CD14 (Yoshimura *et al.*, 1999; Dziarski *et al.*, 2001; Schroder *et al.*, 2003b). Stimulation via TLR-2 causes activation of a MyD88-dependent pathway that is common to all TLRs (Takeda and Akira, 2001), as well as activation of a Rac1-dependent pathway (Arbibe *et al.*, 2000).

Not all Gram negative bacteria have LPS that interacts with TLR-4; some Gram negative bacteria have LPS known to signal primarily via TLR-2, including *Porphyromonas gingivalis* (Kikkert *et al.*, 2007). Reports that other Gram negative bacteria are known to stimulate via both TLR-4 and TLR-2 are not surprising given that minor components of the Gram negative bacterial cell wall include the TLR-2 ligands peptidoglycan and lipoprotein (Kikkert *et al.*, 2007). Activation via TLR-4 results in increased sensitivity for TLR-2 ligands, as evidenced by the fact that susceptibility to Gram negative bacteria-mediated septic shock can be inhibited most effectively by blockade of both TLR-2 and TLR-4 (Spiller *et al.*, 2008). Thus, it seems likely that the host immune response to *C. burnetii* may involve TLR-2, TLR-4, or both receptors in either initial recognition of infection or in subsequent activation of sensitised host cells.

There is conflicting evidence suggesting a role for either TLR-2 and/or TLR-4 as receptors involved in sensing *C. burnetii*. A TLR-4 response pattern may be viewed as preferential for fighting *C. burnetii* infection given that TLR-4 signalling via the MyD88-independent pathway is effective in generating IFN-β, a key first step in the induction of nitric oxide

production known to be critical in clearing infection, while TLR-2 ligands are poor inducers of IFN-β (Toshchakov *et al.*, 2002). Furthermore, TLR-4 signalling in DCs results in a Th1-type immune response, which has been shown to be more effective than a Th2-type response in controlling or clearing infections of intracellular pathogens such as *Mycobacterium tuberculosis* (Stenger and Modlin, 2002), *Leishmania major* (Scott, 1998) and *Brucella suis* (Dornand *et al.*, 2002).

The evidence suggesting a role for TLR-2 or TLR-4 in the innate immune response to *C. burnetii* is conflicting, and largely derived from data in mice, which may not necessarily translate to human infection. In murine models, phagocytosis of phase I organisms, but not of phase II organisms, is impaired in the absence of TLR-4 (Honstettre *et al.*, 2004), presumably because filamentous actin rearrangement was prevented in TLR-$4^{-/-}$ macrophages. TLR-4 is not associated with *C. burnetii* survival in macrophages, but is essential for granuloma formation *in vivo* (Honstettre *et al.*, 2004). The TLR-$4^{-/-}$ genotype is associated with altered cytokine response patterns, resulting in decreased IFN-γ, TNF-α and IL-10 production in mouse splenocytes and macrophages in response to *C. burnetii* (Honstettre *et al.*, 2004). The lipid A component of *C. burnetii* LPS is not a ligand for TLR-4 (Zamboni *et al.*, 2004; Shannon *et al.*, 2005b). Rather, lipid A appears to be an antagonist of TLR-4, acting to reduce TNF-α production in response to *E. coli* LPS stimulation (Zamboni *et al.*, 2004). From these limited studies, the precise role of TLR-4 in the host response to *C. burnetii* remains to be elucidated, particularly in humans.

There is also evidence in murine models of *C. burnetii* infection to suggest a role for TLR-2 in phagocytosis of the organism and induction of cytokine responses. Engagement of TLR-2 may compensate for the impaired phagocytosis of phase I *C. burnetii* in TLR-$4^{-/-}$ mice (Honstettre *et al.*, 2004). In a CHO cell line transfected with CD14 and stimulated with *C. burnetii*, lack of TLR-2 resulted in less TNF-α and IL-12 production than that induced in cells where TLR-4 was lacking (Zamboni *et al.*, 2004). Furthermore, CHO cells lacking TLR-2 were highly permissive of bacterial replication, demonstrating a greater number of large replicative vacuoles, each with a higher bacterial load, in comparison to CHO cells that had been transfected with either TLR-2 alone or both TLR-2 and TLR-4 (Zamboni *et al.*, 2004). Whole, killed *C. burnetii*, or LPS, of either phase I or phase II chemotype, all activate macrophage cytokine responses via TLR-2 and not TLR-4 (Zamboni *et al.*, 2004). Mice lacking TLR-2 display poor ability to form granulomas in response to *C. burnetii* infection, indicating that TLR-2 is important in generating an inflammatory response (Meghari *et al.*, 2005). Lack of TLR-2 does not prevent bacterial clearance by mice (Meghari *et al.*, 2005) although it does make these animals susceptible to febrile illness when exposed to non-virulent phase II strains (Ochoa-Reparaz *et al.*, 2007). Despite being susceptible to phase II infection, adaptive immune responses in TLR-$2^{-/-}$ mice are sufficient to protect these immunocompromised animals from subsequent challenge (Ochoa-Reparaz *et al.*, 2007).

The precise role of TLR-2 and TLR-4 in the human macrophage response to *C. burnetii* remains to be determined, however there are some data suggesting that signalling via one or both of these receptors may be important in cases of human infection. *In vitro* treatment of

the human monocytic cell line, MonoMac-1 with either TLR-2 or TLR-4 agonists causes activation of these macrophages and enhances their ability to kill intracellular phase II *C. burnetii* (Lubick *et al.*, 2007). However, *C. burnetii* killing by activated macrophages is not dependent on TLR stimulation; TLR-independent macrophage activation induces comparable bactericidal effects as the TLR agonists (Lubick *et al.*, 2007). Thus, TLR signalling represents one mechanism by which macrophages may be activated to kill *C. burnetii*, but is not a requirement for killing of this intracellular pathogen, and regardless of whether *C. burnetii* interacts with host innate cells via TLR-2 or TLR-4, it is clear that the activation profile of *C. burnetii*-stimulated macrophages varies substantially with that induced by LPS (Benoit *et al.*, 2008a).

A number of polymorphisms have been identified within the human genes for TLR-2 and TLR-4. In particular, single nucleotide polymorphisms (SNPs) have been identified that result in non-synonymous amino acid sequences, and are therefore proposed to confer functional changes. The Arg753Gln polymorphism in TLR-2 has been found in approximately 3-10% of Caucasian populations, and has been associated with reduced responsiveness to bacterial lipopeptides as determined by reduction in the downstream activation of the transcription factor, NFκB (Lorenz *et al.*, 2000). While macrophages from homozygotes with the Arg753Gln polymorphism have been shown to lose lipoteichoic acid responsiveness, heterozygotes showed no loss of function, suggesting a single wild type copy may be sufficient for normal TLR-2 function (von Aulock *et al.*, 2004). The Asp299Gly polymorphism in TLR-4 has also been associated with reduced induction of transcription factors in response to LPS (Schnare *et al.*, 2001). PBMC from carriers of the

TLR-4 Asp299Gly polymorphism (heterozygotes) do not differ in endotoxin sensitivity with regard to cytokine production or mitogen-activated protein kinase activity in PBMC, suggesting that if the polymorphism does result in functional impairment, a single wild-type allele may be sufficient for full TLR-4 function (Imahara *et al.*, 2005).

Polymorphisms in the TLR-2 or TLR-4 genes may affect susceptibility to, or outcomes from, infectious diseases that signal via those receptors. Some studies report correlations between disease incidence and the presence of polymorphism in those genes. For instance, SNPs in the TLR-2 gene (Arg677Trp and Arg753Gln) have been associated with the mycobacterial infections, tuberculosis and leprosy (Kang and Chae, 2001; Ben-Ali *et al.*, 2004), and in the TLR-4 gene (Asp299Gly) with Gram negative sepsis (Lorenz *et al.*, 2002). The possible role of TLR polymorphisms in affecting Q fever susceptibility or illness course has not been investigated.

3.6 NK cells, NKT cells, γδ T cells

Natural killer (NK) cells, natural killer T (NKT) cells, and gamma/delta (γδ) T cells are all cells that initiate anti-microbial or tumour responses via mechanisms that are either antigen non-specific, or directed against conserved antigen motifs, and as such can be described as responding to pathogen patterns. The early kinetics of their induction suggests a role for these cells in the host innate immune response. In comparison to other components of the host immune response, the function and activity of these cells is poorly understood.

NK cells are a component of the innate immune system that act in antigen non-specific ways to eliminate host cells that display altered expression of MHC (Moretta *et al.*, 1994), and are considered an important host defense against tumours. NK cells also play an important role in clearing virally-infected host cells, such as those infected by *Herpes simplex virus* (Ching and Lopez, 1979), and are recognised as an important innate host defense against microbial pathogens (Zucchini *et al.*, 2008). These cells are cytotoxic, release cytokines, and can co-stimulate cells of the adaptive immune system (Orange and Ballas, 2006). Activated NK cells are present in *M. tuberculosis* infection, but do not appear to be critical in controlling infection (Junqueira-Kipnis *et al.*, 2003), however NK cells have been documented to play a protective role in murine infection by *Listeria monocytogenes* (Tripp *et al.*, 1993). It is not clear why NK cell activity may be relevant in fighting one intracellular pathogen yet dispensible in the host response to another similar pathogen.

Natural killer T (NKT) cells elicit early, first line defenses in the absence of typical antigen processing and presentation via MHC. These CD1d-restricted cells are also known to play an important role in tumour immunity (Terabe and Berzofsky, 2008). Rapid expansion and activation of NKT cells occurs in response to microbial infection, but is rapidly down regulated via marked unresponsiveness or programmed apoptosis of NKT cells (Chiba *et al.*, 2008), suggesting these cells may play a key early role in the host response to infection but do not have long term involvement. NKT cells can drive a Th2 immune response by DCs in murine models of *Leishmania major* (Wiethe *et al.*, 2008), highlighting their potential role in skewing the immune response towards one that favours intracellular

pathogens. Furthermore, NKT cells have been implicated in the generation of pathogenic T cell responses to *Erlichia* via apoptosis of macrophages and upregulation of costimulatory molecules on T cells (Stevenson *et al.*, 2008). NKT knockout mice have comparable ability to control *M. tuberculosis* infection as wild type mice, although splenic cells from NKT knock out mice display decreased ability to induce cytokines (Sugawara *et al.*, 2002). The role of NKT cells in mediating pathology or protective immune responses to intracellular pathogens remains unclear.

Gamma delta (γδ) T cells differ from traditional αβ T cells in their TCR composition: the γδ T cell TCR consists of one γ-chain and one δ-chain while the αβ T cell TCR has α- and β-chains. These γδ T cells undergo TCR gene rearrangement and can generate immunological memory, giving them properties suggestive of an adaptive immune response (Xiong and Raulet, 2007), however the restricted TCR present in some γδ T cells subsets suggests that they are a type of pattern recognition receptor, and therefore γδ T cells may also be said to contribute to early responses that are innate in nature (Konigshofer and Chien, 2006). The presence of phosphoantigens in microbial infections can trigger γδ T cell activation, resulting in pro-inflammatory cytokine production including TNF-α and IFN-γ (Beetz *et al.*, 2008). Furthermore, γδ T cells have been documented to play a protective role in murine infection by *Listeria monocytogenes* (Hiromatsu *et al.*, 1992).

Studies of macrophage function indicate the critical requirement for activation of microbicidal activity in order for macrophages to clear *C. burnetii* infection. NK, NKT and γδ T cells are known producers of cytokines including IFN-γ, and may therefore play a role

in macrophage activation prior to the induction of adaptive cellular responses. *C. burnetii* is known to activate NK cell activity (Tokarevich *et al.*, 1990), although NK cell deficiency does not alter bacterial clearance or clinical diseases in murine models of *C. burnetii*-infection, despite increased histopathological changes being evident in NK-deficient mice (Andoh *et al.*, 2007). The proportion of γδ T cells increases rapidly in the acute phase of *C. burnetii* infection, indicating that these cells likely play an important part in the early response to this pathogen (Schneider *et al.*, 1997).

3.7 B lymphocytes and antibody production

The adaptive immune response has two key characteristics: specificity for a particular pathogen, and the potential for the generation of immunological memory. Both T and B cells possess receptors which determine their specificity for a particular pathogen-derived epitope; those with the same antigen specificity work together in a coordinated way to drive the adaptive immune response. The primary contribution of B cells to an adaptive immune response is the production of antibody. Resting B cells become activated to proliferate following direct contact with activated T cells (Clement *et al.*, 1984), and then antibody secretion may be triggered via helper T cell-mediated antigen specific or polyclonal mechanisms (Friedman *et al.*, 1983). Humoral responses to bacterial pathogens may also be triggered directly in a T cell-independent manner, involving the cross-linking of B cell antigen receptors by highly repetitive and repeated antigens that are present on the bacterial cell wall (Vos *et al.*, 2000). Thus, antibody secretion may be triggered in an antigen-

specific manner by T cell-dependent and T cell-independent mechanisms, or polyclonal activation may occur in response to LPS.

Immunoglobulin occurs in five different forms, with each playing a different role in the host immune response. IgD occurs on the surface of resting B cells, however its role is not well characterised. IgA and IgE are not generally associated with the immune response to bacterial infection; IgA is a component of mucosal immunity which may sometimes be associated with the host response to bacteria in the case of ingested or inhaled pathogen, while IgE is associated with allergy or the immune response to parasites. By contrast, IgM and IgG are important in the host response to bacterial pathogens. Antigen-specific stimulation of naïve IgM-expressing B cells induces secretion of high affinity IgM that plays a role early in the B cell response to infection. Stimulation also triggers a maturation pathway resulting in a class switch to IgG expression and secretion (Coffman and Cohn, 1977). Thus, the kinetics of antibody production vary by antibody class, as expected for any infection, with IgM occurring earlier, and IgG later, including in *C. burnetii* infection (Kazár *et al.*, 1977). Assessment of the change from an IgM-dominated response to an IgG-dominated response is therefore suggestive of acute, primary infection, while an early IgG response is indicative of a memory response or secondary infection, as memory B cells typically express and secrete IgG.

Antibody production is considered a critical component of the host adaptive immune response to bacteria, and can assist in bacterial killing via different mechanisms. Antibody bound to a bacterium can trigger the complement cascade. Complement-mediated

opsonisation targets a bacterium for phagocytosis, but complement may also form a membrane attack complex (MAC) to directly kill the bacterium. Antibody-mediated agglutination of bacteria can activate microbicidal activity in effector cells, such as degranulation of mast cells or neutrophils, or phagocytosis by macrophages or monocytes. Neutralising antibody may also bind to a bacterium and prevent it from adhering, and infecting, host cells, or it may bind and neutralise toxins (Janeway *et al.*, 2001).

The majority of successful vaccines enable protective immunity against extracellular pathogens by triggering specific antibody production by B cells, suggesting that antibody production may also be critical in clearing primary infection (Plotkin, 1999). In the case of intracellular infections, the importance of antibody production in control of infection is less clear. It has been shown for a number of intracellular pathogens, including *Mycobacterium tuberculosis* and *Listeria monocytogenes*, that T cell responses, and not antibodies, are associated with protective immunity (Harty and Bevan, 1992; Kaufmann, 2002). Given that transfer of sera from immune animals does not protect immunodeficient SCID mice from infection, despite affording considerable protection in naïve, immunocompetent BALB/c mice (Zhang *et al.*, 2007), it appears that the presence of specific antibody may not provide protective immunity to *C. burnetii* in the absence of cellular immunity.

Phase specific variance in the antibody response to vaccination appears to be critical in the generation of protective immunity to *C. burnetii*. The humoral response to *Coxiella burnetii* varies depending on whether it is specific for the phase I or phase II organism, with only some shared antigens being present on both phases (Hackstadt *et al.*, 1985). Furthermore,

antigenic variations exist across different strains of the same phase (Hackstadt, 1986). The kinetics of antibody production vary depending on their phase specificity: Following infection with phase I organisms, phase II-specific antibodies appear more rapidly than phase I-specific antibodies in guinea pig (Kishimoto and Burger, 1977) and rabbit models of infection (Fiset, 1957; Kazár *et al.*, 1977). Only minor differences in antibody production occur when live phase II organisms are used rather than phase I for vaccination or infection (Fiset, 1957), presumably because "phase II" preparations will quickly revert to phase I in an adult host animal due to the preferential replication of any contaminating phase I organisms that are present in the phase II preparation.

In murine models, B cell deficiency results in increased tissue damage during infection, but does not affect disease course or *C. burnetii* clearance (Andoh *et al.*, 2007). However, antibodies form an important component of the immune response to Q fever, with sera from immune animals and from humans having a protective effect in naïve animals (Abinanti and Marmion, 1957). This protective effect has been shown to be largely mediated by phase I specific antibodies (Ormsbee *et al.*, 1968). Interestingly, antibodies directed against phase I appear after the resolution of symptoms such as fever and weight loss (Kishimoto and Burger, 1977), suggesting they are unlikely to be critical in the resolution of acute phase disease.

In human subjects with acute Q fever, the antibody response to phase II antigen is generally greater and earlier than the phase I antibody response (Embil *et al.*, 1990). IgM occurs early in acute infection with a peak level reported to occur 10-14 days after onset of

symptoms (Murphy and Magro, 1980). Given that phase I-specific IgM is absent during early acute Q fever (Embil *et al.*, 1990), the reported early high IgM titres are phase II-specific. IgG antibodies occur later in acute Q fever (Worswick and Marmion, 1985), with IgG1 reported to be the predominant IgG subclass and demonstrating significant affinity maturation over the course of infection (Guigno *et al.*, 1992). Phase I IgM, and phase II IgM, IgG, IgA and complement fixing antibodies are present in the convalescent phase of acute Q fever, while patients with chronic, localised Q fever are reported to have IgG and IgA, but little or no IgM (Worswick and Marmion, 1985). Secondary *C. burnetii* challenge has been shown to result in the induction of phase I antibodies of IgM and IgG isotype (Peacock *et al.*, 1979; Zhang *et al.*, 2007). Immunity in vaccinated mice has been associated with the induction of IgG2a antibodies, which are generally associated with a Th1 immune response (Zhang *et al.*, 2007).

Diagnosis of Q fever is generally based on serology, however significant cross-reactivity between *C. burnetii* and *Legionella micdadei* (Musso and Raoult, 1997) has been reported. Furthermore, analysis of paired sera from control subjects with Epstein-Barr virus, *Mycobacterium pneumoniae* or *Bordetella pertussis* infections showed that elevated *Coxiella*-specific anti-phase II IgM or IgA antibody responses can develop in the course of these infections (Devine *et al.*, 1997). Phase II antibody titres (by microimmunofluorescence) of IgM $\geq$ 1:50 and IgG $\geq$ 1:200 are considered to be diagnostic of acute Q fever (Dupont *et al.*, 1994). Phase I IgG and IgA antibodies are generally associated with chronic, localised Q fever, however one study with more than 2000 patients suggested that IgA antibody titres do not contribute to diagnosis, while high IgG titre is

highly predictive of chronic Q fever (Dupont *et al.*, 1994). In rare cases, phase II IgA antibodies are detected in acute Q fever (Fournier and Raoult, 1999). One study found a phase I IgA titre ≥ 1:320 by immunofluorescence and a complement fixation titre ≥ 128 to be predictive of endocarditis in a cohort of patients with acute Q fever and chronic endocarditis (Soriano *et al.*, 1993), however this study had relatively few patients (n=10 for each group). Another study found that phase II IgA2 antibodies were only present in patients with Q fever endocarditis (Camacho *et al.*, 1998). Given the somewhat contradictory nature of these data, it is reasonable to conclude that quantitation of antibody titres from a single serum sample provides questionable diagnostic assistance in discriminating acute from chronic Q fever, even when phase and class specific determinations are done.

There are varied reports of the duration of the individual humoral responses following *C. burnetii* infection. A study of previously infected or vaccinated individuals, including one individual who became infected in the course of the study, found that while the presence of specific antibody is useful in diagnosis of Q fever, these antibodies do not remain detectable by microagglutination in sera tested up to eight years after convalescence (Jerrells *et al.*, 1975). By contrast, in another study phase II-specific IgG persisted for up to 12 years post-infection (Marmion *et al.*, 2005). Repeated exposure does not seem to be a likely cause of this long term antibody persistence, as the study included a subset of subjects who were not likely to experience ongoing exposure to *C. burnetii* (Marmion *et al.*, 2005). The large variation in the duration of antibody persistence reported by these two studies may instead relate to differing sensitivities in the antibody detection methods. Very

low levels of antigenic challenge have been shown to be sufficient in boosting pre-existing humoral responses, with application of the delayed-type hypersensitivity (DTH) skin test of killed phase I antigen being sufficient to induce measurable increases in antibody titre in subjects who were skin test positive (Luoto *et al.*, 1963).

Analysis of the differences in humoral immunity that occur in patients with acute and chronic infection has provided insights into the role of *C. burnetii* in the pathogenesis of chronic disease. An immunodominant outer membrane protein of *Coxiella burnetii* was identified as being reactive with sera from acute, but not chronic, Q fever patients (To *et al.*, 1998a), and characterised by Zhang *et al* (2005). The gene encoding this protein was designated acute disease antigen A (*adaA*). A putative DNA sequence encoding the N-terminal amino acid sequence of the protein was amplified by PCR and used to screen a *C. burnetii* genomic library. The putative *adaA* gene was then found in isolates from patients with acute Q fever but was not found in isolates from patients with chronic Q fever. Antibodies specific for AdaA reacted with the outer membrane fraction of *C. burnetii*. PCR or a recombinant serodiagnostic test targeting *adaA* may be useful for diagnosis of acute Q fever (Zhang *et al.*, 2005). More significantly, identifying the function of the AdaA protein may help elucidate the mechanism by which *C. burnetii* may establish chronic infection.

Cross-sectional serological testing can not reliably distinguish acute, resolved or chronic Q fever. What is clear from mice models is that in the absence of cellular immunity, specific antibody cannot protect against *C. burnetii* infection, although it contributes to the

immunity conferred by T cell responses in immunocompetent animals. The notion that antibodies do not mediate protection in humans is supported by the finding that protection due to vaccination or prior natural infection is not always associated with seroconversion. T cell responses are therefore likely to play a critical role in resolution of acute Q fever, and in vaccine derived immunity.

3.8 T lymphocytes

There are overwhelming data to suggest that T cell responses play a critical role in resolution of acute infection and in vaccine-generated immunity to Q fever. In murine models, T cell deficiency results in an inability to control or clear *C. burnetii* infection (Andoh *et al.*, 2007). A comparison of mice with different susceptibilities to infection revealed that A/J mice, known to have deficits in innate immune function (Fortier *et al.*, 2005), when infected at high doses with *C. burnetii*, have significant antibody responses but poor T cell responses to *C. burnetii* and readily succumb to infection, while resistant mice strains such as C3H/HeN or C57BL/6J develop both cellular and humoral responses in the absence of disease symptoms (Scott *et al.*, 1987). Findings in other animals support the notion of T cell immunity as critical for protection from *C. burnetii* infection. In guinea pigs for instance, resistance to *C. burnetii* challenge occurs in association with vaccine-induced cellular responses, but not antibody production (Kishimoto *et al.*, 1977).

In humans, vaccine-induced immunity to *C. burnetii* develops with cellular immune responses, but not necessarily with antibody production. Of individuals vaccinated with a

highly effective and protective Q fever vaccine, as many as 85-95% demonstrated lymphoproliferative responses to *C. burnetii*, while only 60% displayed long term seroconversion (Marmion *et al.*, 1990; Izzo *et al.*, 1991). In fact, prior exposure to Q fever cannot be reliably predicted by serological screening alone, while DTH skin testing is a good indicator of natural, or vaccine-induced, sensitivity to *C. burnetii* antigens (Luoto *et al.*, 1963). Cellular immune responses generated by infection or vaccination are long lasting. In humans, cellular immune responses specific to phase I and II whole cell antigens remain detectable using a cellular proliferation assay for periods of up to 8 years post-infection (Jerrells *et al.*, 1975). Thus, cellular immunity appears to confer the high level of protection afforded by vaccination, and likely plays a pivotal role in natural immunity to infection.

Historically, *in vitro* characterization of the cellular immune response to *C. burnetii* has usually been performed by one of two assays: the lymphocyte proliferation assay or macrophage migration inhibition assay. Lymphocyte proliferation (also known as lymphoproliferation or lymphocyte transformation) is usually measured in a mixed PBMC culture, where T cells interact with APCs such as monocytes and B cells, with or without stimulating antigen. Tritiated thymidine is usually added to the cultures, and uptake of this radio-labelled marker reflects its incorporation into the genomes of dividing cells. The cells in each well are harvested onto glass filters and counted in a beta-scintillation counter with data expressed as counts per minute (cpm). Analysis usually involves calculation of the stimulation index (cpm of stimulated well divided by cpm for unstimulated control well) for each assay condition.

The macrophage migration inhibition assay is a bioassay for detecting migration inhibition factor (MIF) secretion by immune cells. To perform this assay, macrophages are placed in a capillary tube in the presence of T cell culture supernatant. Macrophages demonstrate decreased migration when in the presence of supernatant from activated T cell cultures, due to the presence of MIF. MIF was once believed to be primarily a product of activated T cells, however subsequent studies found its production in athymic, T cell deficient mice, by both the anterior pituitary gland and macrophages (Calandra *et al.*, 1994). Induction of MIF production in macrophages has a pro-inflammatory effect, reversing protective glucocorticoid effects such as suppression of cytokine production following LPS stimulation, and therefore MIF plays a critical role in septic shock (Calandra *et al.*, 1995). The mechanism of action of MIF appears to be autocrine; macrophages lacking MIF show decreased TLR-4 expression and an associated decrease in responsiveness to bacterial ligands (Roger *et al.*, 2003). Despite these newly described sources and functions for MIF, it has a well-characterised role in regulation of T cell activation. Production of MIF by activated T cells results in enhanced T cell proliferation and IL-2 production, while blocking MIF with neutralising antibodies inhibits antigen-specific T cell proliferation and antibody production (Bacher *et al.*, 1996). Thus, experiments measuring MIF production in response to *C. burnetii* stimulation are likely to approximate the degree of antigen-specific T cell activation and therefore macrophage migration inhibition assays provide an indirect measure of the cellular immune response to infection. Detection of *C. burnetii* sensitisation via skin test shows high concordance with the presence of lymphoproliferative responses to *C. burnetii*, but not with a positive migration inhibition test (Kazár *et al.*,

1984), suggesting that detection of cellular responses by lymphoproliferation assay may be a more sensitive detection method.

Assays of cellular immunity usually make use of whole cell antigens (phase I or phase II), or components of the whole cell antigen obtained by different extraction processes, as recall antigens for *in vitro* stimulation assays, or as vaccines for *in vivo* experiments. Whole phase I antigen is highly immunogenic, causing activation of macrophages (Kelly, 1977) while also inducing production of phase I and phase II specific antibody responses and lymphocyte proliferation (Williams and Cantrell, 1982). Whole phase I antigen also induces non-specific immune responses, having been demonstrated to protect mice from protozoan infection (Clark, 1979) and enhance resistance to tumours (Kelly *et al.*, 1976), however it can also elicit deleterious tissue reactions (Williams and Cantrell, 1982). Screening potential seronegative vaccinees for prior exposure to *C. burnetii* via DTH skin testing reduces the rate of vaccine-associated side effects (Ascher *et al.*, 1983a).

Interest in extracted fractions of the whole cell antigen arose out of a need to develop vaccines with decreased risk of localised tissue reactions. Extraction using trichloroacetic acid (TCA) yields a soluble fraction that includes both protein and carbohydrate; re-stimulation of T cell responses in previously infected subjects is dependent on only the protein fraction of the TCA extract (Jerrells *et al.*, 1975). In murine studies, vaccination with a TCA extract induced protection that was only slightly less effective than the whole phase I organism from which is was derived (Gajdošová *et al.*, 1994). Chloroform-methanol extraction (CME) yields a soluble component of branched fatty acids and lipids

that does not stimulate an immune response (Williams and Cantrell, 1982), and an insoluble residue (CMR) that has been demonstrated to elicit potent, antigen non-specific immune responses including IFN-γ, IL-1α and IL-6 production (Zvilich *et al.*, 1995). CMR does not seem to elicit dermal granulomatous reactions that can be problematic when sensitised individuals are vaccinated (Ascher *et al.*, 1983b). These different whole cell antigens or their extracts have been used in a variety of studies to investigate the role of T cells in providing protective immunity to Q fever.

Conflicting data exist regarding the kinetics of T cell activation in response to *C. burnetii* infection. One study found that cellular responses develop during recovery from natural infection, but are not evident during the acute phase of Q fever (Jerrells *et al.*, 1975), while vaccine-mediated immunity has been proposed to take approximately 9-13 days to develop (Izzo *et al.*, 1988; Marmion *et al.*, 1990). Some suggest that generation of cellular responses may take as few as 3 days (Kishimoto and Burger, 1977), however these studies make use of migration inhibition assays, which as discussed earlier may reflect general immune activation rather than being specifically indicative of T cell responses. Furthermore, lymphoproliferative responses detected in a similar study by the same researchers were found to appear much later - from day 21 of infection (Kishimoto *et al.*, 1978). Thus, it appears that generation of T cell responses during *C. burnetii* infection occurs over a period of weeks following initial exposure to the pathogen.

It is unknown how long *C. burnetii*-specific cellular responses persist following the initial antigenic challenge. Individuals vaccinated with QVax^{TM} demonstrate cellular responses

for at least eight years, however the subjects of such reports are in occupations such as abattoir work which have a high risk of exposure to *C. burnetii* and possibly receive ongoing, low level antigenic stimulation due to contact with infected animals or their waste products that could act to boost cellular responses (Marmion *et al.*, 1990). It is not clear whether vaccine-induced cellular responses persist in the absence of periodic natural exposure (Ackland *et al.*, 1994). In a study of only three individuals vaccinated more than 15 years earlier, none remained skin test positive, and only one of the three subjects retained proliferative responses to phase I antigen stimulation (Gajdošová and Brezina, 1989), suggesting vaccination may not intrinsically provide life long immunity to infection. By contrast, natural infection appears to provide longer lasting cellular responses than vaccination; subjects who had a prior Q fever illness demonstrated long term lymphoproliferative responses to phase I antigens, while some also retained phase II-specific proliferation (Gajdošová and Brezina, 1989).

The importance of phase variation in generation of protective immune responses is unclear. Phase I organism has been clearly demonstrated to generate long lasting and protective immunity, while phase II-derived vaccines of the same *C. burnetii* strains are poorly immunogenic, differing in their protective end point (PD_{50}) by a factor of 100-300 fold (Ormsbee *et al.*, 1964). Vaccination with a phase I preparation results in responses that are both phase I and/or phase II specific; vaccination with phase II vaccine results in phase II specific responses only (Ormsbee *et al.*, 1964). The importance of phase specificity in protective immunity is unclear: either phase I-specific responses (Gajdošová and Brezina, 1989) or phase II-specific responses (Izzo *et al.*, 1988; Izzo and Marmion, 1993) have been

found to be at higher levels, or were more consistently identified, in individuals who have vaccine-induced protective immunity. The differences in these studies may relate to the immunosuppressive effects of phase I LPS. Separation of proteins from LPS for use as antigenic stimulation results in greater IFN-γ production *in vitro*, while addition of purified phase I LPS to culture systems reverses this trend, confirming that *C. burnetii* phase I LPS-mediates inhibition of antigen-specific IFN-γ production (Izzo and Marmion, 1993). Thus, the low levels of T cell activation detected in response to phase I antigen may relate to limitations of the assay system rather than a real lack of phase I-specific T cells *in vivo*.

The nature of antigen-specific T cell responses in Q fever is poorly characterised. It remains to be elucidated whether specific epitopes within the protein component of *C. burnetii* are particularly significant in protective immunity (i.e. immunodominant). The size and complexity of the *C. burnetii* genome makes it challenging to identify epitopes by the screening methods currently used for the study of smaller pathogens such as viruses, where peptides spanning the entire genome can be synthesised and used to map responses to specific epitopes (Meddows-Taylor *et al.*, 2007). Instead, research has tackled the issue of peptide-MHC specificity in generating T cell immunity by examining the role of MHC diversity in affecting Q fever severity or outcome of infection. Given the importance of T cell responses in control of *C. burnetii* responses, and the pathogen's tendency to infect host monocytes and macrophages, it is likely that allelic variation in HLA class II, present on antigen presenting cells, may contribute to disease susceptibility or severity. A comparison of Q fever subjects (with either active Q fever, or recovered patients) and control subjects found a significantly higher prevalence of HLA-DRB1*11 among Q fever

subjects (Helbig *et al.*, 2003). PBMC from subjects carrying the HLA-DRB1*11 allele produced less IFN-γ following phytohaemagglutinin (PHA) stimulation and less IL-2 following stimulation with *C. burnetii* antigens (Helbig *et al.*, 2005). These deficits in T cell mediated cytokine production were proposed to contribute to the higher susceptibility to Q fever found for subjects carrying HLA-DRB1*11.

It appears that although *C. burnetii* generates T cell responses that assist in bacterial clearance and contribute to protective immunity *in vivo*, vaccination or infection may also result in suppression of lymphoproliferative responses in both antigen-specific and antigen-non-specific ways. Mice have been shown to develop lymphocyte non-responsiveness to T cell mitogens when infected with phase I organisms (Scott *et al.*, 1987). While higher suppression rates were noted in animals that were injected with viable organisms, immunosuppressive effects were also observed in mice vaccinated with whole inactivated phase I organisms (Damrow *et al.*, 1985). This suppressive effect is not limited to mitogen responsiveness. In fact, intra-peritoneal vaccination of an inactivated whole cell phase I preparation resulted in spleen cells that displayed significantly decreased responsiveness to antigen-specific stimulation *in vitro*, perhaps due to the presence of regulatory or suppressive T cells (Damrow *et al.*, 1985). A small study found that antigen-specific immunosuppression was present in patients with Q fever endocarditis in comparison with those with Q fever hepatitis and acute Q fever, with *C. burnetii* antigen-specific lymphoproliferation among those with Q fever endocarditis being comparable with that found in uninfected controls (Koster *et al.*, 1985b). It was deduced that a suppressor cell contributed to the immunosuppression seen in these patients, and due to the retention of

antigen-specific responses to *Candida* antigens, that this immunosuppression was *C. burnetii* antigen-specific (Koster *et al.*, 1985b). It has been hypothesised that *C. burnetii* exposure over a period of time results in clonal expansion of antigen-specific suppressor CD8+ T cells (Koster *et al.*, 1985a). Suppressor cells are believed to be generated in an antigen-specific manner, and subsequently act by down-regulating costimulatory activity on APC that present their cognate antigen (Suciu-Foca *et al.*, 2005). Although suppressor cells (now more commonly referred to as T regulatory cells) are considered important in regulation of the immune response to prevent immunopathology, it appears that in the context of Q fever, suppressor T cells may be associated with an inability to clear *C. burnetii*, given that antigen-specific immunosuppression is associated with chronic disease.

The importance of both antigen presentation by MHC and CD28 co-stimulation in inducing specific T cell activation has been well characterised (Harding *et al.*, 1992; Gimmi *et al.*, 1993), however it appears that generation of a T cell response to *C. burnetii* infection is not dependent on CD28 co-stimulation. There were no measurable differences in granuloma formation, IFN-γ production or TNF-α production by $CD28^{-/-}$ mice in comparison to wild type mice, suggesting that co-stimulation, usually associated with prevention of T cell anergy, was not required for a robust cellular immune response (Honstettre *et al.*, 2006). In fact, mice lacking CD28 demonstrated lower bacterial levels in the spleen and liver, perhaps due to impaired IL-10 production in comparison to that seen in wild type mice (Honstettre *et al.*, 2006). The finding that CD28 is not essential for clearance of *C. burnetii* infection may not be surprising when considered alongside evidence that $CD28^{-/-}$ mice are able to control infections of *Mycobacterium bovis* (Hogan *et al.*, 2001) and were able to

generate antigen-specific T cell responses to *Listeria monocytogenes* infection (Mittrucker *et al.*, 2001). While it has not been demonstrated in these mice that the T cell responses are generated via non-specific monocyte-mediated activation, the mitogenic capacities of *C. burnetii* LPS have been well characterised and may drive the protective immune responses seen in *C. burnetii*-infected $CD28^{-/-}$ mice.

LPS derived from bacteria other than *C. burnetii* have been documented to have indirect mitogenic effects on T cell responses, however LPS-stimulated T cell proliferation, accompanied by IFN-γ and IL-2 gene expression, has been reported to be donor specific, with only 50% of subjects undergoing T cell proliferation in response to LPS or synthetic lipid A (Mattern *et al.*, 1994). In these LPS-responsive subjects, LPS-activated monocytes are capable of activating T cells *in vitro*, even when LPS is no longer present in culture and only minor differences exist between memory and naïve T cell LPS responsiveness, confirming that T cell activation by LPS is not an antigen-specific process (Mattern *et al.*, 1994). Unlike B cells, which may show some mitogenic tendencies in response to *C. burnetii* LPS, T cell activation by LPS is thought to be dependent on contact with monocytes (Izzo *et al.*, 1991), although studies of T cell lines suggest that a subset of T cells may be capable of responding to LPS directly, without the involvement of monocytes or B cells (Vogel *et al.*, 1983). A number of studies suggest that while pathogen-derived peptides drive antigen-specific T cell responses to *C. burnetii* (Jerrells *et al.*, 1975), indirect LPS-monocyte mediated T cell responses are also likely to be important *in vivo*. Phase I LPS has been shown to be mitogenic in guinea pig leucocytes and induces non-specific resistance to infection in mice (Paquet *et al.*, 1978). In mixed cultures of lymphocytes and

phagocytes, lymphocyte responsiveness to both protein and carbohydrate components likely relates to antigen-specific T cell responses and non-specific monocyte activation of T cells respectively (Heggers *et al.*, 1974). The overall T cell response to *C. burnetii* in an individual is therefore likely to be the result of antigen-specific T cell responses, monocyte driven antigen non-specific T cell activation, and balanced by regulatory T cell activity.

3.9 Summary of host response to infection

C. burnetii displays an ability to modify or suppress the immune response, inducing changes that enhance bacterial replication and survival within the host. For example, dendritic cells, important for antigen processing and presentation to prime responses in naïve T and B lymphocytes, display inhibited maturation in the presence of *C. burnetii* and have deficient antigen presenting capabilities (Shannon *et al.*, 2005a). Furthermore, although infected phagocytes such as monocytes and macrophages are able to express soluble *C. burnetii* antigens on MHC class II molecules and prime responses in naïve T cells, macrophage interaction with *C. burnetii* phase I LPS also results in prostaglandin production and subsequent suppression of IFN-γ and IL-2 production by CD4+ and CD8+ T cells (Izzo and Marmion, 1993).

The importance of the humoral immune responses to *C. burnetii* is unclear, although many studies have proven that sera from immune animals have functional effects *in vitro* and that humoral responses have some protective effects *in vivo*. For instance, antibody-mediated opsonisation has been shown to enhance *C. burnetii* phagocytosis by host macrophages

(Kazár *et al.*, 1975), and the production of specific antibody appears to play a role in vaccine generated immunity (Zhang *et al.*, 2007). However, the development of specific antibody is not always detectable in cases of *C. burnetii* infection, and it is thought that T cell responses play a greater role in limiting Q fever.

Generation of a strong Th1 type (Mosmann and Coffman, 1989) immune response is generally critical in the clearing of intracellular pathogens, including *M. tuberculosis* (Rook, 2007) and *C. burnetii* (Zhang *et al.*, 2007). The Th1 cytokine, IFN-γ, has potent immunomodulatory effects on the *C. burnetii*-infected macrophage, acting to restore bactericidal activity and enhance control of infection (Howe *et al.*, 2002; Brennan *et al.*, 2004). Vaccine-induced protection from *C. burnetii* infection occurs in association with a strong pro-inflammatory Th1 type immune response (Zhang *et al.*, 2007). *C. burnetii* is able to actively inhibit the generation of Th1 cytokines, thereby skewing the response towards an ineffective Th2-biased response. The ability of phase I organisms to suppress IFN-γ and IL-2, both key Th1 cytokines, is important for *C. burnetii*'s pathogenesis (Izzo and Marmion, 1993).

More recent research has demonstrated that stimulation of monocyte-derived macrophages with *C. burnetii* results in an atypical M2 polarisation, resulting in the down regulation of pro-inflammatory cytokines such as TNF-α, upregulation of the anti-inflammatory cytokine TGF-β1, and in suppression of nitric oxide production, making these macrophages less able to clear intracellular pathogens (Benoit *et al.*, 2008a). Thus, skewing of the immune

response towards an anti-inflammatory biased response, in terms of both T cell responses and macrophage activity, appears to be associated with the pathogenesis of Q fever.

C. burnetii survives in inhospitable intracellular environments, targeting immune cells as sites of infection and replication, and subverts the immune system, driving responses that are inappropriate for its clearance and permissive of infection. Despite the organism's ability to modify host responses and subvert the immune response, chronic infection affects only a subset of human hosts, suggesting that the immune response is typically sufficient to clear infection. The mechanisms that mediate its virulence remain quite poorly understood, particularly as they relate to cases of human infection. It remains unclear whether factors relating to the pathogen, or the host response to infection, contribute to the complications and variable illness course reported in subjects with acute Q fever.

3.10 Aims and hypotheses

Acute Q fever is often a severe and debilitating illness, however significant inter-patient variance in symptom severity has been reported. It was hypothesised that demographic factors or patterns of symptom prevalence may provide an indication of an individual's likelihood of suffering from severe or prolonged duration of acute Q fever illness. Thus, the first aim of this study was to characterise the manifestations of *C. burnetii* infection in an Australian prospective cohort, to compare the prevalence and severity of physiological, psychological and fatigue related symptoms amongst subjects with varied illness course, and to determine whether demographic variables may be associated with the risk of severe or prolonged acute Q fever.

There are varied reports suggesting a role for persistence of *C. burnetii* in the development of chronic Q fever, and in some subjects *C. burnetii* remains detectable for years after acute infection. It was hypothesised that prolonged symptomatic Q fever may relate to an ongoing immune response due to the persistence of *C. burnetii* organisms within the host providing ongoing antigenic challenge. An aim of this work was to determine whether persistence of *C. burnetii* is associated with illness course in this cohort.

The study also aimed to elucidate the role of Toll-like receptors in the host innate immune response to *C. burnetii*. Conflicting reports suggest a role for TLR-2 or TLR-4 in the interaction of *C. burnetii* with the innate immune system. It was hypothesised that the role of these receptors in the host response to infection could be elucidated by examing the *C.*

burnetii-induced cytokine production when one or both of these receptors was inhibited by monoclonal blocking antibody treatment, and that the patterns of cytokine production would provide information about the signalling transduction pathways that are triggered in response to *C. burnetii*. If TLR-2 and TLR-4 are important in infection, then it was hypothesised that functional polymorphisms in these genes may be associated with an increased susceptibility to symptomatic illness. Evidence for a role of polymorphisms in TLR-2 and TLR-4 were sought by genotypic screening for three documented functional polymorphisms.

Finally, this work aims to characterise the adaptive immune response in subjects who suffer prolonged illness or who recover promptly from infection. In particular, it was hypothesised that *C. burnetii*-specific circulating antibodies may be elevated or show specific patterns of isotype titres in subjects with prolonged illness, or that ongoing symptoms may be associated with the level or activation state of circulating leukocyte subsets. This work aimed to quantify factors of the host immune response in individuals with varied illness course using longitudinally collected peripheral blood samples and to determine whether differences in immune activation are associated with illness severity or course.

Part II: Research Studies

4. Natural history of Q fever: DIOS cohort

4.1 Introduction

Q fever is a highly infectious zoonotic disease which is endemic worldwide (Norlander, 2000). Domestic animals such as sheep and cattle that are infected by *C. burnetii* excrete infectious particles in their blood and body fluids, resulting in formation of infectious dust-like particles (Stoker and Marmion, 1955) which when airborne cause infection in humans via inhalation of very low infective doses (less than 10 organisms) (Tigertt *et al.*, 1961; Moos and Hackstadt, 1987). Acute Q fever and its chronic sequelae are among the most serious infective hazards for occupational health.

In Australia, Q fever is a notifiable disease with annual infection rates of 2.7-4.9 per 100,000 population reported for 1991-2002. These figures are likely to be a substantive under-estimate of the true incidence due to the non-specific "influenza-like" disease manifestations, the lack of awareness of Q fever among general practitioners, and resistance to seeking treatment among rural workers who become unwell (Owen *et al.*, 2007). The prevalence of DTH skin test reactivity and/or Q fever antibodies amongst adults in rural communities in eastern Australia is 20-30% (Garner *et al.*, 1997; Hutson *et al.*, 2000; Taylor *et al.*, 2001), suggesting that subclinical seroconversion may be common in regions where exposure to infected animals provides a high risk of *C. burnetii* infection. Effective immunization against Q fever is available only in Australia via the purified, killed phase I *C. burnetii* vaccine (QVax^{TM}, CSL Pty Ltd, Australia) which confers long-lasting and high levels of protective cellular immunity (Marmion *et al.*, 1984; Ackland *et al.*, 1994). Since

2002, the annual incidence of Q fever has recently been declining with the implementation of a national vaccination strategy targeting workers of the meat and livestock industries who are most at risk of exposure (Owen *et al.*, 2007). Prior to this vaccination campaign, Q fever had been estimated to cost the Australian meat industry approximately A$1 million in workers' compensation and 1700 weeks in lost work time annually (Garner *et al.*, 1997).

The clinical manifestations of acute Q fever vary widely from individual to individual. At the mild end of the spectrum, Q fever is often asymptomatic, or minimally symptomatic and unrecognised, while the acute illness may be a severe debilitating disease with fever, headache, myalgia and drenching sweats. In patients with symptomatic illness, acute Q fever may be complicated by pneumonia, hepatitis and more rarely by neurological or cardiac manifestations. In a small minority of patients the organism may establish chronic localised infection, most commonly with vegetations containing viable organisms on the heart valves (endocarditis), but may also affect the liver, kidneys, joints and bone marrow with granulomatous inflammation (Karakousis *et al.*, 2006). Prolonged illness occurs in approximately 10% of subjects who suffer acute Q fever, marked by fatigue and constitutional symptoms with associated disability lasting 6 months or more (Hickie *et al.*, 2006). This prolonged illness is consistent with the post-infective fatigue syndrome described following Epstein-Barr virus infection (White *et al.*, 1995). In the context of Q fever infection this has been referred to as post Q fever fatigue syndrome (QFS) (Penttila *et al.*, 1998).

The Dubbo Infection Outcomes Study (DIOS) aims to examine factors contributing to severity and course of infectious illness in a prospective cohort of subjects diagnosed with acute Epstein-Barr virus (EBV), Ross River virus (RRV) or Q fever (QF) infections. The study has been established for more than a decade, recruiting individuals from the rural township of Dubbo (New South Wales, Australia) and surrounding regions. A significant proportion of the working population in that region has a high risk of exposure to *C. burnetii* due to employment in the farming industry, or in meat processing in abattoirs in the region. Liason between local pathology services and general practitioners allows the identification of individuals within the study region who are suspected to be suffering acute illnesses caused by the three pathogens of interest: *C. burnetii* (Q fever), Ross River virus (RRV) or Epstein-Barr virus (EBV).

The work presented here examined the impact of acute Q fever in the DIOS cohort, and makes comparisons of the clinical features of Q fever in subjects suffering from "high" or "low" severity of acute Q fever (see definitions following). A number of studies have suggested that a protracted illness course following acute Q fever may be driven by persistence of *C. burnetii* within the host (Harris *et al.*, 2000; Iwakami *et al.*, 2005; Marmion *et al.*, 2005). In addition, it has been hypothesised that chronic antigen stimulation may drive aberrant cytokine responses in subjects with prolonged symptoms of fatigue (Penttila *et al.*, 1998; Wildman *et al.*, 2002). Sensitive real time PCR assays have been used on DNA derived from patient blood or bone marrow samples to demonstrate the presence of *C. burnetii* genomes. However, culture of the organism has been largely unsuccessful, raising questions as to whether intact organisms, alive or dead, remain within

previously infected subjects (Marmion *et al.*, 2005). Two sensitive and validated real time PCR assays were therefore conducted on longitudinally-collected DNA samples from subjects with varied illness duration to examine whether persistence of *C. burnetii* genomic DNA was associated with symptoms of post Q fever fatigue in the DIOS cohort.

4.1.1 Aims and hypotheses

It was hypothesised that the varied clinical course of acute Q fever may be explained by demographic factors, or by persistence of *C. burnetii* within the host.

The aims of this chapter were to:

(1) characterise the natural history of acute Q fever in a prospective Australian cohort

(2) seek evidence for demographic variables that may be associated with severe or prolonged acute Q fever illness

(3) determine whether prolonged symptomatic illness may relate to persistence of the infecting pathogen

4.2 Subjects and methods

4.2.1 Subjects

Subjects suffering acute Q fever, EBV or RRV infections were recruited from the rural community surrounding Dubbo, approximately 400km west of Sydney, Australia.

Individuals presenting to their general practitioner with an illness suggestive of one of these three infections were serologically tested, and those with pathogen-specific IgM titres diagnostic for the relevant infection type were approached by the research team for enrolment into the study.

Registered nurses employed in Dubbo by the study coordinated enrolment of subjects and the collection of blood samples and clinical data. Blood samples were either processed at a laboratory established for DIOS in Dubbo, or transported to Sydney for processing within 8h of collection. The study employs research nurses and research assistants to collect and process questionnaire data and peripheral blood samples. These employees managed data entry, blood processing and sample storage (plasma, serum, peripheral blood mononuclear cells, DNA, RNA) according to the protocols described here.

The Dubbo Infection Outcomes Study has been funded by the National Health and Medical Research Council of Australia, the Centers for Disease Control and Prevention (USA), the Australian Meat and Livestock Council, the Australian Rotary Health Research Fund and the Mason Foundation Australia. The study has human research ethics approval from the University of New South Wales.

4.2.2 Serological confirmation

Serological testing was conducted on study samples to confirm that each subject was suffering acute Q fever. Sera collected at enrolment and later time points (3 months, 12

months) were tested for the presence of Q fever specific IgG and IgM using standard diagnostic ELISA kits according to the manufacturer's instructions (PanBio, Sinnamon Park, Australia) and/or by complement fixation (CF) or immunofluorescence assay (IFA) using assays developed in the Australian Q fever reference laboratory at the Institute of Medical and Veterinary Science (IMVS). Confirmation of acute Q fever infection required one or more of the following: IgG seroconversion, or a four fold increase in IgG antibody titre (CF or IFA), or a high CF titre (≥32) plus IgM positive for at least one time point in addition to that recorded by the general practitioner, or anti-complementary CF, but IgM positive at two or more time points, or a four fold decrease in CF titre between baseline and late time points. Only subjects with serologically confirmed acute Q fever were included in the studies reported here.

4.2.3 Symptom data

Detailed information regarding the clinical course of illness was obtained for each subject, using a combination of self-report questionnaires and semi-structured interviews. A symptom onset date was recorded for each patient, no greater than 6 weeks prior to enrolment. A detailed medical history interview was conducted at enrolment, and subjects who reported excessive alcohol consumption, or who were currently taking medications with known hepatotoxic effects, were excluded from further analysis in this study. After enrolment, subjects were followed up at regular intervals by a study nurse to conduct clinical interviews, to ensure completion of self report questionnaires and to collect blood samples. Each subject was assessed at enrolment, 2-3 weeks, 4-6 weeks, and 3 months time points. Subjects still unwell at 3 months were further assessed at 6 months and 9

months. All subjects were reviewed at 12 months, with further half-yearly visits for those not yet recovered from their illness.

The questionnaires used to collect symptom data include the Somatic and Psychological HEalth REport (SPHERE, 34 items, self-report, see Appendix 1), the Physical Symptom Checklist (PSC, 17 items, self-report, see Appendix 2) and Brief Disability Qustionnaire (BDQ, 8 items, self-report, see Appendix 3). The SPHERE consists of 34 questions relating to the presence of physical and psychological symptoms associated with acute illness (Hickie *et al.*, 2001). The SPHERE was developed to concurrently screen for fatigue states and mood disorders in primary care settings. The questions were derived from the GHQ-30 (General Health Questionnaire), a widely used screening instrument for major depression (Goldberg and Williams, 1988), and the SOFA questionnaire (or "SOMA") which was developed and validated for the identification of patients with clinically significant fatigue states (Hadzi-Pavlovic *et al.*, 2000).

The PSC was devised by the DIOS investigators to assess the presence of symptoms associated with acute illness that were not otherwise captured in the SPHERE (for example, night sweats) (Hickie *et al.*, 2006). The BDQ enabled the assessment of social and occupational role impairment, by documenting difficulty in performing vigorous exercise such as running, or self-help activities such as bathing (von Korff *et al.*, 1996). Items in the BDQ also collated information about illness-associated confinement to bed rest ("days in bed") or inability to fulfill their usual activities ("days out of role").

The standardised responses for each item in the SPHERE and PSC questionnaires are scored 0 for “never or some of the time”, 1 for “a good part of the time” and 2 for “most of the time”. In some instances, these data were simplified into “presence” (score of 1 or 2) or “absence” (score of 0) of each item. A score of 0 (or “absence”) in this algorithm actually reflected a statement of “never or some of the time”, making these measures conservative estimates of symptom incidence.

SPHERE data collected at enrolment for n=73 subjects with serologically-confirmed acute Q fever were collated to determine the ten most commonly reported physical and psychological symptoms (using the “presence” / ”absence” designation). These data were then collated across all time points by “days post symptom onset” rather than by the arbitrary study time point labels (“2 weeks”, “6 weeks” etc) due to significant inter-subject variance in the time taken to enrol into the study after illness onset. Missing data were extrapolated in a conservative fashion using the following rules:

- Subjects with data for fewer than 3 time points were excluded from analysis (n=6);
- A single negative response resulted in that symptom being designated as permanently resolved for that subject (i.e. later time points were zero-ed);
- Missing data points take the value of the next available data point;
- Subjects lost to follow up were designated as recovered.

The proportion of subjects who continued to report each symptom at each time point was then determined.

Within the SPHERE, the six somatic items constituting the SOMA subscale that best predict clinically relevant fatigue states have been validated by physician and psychiatric review (Hickie *et al.*, 2001; Hickie *et al.*, 2006). The SOMA score (see Appendix 4), calculated by summing the numerical values assigned to each of the six questions (values of 0, 1 or 2 for each question), is predictive of both patient- and clinician-assessments of disability ($r=0.61$, $P<0.001$) with a validated cut-off score of 3 (out of a possible 12) on the SOMA subscale indicating a 'clinically-significant' fatigue state (Hickie *et al.*, 1999; Koschera *et al.*, 1999; Hadzi-Pavlovic *et al.*, 2000). A subject was designated as "SOMA positive" (i.e. a case of clinically significant fatigue) at any time point where their SOMA score was 3 or more.

In this study, illness duration was estimated as the length of time for which the subject remained fatigued (i.e. consistently SOMA positive), or more specifically, the number of days between the symptom onset date to the mid-point of the first SOMA negative time point and the closest preceding SOMA positive time point. This conservative estimate of ongoing fatigue due to illness ensured that the duration measure reflected the persistence of clinically significant illness in each subject. Subjects remaining unwell (i.e. consistently SOMA positive) six months after symptom onset were examined by a physician and a psychiatrist, and laboratory investigations were undertaken, to ensure that ongoing symptoms were associated with Q fever and not a result of another underlying medical or psychiatric disorder (e.g. hypothyroidism, sleep disorder). Each subject with ongoing symptoms of fatigue as a result of Q fever was designated as a "case" of post Q fever fatigue syndrome. Age and sex matched subjects who had recovered promptly from acute

Q fever were also invited to a six month medical and psychiatric examination, and acted as a "control" for each case.

A principal components analysis (PCA) of data from the SPHERE questionnaire was conducted to derive a severity score for each subject at enrolment. Data collected at baseline in the SPHERE questionnaire were selected for possible inclusion in the PCA. Items were excluded if more than 60% of the group reported a "0" ("never or some of the time") for that particular symptom. Items given low coefficients (e.g. <0.3) in the first component were sequentially removed from the analysis. This process was repeated until the first component accounted for >40% of the variance in the dataset. The items included in the severity index included physical symptoms, psychological symptoms and fatigue-related items. The entire cohort was divided by mean split of this PCA severity index into "low" severity and "high" severity groups. In confirmation of this designation, the high severity group was found to have significantly increased reported "days out of role" and "days in bed" in the last month (at enrolment) in comparison to the low severity group (student's t test, $p<0.05$, see section 4.3 Results).

4.2.4 Blood collection, processing and cryopreservation

All processes described in this section were performed by staff employed by the study. Peripheral blood was collected from each subject at each visit under endotoxin-minimised conditions. Anti-coagulated blood (approximately 80mL, in acid-citrate-dextrose or ACD) and coagulated blood (approximately 15mL) was generally collected in the subject's home, then transported within an insulated esky (in summer with an ice pack included) to ensure

the samples were not exposed to extremes in temperature. This was confirmed by the inclusion of a TinyTag Ultra temperature recorder in the esky, which monitors temperature and records data every 15min. Data from these temperature recorders indicate that temperatures within the esky varied between 12-26°C with an average temperature of 20°C.

Bloods were processed either in a dedicated laboratory in Dubbo, or couriered by air to Sydney and processed within 8h of collection. From every blood collection, plasma, serum and peripheral blood mononuclear cells (PBMC) were separated and stored. For baseline samples, genomic DNA was also extracted using the Wizard DNA extraction kit (Promega, Madison, USA) according to the manufacturer's instructions. DNA was quantitated using a NanoDrop™ 1000 spectrophotometer and ND 1000 software package (Fisher Thermo Scientific). DNA purity was confirmed by measuring the optical density ratio (260nm/280nm, a value of 1.8-1.9 being acceptable). DNA quality was confirmed by agarose gel electrophoresis; a single band indicated intact DNA, while a smear indicated that degradation had occurred. DNA samples that were degraded or did not meet purity standards were re-extracted from stored PBMC.

Blood processing involved centrifugation of all tubes (400g, 10min) to allow removal of plasma from ACD tubes, and serum from coagulated blood tubes. Aliquots of plasma and serum were stored in Nunc cryovials (Thermo Fisher Scientific, Suwanee, USA) at -80°C prior to transfer to either liquid nitrogen or nitrogen vapour phase storage units. A minimum of 5mL plasma was retained at 4°C for later use in cryopreservation of PBMCs. The remaining red cells and buffy coat in each ACD tube were pooled into two Falcon™

50mL polypropylene tubes (BD Biosciences, San Jose, USA), and diluted to 35mL in phosphage buffered saline (PBS, GIBCO® Invitrogen, Carlsbad, USA). In each tube, 15mL of Lymphoprep™ (Axis-Shield, Oslo, Norway) was under-laid beneath the blood/PBS mixture using a sterile syringe and canula, and then tubes were centrifuged (400g, 25min, no brake). The buffy coat was aspirated using a sterile transfer pipette to a fresh 50mL tube, and washed twice with PBS with centrifugation (400g, 10min) and removal of supernatant between each wash.

If the PBMC were not for immediate use, they were cryopreserved as follows: the PBMC were resuspended in autologous plasma and quantitated using a COULTER Ac·T™ analyser (Beckman Coulter, Fullerton, USA). The cell suspensions were adjusted to a concentration of 10-15x10^6cells/mL in autologous plasma, and then mixed with an equal volume of complete medium (RPMI 1640 medium supplemented with penicillin (100U/mL), streptomycin (100µg/mL), L-glutamine (2mM) (all GIBCO® Invitrogen))/20% DMSO (Sigma Aldrich, St Louis, USA). Aliquots of PBMC were transferred to Nunc cryovials, placed in a Nalgene® Mr. Frosty freezing container (Sigma Aldrich) and stored at -80°C for a minimum of 4h before vials were transferred to liquid nitrogen or nitrogen vapour phase storage vessels for long term storage.

4.2.5 *C. burnetii* detection

To determine whether persistence of *C. burnetii* was associated with prolonged illness in this cohort, *C. burnetii* genomes were sought in longitudinally collected peripheral blood samples from patients with varied illness course. Two sensitive real-time PCR assays were

used. These assays targeted the *com1* and *IS1111a* sequences in *C. burnetii*, and were developed by the Australian Rickettsial Reference Laboratory, in Geelong, Victoria, for use in clinical diagnostic tests for Q fever in peripheral blood and tissues samples.

The *com1* assay utilises forward (5' AAAACCTCCGCG TTGTCTTCA 3') and reverse (5' GCTAATGATACTTTGGCAGCGTATTG 3') primers to amplify a 76bp region. The probe (5'AGAACTGCCCATTTTTGGCGG CCA 3') was dual-labeled with fluorophore (5' 6-FAM) and quencher (3' BHQ-1). As the probe binds to the complementary target DNA sequence, the quencher is cleaved by enzymatic primer extension, such that increases in fluorescence accompany target sequence amplification. Each 25µL reaction included 5µL DNA sample, 400nM of each primer (Invitrogen) and 200nM of probe (Biosearch Technologies, Novato, USA) in Platinum® Quantitative PCR Supermix-UDG (Invitrogen). Reactions were performed using a Rotor Gene RG-3000 machine, set to hold the sample at 50°C for 3min, then polymerase activation at 95°C for 5min, followed by 65 cycles of denaturation at 95°C for 20s, annealing and extension at 70°C for 40s. Fluorescence was recorded during the annealing and extension stage of each cycle.

A second assay targeting *IS1111a*, an insertion sequence found at multiple copy number in most strains of *C. burnetii*, was developed to enhance sensitivity of *C. burnetii* detection. The forward (5' GTTTCATCCGCGGTGTTAAT 3') and reverse (5' TGCAAGAATACGGACTCACG 3') primers amplified a 105bp fragment of the insertion sequence, which was the target of the dual-labeled probe (5' 6-FAM-CCCACCGCTTCGCTCGCTAA-BHQ-1 3'). Each 25µL reaction included 5µL DNA

sample, 1000nM forward primer (Invitrogen), 800nM reverse primer (Invitrogen), 50nM of probe (Biosearch Technologies) and 4.5mM $MgCl_2$ in Platinum® Quantitative PCR Supermix-UDG (Invitrogen). Each sample was held at 50°C for 2min, then polymerase activation at 95°C for 2min, followed by 65 cycles of denaturation at 95°C for 10s, annealing and extension at 60°C for 20s. Fluorescence was recorded during the annealing and extension stage of each cycle.

Subjects were classified as having "short" (SOMA negative within 6 weeks), "intermediate" (became SOMA negative after 3-6 months), or "prolonged" (SOMA positive for greater than 6 months) duration of illness. DNA was extracted from nine subjects with short illness duration, four subjects with intermediate duration and 17 subjects with prolonged duration of illness. All subjects had PBMC samples available for three or more time points, including one sample within two weeks of enrolment, and one sample from at least six months post symptom onset. As a negative control, DNA samples from five individuals who were seronegative and Q fever delayed type hypersensitivity (DTH) skin test (QVax, CSL Ltd) negative were included in each run. All DNA was extracted from a minimum of $5x10^6$ PBMC, resulting in a minimum concentration of 50ng/µL, and a maximum concentration of 200ng/µL, with an OD260/280 ratio range 1.7-2.0. Approximately 25,000 monocytes (the target cell for coxiella infection) were thereby represented in each reaction.

4.2.6 Statistical analyses

Statistical analyses were performed using SPSS for Windows (v17). Multiple regression analysis was used to confirm independence of the PCA-derived severity index from age and gender. Pearson correlations were sought between illness severity and disability. Time course analyses (Kaplan Meier) were used to assess the impact of illness severity on illness duration.

4.3 Results

4.3.1 Demographics and illness characteristics

Cases of acute Q fever were detected by coded monitoring of all positive Q fever IgM serology tests reported by pathology services in the study region. These potential study subjects were contacted through their general practitioner and invited to enrol in the study. A total of 115 subjects were enroled with suspected Q fever, of whom 94 were males (82%) with a median age of 38 years (range 16-73 years). Of the subjects who were provisionally enroled with Q fever, 73 met strict serological diagnostic criteria for acute Q fever infection. These confirmed acute Q fever cases form the basis of this study; unconfirmed subjects were excluded from further analysis. A comparison of the illness characteristics of the acute Q fever cohort and the control EBV and RRV subjects is given in Table 4.1.

Table 4.1 Characteristics of the DIOS cohort

Illness characteristic	Q fever	EBV	RRV
Number of subjects	73	126	96
Mean fatigue score at enrolment	6.2	6.0	6.3
Mean number of days in bed in the last month	8.5	7.5	2.5
Mean number of days out of role in the last month	15	13.7	10.1
Mean illness duration (days)	143	112	129

The median age for the cohort with confirmed acute Q fever was 39 years (range 16-73 years). Men were substantially over-represented (n=62, 85%), likely due to occupational biases in exposure rates. To confirm the role of occupational exposure as a risk factor for contracting Q fever, demographic data were collected to record employment in rural positions. Midway through the study, a variation was made to the demographics questionnaire and therefore this occupational risk information was not available for the entire cohort, however 36 of 42 subjects with confirmed acute Q fever (86%) reported having worked as a shearer, grazier, abattoir worker, or in other rural positions. This contrasts with the data obtained for other subjects in the study (EBV or RRV) where 105 of 193 (54%) reported working in these occupations. In the case of Q fever subjects enroled later in the study, 26 of 28 confirmed Q fever cases (93%) were exposed to livestock in the 6 weeks prior to symptom onset, in contrast with 44% (44 of 101 subjects) of the control cohorts who reported exposure to livestock in the previous 6 weeks. In this cohort,

exposure to animals, through occupation or otherwise, likely represents a key risk factor in contracting *C. burnetii* infection.

Q fever was often severe, with 23 subjects (32%) being hospitalised during the acute illness. Given the known variance in the clinical manifestations of Q fever, all subjects were screened for pneumonia and hepatitis. Pneumonia was identified by chest x-ray in only two subjects (3%). Blood tests were used to quantitate liver enzyme levels in peripheral blood from each subject, including markers of hepatocyte integrity (alanine aminotransferase or ALT, normal range 0-40IU/L, and aspartate aminotrasferase or AST, normal range 0-40IU/L), and markers of cholestatic damage (alkaline phosphatase or ALP, normal range 50-150IU/L, and γ-glutamyl transferase or GGT, normal range 0-50IU/L). Biochemical hepatitis (defined here as elevation of any of these liver enzyme levels) was present in 47 subjects (64%) during the acute phase of the Q fever illness.

Health care utilisation due to acute Q fever was assessed at six weeks post illness onset in a subset of patients who were asked to recall all medical care received from the onset of acute Q fever symptoms. Due to changes in reporting methods, this information was only available for 13 subjects. The acute illness resulted in an average of three visits (range 0-10) to a general practitioner, and an average of five visits (range 0-15) in total to any health care professional, including visits to general practitioners, specialists, allied health care professionals and alternative therapists. Forty-eight subjects (66%) reported use of analgesics (predominantly paracetamol) to manage symptoms of the acute illness. Although antibiotic therapy was prescribed for 51 patients, these subjects tended to report a

longer duration of illness (mean of 167 days vs 91 days for the untreated subjects, p=0.074, student's t test). The patients receiving antibiotic treatment also tended to report more severe acute illness (mean severity index of 0.14 vs -0.19 for the untreated subjects, p=0.237, student's t test). This suggests a prescribing bias, where subjects suffering more severe illness were more likely to be treated with antibiotics, and therefore a direct effect of antibiotic therapy on the duration of illness could not be determined.

The BDQ questionnaire provided information about the disability experienced by each subject as a result of acute Q fever. At enrolment, Q fever subjects reported a mean of 8 days in which they were confined to their bed ("days in bed") and a mean of 15 days away from work or out of their usual role ("days out of role") in the acute illness, indicating that acute Q fever is associated with significant disability. This was confirmed by self-reported inability or difficulty in performing tasks, including vigorous activity (84% of subjects), moderate activity (66%) and even basic self-care tasks such as bathing (41%). Furthermore, the acute illness resulted in decreased motivation to do work (70% of subjects), a perceived decrease in efficiency when performing tasks at home or work (63%), and deterioration in social relationships (57%). Thus, it can be inferred that in a majority of subjects, acute Q fever resulted in substantive physical as well as psychological and social impairment.

To assess the severity of the acute illness, self-report data from the SPHERE questionnaire at enrolment to the study (baseline) were collated for the 73 confirmed acute Q fever subjects. Principal component analysis was conducted to determine which items from these questionnaires contributed the most variance to this dataset, and to derive a single "severity

score" for each subject that reflected this variance in symptom reporting. The PCA-derived severity index featured typical dimensions of acute Q fever including fever, headache, sleep disturbance, pain, and fatigue. The questions that were included in the index are listed in Table 4.2. The resultant severity index accounted for 42.5 % of the variance in the dataset. The components loadings given for each item that contributes to the severity index indicate the correlation between each item and the derived severity index.

Table 4.2 Items included in the PCA-derived severity index

Questionnaire	Item #	Item description	Component loading
SOMA	1	muscle pain after activity	0.615
SOMA	2	needing to sleep longer	0.616
SOMA	3	prolonged tiredness after activity	0.564
SOMA	4	poor sleep	0.613
SOMA	6	tired muscles after activity	0.524
SPHERE	1	headaches	0.666
SPHERE	2	feeling irritable or cranky	0.705
SPHERE	4	pains in your arms or legs	0.750
SPHERE	5	feeling nervous or tense	0.682
SPHERE	7	rapidly changing moods	0.613
SPHERE	9	nausea	0.640
SPHERE	10	arms or legs feeling heavy	0.675
SPHERE	13	fevers	0.630

Questionnaire	Item #	Item description	Component loading
SPHERE	14	back pain	0.698
SPHERE	18	joint pain	0.763
SPHERE	19	weak muscles	0.656
SPHERE	20	feeling frustrated	0.682
SPHERE	22	getting annoyed easily	0.632
SPHERE	23	everything getting on top of you	0.684
SPHERE	24	dizziness	0.643
SPHERE	25	feeling tired after rest or relaxation	0.583

The severity scores were normalised around a mean value of zero, and the cohort was divided into groups suffering from either high or low severity of acute Q fever (mean split). A comparison of these two groups is provided in Table 4.3. The severity index correlated with "days in bed" ($r=0.34$, $p=0.011$) and "days out of role" ($r=0.270$, $p=0.049$) (both Pearson correlations). Multiple regression analysis determined that age ($r=-0.065$, $p=0.584$) and gender ($r=0.222$, $p=0.059$) were not predictors of severity, although women in the Q fever cohort tended to report greater severity of the acute illness than men. The subjects requiring hospitalisation were evenly distributed amongst the "high" and "low" severity groups, perhaps suggesting a tendency in this cohort to under-report the symptoms of their illness.

Table 4.3 Characteristics of Q fever-associated disability in subjects with "high" and "low" severity of acute illness

Disease parameter	High severity mean (SD)	Low severity mean (SD)
Total number of subjects	33	40
Median age (range)	38 (18-73)	39.5 (16- 66)
Number of women (%)	8 (24)	3 (8)
Number who were hospitalised (%)	12 (36)	11 (28)
Number with biochemical hepatitis (%)	23 (70)	24 (60)
Number with pneumonia (%)	2 (6)	0 (0)
Mean fatigue score at enrolment (SD)	8.8 (1.8)	3.7 (2.3)
Mean number of days in bed in the last month (SD)	10.1 (8.4)	6.2 (5.2)
Mean number of days out of role in the last month (SD)	17.2 (10.8)	12.8 (10.4)

The median delay between onset of Q fever illness and enrolment to the study was 28 days (range 3-70 days). To ensure that delays to enrolment did not prevent the capture of clinical data relating to the acute phase of Q fever, attempts were made to correlate this delay with reported or derived measures of the acute illness. No correlation was observed between the delay in recruitment and severity (Pearson correlation, r=0.156, p=0.187). This confirmed that the severity index was independent of any significant effect arising from delays in subject enrolment into DIOS. Importantly, delays to enrolment also did not

correlate with other reported measures of the acute illness, including disability measures ("days out of role", "days in bed", both Pearson correlations, r=-0.015, p=0.913 and r=0.158, p=0.253 respectively) and SOMA score (Pearson correlation, r=-0.180, p=0.193) at the "baseline" time point. The reported variance in severity and disability due to Q fever across different subjects was therefore not likely to be a consequence of the varied time taken to enrol subjects into the study.

The duration of acute Q fever was compared in subjects with "high" or "low" severity of acute Q fever (Figure 4.1). Individuals with "high" severity of the acute phase of illness had a significantly longer duration of post Q fever illness, remaining positive for fatigue caseness (SOMA positive) for a mean of 241 days in comparison with individuals with "low" severity illness (mean of 68 days) (Breslow test, χ^2=28.555, p=0.000). Approximately 20% of subjects with "high" severity of illness at enrolment remained symptomatic for one year or longer, while only one subject with "low" severity of acute illness remained unwell for a comparable period.

Analysis of the longitudinally collected self-report data regarding ongoing symptoms revealed that the most frequently reported illness manifestations included physical symptoms ('fever', 'headache', 'joint pain'), prominent symptoms of fatigue ('waking up feeling tired', 'weak muscles', 'heavy arms and legs', 'feeling tired after rest'), and psychological symptoms ('feeling frustrated', 'irritable', 'easily annoyed'). The proportions of the group reporting these symptoms at each time interval post illness onset

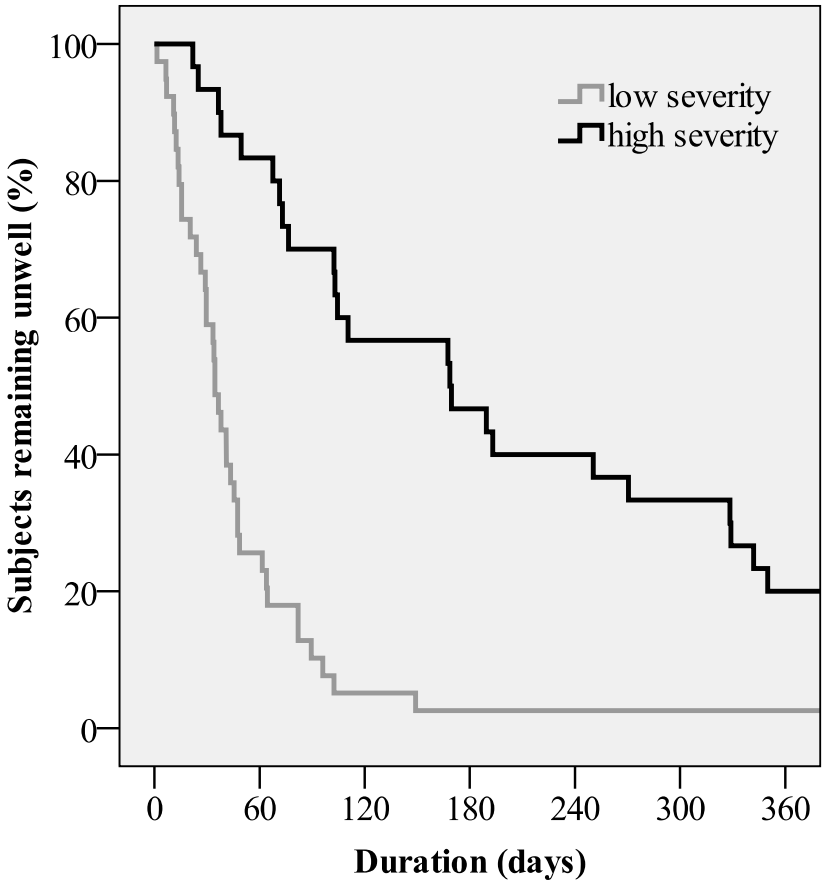

Figure 4.1: Severity predicts duration of acute Q fever. A severity factor score derived by principle components analysis was used to divide the cohort by mean split into those suffering from 'low' or 'high' severity of acute illness. Duration was calculated from the number of days each subject remained symptomatic following the self-reported symptom onset date. Duration data was available for n=30 subjects with 'high' severity and n=39 subjects with 'low' severity of acute Q fever.

are shown in Figure 4.2. The patterns of symptom resolution were broadly comparable across physical, psychological and fatigue symptoms, although there were subtle differences in the resolution of self-reported fevers, which were no longer present in the vast majority of patients after 6 weeks of illness, in comparison to the slower resolution of fatigue-associated symptoms.

A significant proportion of subjects reported ongoing symptoms, including physical symptoms, 3 months or more after the illness onset. This pattern of persistent symptoms after acute Q fever was associated with a significant impact on daily activities, even up to 6 months after the onset of illness. Subjects who remained as fatigue cases 3 months after enrolment into the study reported an average of 9 days out of their usual role in the last month, and an average of 1 day in bed. At 6 months after enrolment, subjects who remained as fatigue cases reported an average of 5 days out of their usual role and approximately 2 days in bed in the last month due to ongoing Q fever illness.

Subjects who remained unwell were examined by a physician and psychiatrist at the 6 month time point to confirm that ongoing symptoms were a result of their initial Q fever illness and not due to underlying physical or psychiatric illness. The cohort was also specifically evaluated for clinical or laboratory signs of chronic, localised Q fever. Five subjects retained high phase I-specific antibody titres that were suggestive of possible chronic, localised infection. One individual, who had a congenital heart defect and a heart murmur, was referred for a transoesophageal echocardiogram. A diagnosis of Q fever endocarditis was excluded. One individual with clinical signs of hepatitis was screened for

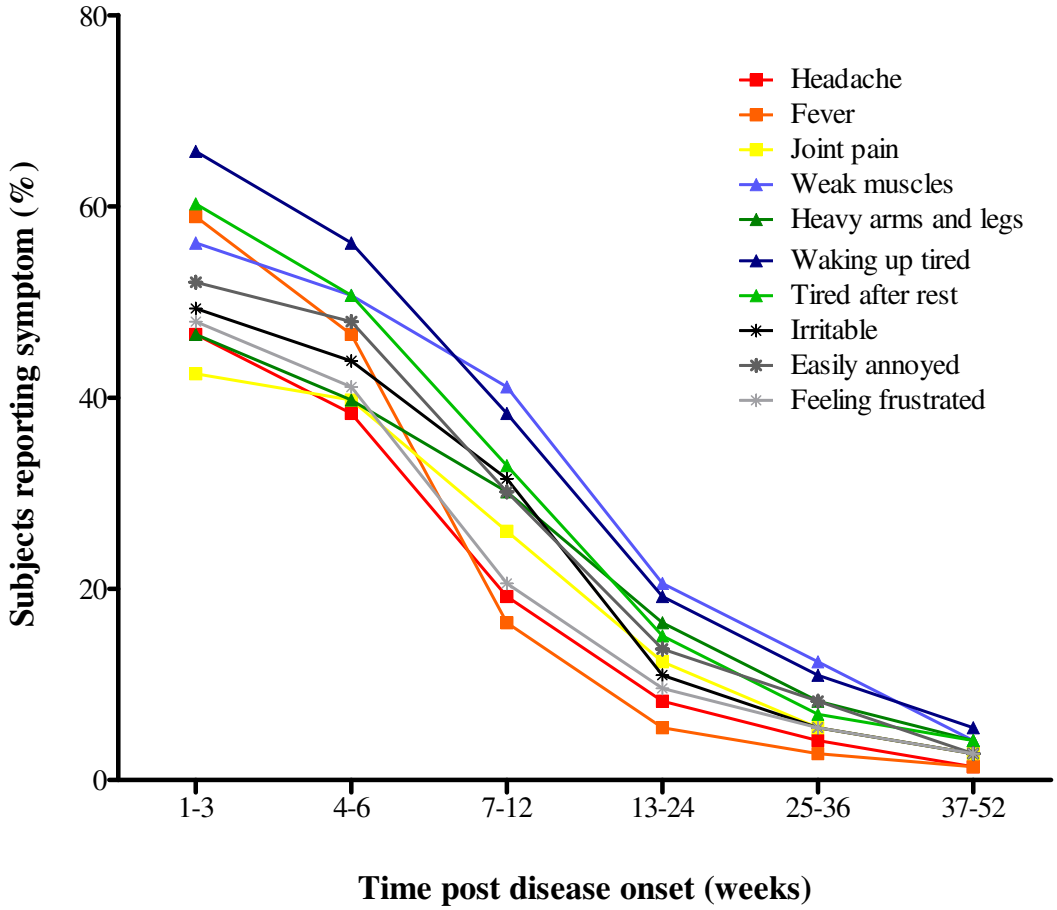

Figure 4.2: Persistence of symptoms in acute Q fever. Data was collected from longitudinal self-reported SPHERE questionnaire (n=73) for the ten most frequently reported symptoms at enrolment, and grouped by days post self-reported symptom onset date. The graph represents the proportion of subjects who continue to report each symptom at each time point.

granulomatous hepatitis, however histological examination of liver tissue taken by biopsy revealed non-alcoholic steato-hepatitis (NASH) and liver function test changes resolved with dietary modification as ongoing symptoms of fatigue relating to Q fever remained. The remaining three subjects underwent further serological testing; all demonstrated reducing phase I-specific antibody titres in subsequent time points, thus chronic, localised infection was no longer suspected. Therefore, there were no cases of chronic, localised Q fever infection identified in this cohort.

4.3.2 Persistence of *Coxiella burnetii* within the host

It has previously been hypothesised that ongoing symptoms of illness in subjects with acute Q fever are a result of ongoing antigenic stimulation due to persistence of low levels of *C. burnetii* within the host (Marmion *et al.*, 2005). To test this hypothesis in the DIOS cohort, two sensitive real-time PCR assays were utilised for detection of *Coxiella burnetii* within PBMC samples. Target sequences were chosen within the *C. burnetii com1* gene, and within an insertion sequence (*IS1111a*) that is found at multiple copy number within the *C. burnetii* genome (Klee *et al.*, 2006). Real time PCR assays to detect these target sequences were optimised by Michelle Lockhart at the Australian Rickettsial Reference Laboratory, Geelong, Australia, but were conducted by the candidate.

Each PCR target sequence was cloned into a plasmid vector for use as a positive control, and for determination of each assay's range of sensitivity. Plasmid concentration was estimated by spectrophotometric measurement of the absorbance at 260 nm (conc = A_{260} x

50μg/mL), and copy number was then estimated using the following formula, assuming an average base pair molecular weight of 660mol/g:

Copy number (mol/mL) = concentration (μg/mL) x 1mol/660g x 1/N

where N = number of base pairs of plasmid; then

Copy number (/mL) = copy number (mol/mL) x Avagardro's constant (6.022×10^{23})

Serial dilutions of this stock produced a standard curve ranging from an estimated 0.01 copies per reaction up to 1,000,000 copies per reaction, which was then used to determine the detection limit for each reaction. A minimum of 10 target copies per reaction was required for successful detection in both the *com1* and *IS1111a* assays (for example, see Figure 4.3). Although both assays had similar sensitivities (10 copies per reaction), given the multiple copy number of *IS1111a* in *C. burnetii*, this assay was considered potentially more sensitive in its capacity to detect *C. burnetii* genomes.

To confirm that the presence of contaminating genomic DNA in a subject's sample would not inhibit detection of the *C. burnetii* target sequences, plasmid DNA was spiked into samples of genomic DNA from three individuals with no exposure to Q fever. In Table 4.4 it can be seen that at low target copy number, human genomic DNA did not prevent detection of the *IS1111a* target by real time PCR, and that excess genomic DNA did not result in any false negative determinations. Similarly, human genomic DNA did not inhibit the *com1* assay (data not shown).

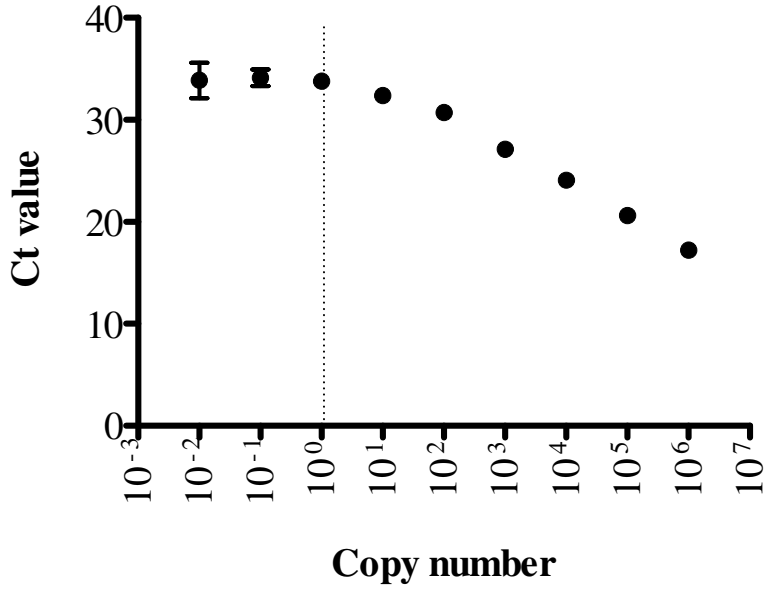

Figure 4.3: Sensitivity of *com1* PCR assay. Serial dilutions of a 4005bp plasmid containing the *com1* gene target sequence were assayed by real time PCR. The dashed line indicates limit of quantitative range for target sequence detection (10 copies per reaction). Values are mean and standard error of two replicates.

Table 4.4 Human genomic DNA does not inhibit *IS1111a* detection by real time PCR

IS1111 target copies/reaction	Expected Ct value	Observed Ct values			Average observed Ct value (SD)
		Donor 1	Donor 2	Donor 3	
2.83	34.57	32.37	31.75	32.84	32.32 (0.54)
28.27	31.85	34.36	31.85	35.28	33.83 (1.78)
282.72	29.12	30.76	31.56	30.32	30.88 (0.63)
2827.17	26.39	28.01	28.02	27.73	27.92 (0.16)

Having validated the sensitivity of the assay and confirmed that genomic DNA did not prevent detection of the target sequences, these two assays were used to detect *C. burnetii* genomes in longitudinal DNA samples from subjects with "short", "intermediate", or "prolonged" duration of Q fever. The number of positive results obtained from either assay is summarised in Table 4.5. *C. burnetii* detection was seen more frequently in samples taken from subjects with prolonged illness. *C. burnetii* was undetectable in late time points from subjects whose Q fever symptoms had resolved, raising the possibility that persistence of the organism may play a role in triggering ongoing symptoms in those patients with prolonged illness.

Table 4.5: Detection of *C. burnetii* DNA by duration of Q fever and sample time point

Illness Duration	Sample time point[#]		
	0w-2w	4w -3m	6m - LFU
Prolonged (>6 months, n=9)	7 (10)	3 (8)	6 (15)
Intermediate (>6 weeks but <3 months, n=4)	0 (7)	0 (6)	0 (4)
Short (<6 weeks, n=17)	4 (22)	2 (16)	0 (16)

Samples positive in one or more assay (total samples tested)

While positive results were seen in some samples with both PCR assays, many samples were positive in only one of the PCR assays. Furthermore, detection of *C. burnetii* was not always consistent across longitudinally collected samples from the same patient. Thus, detection of *C. burnetii* genomes in a late time point was often accompanied by a lack of *C. burnetii* detection in earlier time point samples from the same subject. It was perhaps unexpected that *C. burnetii* genomes were not detectable in any of the early time point samples from the subjects with intermediate duration of illness, although the early time point DNA samples that were screened for *C. burnetii* (designated "0w-2w") were in fact an average of 4-5 weeks post symptom onset, by which time bacterial clearance may have occurred. In general, Ct values were high, and although melting curves were indicative of genuine target sequence amplification, the copy number per assay was believed to be very close to each assay's limit of detection. The rare detection of *C. burnetii*, and the high Ct values that accompanied positive detection, would be consistent with the notion that *C. burnetii* genomes occur at very low copy number in the peripheral blood of subjects who have recently had Q fever. Comparisons of the rates of detection between subjects with

varied duration of illness might suggest that pathogen persistence drives the ongoing symptoms of fatigue that are seen in subjects with prolonged illness, however it is plausible that *C. burnetii* persists at extremely low levels in all subjects, regardless of clinical outcome, and that assay limitations prevented their detection. A conservative interpretation of the data would therefore prevent drawing conclusions about the role of persisting organisms in driving ongoing symptoms in subjects with prolonged illness.

4.4 Discussion

This study provides the first systematic documentation of the prolonged natural history of serologically-confirmed acute Q fever infection. The prospective cohort described here includes 73 individuals with serologically confirmed acute Q fever cases. Although 115 subjects were provisionally diagnosed with Q fever on the basis of a consistent illness and a single positive IgM ELISA test result, 42 were not confirmed to be attributable to acute Q fever as IgG seroconversion, or fourfold changes in titre, were not found in longitudinally collected sera. This finding affirms previous assertions that a definitive diagnosis of acute Q fever requires paired testing of acute and convalescent sera to detect changes in antibody production consistent with recent infection (Dupont *et al.*, 1994). False-positive Q fever IgM antibody results have been found in subjects suffering from other infectious diseases, including those resulting from *Mycoplasma pneumoniae*, *Bordetella pertussis*, or *Legionella* sp infections (Finidori *et al.*, 1992; Devine *et al.*, 1997; Musso and Raoult, 1997). Use of Q fever specific ELISA to detect IgM has been demonstrated to have considerably lower specificity than equivalent tests for IgG (Slaba *et al.*, 2005). High titres

of *C. burnetii*-specific IgG may also interfere with serological testing and cause false positive IgM test results (Soriano *et al.*, 1993), such that individuals with prior Q fever infection may be more likely to return false-positive IgM test results. False-positive IgM antibody results are recognised to also be prevalent in the context of acute viral infections associated with polyclonal hypergammaglobulinaemia, notably in EBV infection (Robertson *et al.*, 2003).

In this cohort, contact with livestock was the predominant risk factor for contraction of Q fever, reflected by an over-representation of men within the cohort who reported occupational exposure in the meat and livestock industries. A subset of subjects (15%) did not report any contact with livestock in the 6 weeks prior to illness onset, suggesting alternative exposure mechanisms, perhaps via inhalation of windborne contaminated dust. Wind has been implicated in other studies of *C. burnetii* transmission, where there has been no direct contact between infected animals or their waste products and the humans who become infected (Jorm *et al.*, 1990; Hawker *et al.*, 1998; Tissot-Dupont *et al.*, 1999; Brouqui *et al.*, 2004; Tissot-Dupont *et al.*, 2004).

The most common end-organ complication of acute Q fever in this cohort was biochemical hepatitis which occurred in almost half of cases, whereas pneumonia occurred in only two subjects. Clinical manifestations of Q fever have been documented to vary geographically. In the south of France, hepatitis complicating acute Q fever is more common than pneumonia, while in Canada and the Basque region of Spain, pneumonia is a more common manifestation of infection (Marrie *et al.*, 1985; Montejo Baranda *et al.*, 1985; Raoult *et al.*,

2000). Our findings are consistent with an early report of Q fever in Australia which documented cases of pneumonia to be rare (Spelman, 1982). It has been suggested that pre-existing bronchitis may be associated with the development of Q fever pneumonia (Domingo *et al.*, 1999), so it is possible that the clinical manifestations vary in parallel to differing population health in various geographical regions. Alternatively, the biological basis of these varied manifestations may result either from strain variation in *C. burnetii* or genetic factors of the host population.

In the subjects described here, acute Q fever was often severe, as evidenced by a high rate of hospitalisation and ongoing health care utilisation following diagnosis in the majority of subjects. Furthermore, a significant proportion of subjects reported fatigue and other physical or psychological symptoms of illness lasting six months or longer. The disability associated with Q fever has been documented to have substantive economic impact. More than 50% of Q fever cases in New South Wales, Australia in 1993-94 resulted in workers compensation claims, costing an average of $2,190 per claim (Garner *et al.*, 1997). Estimation of the total economic impact in Australia is problematic as data is not available for the medical costs and lost work time in cases where occupational exposure was not the cause of Q fever acquisition, although the cost to the meat industry in 1993-94 was proposed to be nearly A$1 million (Garner *et al.*, 1997). The implementation of a vaccine strategy targeting individuals in high risk occupations is now complete, and has led to a reduction in Q fever notifications in Australia and presumably this has been accompanied by a decrease in the economic cost of this illness (Owen *et al.*, 2007). Despite the availability of a protective vaccine, there was continued identification of individuals within

the study region who had contracted acute Q fever, suggesting that uptake of this preventative option remains sub-optimal.

In comparison to men, very few women were enroled into DIOS with acute Q fever. Gender differences in infectious disease diagnoses and illness severity may derive from genuine differences in susceptibility and symptom severity, or may result from socioeconomic factors resulting in differing health care access and/or illness reporting. Sex differences in severity of infectious illnesses have been observed in leptospirosis: incidence rates are higher in men, and men suffer from more severe illness than women when the prevalence of symptoms such as jaundice, hemorrage and renal impairment were compared (Jansen *et al.*, 2007), suggesting that biological factors may contribute to this sex difference. By contrast, many studies in low income countries find that women are less likely than men to access health care for the treatment of tuberculosis and that this accounts for varied incidence rates between men and women (Connolly and Nunn, 1996). In Australia, where a diagnosis of Q fever carries no stigma and where health care is freely accessible, it seems likely that a difference in the diagnosis of Q fever between men and women reflects true differences in the rate of infection. The higher number of men enroled into DIOS with Q fever can likely be attributed to higher rates of occupational exposure to *C. burnetii*, rather than increased susceptibility to illness in comparison to women.

Although not statistically significant, women tended to report higher severity of the acute illness. This is surprising, given that women are reported to be less likely than men to suffer symptomatic illness when exposed to *C. burnetii*. Q fever seroprevalence rates have been

reported to be approximately equal in men and women, despite clinical cases being more commonly identified in men (Tissot Dupont *et al.*, 1992). Furthermore, oestradiol, a female sex hormone, has been shown in *C. burnetii*-infected mice to reduce bacterial loads (Leone *et al.*, 2004), suggesting that women might be better able to control infection than men. It is therefore unknown why women in this cohort tended to report greater severity of acute Q fever. It is possible this relates to gender bias in the pattern of reporting symptoms and severity. Expansion of the cohort to include more subjects would confirm whether this trend was significant.

In this cohort, higher severity of acute Q fever correlated strongly with prolonged illness and with the reported level of disability due to Q fever. Given that the production of pro-inflammatory cytokines in acute Q fever has been shown to correlate with symptom severity (Vollmer-Conna *et al.*, 2004), it appeared plausible that prolonged symptoms of fatigue following infection may be a result of host immune factors. There are two scenarios in which the immune response may drive prolonged symptoms following infection. Firstly, that infection is not completely cleared, and the presence of *C. burnetii* within the host drives low level antigen-specific immune responses and hence symptoms remain unresolved. Secondly, that the early host immune response to infection induces lasting changes in the body that result in ongoing disease symptoms in the absence of ongoing infection.

In contrast to previous reports (Harris *et al.*, 2000; Iwakami *et al.*, 2005), this study provides ambiguous support for the assertion of persistence of *C. burnetii* in association

with post Q fever fatigue. *C. burnetii* was detected in a minority of samples, and was not consistently detected across longitudinal samples from a single patient, suggesting that if *C. burnetii* remains in the blood of subjects who have had Q fever, it does so at very low copy number. Thus, it remains possible that persistence of very few organisms or their antigenic remnants may still drive ongoing disease manifestations, or that tissue-based infection (eg in bone marrow or spleen) may harbour persistent organisms without comparable infection in circulating leucocytes. This hypothesis is supported by the previously reported detection of *C. burnetii* DNA in 65% of bone marrow aspirate samples, but only 17% of circulating leucocyte samples, from patients with post Q fever fatigue syndrome using PCR techniques capable of detecting a single gene target (Harris *et al.*, 2000). An uncontrolled pilot study of antibiotic treatment indicated benefit in some cases of post Q fever fatigue, suggesting that presence of viable organisms contributes to the ongoing symptoms (Iwakami *et al.*, 2005). However, others have found evidence of *C. burnetii* DNA within bone marrow aspirates up to 12 years post-infection, in a small proportion of subjects, regardless of their clinical outcome (Marmion *et al.*, 2005). The role of persisting organism in driving ongoing symptoms of Q fever in DIOS subjects with prolonged illness therefore remains unclear, although the results reported here do support the notion that levels of *C. burnetii* within the peripheral blood are extremely low, regardless of clinical outcome.

4.5 Conclusions

Q fever is a severe and debilitating illness that often requires substantive medical care, including hospitalisation for a significant proportion of subjects. The predominant

manifestation of acute Q fever in this cohort was fever and constitutional symptoms, with or without biochemical hepatitis, while pneumonia was extremely rare. Of those subjects recruited to DIOS with acute Q fever, the vast majority were men, presumably due to the tendency for men to be employed in occupations with high risk of exposure to *C. burnetii*, including employment in the agriculture or meat industries.

Detection of *C. burnetii* in longitudinal DNA samples from patients with varied illness duration failed to demonstrate a consistent pattern of pathogen persistence in association with prolonged symptoms. Instead, it is possible that *C. burnetii* persists in all subjects, regardless of clinical outcome. It remains to be definitively determined whether fatigue following acute Q fever occurs due to the presence of ongoing, low level, antigenic stimulation.

A key predictor of illness duration was the severity of the acute illness – higher severity correlated with more prolonged illness. A heightened immune response during the acute phase of the illness may induce biological changes in the brain, and drive ongoing fatigue in the absence of ongoing *C. burnetii* infection or immune activation. Further investigation of the factors determining the host immune response to infection is therefore warranted.

5. Innate immune responses to *C. burnetii*

5.1 Introduction

A common feature of all Gram negative organisms, including *C. burnetii*, is the presence of lipopolysaccharide (LPS) in the outer cell wall. There are two serologically distinct phases of *C. burnetii*, designated phase I and phase II, which differ only in the structure of the LPS components which are somewhat analogous to the smooth and rough phase variants of *E. coli* respectively (Hackstadt *et al.*, 1985). Phase II organisms contain one-tenth the quantity of LPS present in phase I organisms (Baca *et al.*, 1980), and the saccharide chain length of phase II LPS is significantly truncated in comparison to phase I LPS (Amano and Williams, 1984).

The virulence of *C. burnetii* depends heavily on the LPS phase, with phase I organisms being virulent in a mammal host, while phase II organisms are avirulent (Moos and Hackstadt, 1987). The variance in virulence observed between organisms of different phase has been proposed to relate to the impaired host immune response that is induced by exposure to phase I organisms in comparison to phase II organisms. Phase I organisms are a poor inducer of dendritic cell maturation (Shannon *et al.*, 2005a), perhaps because the longer, branch LPS structure shields immuno-stimulatory epitopes that are exposed in phase II organisms.

Cells of the innate immune system possess receptors that enable them to identify pathogens and initiate appropriate immune responses, which may include phagocytosis of the

pathogen and the production of cytokines to drive innate and adaptive responses. In the case of Gram negative pathogens, host Toll-like receptor (TLR)-4 typically interacts with LPS in the pathogen cell wall to initiate the host immune responses, and mice deficient in this receptor are hyporesponsive to LPS (Hoshino *et al.*, 1999). By contrast, recognition of lipoteichoic acid (LTA), an abundant component of the Gram positive cell wall, occurs via TLR-2 and does not involve TLR-4 (Schroder *et al.*, 2003b). It could be assumed that as *C. burnetii* is Gram negative and has LPS in its outer cell wall, TLR-4 would be involved in the innate immune response to *C. burnetii*. However, the structure and composition of *C. burnetii* LPS differs significantly with that found in other Gram negative organisms (Amano and Williams, 1984), and it is possible that *C. burnetii* LPS may be so structurally different that it does not interact with TLR-4. This would not be without precedent; there are other Gram negative organisms that have been documented to interact with TLR-2 and not TLR-4 (Kikkert *et al.*, 2007).

There are conflicting data from murine studies to suggest a role for TLR-2 and/or TLR-4 in the host immune response to infection. Both TLR-4 and TLR-2 are required for *in vivo* granuloma formation in mice (Honstettre *et al.*, 2004; Meghari *et al.*, 2005). *In vitro*, TLR-4 appears to be essential for the actin rearrangement that occurs during phagocytosis of *C. burnetii* (Honstettre *et al.*, 2004). Immune cells from mice that are deficient in TLR-4 generate abnormal cytokine responses *in vitro* in response to *C. burnetii* (Honstettre *et al.*, 2004), yet some studies suggest that lipid A from *C. burnetii* LPS actually acts as an antagonist of TLR-4 rather than an agonist, and can inhibit the interaction of TLR-4 with LPS from other bacterial species (Zamboni *et al.*, 2004). *C. burnetii*-induced cytokine

production has been suggested to occur via TLR-2 stimulation rather than via TLR-4, and a lack of TLR-2 has been associated with increased *C. burnetii* replication *in vitro* (Zamboni *et al.*, 2004). Mice lacking TLR-2 are susceptible to febrile illness when challenged with avirulent phase II *C. burnetii* strains and organisms are recoverable from exposed animals (Ochoa-Reparaz *et al.*, 2007). Regardless of whether *C. burnetii* activates host responses via TLR-2 or TLR-4, it is clear that the host response varies considerably with that induced by LPS from other bacterial species (Benoit *et al.*, 2008a).

The ability of cells of the innate immune system to sense bacterial ligands is dependent on the presence of functional TLRs. A number of studies have suggested that non-synonymous single nucleotide polymorphisms (SNPs) within TLR-2 and TLR-4 affect their receptor function, and have been implicated in susceptibility to, or outcomes from, infectious diseases. There are two SNPs within the intracellular domain of TLR-2 that have been implicated in susceptibility to infectious diseases (Schroder and Schumann, 2005). The TLR-2 Arg753Gln polymorphism has been associated with susceptibility to tuberculosis (Ogus *et al.*, 2004), and with acute rheumatic fever in children (Berdeli *et al.*, 2005). The TLR-2 Arg677Trp polymorphism was initially reported to be associated with lepromatous leprosy in a Korean population (Kang and Chae, 2001). More recent publications question whether the Arg677Trp was identified correctly in these subjects, given that a homologous duplicated sequence (including the hypothetical mutation site) occurs upstream of the TLR-2 start codon (Malhotra *et al.*, 2005), however functional deficiencies have been noted in subjects carrying the putative Arg677Trp polymorphism, including reduced capacity to produce IL-2, IL-12, IFN-γ and TNF-α in response to

Mycobacterium leprae in patients with lepromatous leprosy, while overexpressing IL-10 (Kang *et al.*, 2002; Kang *et al.*, 2004). The Arg677Trp polymorphism is also associated with unresponsiveness to *Mycobacterium tuberculosis* (Bochud *et al.*, 2003). Transfection studies confirm the functional deficits that either of these two polymorphisms confer; cells expressing TLR-2 carrying either the Arg677Trp or the Arg753Gln polymorphisms were unresponsive to bacterial lipopeptides (Lorenz *et al.*, 2000; Schroder *et al.*, 2003a). The prevalence of the TLR-2 Arg753Gln mutation is relatively low; it has been reported to be carried by approximately 3-9% of individuals in healthy Caucasian populations (Lorenz *et al.*, 2000; Schroder *et al.*, 2003a). By contrast, no cases of the Arg677Trp polymorphism have been found in Caucasian populations (Schroder *et al.*, 2003a; Texereau *et al.*, 2005), suggesting it may not be of relevance in the cohort described in this thesis which is predominantly Caucasian.

Polymorphisms in TLR-4 are associated with increased susceptibility to infectious disease, and carriage of polymorphic alleles has been associated with decreased responsiveness to LPS (Arbour *et al.*, 2000). Two SNPs in the extracellular domain of TLR-4 (Asp299Gln and Thr399Ile) have been reported to co-segregate (Hawn *et al.*, 2005). Studies reporting associations to one or both of these alleles do not necessarily indicate which allele confers functional differences, although overexpression studies suggest that it is the Asp299Gln polymorphism that confers decreased LPS responsiveness (Arbour *et al.*, 2000). Polymorphisms in TLR-4 have been implicated in susceptibility to septic shock, in particular in response to infections caused by Gram negative (i.e. LPS containing) organisms (Lorenz *et al.*, 2002). In one study, the presence of these polymorphic alleles

was associated with protection from Legionnaire's disease, suggesting that deficiencies in TLR-4 receptor function might reduce the immunopathology that is characteristic of some infectious diseases caused by Gram negative pathogens (Hawn *et al.*, 2005).

The ability of *C. burnetii* to establish infection within host macrophages and to replicate within the phagolysosome is dependent on inadequacies of the host immune response and subversion strategies evolved by the pathogen. A robust cellular response to infection can activate microbicidal activity in infected macrophages and lead to rapid *C. burnetii* clearance (Howe *et al.*, 2002; Brennan *et al.*, 2004; Zhang *et al.*, 2007). Comprehensive understanding of the way in which the host interacts with *C. burnetii* to activate appropriate immune responses requires identification of the receptors involved in pathogen recognition.

5.1.1 Aims and hypotheses

It was hypothesised that TLR-2 and/or TLR-4 play a role in pathogen recognition and induction of the monocytic response to *C. burnetii* infection.

The aims of this chapter were to:

(1) identify whether TLR-2 and/or TLR-4 interact with *C. burnetii* to cause induction of cytokine responses

(2) identify whether functional polymorphisms in TLR-2 and TLR-4 are associated with susceptibility to Q fever, or with severity of the acute Q fever illness

5.2 Materials and methods

5.2.1 Whole inactivated *Coxiella burnetii*

Antigens were required in order to examine the immune response that was recalled (*ex vivo*), or induced (*in vitro*), in response to *C. burnetii*. Whole, formalin inactivated phase I and phase II *C. burnetii* (Henzerling strain) was obtained at a concentration of 1mg/mL in PBS (PanBio, Brisbane, Australia). These preparations were supplied in highly pure form, having been extracted using Renografin density gradient centrifugation (Renografin is usually used clinically as an x-ray contrast medium; Bracco Diagnostics Inc, Princeton).

An aliquot of each vial of phase I and phase II organisms was examined using scanning electron microscopy (SEM) to verify the presence of coxiellas. Round glass coverslips (diameter 11mm) were coated with poly-L-lysine (Sigma Aldrich) according to the manufacturer's instructions. Ten micrograms of whole antigen in 50μL of PBS was loaded on to each coverslip, each contained in a Petri dish, and left undisturbed for 10min. Coverslips were then flooded with a fixative comprising 2.5% gluteraldehyde in 0.1M phosphate buffer, and incubated at room temperature for 2h. Using a transfer pipette, fixative was gently removed from the first coverslip and then replaced with phosphate buffer wash. This step was repeated for each coverslip. Following a second phosphate buffer wash step, a series of graded ethanol washes, from 30% to 100% ethanol in PBS, was conducted to gradually dehydrate the specimens. To ensure total dehydration, 100% ethanol washes were conducted twice further.

Specimens were then loaded with 100% ethanol in a critical point dryer (Bal-Tec, model CPD-030). Approximately 15-20 cycles were required to completely replace the ethanol in the chamber with liquid carbon dioxide. Heat and pressure were then increased so that the samples passed through the critical point of carbon dioxide (31.1°C, 73atm). The chamber was then depressurised under hot conditions, and dehydrated samples were coated immediately to prevent rehydration. To do this, each coverslip was mounted to an aluminium stub, and coated using chrome sputter coating (Emitech K-575X).

Specimens were visualised using a Hitachi S3400-I scanning electron microscope under high vacuum, using a 15kV electron beam and probe current size of 20nA. Multiple images were taken across the surface of the specimen stub at 100-10,000 fold magnification, corresponding to maximal resolution of 0.1μm.

5.2.2 Stimulation assays – THP-1 cells

A significant portion of this work aimed to examine the role of TLR-2 and/or TLR-4 in mediating the host response to *C. burnetii*. Monocytes were of particular interest given their role as the principal cellular target of *C. burnetii* infection. The monocytic cell line, THP-1 (ATCC number TIB-202^{TM}), derived from a 1 year old human male suffering from acute monocytic leukaemia, was used as a model of primary monocytes. This cell line was expanded in culture in complete medium supplemented with 10% foetal calf serum (FCS) and grown in suspension at $2x10^4$/mL – $1x10^6$ cells/mL with media replacement twice weekly. Aliquots of cells ($10x10^6$/cryovial) were cryopreserved in growth media supplemented with 5% DMSO (Sigma Aldrich) and stored in liquid nitrogen. Cells were

maintained at low passage number and discarded when visual inspection by light microscopy revealed morphological changes such as the formation of giant cell structures. Experiments were conducted on cells in logarithmic growth phase which was generally two days after media replacement.

The cell surface expression of TLR-2 and TLR-4 on these cells was confirmed by flow cytometry and compared with that of PBMCs from a healthy donor (Figure 5.1). THP-1 cells have abundant expression of TLR-2, while TLR-4 is less highly expressed, an expression pattern that is comparable in primary monocytes. THP-1 cells also express the monocyte markers CD14 and CD16, making them most comparable to the "inflammatory" monocyte subset. Interestingly, CD14 and CD16 expression appear to be much higher in primary monocytes in comparison to THP-1 cells, although this variance may relate to differing staining efficiencies in different cell types. Having confirmed the presence of the TLRs of interest and their co-receptor, CD14, THP-1 cells were deemed to be a reasonable surrogate for primary monocytes.

To assess the role of Toll-like receptors (TLR) in host recognition of *C. burnetii* infection and immune activation, THP-1 monocytes were stimulated in culture with whole inactivated antigen, with or without the addition of blocking antibodies to TLR-2 and TLR-4. THP-1 cells were cultured at either $1x10^6$ per mL per well in 24 well Costar flat-bottomed tissue culture treated polystyrene cell culture plates (Corning, Corning, USA), or $1x10^5$ per 100μL per well in 96 well Costar flat-bottomed tissue culture treated polystyrene

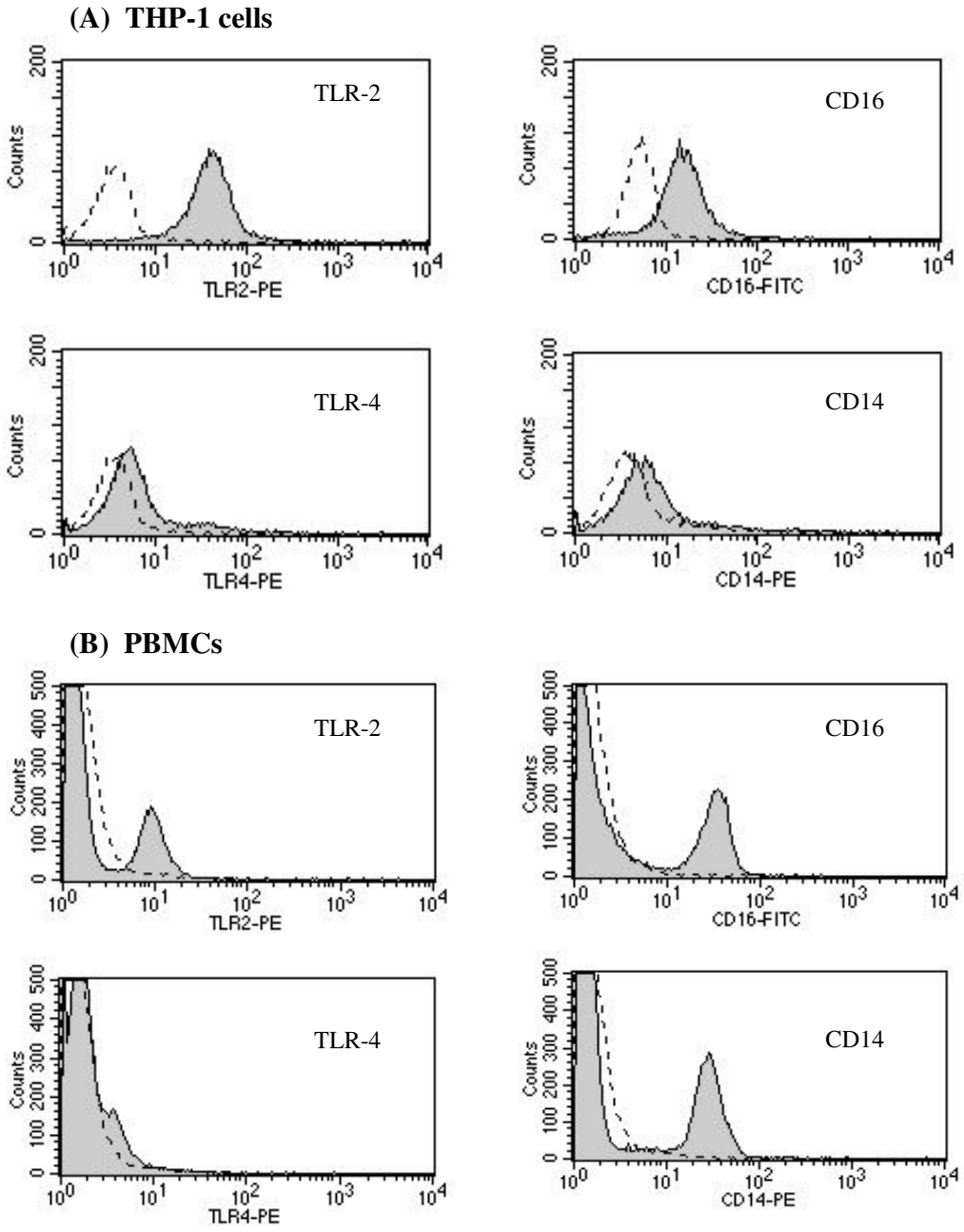

Figure 5.1: Expression of TLR-2 and TLR-4 on THP-1 cells and PBMCs. Cell surface expression of Toll-like receptors, as well as the monocyte markers CD14 and CD16, was assessed by flow cytometry of (A) THP-1 cells (top) and (B) PBMCs (bottom). Dashed lines are isotype controls. Grey shaded areas represent staining for each marker.

cell culture plates (Corning). Stimulus concentration, incubation time, and blocking antibody concentrations were optimised from dose-response and time course experiments.

Monoclonal blocking antibodies were used at 20µg/mL for clones HTA125 (anti-TLR-4) and TL2.1 (anti-TLR-2) (both eBioscience, San Diego, USA) (Tabeta *et al.*, 2000; Bes-Houtmann *et al.*, 2007). An equivalent quantity of an isotype control mouse IgG2a antibody (eBioscience) was used to control for non-specific antibody effects. THP-1 cells were pre-incubated with blocking antibody for 30 min at room temperature prior to stimulation. Whole inactivated phase I organisms or phase II organisms were used at 12.5µg/mL. Positive control stimuli were LPS (from *Escherichia coli* 0111:B4, 10ng/mL) and peptidoglycan (250ng/mL) (both Sigma Aldrich), for stimulation via TLR-4 and TLR-2 respectively. Cells cultured in complete media supplemented with 10% FCS acted as a "no stimulus" control. Stimulation occurred over 12h, incubated at 37°C/5% CO_2. Cell-free supernatants were stored at -80°C and later assayed by ELISA for detection of TNF-α, IL-10 and IFN-β that had been secreted into the cell culture media. Cell pellets were lysed in Trizol and stored at -80°C for later extraction of RNA and quantitation of mRNA via qPCR. When using a 96 well plate format, cells and supernatants from 3-6 replicate wells were pooled to give sufficient supernatant for ELISA measurements.

5.2.3 Stimulation assays – PBMC

While THP-1 cells are a frequently used model of monocyte activity, the project also aimed to confirm the findings in the cell line in primary cells. PBMC from healthy control subjects (n=3) were isolated from peripheral blood samples in ACD as described previously

(Section 4.2.4). Differential cell counts were conducted using a COULTER Ac·T™ analyzer. PBMC were seeded such that there were $2x10^5$ monocytes per well in complete medium/10% FCS. Stimulation and TLR blocking experiments were conducted as for THP-1 cells, with the exception that *C. burnetii* whole inactivated phase I and phase II antigens were used at a lower concentration of 2µg/mL. PBMC were stimulated for 3, 4.5, 6 or 12h. In a variation to this protocol, monocytes were isolated by adherence during a 12h incubation, with non-adherent cells washed off with warm PBS. Adherent monocytes were then used in blocking and stimulation assays. Cytokine induction was determined for each experiment by qPCR to quantitate mRNA, and ELISA to quantitate protein secretion.

5.2.4 Stimulation assays – primary monocytes

Given the difficulties encountered in working with mixed PBMC cultures (see following), experimentation was also conducted using cell preparations that were enriched for monocytes. Primary monocytes were enriched from PBMC samples from a single healthy donor using a MACS magnetic bead-based negative selection kit following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolation buffer (PBS supplemented with 0.5% FCS and 2mM EDTA) was de-gassed prior to use by placing the bottle under vacuum for at least 60min, and then cooled to 4°C. Meanwhile, PBMC were isolated from peripheral blood samples in ACD as described previously (Section 4.2.4). PBMC were washed twice in PBS using a long slow centrifugation step to allow removal of platelets (200g, 15min). Total cell counts were obtained using a Coulter counter (Beckman) and cells were resuspended in 30µL of isolation buffer per 10^7 cells in a 15mL Falcon™ tube (BD Biosciences). For every 10^7 cells, 10µL of 'FcR blocker' (human

immunoglobulin) and 10μL of 'biotin-antibody cocktail' (containing biotin-conjugated antibodies specific for CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A) were added, mixed thoroughly by flicking, and incubated at 4°C for 10min. Isolation buffer was then added (30μL per 10^7 cells), followed by 'anti-biotin microbeads' (microbead-conjugated monoclonal antibody, clone Bio3-18E7.2, 20μL per 10^7 cells), the tube mixed thoroughly by flicking and incubated for 15min at 4°C. Cells were then washed in 5mL isolation buffer, centrifuged to pellet the cells (300g, 10min) and resuspended in 500μL isolation buffer per 10^8 cells.

Magnetic separation of monocytes was conducted using a MACS CS column mounted in a VarioMACS™ separation unit (both Miltenyi Biotec). A 3-way stopcock attached to the bottom of the separation column allowed the positioning of a side-mounted 10mL syringe for back-flushing the column with isolation buffer. At the bottom of the stopcock, a flow resistor (23G needle) ensured slow passage of the cells through the column. The column was backfilled from the side-mounted syringe, before 2-3 column volumes (approximately 15mL) of cool isolation buffer were passed through the column prior to monocyte separation. The magnetically-labeled PBMC cell population was applied to the reservoir of the column, and allowed to pass through the column. Additional isolation buffer was applied to the top of the column, ensuring the reservoir never ran dry. A minimum of 6 column volumes (approximately 40mL) of isolation buffer was rinsed through the column to ensure maximal recovery of monocytes in the effluent.

Total cell count and viability was determined by manual cell count using a haemocytometer and the Trypan blue dye exclusion method. The enrichment for monocytes was confirmed via flow cytometry. Briefly, approximately 10^5 cells were stained with 10µL anti-CD14-PE antibody or 10µL isotype control antibody, and proportion of CD14+ cells determined by flow cytometry. In all instances, the resulting cell populations were enriched to >90% CD14+ cells.

The monocyte enriched cell population was then seeded at $2x10^5$ cells per well in 24 well Costar cell culture plates and utilised in TLR blocking and antigen stimulation assays as described for PBMC. A time course experiment was conducted over 24 hours to determine the kinetics of cytokine induction in these primary monocyte cultures, and subsequently blocking experiments were performed using anti-TLR-2 and anti-TLR-4 antibodies.

In a variation to the previously described protocols, one experiment made use of three different culture vessels to examine the role of monocyte adherence and consequent activation in the ability to respond to stimulation. Replicate experiments were conducted in Costar cell culture treated plates (to which monocytes adhere, Corning), Costar ultra-low attachement surface cell culture plates (to which monocytes do not adhere, Corning) or in 15mL Falcon™ tubes (BD Biosciences). The experiment conducted in 15mL tubes involved maintaining the tubes on an angle of approximately 15° from horizontal, on a rotating platform set to the minimum rotation rate. For all culture vessels the reaction volume of 1mL included $2x10^5$ cells per well or tube. Stimulation with or without blocking antibodies was conducted as described for PBMC samples. In all stimulation and blocking

experiments, cytokine induction was quantitated by qPCR (mRNA) and ELISA (protein secretion).

5.2.5 ELISA assays for quantitation of TNF-α, IL-10 and IFN-β

The effect of blocking antibodies in modulating the response of THP-1 cells, PBMC or primary monocytes to *C. burnetii* stimulation was assessed by determining the cytokine production by these cells. Cytokine secretion into culture supernatants was quantitated via ELISA of culture supernatants. Detection of TNF-α and IL-10 was conducted by use of Duoset ELISA development systems (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The provided capture antibodies were diluted to 4µg/mL (TNF-α) or 2µg/mL (IL-10) in PBS. A Maxisorp 96-well Nunc-Immuno Plate was coated with 100µL/well capture antibody and incubated at room temperature overnight. The ELISA plate was then washed three times with 200µl/well ELISA wash buffer (PBS/0.05% Tween) and blocked with 200µL/well ELISA reagent diluent (PBS/1% bovine serum albumin, 0.2µm filtered) at room temperature for 1h.

Culture supernatants were thawed to room temperature. Solutions of standard cytokine concentration were prepared in reagent diluent in doubling dilutions from a maximum concentration of 1000pg/mL (TNF-α) or 2000pg/mL (IL-10). Once blocking was complete, the plate was flicked to remove the reagent diluent, and gently blotted with paper towels. Standards and culture supernatants were added with 100µL/well in duplicate wells. Reagent diluent alone acted as a plate blank (negative control). The plate was incubated for at least 2h at room temperature prior to being washed three times with 200µL/well wash

buffer and gently blotted with paper towel. Detection antibody was diluted to an appropriate working concentration in reagent diluent of 250ng/mL (TNF-α) or 300ng/mL (IL-10) and 100µL added to each well, then the plate was incubated for a further 2h at room temperature.

The ELISA plate was then washed three times with 200µL/well wash buffer and gently blotted on paper towels. Streptavidin conjugated to horseradish peroxidase (Streptavidin-HRP) was diluted 1/200, then 100µl was added to each well. The plate was incubated for 20min at room temperature in dark conditions. The plate was washed as described previously, before addition of 100µL/well substrate solution (H_2O_2 mixed with tetramethylbenzidine in a 1:1 ratio, TMB). The plate was incubated in darkness and checked periodically for colour development as the blue precipitate formed. The required incubation time varied by cytokine, and ranged from as little as 15min for TNF-α assays to as much as 40min for IL-10 assays. The assay was stopped by addition of 50µL/well 2N H_2SO_4, and the plate read immediately on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, USA). The standard endpoint protocol used to read each plate measured the absorbance at a wavelength of 450nm and subtracted the absorbance at a reference wavelength of 540nm. All absorbance values were corrected using the plate blank well absorbance values. Standard curves were constructed, and unknown concentrations calculated, using SoftMax Pro 5 (Molecular Devices).

Quantitation of IFN-β was performed using a commercial kit (PBL InterferonSource, Piscataway, USA) according to the manufacturer's instructions. A standard curve was

constructed by dilution of the IFN-β cytokine standard in sample diluent to give a concentration range of 25-2000pg/mL, and then 100µL of standard or samples were added directly to duplicate wells of pre-coated plates. Sample diluent was added to duplicate wells as a plate blank (negative control). Samples were allowed to incubate in the plate for 1h at room temperature. The plate was then flicked empty, washed three times with 200µL/well wash buffer and gently blotted on paper towel. Antibody concentrate was diluted in concentrate diluent, 100µL was added per well, and the plate was incubated for 1h at room temperature. The plate was again washed as described previously, and 100µL/well of HRP-conjugate concentrate diluted in concentrate diluent was added. The plate was incubated for a further 1h at room temperature, and then washed as previously. Development of the plate was done as described for the TNF-α and IL-10 ELISA assays using TMB as substrate and H_2SO_4 as stop solution. Plates were immediately read exactly as described for the TNF-α and IL-10 ELISA assays.

5.2.6 RNA extraction

Following cell stimulation experiments, cytokine mRNA levels were quantitated to corroborate the data obtained by ELISA. RNA was extracted from the cell pellets resulting from THP-1, PBMC or primary monocyte stimulation experiments to quantitate relative expression of TNF-α, IL-10, IFN-β and iNOS mRNA. Each cell pellet was mixed thoroughly with 1mL of Trizol reagent (Invitrogen) in a sterile, RNase and DNase 1.8mL tube (Eppendorf, Hamburg, Germany) prior to storage at -80°C, or immediately processed for RNA extraction. To each tube, 200µL of chloroform was added, and shaken to mix. After standing at room temperature for 2min, each tube was spun at 12,000g for 15min at

4°C. The aqueous, upper layer was transferred to a clean tube, 0.5mL isopropanol was added to each tube, mixed by inversion and allowed to incubate at room temperature for 15min. The RNA was pelleted by centrifugation at 12,000g for 10min at 4°C, the supernatant discarded and the pellet washed in 1mL of 75% ethanol in DEPC treated dH_2O. Each tube was quickly vortexed, then the RNA pelleted by centrifugation at 7,500g for 5min at 4°C. The supernatant was carefully aspirated and the RNA pellet allowed to air dry. The dry RNA pellet was dissolved in dH_2O by heating to 55-60°C for 10min in a heat block. Samples were immediately cooled on ice and RNA quantitated using a NanoDrop™ 1000 spectrophotometer and ND 1000 software package (Fisher Thermo Scientific).

5.2.7 cDNA synthesis

Cytokine messanger RNA (mRNA) levels were quantitated by reverse transcription from single-stranded RNA to form double-stranded copy DNA (cDNA), and relative cDNA copy numbers were measured in a quantitative real-time PCR assay. Firstly, the extracted RNA was DNase treated using a Turbo DNase kit (Applied Biosystems/Ambion, Texas, USA) to remove any contaminating genomic DNA that may be present. In 200µL PCR tubes, each 10µL reaction included 2µg of RNA, 1µL of Turbo DNase 10x mastermix and 1µL of Turbo DNase enzyme in DEPC-treated dH_2O. Tubes were incubated at 37°C for 30min, then 1µL of 50mM EDTA was added per tube and incubated at 75°C for 10min.

Synthesis of cDNA from DNase-treated RNA was completed using the SuperScript™ III First-Strand Synthesis SuperMix kit (Invitrogen), which utilises a mix of random hexameric and oligodT primers. In 200µL PCR tubes, each 20µL reaction contained 10µL

of 2x reaction mix, 2μL of enzyme mix, 5μL of DNase-treated RNA, and PCR-grade dH_2O. Reactions were run in duplicate, with or without the enzyme mix, and the reactions lacking enzyme mix ("no-RT control") were a control for genomic DNA contamination of each RNA sample. Tubes were heated to 25°C for 10min, then 50°C for 30min, and the reaction terminated at 85°C for 5min. One microlitre (2U) of *E. coli* RNase H was added per tube, and then incubated at 37°C for 20min to remove any residual RNA. The resulting cDNA was stored at 4°C if intended for use within 2d, otherwise cDNA was stored at -20°C until needed.

5.2.8 Semi-quantitative real-time PCR

Changes in cytokine gene expression as a result of stimulation were measured as fold change in normalised expression levels in comparison to unstimulated cells. Sensitive real-time PCR assays were developed and optimised by Dr Ken Hsu and Dr Clovis Palmer, CIIR, for use in quantitation of TNF-α, IL-10, IFN-β and iNOS mRNAs. Quantitation of β-actin expression was also conducted for every cDNA sample included in each PCR run. Where possible, primers were designed to span introns, or individual primers located at an intron-exon boundary, to avoid the potential for amplification of contaminating genomic DNA. Forward and reverse primer sequences are shown in Table 5.1. Primers were purified by desalting, and supplied as lyophilised oligonucleotides (Invitrogen). Primers had melting temperatures of 65-73°C and GC contents of 45-55%. Each primer pair amplified a region of the specified gene of 150-250 base pairs in length.

Table 5.1 Primer sequences (5' – 3') used for qRT-PCR

Gene	Forward primer	Reverse primer
TNF-α	ATGAGCACTGAAAGCATGATCC	GAGGGCTGATTAGAGAGAGGTC
IL-10	TCAAGGCGCATGTGAACTCC	GATGCTAAACTCACTCATGGCT
IFN-β	GTCAGAGTGGAATCCTAAG	ACAGCATCTGCTGGTTGAAG
iNOS	ATCTGCAGACACGTGCGTTA	GATGAGCTGAGCATTCCACA
β-actin	GATCAAGTCATTGCTCCTCCT	TAGAAGCATTTGCGGTGG

Real-time PCR reactions were conducted in either 96-well plate or 384-well plate formats. The majority of experiments were conducted in a 384-well plate format to allow comparison of all genes of interest across all experimental samples. Reaction conditions were identical for either format, with the exception that reagent volumes were doubled when using the 96-well plate format to give a 20μL total reaction volume. For the 384-well plate format, each 10μL reaction included 5μL of 2x SybrGreen FastStart PCR mastermix (Roche Applied Science, Basel, Switzerland), 300nM of forward and reverse primer, and 1μL of cDNA (generated as described above) in dH_2O.

No template control (NTC) wells were included in every run for each primer set. For every cDNA sample, no-reverse transcription (no-RT) control samples were included for each primer set to check for genomic DNA contamination of each cDNA. All cDNA samples were run in duplicate wells for each primer set. To minimise pipetting errors, mastermixes

were made for each primer set and aliquoted into the wells. When possible, real-time PCR plates were set up away from direct light.

Real-time PCR reactions were conducted using a LightCycler® 480 (Roche Applied Science). The amplification program was as follows: denaturation at 95°C for 10min, then 45 cycles of denaturation at 95°C for 15s, annealing at 60°C for 15s, and extension at 72°C for 15s. Melting curve analysis was conducted for every sample. The melting curve cycle began with denaturation at 95°C for 10s and annealing at 65°C for 30s. The temperature was then increased at a rate of 0.11°C/s to 95°C, with continuous acquisition of melting curve data.

Data analysis was conducted using LightCycler® Software 4.0 (Roche Applied Science), Crossing point (Cp) values were determined automatically by the software using the second derivative method (Luu-The *et al.*, 2005). The Cp value corresponds to the cycle number at which the log linear amplification stage was initiated, and has been shown to be more accurate than the cycle threshold (Ct) method which relies heavily on the assumption of equal reaction efficiencies between different samples (Guescini *et al.*, 2008). Individual cDNA samples were excluded from analysis if the no-RT control samples returned values that were within 10 cycles of the sample Cp, or if more than one melting temperature was detected. Data from each sample was first normalised to the housekeeping gene, β-actin, to allow for variation in reverse transcription efficiency, using the formula $2^{(Cp_{(\beta\text{-actin})} - Cp_{(\text{gene of interest})})}$. This normalised value represents the number of gene of interest copies in comparison to the number of β-actin gene copies present in each sample. Gene induction

was then expressed as a ratio of the normalised gene copy number for each sample with respect to a reference sample, such as an unstimulated control and/or zero time point sample, as "mRNA fold change".

5.2.9 TLR genotyping

Given the possible role of TLR-2 and/or TLR-4 in initiating the host response to *C. burnetii*, the role of polymorphisms in these two genes was examined with respect to Q fever susceptibility and illness course. Single nucleotide polymorphisms (SNP) in TLR-2 and TLR-4 were genotyped using restriction fragment length polymorphism (RFLP) assays reported by others (Lorenz *et al.*, 2001; Sánchez *et al.*, 2004). The TLR-2 Arg677Trp (C2029T), TLR-2 Arg753Gln (G2258A) and TLR-4 Asp299Gly (A896G) polymorphisms were determined in all subjects of DIOS (see Section 4.2.1 Subjects) for whom DNA was available at the time of this study. Briefly, the region surrounding each SNP was amplified by PCR, digested with an appropriate enzyme, and the resulting fragments visualised on polyacrylamide gel. The genotype of each individual was designated by assessing the size of each digest product. A subset of amplicons was sequenced to confirm specificity of the PCR reaction for the region of interest. PCR products were purified using ExoSAP-IT (GE Healthcare, Giles, UK), and forward and reverse sequencing reactions were conducted at SUPAMAC (Sydney University, Sydney, Australia). DNA from an individual with homozygous polymorphic alleles at TLR-4 Asp299Gly was available as a control for complete digestion of PCR products, and was included in every experimental run.

TLR-2 genotyping was performed using a single PCR product, with forward (5' CCTT CAAGTTGTGTCTTCATAAC 3') and reverse (5' GGCCACTCCAGGTAGGTCTT 3') primers designed to amplify a region spanning both SNPs of interest. The forward primer introduced a single base mismatch (underlined) in order to create a cleavage site (C**C**GG) for *Hpa*II in the instance of a wild type C nucleotide at the +2029 (Arg677Trp) SNP site. A natural cleavage site for *Pst*I (CTGC**A**G) exists in the instance of a polymorphic A allele at the +2258 (Arg753Gln) SNP site.

Each TLR-2 SNP genotyping assay required a 25µl PCR reaction, which included 10ng of genomic DNA template, 10ng/µL of each primer (Sigma-Proligo), 2.5mM $MgCl_2$ (Applied Biosystems, Forster City, USA), 0.4mM of each dNTP (Invitrogen), 1U of AmpliTaq GOLD DNA polymerase (Applied Biosystems), and 1xABI PCR buffer (Applied Biosystems) in PCR grade dH_2O (Invitrogen). Reactions were conducted in an FTS-960 Thermal Sequencer (Corbett Research, Sydney, Australia). Cycling conditions began with 5min of polymerase activation at 95°C, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 56°C for 30s and extension at 72°C for 45s, then held a further 7min at 72°C before being refrigerated at 4°C.

PCR products for TLR-2 genotyping were digested in two separate assays with either *Hpa*II (Arg766Trp) or *Pst*I (Arg753Gln). Each digest included 5µL of PCR product and 5U of enzyme, in buffer O or buffer Tango for the *Hpa*II and *Pst*I digests respectively (all Fermentas, Burlington, Canada). Each tube was incubated at 37°C for a minimum of 3h prior to running digest products on a 12.5% polyacrylamide gel at 150V for 90min. For the

*Hpa*II digest of the Arg677Trp SNP site, a short digest product was indicative of the wild type allele, a long digest product was indicative of the polymorphic allele, and a combination of both long and short digest product was indicative of heterozygous alleles. For the *Pst*I digest of the Arg753Gln SNP site, a long digest product was indicative of the wild type allele, the short digest product was indicative of the polymorphic allele, and a combination of both long and short digest products was indicative of heterozygous alleles. In Figure 5.2, typical results are shown for subjects who are homozygous wild type or heterozygous at the TLR-2 Arg753Gln site. No instance of the TLR-2 Arg677Trp polymorphic allele was identified therefore genotyping experiments identified a single band in all subjects.

TLR-4 SNP genotyping was done using a forward (5' ATACTTAGACTACTACCTC CATG 3') and reverse (5'TTTTGAGAGATTTGAGTTTCA 3') primer pair where the forward primer contains a single base mismatch (underlined) in order to introduce a cleavage site for *Nco*I (CCATG**G**) in the instance of the polymorphic G allele being present at the +896 (Asp288Gly) SNP site. Each 25µL PCR reaction included 10ng of genomic DNA template, 10ng/µL of each primer (Sigma-Proligo), 1.5mM $MgCl_2$ (Applied Biosystems), 0.3mM of each dNTP (Invitrogen), 1 unit of AmpliTaq GOLD DNA polymerase (Applied Biosystems), and 1xABI PCR buffer (Applied Biosystems) in PCR grade ddH_2O (Invitrogen). Reactions were conducted in an FTS-960 Thermal Sequencer (Corbett Research). Cycling conditions began with 5min polymerase activation at 95°C, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 52°C for 30s and extension at 72°C for 30s, then held a further 7min at 72°C before being refrigerated at 4°C.

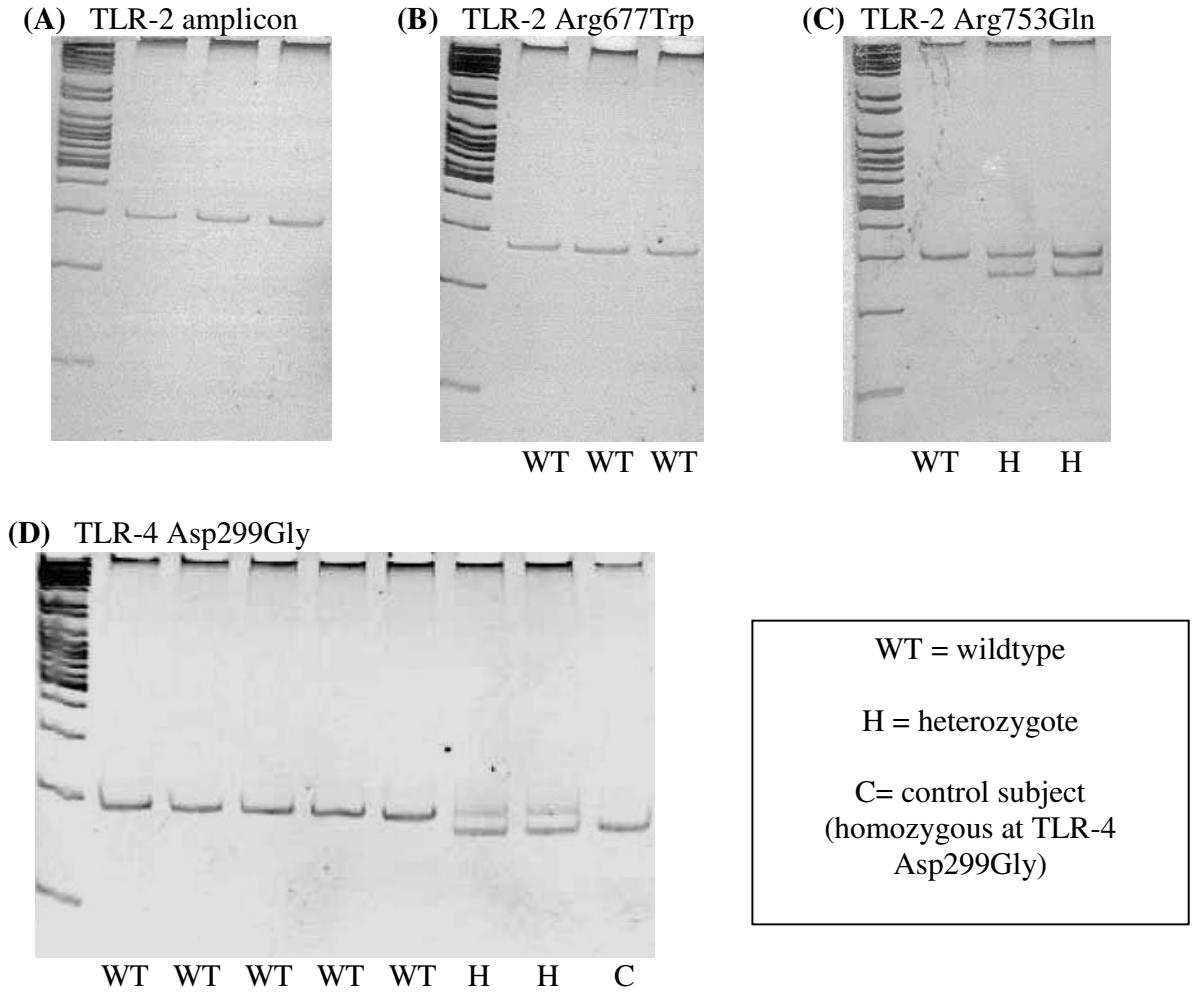

Figure 5.2: TLR genotyping by PCR, enzyme digest and gel electrophoresis. Regions spanning the polymorphic sites in TLR-2 (A-C) or TLR-4 (D) were amplified by PCR from genomic DNA. The resultant amplicons were digested by the enzymes *HpaII*, *PstI* and *NcoI* for the identification of the TLR-2 Arg677Trp, TLR-2 Arg753Gln and TLR-4 Asp299Gly polymorphisms respectively via visual inspection of the resultant amplicon fragments on polyacrylamide gel. The far left lane of each gel is a 100kp ladder to allow estimation of band size. Images of gels with (A) undigested TLR-2 amplicons, (B) *HpaII*-digested TLR-2 amplicons, (C) *PstI*-digested TLR-2 amplicons, (D) *NcoI*-digested TLR-4 amplicons.

Digests of PCR products for TLR-4 genotyping were done as described for TLR-2 using *Nco*I in buffer Tango (Fermentas). For the TLR-4 Asp299Gly SNP genotyping, a long digest product was indicative of the wild type allele, the short digest product was indicative of the polymorphic allele, and a combination of both long and short digest products was indicative of heterozygous alleles. An example of the bands that would be expected to be present for homozygous wild type, heterozygous, or homozygous polymorphic subjects are shown in Figure 5.2.

5.3 Results

5.3.1 Role of TLR-2 and TLR-4 in the host response to infection

5.3.1.1 Stimulation assays - THP-1 cells

Initial experiments in THP-1 cells characterised the kinetics of cytokine induction in response to whole killed *C. burnetii* antigens. The responses to *C. burnetii* were compared with those to control stimuli, known to activate cytokine production in monocytes via TLR-4 (LPS) or TLR-2 (peptidoglycan). Stimulus concentrations were chosen by their ability to elicit TNF-α production within the range of detection by ELISA. For these and subsequent experiments, TNF-α, IL-10 and IFN-β induction were quantified with respect to both mRNA induction by quantitative real time PCR, and protein secretion by ELISA of culture supernatants, while iNOS induction was assessed at the mRNA level only.

The data presented in Figure 5.3 are representative of one of three identical experiments conducted to examine cytokine induction by THP-1 cells over 24h of stimulation. The induction of IFN-β and iNOS mRNA, and of IFN-β protein, was barely detectable (data not shown), while TNF-α and IL-10 were readily measurable. As expected, mRNA induction preceded protein secretion. When used in equal concentrations, phase II antigen was a more potent inducer of TNF-α by THP-1 cells than phase I antigen. The differences in TNF-α production did not relate to differences in the kinetics of response to *C. burnetii* phase I and phase II; rather, the absolute magnitude of responses to phase I was reduced in comparison to the phase II response. This difference was less pronounced in IL-10 induction, but was nevertheless observable. *C. burnetii*-induced TNF-α protein production peaked approximately 6-12h after stimulation, however a noticeable difference in the kinetics of this response was observed when comparing phase I or phase II with the control stimuli (LPS and peptidoglycan). TNF-α production in response to phase I and phase II was delayed by 3-6h in comparison to these control stimuli. Similar delays in *C. burnetii*–induced responses were observed in IL-10 mRNA transcription. Despite differences in mRNA induction, no differences in the kinetics of IL-10 protein secretion were observed when comparing *C. burnetii* with LPS or peptidoglycan as stimuli.

In addition to stimulus-specific differences in cytokine induction kinetics, cytokine-specific differences were identified. TNF-α induction in response to each of the stimuli preceded IL-10 by approximately 6-12h. Having observed the different kinetics of TNF-α and IL-10 induction in response to *C. burnetii*, a 12h stimulation assay was chosen for further work, as this time point represented a compromise for simultaneous examination of TNF-α and

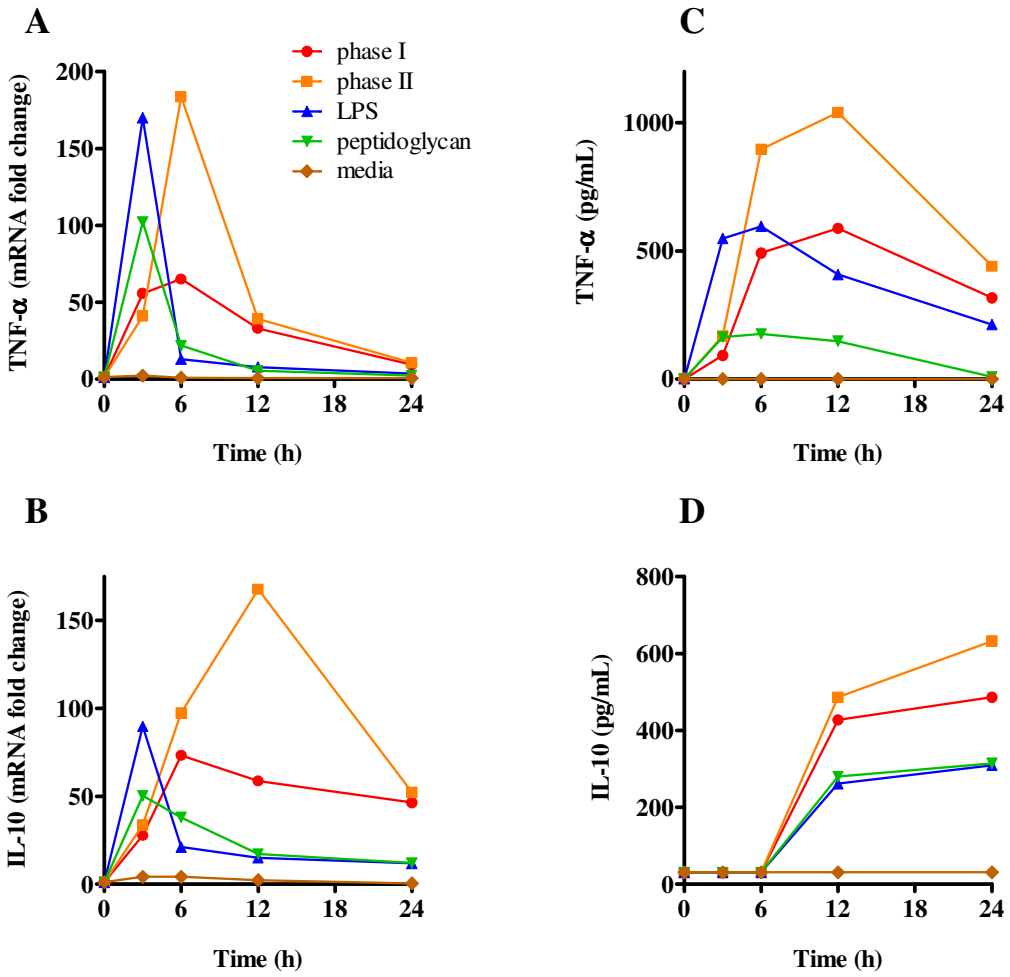

Figure 5.3: Kinetics of cytokine induction by THP-1 cells in response to stimuli. Data are representative of one of three experiments performed. THP-1 cells were cultured in the presence of *C. burnetii* whole inactivated phase I (red) or phase II (orange) antigen, LPS (blue), or peptidoglycan (green). Cells cultured in complete medium (brown) acted as a negative control. Induction of TNF-α (A and C) and IL-10 (B and D) was assessed with respect to increases in mRNA transcription (by qPCR, A and B) and protein secretion into culture supernatants (by ELISA, C and D). In qPCR assays, mRNA quantitation was determined as fold change in mRNA expression relative to the expression of each gene at the zero time point. Protein was quantitated by reference to a standard curve.

IL-10, as it corresponded with the approximate peak of TNF-α protein secretion and of IL-10 mRNA induction. Thus, experiments making use of TLR-specific blocking antibodies were conducted over 12h of stimulation.

The anti-TLR-4 antibody (clone HTA125) has previously been demonstrated to block signalling via TLR-4 (Wang *et al.*, 2003). Similarly, the anti-TLR-2 antibody (clone TL2.1) has been shown to block signalling via TLR-2 (Flo *et al.*, 2000). The ability of these antibodies to block cytokine production by THP-1 cells was tested in comparison with an irrelevant isotype control antibody (mouse IgG2a for both HTA125 and TL2.1). Anti-TLR-4 and anti-TLR-2 antibody treatments were effective in blocking up to 70% of TNF-α production in response to LPS (TLR-4) stimulation and up to 98% of TNF-α production in response to peptidoglycan (TLR-2) stimulation respectively (Figure 5.4). The ability of these antibodies to block TNF-α secretion were statistically significant (no antibody vs anti-TLR-4 antibody, $p=0.002$; no antibody vs anti-TLR-2 antibody, $p=0.003$, student's t tests) and were determined to be TLR specific due to minimal effects seen when cells were treated with the isotype control antibody.

Having established appropriate experimental conditions, blocking antibodies were used to determine whether TLR-2 or TLR-4 play a role in the induction of cytokines in response to *C. burnetii* by THP-1 cells. THP-1 cells were cultured in the absence of blocking antibody to determine the maximal cytokine response to stimulation, and this cytokine response was then compared with that of cells cultured in the presence of blocking antibodies. Anti-TLR-2 and anti-TLR-4 antibodies were used singly or in combination, to determine whether

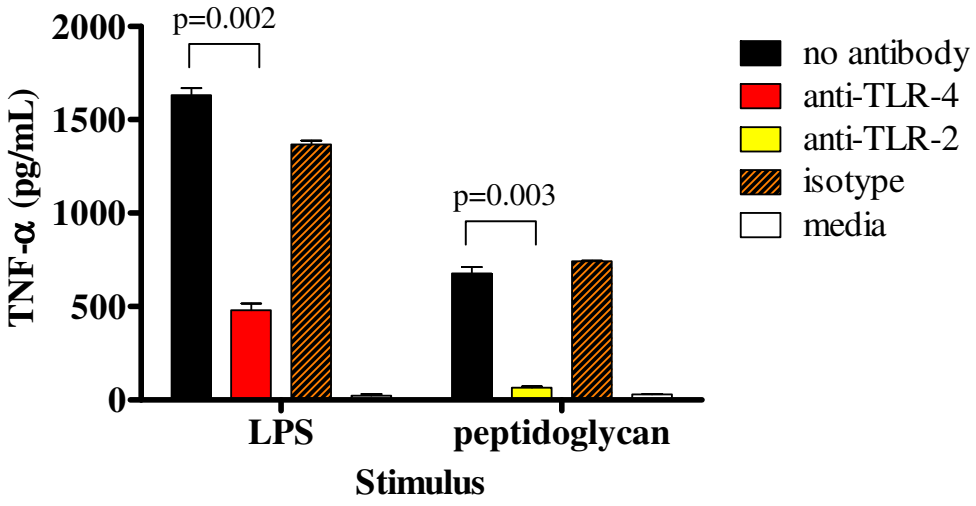

Figure 5.4: Capacity of anti-TLR-4 and anti-TLR-2 antibodies to block TNF-α production by THP-1 cells. THP-1 cells were pre-treated with either no antibody (black bars), or 20μg/mL of blocking antibodies HTA125 (anti-TLR-4, red bars) or TL2.1 (anti-TLR-2, yellow bars) for 30min prior to addition of stimulus. HTA125 and TL2.1 were effective in reducing TNF-α production by THP-1 cells in response to LPS and peptidoglycan respectively (p=0.002 and p=0.003 respectively, student's t tests). An isotype-matched mouse IgG2a antibody to an irrelevant target was used to control for non-specific antibody effects (black bars with orange stripes). Cytokine induction was compared to cells cultured in the absence of stimuli (white bars). Error bars represent the standard error of two replicate culture wells.

these receptors may play a synergistic role in the cytokine response to *C. burnetii*. To control for non-specific antibody effects, cells were also cultured in the presence of an IgG2a murine isotype control antibody. After antibody treatment, cells were stimulated for 12h in the presence of *C. burnetii* whole inactivated phase I or phase II antigen, LPS, peptidoglycan, or complete medium alone. As anticipated by earlier experiments, IFN-β (mRNA and protein) and iNOS (mRNA) were not induced by THP-1 cells in response to any of the stimuli, even in the absence of TLR-specific antibody. TNF-α and IL-10 were induced and measurable at both the protein and mRNA level.

Anti-TLR-2 antibody pre-treatment consistently inhibited TNF-α protein production in response to *C. burnetii* phase I or phase II antigens (Figure 5.5). In phase I-stimulated cultures, blocking of TLR-2 signalling resulted in TNF-α levels that were comparable to those in unstimulated controls (no antibody vs anti-TLR-2 antibody, p=0.002, student's t test). The TLR-2 pathway was similarly critical in the response of THP-1 cells to phase II antigen, with TLR-2 blocking antibodies reducing TNF-α production to approximately one tenth of that seen in the absence of blockade (no antibody vs anti-TLR-2 antibody, p=0.019, student's t test). The anti-TLR-4 antibody had little effect on cytokine induction when used alone and did not enhance cytokine blockade when used in combination with anti-TLR-2. The suppression of cytokine induction seen in cells cultured with anti-TLR-2 antibody was specific as the use of an unrelated isotype control antibody had no effect on *C. burnetii*-induced TNF-α production by THP-1 cells.

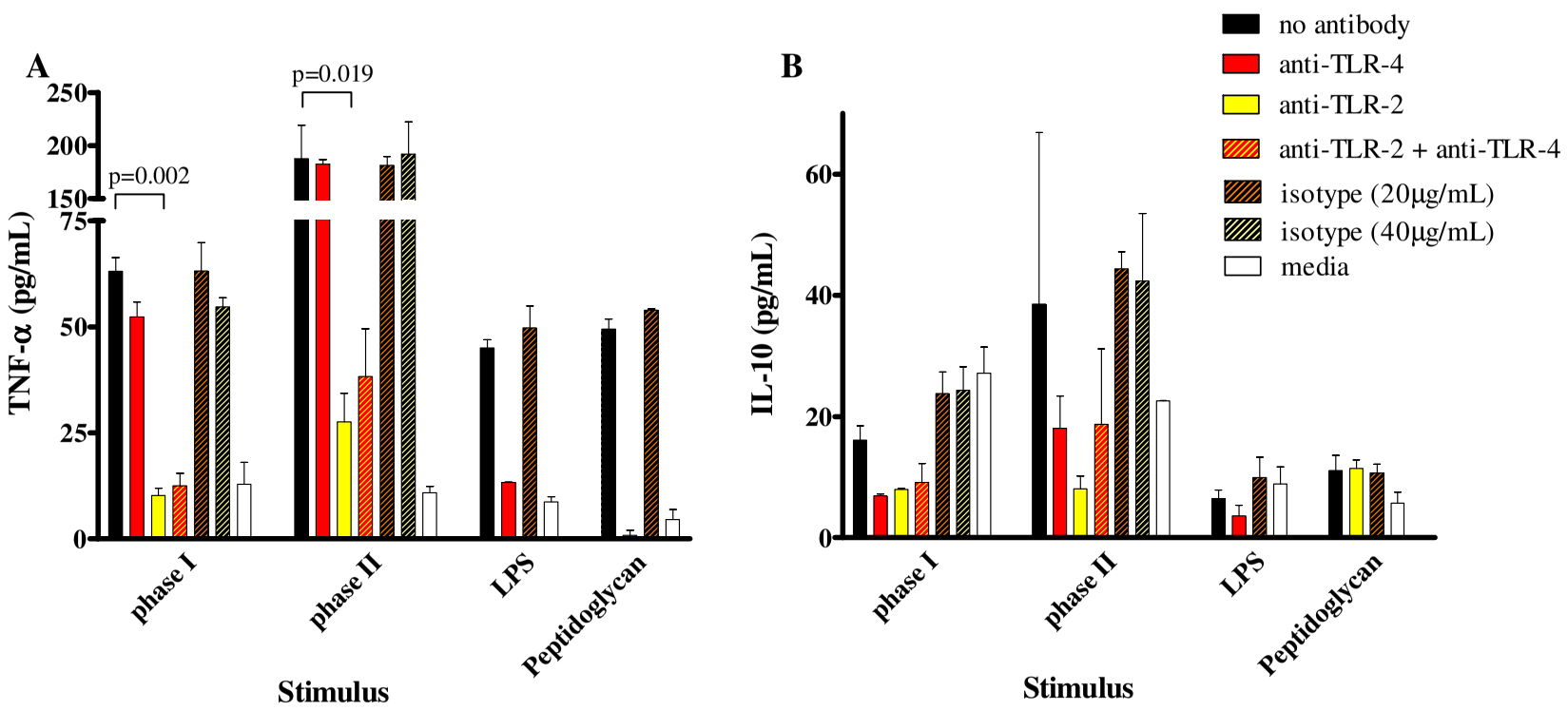

Figure 5.5: Effect of blocking antibodies on TNF-α and IL-10 protein secretion by THP-1 cells in response to stimuli. THP-1 cells were pre-treated for 30min with either no antibody (black bars), anti-TLR-4 antibody (red bars), anti-TLR-2 antibody (yellow bars), or anti-TLR-2 and anti-TLR-4 antibodies combined (red bars with yellow diagonal stripes) at 20µg/mL per antibody, or with an isotype control antibody at 20µg/mL (black bars with orange stripes) or 40µg/mL (black bars with yellow stripes). After antibody treatment, cells were stimulated for 12h in the presence of *C. burnetii* whole inactivated phase I or phase II antigen, LPS, peptidoglycan, or complete medium, and cytokine secretion assessed by ELISA. Error bars represent the standard error in two culture replicates for each set of conditions. Significant blocking of TNF-α protein secretion in response to phase I and phase II stimulation was achieved by anti-TLR-2 antibody treatment (student's t tests).

At the 12h time point, differences in IL-10 protein secretion in the presence of blocking antibody were difficult to ascertain due to the relatively delayed kinetics of IL-10 production. Given that mRNA precedes protein production, it seemed plausible that effects of TLR-2 blockade might be more easily observed for IL-10 when examining mRNA rather than protein secretion. Therefore, the differences in mRNA induction of both TNF-α and IL-10 were also determined in the presence or absence of TLR-2 and TLR-4 blockade (Figure 5.6). All data were normalised to a housekeeping gene (β-actin) and expressed as a "fold change" in comparison to the mRNA expressed by unstimulated cells. Anti-TLR-2 blocking antibody was effective in reducing the TNF-α and IL-10 mRNA transcription to approximately half the induction seen in response to phase I or phase II stimulation in the absence of antibody, but this effect was not significant ($p>0.05$, student's t tests). Anti-TLR-4 antibody had no effect on cytokine mRNA induction in response to *C. burnetii.* The combined data relating to TNF-α protein secretion, and TNF-α or IL-10 mRNA induction in the presence or absence of TLR-specific antibodies point to a critical role of TLR-2, and not TLR-4, in the cytokine production response of THP-1 cells to *C. burnetii*.

5.3.1.2 Stimulation assays - PBMC

Having established the role of TLR-2 signalling within THP-1 cells for the activation of cytokines in response to *C. burnetii*, a similar experimental procedure was adopted for investigation into whether TLR-2 is also critical in the function of primary monocytes exposed to *C. burnetii*. Preliminary experiments were conducted using PBMC, to confirm that stimulus concentrations and time of stimulation would be suitable. The concentration of *C. burnetii* was able to be significantly decreased with comparable cytokine levels in comparison to the experiments utilising THP-1 cells; a final concentration of 1μg/mL was

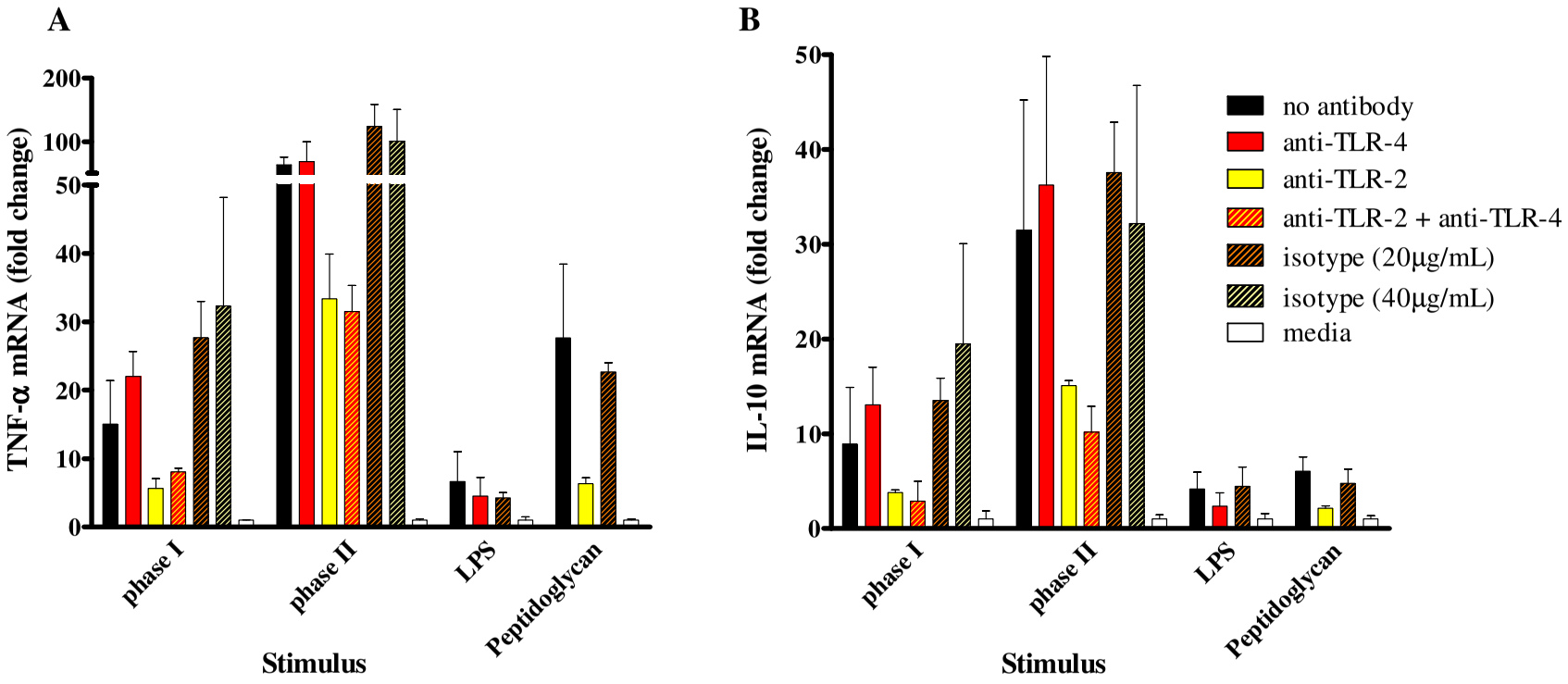

Figure 5.6: Effect of blocking antibodies on TNF-α and IL-10 mRNA induction by THP-1 cells in response to stimuli. THP-1 cells were cultured as described for Figure 5.5. Total RNA was extracted from the cells following 12h of stimulation. RNA was reverse transcribed to cDNA for use in qPCR. Relative amounts of TNF-α (A) and IL-10 (B) mRNA were estimated by normalizing all data to the constantly expressed β-actin gene. Fold changes in mRNA expression were quantitated by comparing gene transcription with that in control THP-1 cells cultured in complete medium alone. Error bars represent the standard error of two replicate cultures for each test condition.

an adequate inducer of TNF-α and IL-10 (Figure 5.7) and was adopted for TLR blocking experiments in PBMC.

Utilising an identical procedure as for THP-1 cell experiments, an attempt was made to block cytokine production in stimulated PBMCs. Anti-TLR-2 and anti-TLR-4 antibodies had virtually no effect on the production of TNF-α in response to any of the stimuli (Figure 5.8). Any decrease in IL-10 protein production, such as that seen in IL-10 secretion when LPS stimulation is blocked by TLR-4-specific antibody, could not be determined to be specific to TLR blockade as isotype control treatment resulted in comparable IL-10 levels to those seen in PBMC treated with TLR antibody. Furthermore, anti-TLR-2 and anti-TLR-4 treatment had no suppressive effect on TNF-α or IL-10 secretion in response to *C. burnetii* antigens. Similarly, no evidence of TLR antibody-mediated suppression of TNF-α or IL-10 mRNA could be detected (data not shown), however extremely low "fold change" values were obtained, suggesting that the 12h time point may have been too delayed in the natural kinetics of the PBMC response to stimulation to detect blocking effects in mRNA. Subsequent attempts to detect blocking of cytokine induction after only 4h of stimulation failed to detect a significant antibody-mediated effect with respect to cytokine mRNA induction (Figure 5.9) or protein secretion (data not shown) by PBMC.

5.3.1.3 Stimulation assays – primary monocytes

It was hypothesised that anti-TLR blocking antibodies may have failed to have an effect in this culture system due to the sequestration of antibody to non-TLR targets via interaction of their Fc regions with Fc receptors (FcR) on cells within the PBMC samples. Specifically,

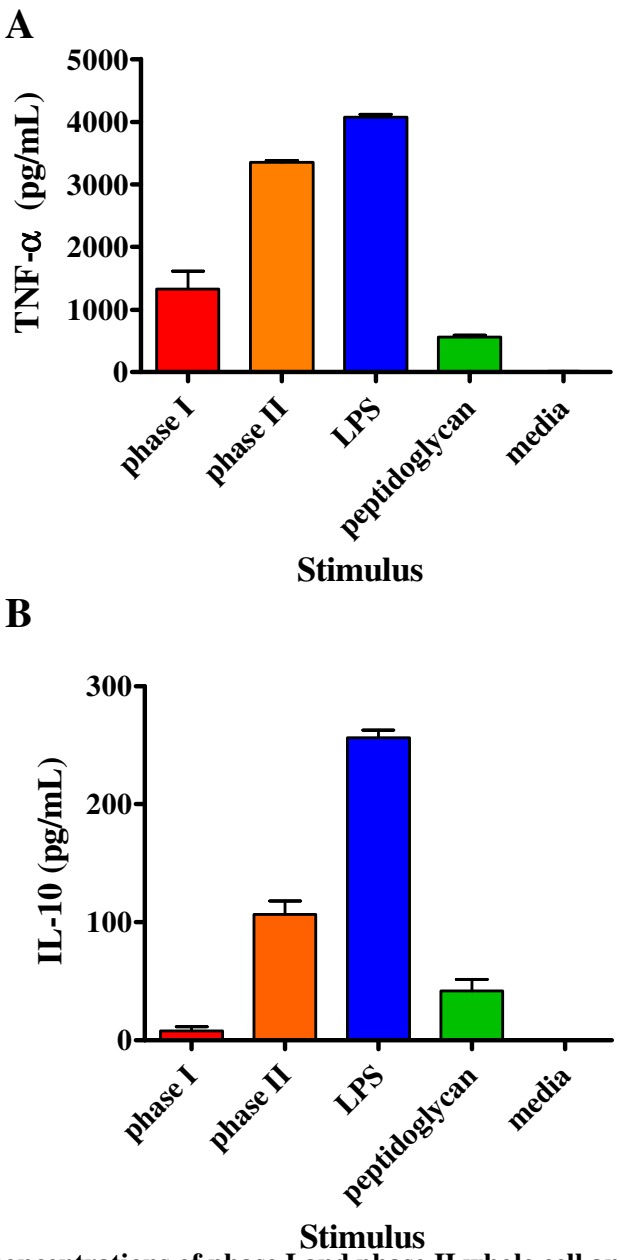

Figure 5.7: Reduced concentrations of phase I and phase II whole cell antigens elicit strong cytokine responses in PBMC. PBMC from a single donor were stimulated for 12h with whole inactivated phase I (red) or phase II (orange) antigens (1μg/mL), LPS (blue, 10ng/mL), peptidoglycan (green, 250ng/mL), or cultured without stimulus (media alone, brown). TNF-α and IL-10 protein secretion were assessed by ELISA. Error bars represent the standard error of two replicate culture wells.

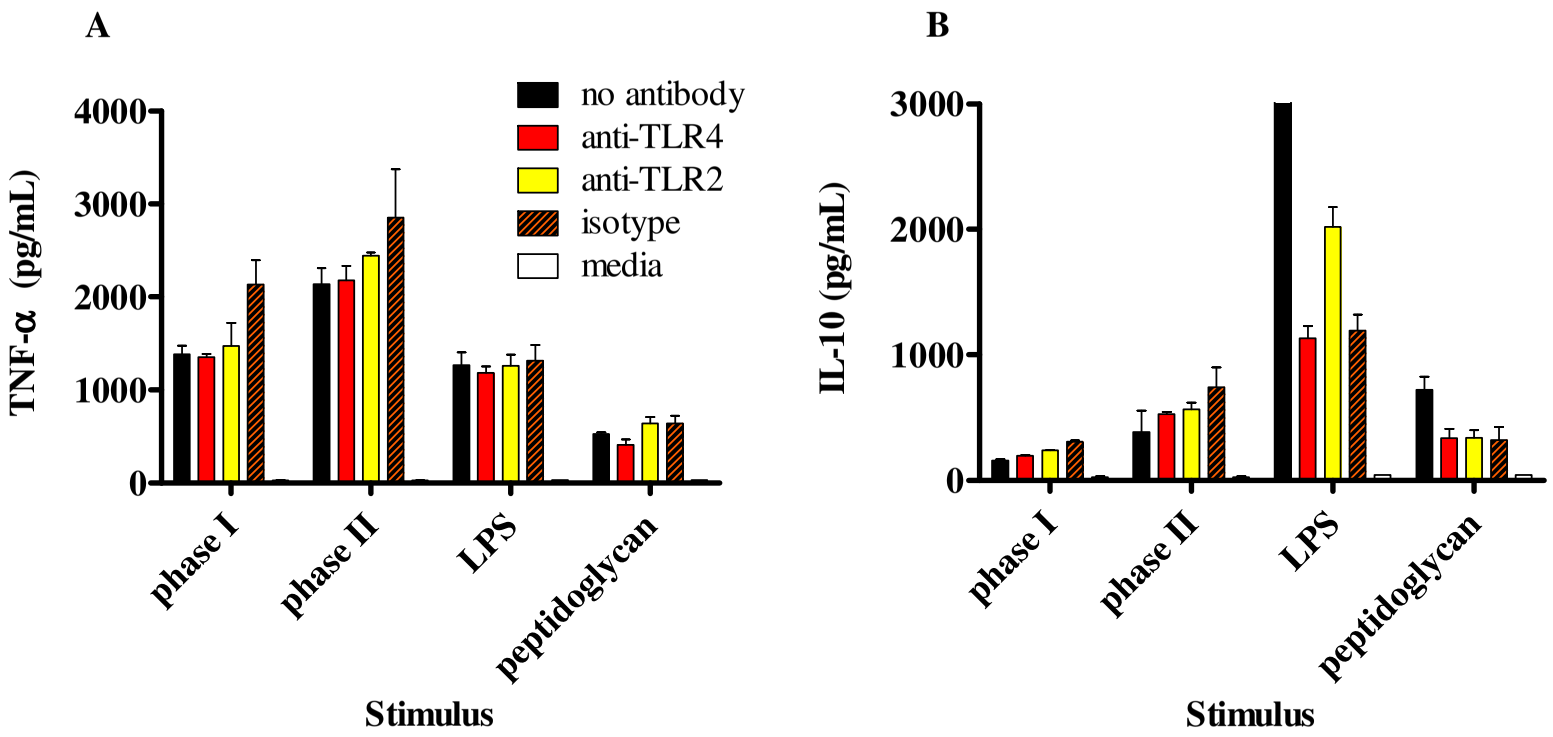

Figure 5.8: Inability of TLR-specific antibody to block cytokine production by stimulated PBMCs. PBMC were pre-incubated with either no antibody (black bars), anti-TLR-4 (red bars) or anti-TLR-2 (yellow bars) antibody, or a mouse IgG2a isotype control antibody (black bars with orange stripes), for 30min prior to stimulation with whole inactivated phase I or phase II antigen, LPS, or peptidoglycan. Cytokine secretion was also assessed in unstimulated cells (media, white bars). TNF-α (A) and IL-10 (B) were quantitated by ELISA. Error bars represent the standard error of two replicate wells per culture condition.

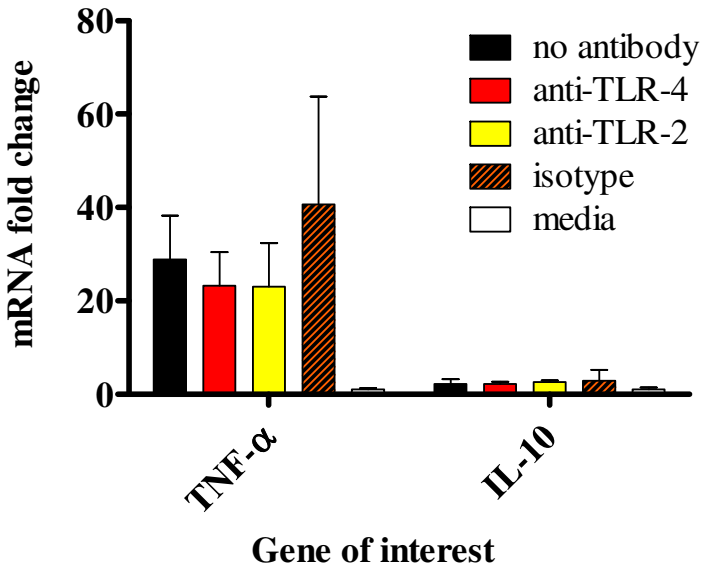

Figure 5.9: Reduced stimulation time does not enable detection of cytokine suppression in PBMC treated with anti-TLR antibodies. PBMC were pre-incubated with antibody as in Figure 5.8 and stimulated for 4h with whole inactivated phase II organism. Changes in TNF-α and IL-10 mRNA induction were determined by qPCR in stimulated samples in comparison to control wells incubated with media alone. Error bars represent the standard error of three replicate wells.

the FcγRII (CD32) receptor, present on most lymphoid cells including monocytes, B cells, some T cell subsets, and neutrophils, is known to interact with murine IgG2a (Gessner *et al.*, 1998), and therefore presumably these receptors could interact with the anti-TLR-2 and anti-TLR-4 murine IgG2a antibodies in use in this experiment. To remove cells that may be sources of unwanted FcR (and which were unnecessary for this work), primary monocytes were isolated by adherence to cell culture plates, non-adherent cells were removed by washing, and stimulation assays were conducted over 6h. Figure 5.10 shows the TNF-α and IL-10 protein and mRNA induction by adherent monocytes in response to phase II stimulation. This method resulted in high inter-well variance, possibly due to differences in monocyte binding and/or washing efficiencies from well to well. This technique was abandoned due to difficulties in obtaining replicable results.

An alternative method of monocyte purification was adopted, involving negative selection of monocytes using a magnetic bead separation kit. This allowed enrichment of a CD14+ monocyte population of at least 90% purity (Figure 5.11). A time course experiment was conducted to examine the kinetics of TNF-α and IL-10 mRNA induction and protein secretion in stimulated monocytes (Figure 5.12). There was rapid induction of TNF-α mRNA, even in unstimulated monocytes, but this did not translate to TNF-α secretion. Such a phenomenon has been reported by others: in the absence of a secondary signal such as LPS, TNF-α mRNA is highly induced by the adherence of monocytes, yet the mRNA is not transcribed into protein (Haskill *et al.*, 1988). Therefore, it seems likely that the early rise in mRNA levels therefore relates to adherence of the monocytes rather than a contaminating stimulus.

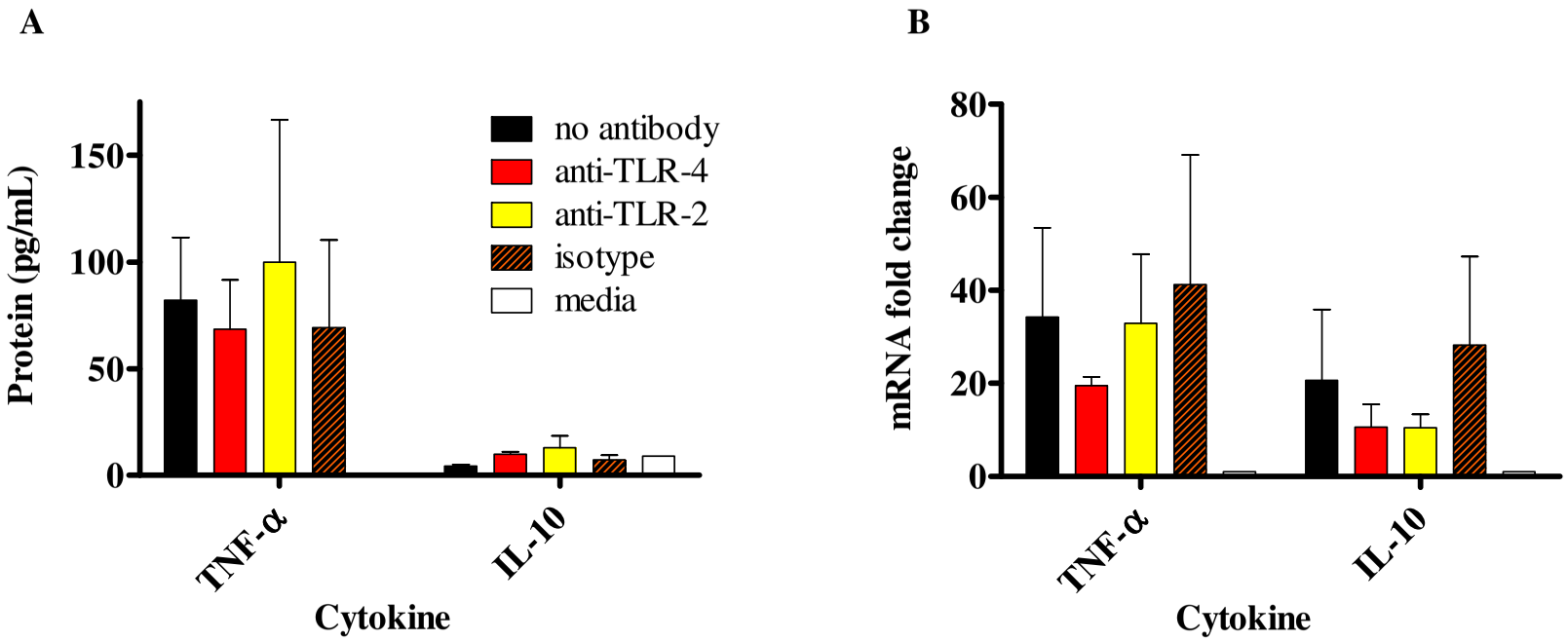

Figure 5.10: Phase II stimulation of adherent monocytes revealed high inter-well variance. PBMC were plated and monocytes allowed to adhere for 12h. Non-adherent cells were removed by washing. The remaining cells were pre-treated with either no antibody (black bars), anti-TLR-4 (red bars) or anti-TLR-2 (yellow bars) antibodies, or with a mouse IgG2a isotype control antibody (black bars with orange stripes) and stimulated with whole inactivated phase II antigen or with media alone (white bars). TNF-α and IL-10 production were assessed with respect to protein secretion (A) and mRNA induction (B). Error bars represent the standard error of three replicate wells.

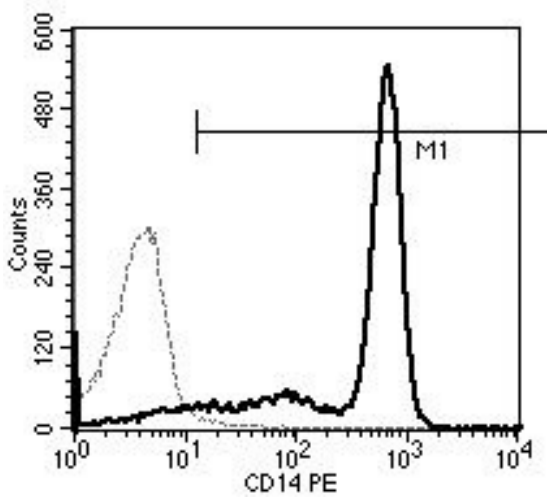

Figure 5.11: Enrichment of CD14+ monocyte cell population using a MACS bead negative selection kit. Following monocyte enrichment, the purity of the resultant cell population was assessed by flow cytometry to detect the proportion of CD14+ cells (black line) in comparison to unstained cells (grey dashed line). For this specific isolation, approximately 92.4% of cells occurred within the CD14+ gate (M1). A minimum enrichment to 90% monocytes was achieved in all separations.

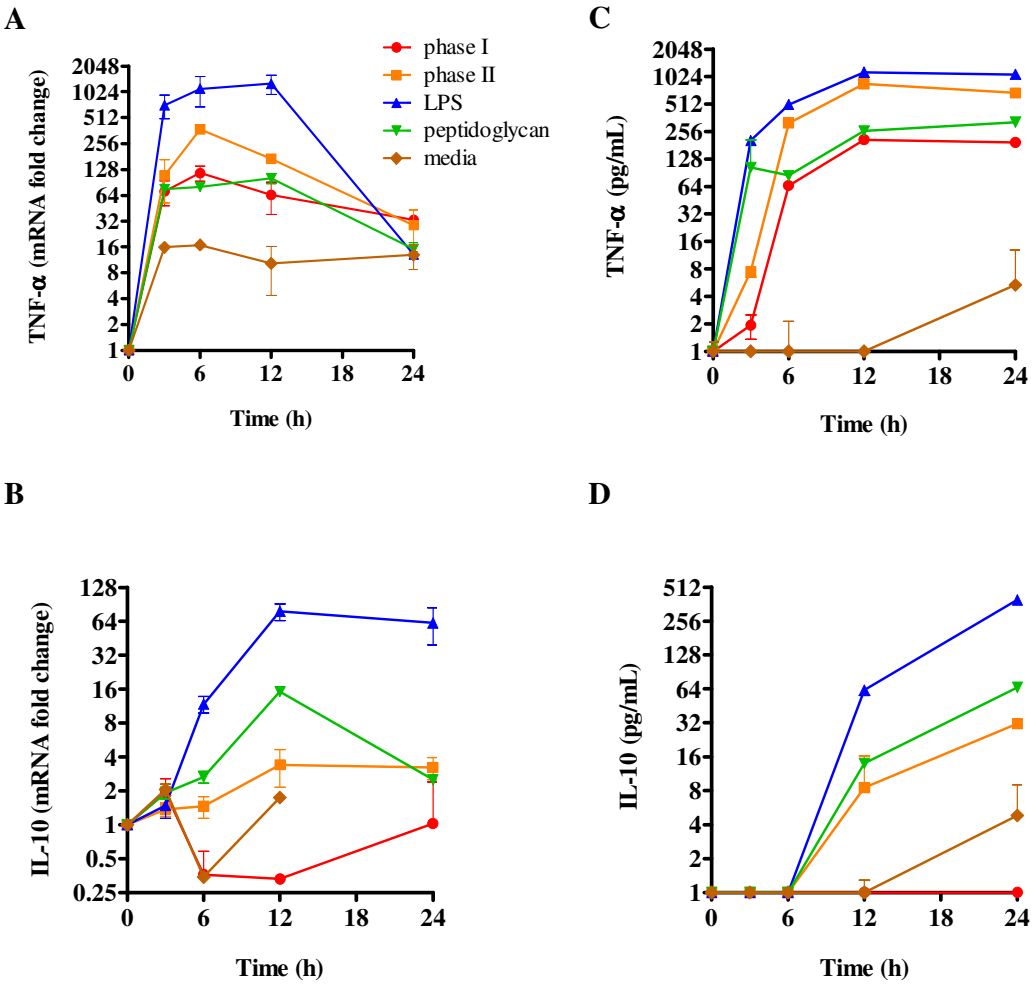

Figure 5.12: Kinetics of cytokine induction by primary monocytes in response to stimuli. Primary monocytes were enriched from PBMC by magnetic bead separation, and were then cultured in the presence of *C. burnetii* whole inactivated phase I (red) or phase II (orange) antigen, LPS (blue), or peptidoglycan (green). Cells cultured in media alone (brown) acted as a negative control. Induction of TNF-α (A and C) and IL-10 (B and D) was assessed with respect to increases in mRNA transcription (by qPCR, A and B) and protein secretion into culture supernatants (by ELISA, C and D). In qPCR assays, mRNA quantitation was determined as fold change in mRNA expression relative to the expression of each gene at the zero time point. Protein was quantitated by reference to a standard curve.

Given the more rapid kinetics of cytokine induction by primary monocytes in comparison to THP-1 cells, an earlier (6h) time point was chosen for TLR blocking experiments. As expected at this early time point, stimulation of negatively-selected monocyte cultures resulted in IL-10 protein secretion that was barely detectable in culture supernatants (data not shown), while TNF-α protein was measurable. When TNF-α levels were assayed in the culture supernatants, no blocking effect of anti-TLR-2 or TLR-4 antibodies was evident, even when monocytes were stimulated with the TLR-2 and TLR-4 control stimuli (peptidoglycan and LPS respectively) (Figure 5.13). Although some reduction in TNF-α was evident in phase I stimulated monocytes that had been blocked with anti-TLR-2 and/or anti-TLR-4 antibodies in comparison with unblocked monocytes, this effect was not significant ($p=0.063$ and $p=0.122$ respectively, student's t test). A comparison of the TNF-α secretion in the presence of blocking antibody or the isotype control found no difference (anti-TLR-2 vs isotype, $p=0.510$, anti-TLR-4 vs isotype, $p=0.803$), suggesting that any trend for reduced TNF-α secretion may be non-specific antibody-mediated effects. Furthermore, subtle reductions in TNF-α observed in phase II stimulated monocytes were not significant (anti-TLR-2 vs no antibody, $p=0.134$; anti-TLR-4 vs no antibody, $p=0.579$; anti-TLR-2 and anti-TLR-4 vs no antibody, $p=0.077$, student's t test). Similarly inconclusive results were obtained when TNF-α and IL-10 mRNA levels were compared between anti-TLR antibody blocked monocytes and control monocytes (data not shown).

It was hypothesised that monocyte adherence had a priming effect in this assay system, causing TNF-α mRNA induction which was then translated into TNF-α protein in the

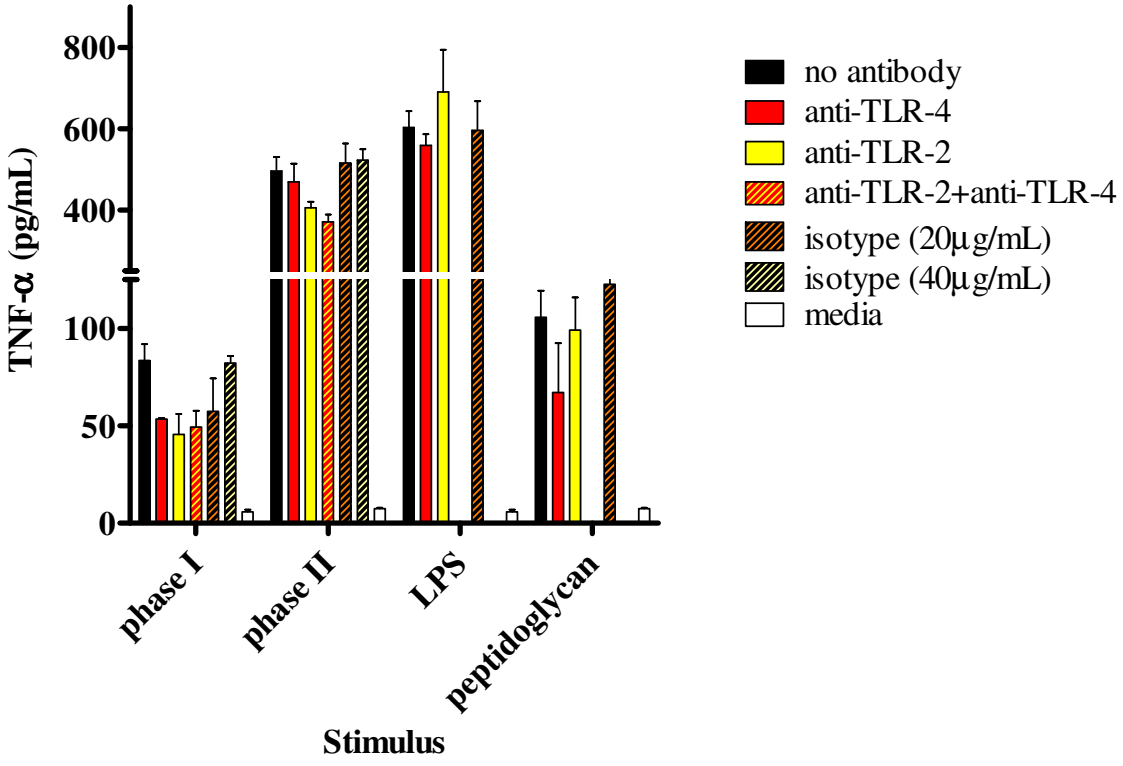

Figure 5.13: Effect of blocking antibodies on TNF-α protein induction by primary monocytes in response to stimuli. Primary monocytes were isolated by negative selection from PBMCs. Monocytes were pre-treated for 30min with either no antibody (black bars), anti-TLR-4 antibody (red bars), anti-TLR-2 antibody (yellow bars), or anti-TLR-2 and anti-TLR-4 antibodies combined (red bars with yellow diagonal stripes) at 20µg/mL per antibody, or with an isotype control antibody at 20µg/mL (black bars with orange stripes) or 40µg/mL (black bars with yellow stripes). After antibody treatment, cells were stimulated for 6h in the presence of *C. burnetii* whole inactivated phase I or phase II antigen, LPS, peptidoglycan, or complete medium, and cytokine secretion assessed by ELISA. Error bars represent the standard error in two culture replicates for each set of conditions.

presence of secondary stimuli such as LPS or *C. burnetii*. As a result, cytokine responses to stimulation were of such high magnitude that TLR-specific antibodies were unable to sufficiently block signalling via TLR-2 or TLR-4. Two alternative culture systems in which adherence would be minimised were therefore tested in parallel with the existing system, involving stimulation assays in cell culture plates designed to allow minimal adherence, or in 15mL Falcon polypropylene tubes that were maintained on a (gentle) shaking platform. Monocytes isolated by negative selection were pre-treated with blocking antibodies and then stimulated by phase II antigen in the three culture systems for a reduced time frame (Figure 5.14). Again, no significant and TLR-specific blocking effects were evident when cells were pre-treated with anti-TLR antibodies.

Although the blocking antibody concentration used in this study was comparable to those reported in the literature and recommended by the manufacturer, a final attempt to block cytokine secretion by isolated monocytes via anti-TLR antibodies was made to examine whether increasing the blocking antibody concentration might result in stronger blocking effects. As can be seen in Figure 5.15, doubling the concentration of TLR-specific antibody did not enhance the suppression of cytokine induction in primary monocytes. This suggested that insufficient antibody concentration was not a factor preventing the detection of antibody-mediated suppression of cytokine production in these cells.

5.3.1.4 Stimulation assays - summary

The data obtained in THP-1 cells provide convincing evidence for TLR-2-mediated signalling in response to *C. burnetii* antigens. Attempts to detect specific effects of anti-TLR antibodies in blocking TLR signalling in primary monocytes were unsuccessful. A

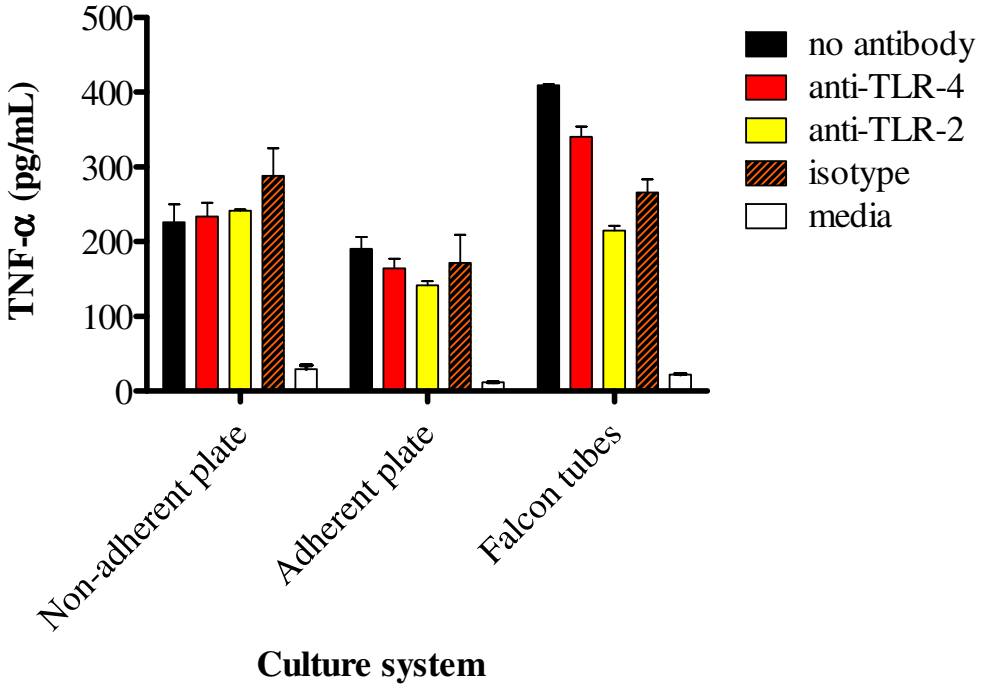

Figure 5.14: Effect of monocyte adherence on phase II antigen-stimulated TNF-α protein secretion. Primary monocytes were isolated by negative selection from PBMCs. Monocytes were pre-treated for 30min with either no antibody (black bars), anti-TLR-4 antibody (red bars), anti-TLR-2 antibody (yellow bars), or an isotype control antibody (black bars with orange stripes, all 20μg/mL). After antibody treatment, cells were stimulated for 3h in the presence of *C. burnetii* whole inactivated phase II antigen or complete medium, in different culture vessels: plates designed for minimal monocyte adherence, normal tissue-culture treated culture plates, or 15mL Falcon tubes. TNF-α protein secretion was assessed by ELISA. Error bars represent the standard error in two culture replicates for each set of conditions.

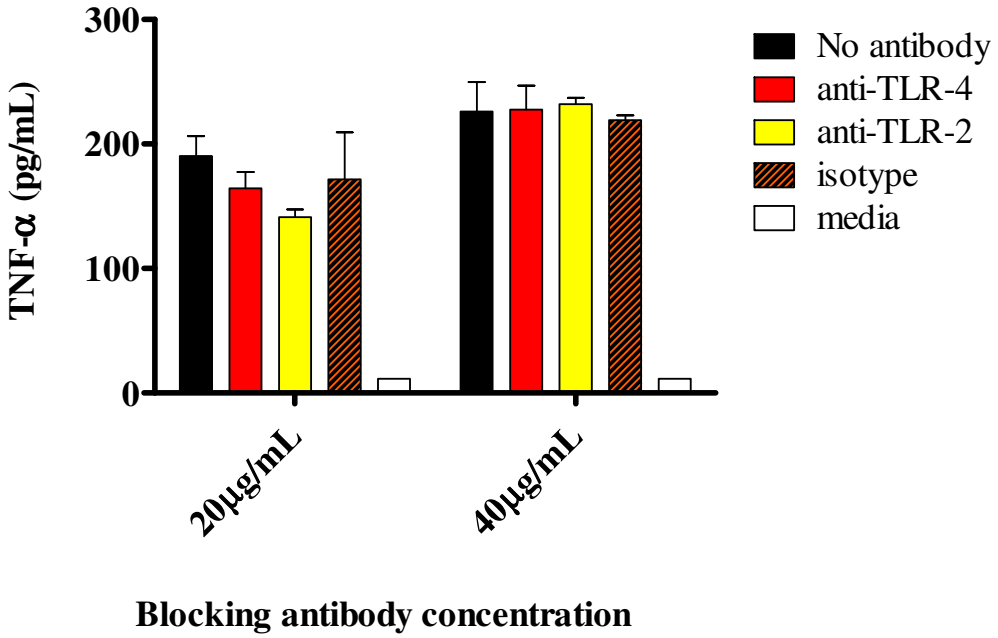

Figure 5.15: Inability of anti-TLR antibodies to block cytokine production by primary monocytes is not due to insufficient antibody concentration. Primary monocytes were isolated by negative MACS bead enrichment, pre-treated for 30min with anti-TLR-4 (red bars), anti-TLR-2 (yellow bars) or isotype control (black bars with orange stripes) antibodies at 20µg/mL or 40µg/mL and then stimulated for 3h with phase II antigen. TNF-α secretion into culture supernatants were assessed by ELISA. Results were compared with unstimulated cells (white bars) or cells that were not treated with antibody (black bars). Error bars represent the standard error of two replicate cultures.

number of conditions were modified in an attempt to optimise the experimental procedure. These included a comparison of the use of PBMC or primary monocytes isolated by negative selection using MACS beads, a comparison of culture vessels with surfaces that enabled varied cell attachment efficiencies, and a comparison of antibody concentrations. It is possible that the phenotype of THP-1 cells is so significantly different from that of "classical" primary monocytes that receptor usage is not concordant. Furthermore, experiments that make use of the CD14+CD16+ "inflammatory" monocyte subset may enable characterisation of the role played by TLR-2 in the host response to *C. burnetii*.

5.3.2 Scanning electron microscopy of whole inactivated *C. burnetii* antigens

Stocks of whole, formalin inactivated *C. burnetii* organisms were purchased for use in the experimental work presented in this thesis. These phase I and phase II organisms were produced by the supplier for use in the manufacture of Q fever-specific IgG and IgM ELISA assay kits. These whole antigens were not produced for commercial distribution but were kindly supplied for use in this study. *C. burnetii* whole inactivated antigens were, to our knowledge, not otherwise available in Australia, and the culture of *C. burnetii* would have required physical containment level 3 (PC3) facilities not available for this study, therefore the supply of these antigens was critical for this research.

Towards the end of the study, production of these antigens was relocated internationally (within the company), and following this change the supplied stocks were visibly different from previous supplies, in particular the new batch was less opaque than previous batches. This raised a concern that either the new batch was of significantly lower concentration

than previous batches, or alternatively, that previous batches had been contaminated with cellular debris from the Vero cells that were used as host cells in the culture of *C. burnetii*. Given the difficulty in observing *C. burnetii* by light microscopy, scanning electron microscopy was used to visualise samples from "old" and "new" batches of whole inactivated antigen to address these issues.

Small aliquots of each batch were adhered to coverslips, fixed, and dehydrated, then coated in gold or chromium and visualised by scanning electron microscopy at a variety of magnifications. Representative images of "old" batches that were opaque in nature are shown in Figure 5.16. Under 2,000 fold magnification, single, free bacteria-like structures are visible, as are larger clumps that warranted further investigation. When these were magnified further (10,000 fold) it became clear that these clumps were aggregates of *C. burnetii* and not cellular debris from the Vero cells that were host cells for the culture of *C. burnetii*. In all vials of old stocks that were tested, it was concluded that the opaque appearance was due to a high concentration of the antigen and not a result of contaminating cellular debris.

By comparison, "new" stocks that were not opaque were demonstrated to contain very few bacteria (Figure 5.17). In comparison to previous stocks, *C. burnetii* was barely detectable in these new stocks. Liaison with the supplier confirmed that the new stocks had in fact resulted in ELISA plates that had significantly reduced sensitivity, confirming suspicions that these stocks were of substantially decreased concentration and not able to be used in these studies, where stimulus concentrations needed to remain constant. Only highly

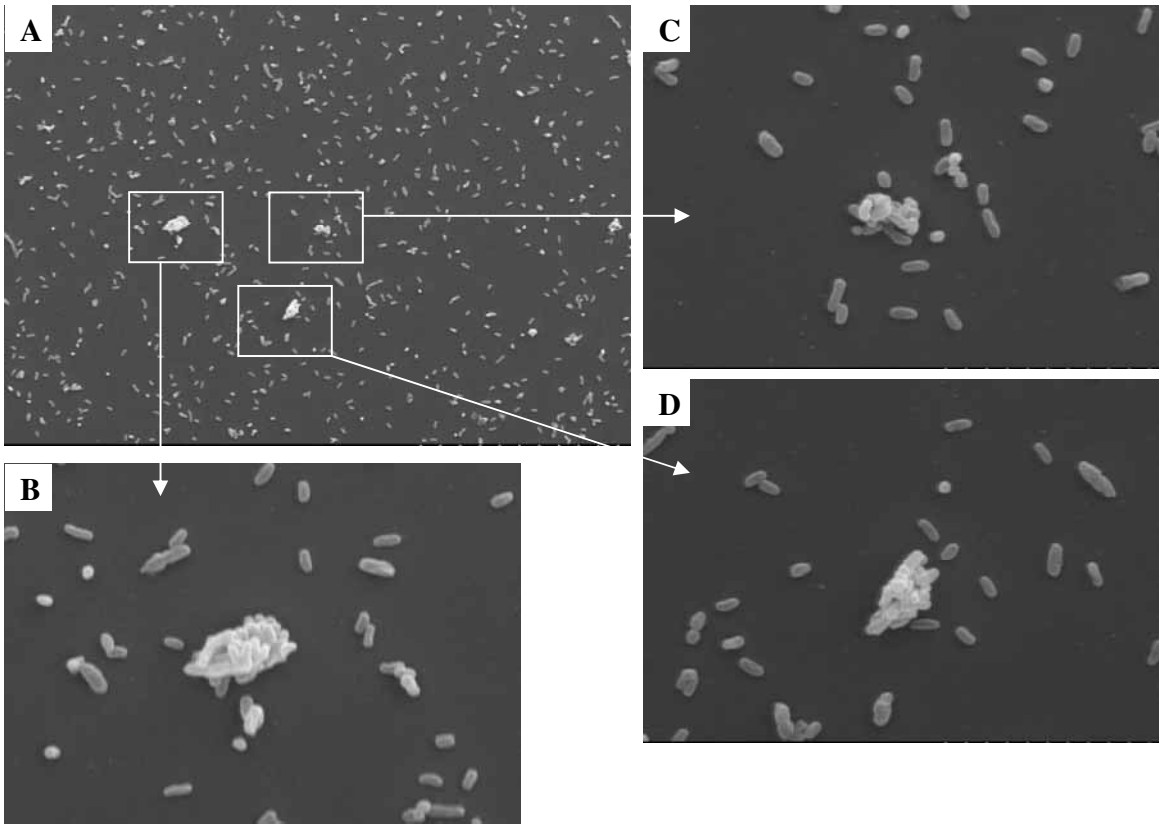

Figure 5.16: Scanning electron micrographs of whole inactivated *C. burnetii* antigen stocks. Opaque stocks were visualised to determine whether turbidity was a consequence of cellular debris. (A) 2,000x magnification showing singular bacteria and boxed clumps warranting further investigation. (B), (C) and (D) 10,000x magnification of clumps, demonstrating them to be bacterial aggregates rather than cellular debris. Images are all of the phase I preparation, but are comparable with those obtained for the phase II preparations.

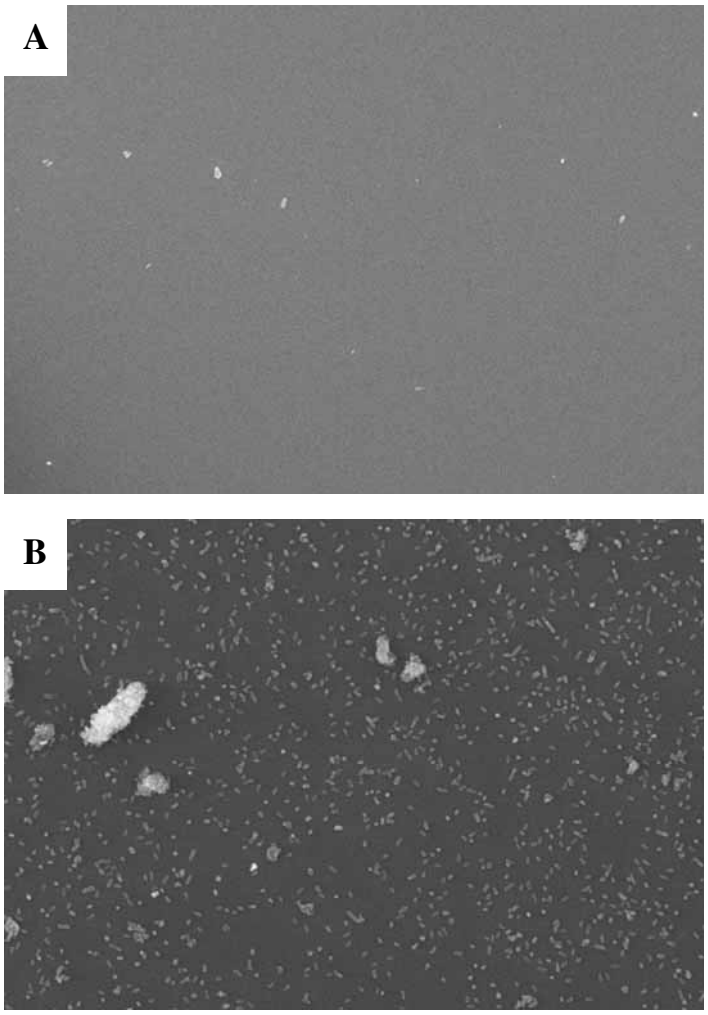

Figure 5.17: Scanning electron micrographs of "new" and "old" whole inactivated *C. burnetii* antigen stocks. "New" batches of whole inactivated *C. burnetii* were visualized in comparison with "old" stocks, to determine why the "new" suspensions appeared to contain less insoluble matter than previous batches of antigen. Aliquots from each batch were adhered to coverslips, dehydrated and gold sputter coated. Specimens were visualized using a Hitachi S34000-I scanning electron microscope. (A) 2,000x magnification of an aliquot from the "new" batch. (B) 2,000x magnification of an aliquot from an "old" batch.

purified and concentrated antigen stocks, as confirmed by electron microscopy, were used in the monocyte stimulation assays reported above and in subsequent work.

5.3.3 Polymorphism in the TLR-2 and TLR-4 genes

Genotyping of the TLR-2 Arg677Trp (C2029T), TLR-2 Arg753Gln (G2258A) and TLR-4 Asp299Gly (A896G) SNPs was done in all subjects for whom DNA was available, using an RFLP technique. No instance of the TLR-2 Arg677TRP polymorphism was found in this cohort. Similarly, no homozygous carriers were detected for either the TLR-2 Arg753Gln or TLR-4 Asp299Gly polymorphisms. Allele frequencies for the Q fever cohort in comparison with EBV and RRV cohorts in DIOS are given in Table 5.2. Two subjects from the RRV and EBV cohorts were excluded as they demonstrated evidence of prior Q fever infection (a serum sample was positive by Q fever IgG ELISA test). There were no differences in allele frequency among subjects presenting with acute Q fever in comparison to subjects with no prior evidence of infection, indicating no likely difference in susceptibility to Q fever infection. In general, comparisons with the RRV cohort are most likely to be valuable as these subjects most closely resemble the Q fever subjects with respect to age, gender and rural exposure risk.

Table 5.2: Observed TLR-2 and TLR-4 genotype frequencies in Q fever and control cohorts[†]

	TLR-2 Arg753Gln (rs5743708)			TLR-4 Asp299Gly (rs4986790)		
	Arg/Arg	Arg/Gln	Gln/Gln	Asp/Asp	Asp/Gly	Gly/Gly
Q fever	82 (80.9)	3 (4.1)	0 (0.1)	71 (72.9)	14 (12.1)	0 (0.4)
RRV	78 (78.0)	4 (3.9)	0 (0.0)	71 (72.0)	13 (12.0)	0 (0.4)
EBV	75 (76.1)	5 (3.9)	0 (0.0)	74 (71.1)	9 (11.9)	0 (0.4)

[†] Observed (expected) allele frequencies by infection type.

Within the confirmed acute Q fever cohort, a comparison of illness severity (PCA-derived severity score or SOMA at enrolment) and duration was made with respect to genotype at the TLR-2 Arg753Gln and TLR-4 Asp299Gly SNP sites (Table 5.3). None of the polymorphic alleles was significantly associated with either severity or duration of acute Q fever, although there was a trend for subjects carrying the TLR-2 Arg753Gln polymorphism or the TLR-4 Asp299Gly polymorphism to experience an increased severity of acute Q fever in comparison to wild type subjects (Kruskal-Wallis test, χ^2=1.862, p=0.172 and χ^2=2.243, p=0.134 respectively). This trend was not strengthened when comparing subjects who were heterozygous at one or both sites of interest with those subjects who were wild type across both polymorphic sites (Kruskal-Wallis test, χ^2=1.489, p=0.222).

Table 5.3: Comparison of acute illness severity and duration by TLR-2 and TLR-4 genotype

	TLR-2 Arg753Gln		TLR-4 Asp299Gly	
	Arg/Arg	Arg/Gln	Asp/Asp	Asp/Gly
Severity[#]	-0.112 (0.879)	0.284 (1.246)	-0.124 (0.843)	0.470 (1.174)
SOMA score[†]	5.7 (3.2)	6.0 (4.0)	5.7 (3.0)	7.5 (3.0)
Illness duration*	157 (235)	101 (125)	157 (247)	145 (138)

PCA-derived severity score at enrolment (normalised to a mean of 0), mean (SD)

† SOMA score at enrolment (possible range 0-12), mean (SD)

* Days SOMA score >3 ("SOMA positive"), mean (SD)

Interestingly, despite the previously described correlation between severity of the acute illness and duration of illness after Q fever (Chapter 4), trends for subjects carrying polymorphisms in TLR-2 or TLR-4 to have more severe acute illness were accompanied by a trend for these subjects to have shorter duration of the acute illness. A greater number of subjects carrying either polymorphism would be required to determine whether such trends were significant.

To confirm functionality of the TLR-4 Asp299Gly polymorphism, cytokine production capacity was determined in PBMC cultures with *Salmonella* LPS stimulation in TLR-4 wild type (n=49) and TLR-4 heterozygote (n=11) subjects. Overnight cultures were harvested and cytokine production assayed by a multiplex bead assay. No significant differences were found between TLR-4 wild type and heterozygote subjects in the

magnitude of *ex vivo* cytokine production for any of the cytokines measured (Mann-Whitney U test, Figure 5.18). Given the low incidence of the TLR-2 Arg753Gln polymorphism across the entire DIOS cohort and the limiting number of PBMC samples available for each subject, comparisons of TLR ligand responsiveness by TLR-2 genotype were not possible.

B cells express TLRs, and in the context of TLR-2 ligation of DCs favouring a Th2 response, may be predicted to enhance humoral immune response. It was therefore hypothesised that polymorphisms in TLR-2 or TLR-4 might affect antibody production in response to Q fever. Antibody production profiles were compared by TLR genotype (Figure 5.19). No significant differences in *Coxiella*-specific (combined anti-phase I and anti-phase II) IgG serum levels were seen with respect to either TLR-2 or TLR-4 genotype (Mann-Whitney U test, $p>0.05$). Comparisons by TLR-2 genotype were limited by the presence of only two subjects with Q fever who carried the TLR-2 Arg753Gln polymorphism.

5.4 Discussion

Toll-like receptors enable pathogen recognition by host innate immune cells. TLR ligation causes activation of microbicidal function in phagocytes, including production of pro-inflammatory cytokines such as TNF-α (Flo *et al.*, 2000) or the induction of respiratory burst (Remer *et al.*, 2005), therefore TLR signalling contributes significantly to the killing of intracellular pathogens (Nacy *et al.*, 1991). Signalling via TLRs has been implicated in

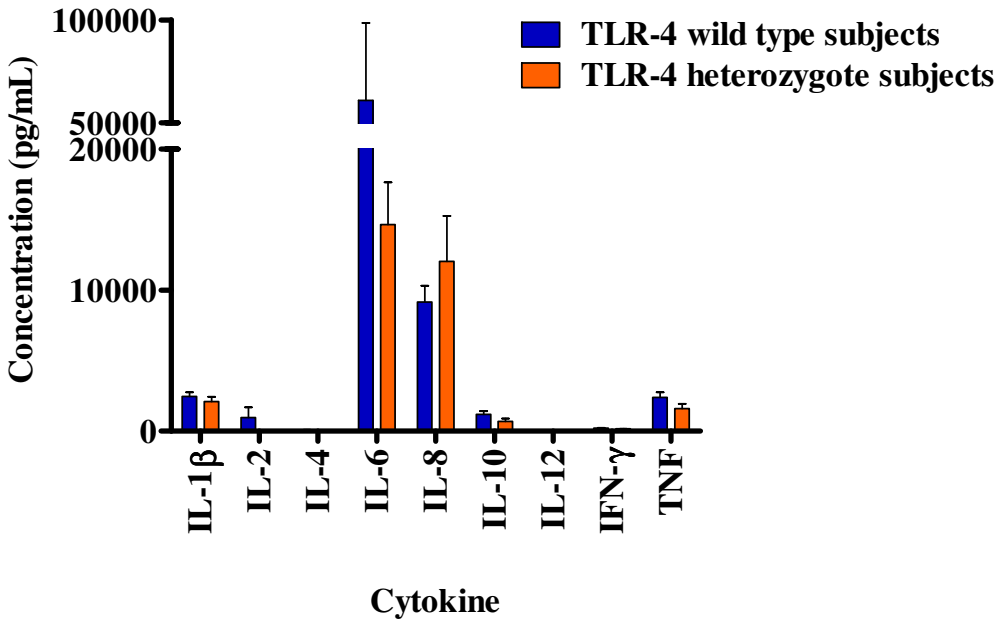

Figure 5.18: Cytokine production by LPS-stimulated PBMC from subjects who carry or do not carry the TLR-4 Asp299Gly polymorphism. Subjects carrying the TLR-4 Asp299Gly polymorphism (heterozygote, n=11) were compared with subjects who did not carry the polymorphism (wild type, n=47) for their capacity to produce cytokines in response to LPS stimulation. Data presented are means for each subject group (error bars represent the standard error).

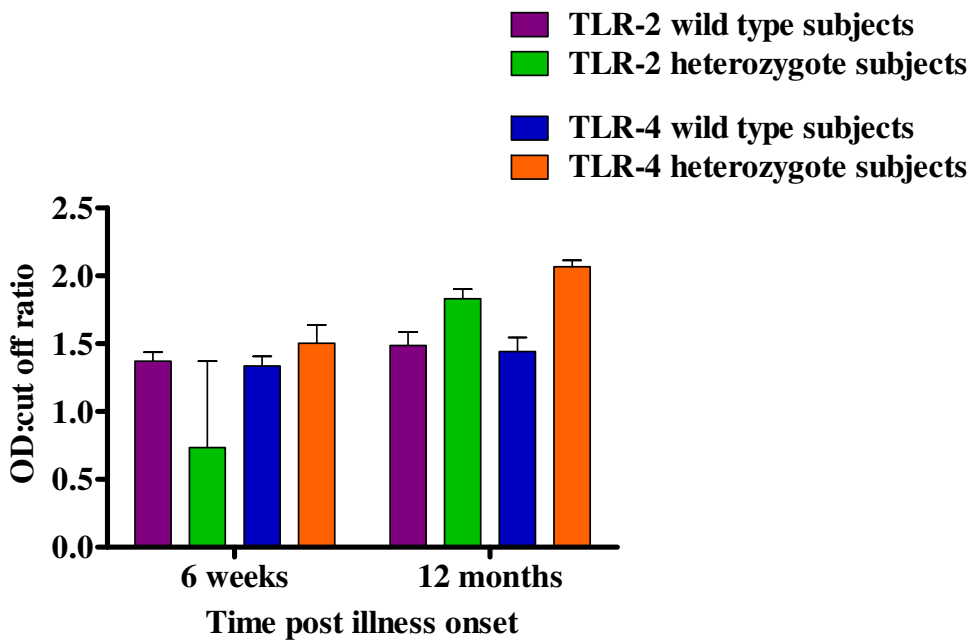

Figure 5.19: Q fever specific IgG production by TLR-2 and TLR-4 genotype. Total *C. burnetii*- specific antibody was detected by ELISA in serum samples taken 6 weeks or 12 months after enrolment to the study. Subjects with confirmed Q fever were grouped according to whether they carry the TLR-2 Arg753Gln polymorphism (TLR-2 heterozygotes, n=2) or do not carry the TLR-2 polymorphism (TLR-2 wild type, n=73). The same subjects were also grouped according to whether they carry the TLR-4 Asp299Gly polymorphism (TLR-4 heterozygotes, n=8) or do not carry the TLR-4 polymorphism (TLR-4 wild type, n=67). Results are presented as mean optical density to cut-off ratios for each group. Error bars represent the standard error.

the DC-driven generation of a strong Th1-type immune response and suppression of Th2-type responses (Sun *et al.*, 2005). Some studies suggest that TLR-2 ligands drive DC responses that favour the development of Th2-type responses, while TLR-4 ligands drive Th1-type favouring responses (Dillon *et al.*, 2004). Elucidation of the TLR(s) involved in the host response to *C. burnetii* is critical in furthering our understanding of the pathogenesis of this macrophage-tropic organism.

There exists increasingly convincing data to suggest that cytokine production by murine macrophages in response to *C. burnetii* infection, and subsequent control of infection, occurs in a TLR-2 dependent (and TLR-4 independent) manner (Zamboni *et al.*, 2004; Meghari *et al.*, 2005; Ochoa-Reparaz *et al.*, 2007). Whether the same holds true for the interaction of human cells with *C. burnetii* remains to be demonstrated. Although TLRs in both mice and humans may direct bactericidal activity against pathogens such as *Mycobacterium tuberculosis*, they do so using distinct mechanisms; in mice, TLR-2 mediated induction of bactericidal function is dependent on nitric oxide production, while in humans the process does not involve nitric oxide (Thoma-Uszynski *et al.*, 2001). The finding that expression of inducible nitric oxide synthase and subsequent nitric oxide production is essential for control of *C. burnetii* replication by murine macrophages (Howe *et al.*, 2002; Zamboni and Rabinovitch, 2003; Brennan *et al.*, 2004) may not necessarily be comparable to the process that occurs in human macrophages. Therefore, an aim of this study was to characterise the human innate immune response to *C. burnetii* and to determine whether TLR-2 or TLR-4 are involved in host recognition of *C. burnetii* infection.

The work presented here used the THP-1 cell line as an *in vitro* model of human monocytes. These cells were demonstrated to possess cell surface expression of TLR-2 and TLR-4, the TLR-4 co-receptor and monocyte marker, CD14, as well as the Fc receptor, CD16. This cell surface receptor expression pattern would suggest that THP-1 cells possess a phenotype comparable to that of the CD14+CD16+ inflammatory monocyte subset. While these cells are not proposed to behave exactly as primary monocytes would, they display similar responses on exposure to pathogens as those produced by primary monocytes, including cytokine induction, production of lysozymes and phagocytosis (Tsuchiya *et al.*, 1980). Their ease of culture made them an ideal tool for this work.

Measurement of cytokine production in THP-1 cells enabled characterisation of an important aspect of the host response to *C. burnetii* exposure. While many proteins may be upregulated in response to infection, it was decided to measure changes in mRNA and protein secretion for a select number of molecules. The measurement of TNF-α and IL-10 mRNA and protein served as markers of the pro-inflammatory and anti-inflammatory activity of monocytes. IFN-β was quantitated at both the protein and mRNA level because of its known participation in the induction of iNOS, and to assess utilisation of the MyD88-independent pathway following *C. burentii* exposure. Quantitation of iNOS mRNA was conducted as a surrogate measure of the potential for monocytes to produce nitric oxide in response to *C. burnetii*.

Many studies point to the important role that TNF-α and IL-10 play in the host response to infection, making them of particular interest in this work. Promotion of *C. burnetii* killing by IFN-γ treatment of THP-1 cells is TNF-α dependent (Dellacasagrande *et al.*, 1999; Dellacasagrande *et al.*, 2002) while IL-10 enhances replication of *C. burnetii* in primary human monocytes via down regulation of TNF-α (Ghigo *et al.*, 2001). Direct treatment of an infected murine macrophage cell line with TNF-α protein has been shown to be bacteriostatic (Howe *et al.*, 2002) and TNF-α production is critical in controlling early bacterial replication in *C. burnetii*-infected mice (Andoh *et al.*, 2007).

In Q fever there is evidence to suggest TNF-α facilitates *C. burnetii* pathogenesis. Overproduction of TNF-α (and IL-10) by unstimulated PBMC isolated during the acute phase of Q fever was associated with subsequent development of chronic Q fever among subjects with valvulopathy (Honstettre *et al.*, 2003). Monocytes from patients with ongoing Q fever endocarditis display an inability to control *C. burnetii* replication in association with high levels of spontaneous TNF-α protein secretion (Dellacasagrande *et al.*, 2000; Honstettre *et al.*, 2003) and high secretion of the soluble 75kDa TNF-α receptor (Ghigo *et al.*, 2000). TNF-α has been shown to promote bacterial uptake by monocytes, thereby contributing to *C. burnetii* virulence (Dellacasagrande *et al.*, 2000). A similar phenomenon has been demonstrated in the pathogenesis of tuberculosis; highly pathogenic strains of *M. tuberculosis* elicit greater TNF-α responses than less pathogenic strains (Manca *et al.*, 1999), and TNF-α production is associated with increased bacterial replication within human monocytes (Silver *et al.*, 1998). Thus, it seems likely that

although TNF-α enhances killing of intracellular *C. burnetii* it also drives uptake of the bacterium and may contribute to the ongoing immunopathology of chronic Q fever.

IL-10 has been implicated by many studies in the pathogenesis of Q fever. Clinically, high spontaneous IL-10 production by PBMC from subjects with acute Q fever has been associated with subsequent development of chronic infection (Honstettre *et al.*, 2003). While both IL-10 and TGF-β are highly secreted by PBMC from patients with chronic Q fever, only IL-10 correlates with clinical status (Capo *et al.*, 1996). Defects in transendothelial migration of leucocytes that are seen in patients with chronic Q fever are IL-10 dependent, and IL-10 likely accounts for the poor cellular response and lack of granuloma formation seen in patients with chronic Q fever (Meghari *et al.*, 2006). Persistent infection of IFN-γ knockout mice by avirulent phase II organisms is associated with production of IL-10 by infected peritoneal macrophages (Ochoa-Reparaz *et al.*, 2007). Furthermore, IL-10 treatment of *C. burnetii*-infected monocytes has been shown to enhance bacterial replication (Ghigo *et al.*, 2001) and impair phagosome maturation (Ghigo *et al.*, 2004). Mice lacking CD28, which are expected to be unable to mount antigen-specific T cell responses, are able to control *C. burnetii* infection due to their impaired ability to produce IL-10 (Honstettre *et al.*, 2006). IL-10 is therefore a key regulator of the host immune response to infection, and is often associated with chronic infection due to its role in promoting *C. burnetii* replication within infected monocytes.

Stimulation via TLR-4 and the MyD88-independent pathway, but not via TLR-2, results in the induction of IFN-β (Toshchakov *et al.*, 2002). This type I interferon is believed to be

critical in the activation of bactericidal activity in monocytes and macrophages via the activation of the STAT1 transcription factor (Punturieri *et al.*, 2004). IFN-β-mediated activation of STAT1 appears to be important in the LPS-mediated induction of key host immune defense molecules, including IFN-inducible 10-kDa protein (IP-10), IFN regulatory factor-1 (IRF-1), and inducible nitric oxide synthase (iNOS) (Ohmori and Hamilton, 2001). Given the known importance of nitric oxide in the host defense against *C. burnetii* (Howe *et al.*, 2002; Zamboni and Rabinovitch, 2003; Brennan *et al.*, 2004), it was important to identify whether *C. burnetii* induces IFN-β upregulation in host monocytes and macrophages and to determine whether any increases in IFN-β are associated with the induction of iNOS.

THP-1 cells stimulated with whole, inactivated *C. burnetii* antigens upregulated the transcription of both TNF-α and IL-10 mRNA, and secreted TNF-α and IL-10 protein into the culture medium. By contrast, IFN-β mRNA and protein were not upregulated by THP-1 cells stimulated with *C. burnetii*, regardless of antigen concentration. Furthermore, induction of iNOS mRNA was not detectable via real time PCR assay. This suggests that *C. burnetii* is unlikely to interact with TLR-4 on human cells. Alternatively, *C. burnetii* may interact with TLR-4 but trigger only the MyD88-dependent pathway, resulting in NF-κB induction and pro-inflammatory cytokine expression, but not activation of the MyD88-independent pathway that drives IFN-β and subsequent iNOS induction. Inability of monocytes or macrophages to be activated via the MyD88-independent pathway may therefore result in uncontrolled replication of *C. burnetii* within the host immune cells.

Early characterization experiments revealed that cytokine induction by THP-1 cells in response to *C. burnetii* was considerably delayed in comparison to the control stimuli. The kinetics of TNF-α production by THP-1 stimulated with LPS reported here were very similar to those reported for whole blood stimulated with LPS derived from the same strain of *E. coli* (DeForge and Remick, 1991). The observed differences in the kinetics of TNF-α induction kinetics between *C. burnetii*-stimulated THP-1 cells and LPS-stimulated THP-1 cells may relate to the reduced potency of *C. burnetii* LPS in comparison to other bacterial LPS strains (Amano and Williams, 1984), however this is unlikely given that the dose of *C. burnetii* antigen used here is more than 1000 fold the concentration of *E. coli*-derived LPS used as a control stimulus. Furthermore, while *C. burnetii*-driven cytokine production was delayed, the peak magnitude of cytokine induction was greater than that induced by control stimuli suggesting that stimulus concentration was not a likely reason for the varied kinetics of TNF-α induction. TNF-α induction was rapid in comparison to IL-10, presumably due to IL-10 production requiring a stronger TLR stimulus than that needed for TNF-α production (Dehus *et al.*, 2008), and these differences were not unexpected given previous reports of rapid TNF-α production but delayed IL-10 production by human monocytes in response to LPS (de Waal Malefyt *et al.*, 1991).

When used at the same concentration, whole inactivated phase I antigen induced less TNF-α and IL-10 than phase II antigen. The finding that phase II is a more potent inducer of cytokines than phase I is consistent with the notion that phase I LPS shields immunostimulatory epitopes, preventing immune recognition and activation (Shannon *et al.*, 2005b). Phase I LPS is also thought to be an antagonist of TLR-4-mediated signalling

(Zamboni *et al.*, 2004), therefore it is not surprising that phase I organisms were less potent inducers of cytokine responses in THP-1 cells than phase II organisms, despite the fact that they differ only in the presence of longer, more complex LPS structures.

TLR-specific antibody clones were chosen for this work based on previous reports that they substantially inhibit TLR-2 and TLR-4-mediated signalling respectively. Treatment with the TLR-2 specific antibody clone, TL2.1, has been demonstrated to decrease cytokine production in human monocytes in response to the Gram-positive bacterium, *Listeria monocytogenes*, but not TLR-4 ligands such as LPS or the Gram-negative Group B Streptococci (Flo *et al.*, 2000). Treatment with the TLR-4 specific antibody clone, HTA125, has similarly been demonstrated to inhibit IL-6 induction by fibroblasts in response to *Porphyromonas gingivalis* LPS (Tabeta *et al.*, 2000). It is worth noting that some studies suggest the binding affinity of HTA125 to be weak and readily blocked by the presence of non-specific murine or human IgG, and that treatment with HTA125 may result in engagement via the monocyte Fc receptor (Wang *et al.*, 2003). Preliminary experiments in THP-1 cells confirmed that despite these potential limitations, TL2.1 and HTA125 were able to significantly down-regulate the induction of TNF-α in response to TLR-2 and TLR-4 ligands respectively.

The data generated in THP-1 cells provide clear evidence for the role of TLR-2 in TNF-α and IL-10 production in response to stimulation with phase I and phase II whole killed antigen. Use of antibody treatment to block signalling via TLR-2 (clone TL2.1) but not TLR-4 (clone HTA125) was able to substantially decrease TNF-α protein secretion and IL-

10 mRNA induction in THP-1 cells in response to *C. burnetii* whole cell antigens. A 12h stimulation protocol was chosen to enable the detection of antibody-mediated suppressive effects on both TNF-α and IL-10 production by THP-1 cells; at this time point measurable differences were detectable in TNF-α protein, TNF-α mRNA and IL-10 mRNA but not in IL-10 protein due to differences in the kinetics of induction for these two cytokines.

THP-1 cells are often utilised as a model of primary monocytes, however some studies have noticed significant functional differences in these cells in comparison to primary monocytes. THP-1 cells have been used extensively in the study of Hantaviruses, yet when infected, THP-1 cells express different chemokine receptors to those expressed by primary monocytes (Markotic *et al.*, 2007). THP-1 cells have also been used to elucidate signalling pathways in primary monocytes, yet conditions that induce strong increases in NADPH oxidase activity in primary monocytes have significantly reduced effect in THP-1 cells (Almeida *et al.*, 2005). By contrast, functional studies comparing THP-1 cells with primary monocytes have indicated comparable requirements for the induction of G-CSF (Hareng *et al.*, 2003). Therefore in some cases THP-1 cells have proven to be a good model of primary monocytes, yet when examining other functional aspects they may be less than ideal. It is always preferable that data obtained in THP-1 cells be replicated in primary monocytes to confirm the data's relevance *in vivo*.

Replication of the finding that TLR-2 is involved in the host response to *C. burnetii* in primary monocytes was unsuccessful. Cytokine release from peripheral blood mononuclear cells has been demonstrated to be highly dependent on experimental

conditions, including whether the cultures occur with continual shaking (rapid kinetics in comparison to stationary cultures), the stimulatory dose (higher concentration of stimulus results in faster kinetics) and the culture volume (larger culture volumes result in delayed kinetics) (Chaka *et al.*, 1997). In the experiments reported here, it appeared that the process of primary monocyte adhesion to culture plate surfaces was inducing upregulation of TNF-α mRNA, such that when secondary stimuli (such as *C. burnetii*) were added, TNF-α production was amplified. Thus, adhesion seemed to prime monocytes to induce cytokine responses that could not be sufficiently blocked with anti-TLR antibodies. Attempts to inhibit monocyte adhesion binding to the culture vessel surfaces by the use of culture plates with low-attachment surfaces, or to inhibit monocyte clumping by the use of falcon tubes on a rotating platform, did not enhance antibody-mediated suppression of TLR signalling. Failure to inhibit TLR signalling was not a result of insufficient antibody, as a two fold increase in the recommended concentration did not enhance blocking. Despite reports that the TL2.1 and HTA125 anti-TLR antibodies can be used to block TLR signalling in human monocytes (Flo *et al.*, 2002), it was not possible to demonstrate TLR-specific effects of anti-TLR antibodies in this system. It therefore remains to be definitively proven that TLR-2 plays a critical role in the response of primary monocytes to *C. burnetii*.

Negative isolation of monocytes using magnetic polymers has been widely used as a method for the efficient purification of monocytes with intact morphology and function (Flo *et al.*, 1991). The kit chosen here includes antibodies to positively select and remove B and T lymphocytes, NK cells, macrophgages, dendritic cells, mast cells, basophils and red blood cells. It is noteworthy that in response to infection, a subset of pro-inflammatory

monocytes designated CD14+ CD16+ increase substantially in number in comparison to classical monocytes (CD14++) (Ziegler-Heitbrock *et al.*, 1993). These monocytes are believed to be a more mature form of classical monocyte, and are known to be generated in response to bacterial infections (Fingerle *et al.*, 1993). Given that the monocyte isolation kit utilised in this study makes use of anti-CD16 antibodies (for the positive selection and removal of NK cells), it is possible that the monocyte population being used in the experiments here is predominantly of the classical phenotype (CD14++), and not the pro-inflammatory population (CD14+ CD16+) that would arguably be active in the host response to *C. burnetii*. Negative isolation is considered important in preventing non-specific activation of monocytes as they are separated from whole blood, yet it may be important to retain the CD14+CD16+ population (by positive selection) as these cells may be the ones of greatest interest in the study of Q fever infection.

Having found preliminary data in THP-1 cells to suggest that TLR-2 may play a role in the human monocyte response to *C. burnetii*, we sought evidence for a role of functional TLR polymorphisms in susceptibility to, or outcome from, acute Q fever in the DIOS cohort. Of primary interest was the TLR-2 Arg753Gln (G2258A) polymorphism. A second polymorphism in TLR-2 (Arg677Trp, C2029T) was sought but not identified in this cohort, which was not unexpected given other reports that this polymorphism is absent in Caucasian subjects (Schroder *et al.*, 2003a). Presence of the TLR-4 Asp299Gly (A896G) SNP was also determined, given that presence of LPS as a major cell wall constituent of *C. burnetii* suggests the possible role of TLR-4 in pathogen sensing.

Instances of the TLR-2 Arg753Gln and TLR-4 Asp299Gly polymorphism were only found in the heterozygous state in the DIOS cohort. These polymorphisms in TLR-2 or TLR-4 were not more prevalent in the Q fever cohort than in the control cohorts (EBV, RRV), nor were they associated with the severity or duration of acute Q fever.

The functionality of polymorphisms within TLR-2 and TLR-4 remain a point of contention within the literature. Studies using transfection or overexpression models suggest that both the TLR-2 Arg753Gln and TLR-4 Asp299Gly polymorphic alleles demonstrate reduced sensitivity to TLR ligands (Arbour *et al.*, 2000; Lorenz *et al.*, 2000; Schroder *et al.*, 2003a). The relevance of these functional defects in individuals with heterozygosity at the TLR-4 Asp299Gly allele remains controversial, with some suggesting a single functional TLR-4 copy is sufficient for LPS responsiveness (von Aulock *et al.*, 2003). It has been hypothesised that the TLR-4 polymorphism merely alters the kinetics of LPS responsiveness, specifically the kinetics of IL-10 release, and that difficulties in demonstrating functional deficiencies in immune cells from individuals who are heterozygous at this allele may result from the specific nature of those cytokine induction defects (Dehus *et al.*, 2008). In subjects from DIOS, carriage of the TLR-4 Asp299Gly allele did not confer reduced responsiveness to LPS with respects to cytokine production, which is in concordance with the notion that this polymorphism only results in functional defects when it occurs in the homozygous state.

Associations between the TLR-4 Asp299Gly polymorphism and susceptibility to septic shock are reported, but the polymorphism may also increase resistance to Legionnaire's

disease (Lorenz *et al.*, 2002; Hawn *et al.*, 2005), suggesting that deficiencies in TLR-4 signalling may convey protection in some contexts from harmful immunopathology. In subjects from the DIOS cohort it was not possible to demonstrate a role for the TLR-4 Asp299Gly polymorphism in conferring either increased susceptibility to infection or resistance to severe or prolonged Q fever disease.

The functional significance of heterozygosity at TLR-2 Arg753Gln remains uncertain, with some studies suggesting that presence of a single functional TLR-2 allele to be sufficient for lipopeptide responsiveness (von Aulock *et al.*, 2004). Very few subjects in DIOS were carriers of the TLR-2 Arg753Gln allele, therefore it was not possible to determine whether heterozygous individuals in this study were hyporesponsive to TLR-2 ligands. Despite previous studies which have variously found associations between TLR-2 polymorphisms and susceptibility to bacterial infections (Lorenz *et al.*, 2000; Texereau *et al.*, 2005), no association was found between carriage of the TLR-2 Arg753Gln polymorphism and susceptibility to acute Q fever in the DIOS cohort.

TLRs are primarily expressed by cells of the innate immune system, however TLR-2 and TLR-4 are also known to be expressed at low levels on B cells (Dasari *et al.*, 2005). Furthermore, TLR ligands including LPS are known to induce polyclonal expansion of B cells (Quintans and Lefkovits, 1974). Despite the possible involvement of TLR engagement in the induction of *C. burnetii*-specific antibody responses, no correlation was found between carriage of polymorphisms of TLR-2 or TLR-4 and the magnitude of antibody responses in subjects with acute Q fever.

5.5 Conclusions

Monocytes mount a response to *C. burnetii* that involves the induction of both pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines. There was no evidence that *C. burnetii* interaction with host monocytes was able to trigger MyD88-independent pathways, and stimulation of monocytes with whole inactivated *C. burnetii* did not lead to the expression of IFN-β or iNOS. This pathway is believed to be important for the enhancement of microbicidal activity of *C. burnetii*-infected monocytes and macrophages, and thus the pathogenesis of *C. burnetii* likely relates to the poor activation of this pathway by host immune cells.

Toll-like receptors are known to play a critical role in the host response to bacterial infections. Convincing data were obtained to suggest a role for TLR-2 in the induction of cytokine responses to *C. burnetii* by the monocytic cell line, THP-1. Despite the long standing notion that LPS from Gram negative bacteria interacts with innate immune cells via TLR-4, there was no evidence in these experiments to suggest a role for TLR-4 in the triggering of the immune response to *C. burnetii*.

The functionality of non-synonymous polymorphisms in TLR-2 (Arg753Gln) and TLR-4 (Asp299Gly) remains debatable. In the DIOS cohort there was no evidence that carriage of polymorphic TLR alleles resulted in increased susceptibility to acute Q fever. Subjects from the acute Q fever cohort who carry polymorphic TLR alleles did not suffer from an

illness that was more severe or prolonged than individuals with homozygous wild type TLR alleles. Heterozygosity at the TLR-4 Asp299Gly allele did not result in deficits in responsiveness to LPS. Quantitative differences in Q fever-specific antibody were not apparent when comparing subjects by TLR genotype. It therefore seems unlikely that polymorphisms in TLR-2 or TLR-4 play a significant role in the pathogenesis of acute Q fever, despite the probable importance of TLR-2 in the host response to *C. burnetii*.

6. Adaptive immune responses to *C. burnetii*

6.1 Introduction

C. burnetii is a pathogen that survives and replicates within the phagolysosome of innate immune cells such as macrophages and monocytes. As a result, a significant body of research into the pathogenesis of Q fever focuses on the interaction of *C. burnetii* with the innate immune system. From this research it has become clear that in the absence of stimulation by adaptive cellular immune responses, macrophages are poorly able to kill this intracellular pathogen (Ghigo *et al.*, 2002; Andoh *et al.*, 2007). Furthermore, the generation of adaptive immune responses by vaccination has been demonstrated to protect against disease symptoms upon *C. burnetii* re-challenge, making both antibody and cellular responses of particular interest to researchers (Gajdošová *et al.*, 1994; Zhang *et al.*, 2007). The aim of this work was to determine whether the characteristics of the host adaptive immune response correlate with severe or prolonged illness in Q fever.

In vaccinees or subjects who have been naturally infected, *C. burnetii*-specific responses are classified as being phase I or phase II-specific, with some shared epitopes also identified (Hackstadt *et al.*, 1985). Phase II-specific humoral responses occur earlier, and at higher magnitude, in acute Q fever, while phase I-specific responses occur later in infection (Embil *et al.*, 1990) and it is these phase I-specific responses that may be re-stimulated on secondary exposure to *C. burnetii* (Peacock *et al.*, 1979; Zhang *et al.*, 2007). Temporal variance in immunoglobulin isotype is also apparent in subjects with Q fever: primary infection results in an early IgM response, while IgG is delayed until early

convalescence, and IgA is generally restricted to individuals with chronic infection (Murphy and Magro, 1980; Worswick and Marmion, 1985). Although humoral responses do not appear to be sufficient for protection against infection in the absence of cellular responses, immune sera can confer protection against *C. burnetii* in an immunocompetent host, highlighting the potential importance in the host response to infection or vaccination (Zhang *et al.*, 2007). It is therefore possible that variance in the kinetics or magnitude of humoral responses may confer susceptibility to severe or prolonged acute Q fever.

Activation of cellular responses appears to be critical in triggering microbicidal activity in *C. burnetii*-infected phagocytes (Ghigo *et al.*, 2002; Benoit *et al.*, 2008a). More specifically, production of IFN-γ is known to be important in inducing killing of intracellular *C. burnetii* and in driving a Th1-type immune response (Mosmann and Coffman, 1989) that is effective in countering intracellular pathogens. In related infections such as those caused by *Leishmania major* or *Mycobacterium tuberculosis*, the generation of Th1-type immune responses is associated with pathogen clearance, while Th2-type responses are associated with failure to control infection (Scott, 1998; Stenger and Modlin, 2002).

Cellular responses remain detectable for 15 years or more following natural infection or vaccination (Gajdošová and Brezina, 1989) and are proposed to confer the long term protection afforded by the QVax^{TM} vaccine (Marmion *et al.*, 1990). It is believed that phase I-specific cellular responses are critical in the generation of protective immunity and that these responses may only be generated by exposure to phase I organisms, while phase

II-specific responses are generated in response to either phase of *C. burnetii* (Ormsbee *et al.*, 1964). Attempts to characterise antigen-specific cellular responses in individuals who have been exposed to *C. burnetii* are hampered by the presence of LPS in whole inactivated *C. burnetii* organisms when used as recall antigens. LPS-stimulated monocytes are known to trigger non-specific T cell activation in a subset of the human population (Mattern *et al.*, 1994), although the mitogenic capacities of *C. burnetii* LPS are unclear. There are suggestions that phase I LPS may actually inhibit the detection of cellular responses, either via shielding immunostimulatory epitopes (Shannon *et al.*, 2005b), or due to a direct immunosuppressive effect of phase I LPS (Izzo and Marmion, 1993). Phase I organisms have been shown to trigger both antigen-specific and antigen-non-specific T cell suppression (Damrow *et al.*, 1985; Koster *et al.*, 1985b; Scott *et al.*, 1987), yet exposure to phase I organisms is essential for the generation of protective immunity (Ormsbee *et al.*, 1964).

The adaptive immune response to Gram negative bacteria, including *C. burnetii*, therefore appears to derive in part from antigen non-specific effects, either by the action of LPS directly on B lymphocytes (Gronowicz and Coutinho, 1974), or by the indirect activation of T lymphocytes by LPS-stimulated monocytes (Vogel *et al.*, 1983). In fact, exposure to *C. burnetii* can induce non-specific humoral and cellular immunity to unrelated pathogens (Zvilich *et al.*, 1995), and cellular immunity does not seem to be impaired even in mice lacking the co-stimulatory CD28 molecule traditionally thought to be essential for the induction of antigen-specific T cell responses (Honstettre *et al.*, 2006). This further

complicates the task of assessing the contribution of antigen-specific T cell responses to *C. burnetii* clearance and illness course.

One means of implicating antigen-specific responses to *C. burnetii* is to investigate whether human leucocyte antigen (HLA) alleles are associated with susceptibility or resistance to Q fever. Priming of antigen-specific responses in T and B cells is dependent on antigen presentation by HLA class II molecules on the surface of antigen presenting cells, or by HLA class I on the surface of infected host cells. The HLA loci are highly polymorphic, with individual alleles having generally different peptide binding specificities. The host adaptive immune response to *C. burnetii* is likely to vary depending on the HLA alleles carried by each individual, as different alleles confer responsiveness to a different range of pathogen-derived peptides. In particular, HLA class II alleles are of interest due to the known interaction between Q fever antigens and HLA-DR and -DM in the HLA class II loading compartment, where enhanced interaction of HLA-DR and -DM results in instability and slower loading of antigenic peptides (Lem *et al.*, 1999). In particular, the HLA-DRB1*11 allele has been reported to be over-represented in subjects who develop Q fever syndrome (Helbig *et al.*, 2003; Helbig *et al.*, 2005). An aim of this work was to compare the HLA allele carriage by subjects with Q fever with subjects in the control cohorts (EBV, RRV) and to determine whether HLA alleles carried by the Q fever cohort were associated with protection or susceptibility to severe acute illness.

Studies of viral infections such as HIV-1 make use of synthesised peptides, designed to replicate every possible epitope present in a viral genome by the use of overlapping

sequences of 8-15 amino acids in length, as stimuli for the detection of antigen-specific responses and for the identification of immunogenic epitopes within the pathogen of interest (Meddows-Taylor *et al.*, 2007). Although the genome of *C. burnetii* has been sequenced, it is more than 200-fold larger than the HIV-1 genome, making this type of approach non-feasible due to prohibitive cost (Seshadri *et al.*, 2003). An alternative method is to identify regions of a *C. burnetii* protein that are likely to be processed appropriately by the proteasome to create peptide epitopes and that are predicted to bind to a specific HLA allele. Complex algorithms have been created to predict HLA-restricted epitopes from an amino acid input sequence, such as the *in silico* "ProPred" (Singh and Raghava, 2003). This algorithm draws on data relating to known HLA-peptide binding affinities and on data that have been obtained regarding cleavage sites for proteasome-mediated protein degradation. The work presented here makes use of such algorithms to predict HLA-A2 and HLA-A3 restricted epitopes within *C. burnetii*.

A single *C. burnetii* protein was chosen as a target for peptide design. Heat shock protein B, (HspB, encoded by the *htpB* gene, CBU1718) is a 60kDa heat shock protein with chaperone function. Heat shock protein B is a homologue of the *E. coli* GroEL protein, and has been shown to have high identity across *C. burnetii* isolates (Fernandes *et al.*, 2009). It is expressed in both large and small cell variants of *C. burnetii*, although it is found more abundantly in the large cell variant indicating that it is induced in response to environmental stressors in the phagolysosome of host macrophages (Coleman *et al.*, 2007). It is therefore likely to be produced in abundance during host infection. HspB has also been shown to be antigenic when used as a vaccine in mice (Vodkin and Williams, 1988), while

vaccination with a fusion protein including HspB induced both cellular and humoral immune responses and protected mice from *C. burnetii* challenge (Li *et al.*, 2005). In human infection HspB acts as an antigen, and HspB-specific antibody responses can be detected in sera from naturally infected individuals (Coleman *et al.*, 2007). A total of ten peptides with predicted epitopes for HLA-A2 or HLA-A3 were synthesised and utilised in IFN-γ ELISpot assays to detect peptide-specific responses in subjects who carry HLA-A2 or HLA-A3. The major goal of this work was to identify CD8+ T cell epitopes within the *C. burnetii* HspB protein, and to determine whether responses to these epitopes vary in relation to the course of infection.

Aspects of humoral and cellular immunity were therefore examined in a subset of subjects suffering prolonged illness after acute Q fever, in comparison to age and sex matched subjects who had quickly resolving symptoms. In this case-control series, humoral responses were quantitated in longitudinally collected serum samples, allowing comparisons of antibody titres and their kinetics of induction, and an assessment of the role of phase-specificity and antibody isotype. General immune activation was assessed as a correlate of illness course, by quantitating the proportions of leucocyte subsets and their activation states by flow cytometric analysis of PBMC sampled at regular intervals over the 12 months following acute Q fever.

The importance of antigen-specificity was considered indirectly, by examining the HLA allele frequencies within the Q fever and the control (EBV, RRV) cohorts. For any HLA allele that was identified to be over-represented or under-represented in the Q fever cohort,

further analysis was conducted to determine whether these alleles were associated with illness severity or duration. Finally, HLA-A2 and HLA-A3-restricted *C. burnetii*-specific peptides were synthesised and used as a tool to identify potential epitopes within a *C. burnetii* protein, HspB, that is known to be immunogenic *in vivo*.

6.1.1 Aims and hypotheses

It was hypothesised that the severity or duration of acute Q fever illness may relate to the quality or magnitude of the adaptive host response to infection.

An aim of this chapter was to examine the following factors of the adaptive immune response in subjects who experienced promptly resolving or prolonged symptoms of acute Q fever:

(1) the patterns of antibody production

(2) the circulating leucocyte populations, including relative proportions of different cell types and their activation states

(3) the patterns of cytokine production by PBMC when stimulated in culture

A futher aim was to conduct genotypic analysis of the polymorphic HLA alleles for the entire DIOS cohort to determine whether specific alleles may be associated with susceptibility to, or outcome from, acute Q fever.

6.2 Methods

6.2.1 Serology

Serological screening was conducted on all subjects with suspected Q fever to confirm the occurrence of acute illness. More detailed serological testing was conducted on a subset of subjects by the Institute of Medical and Veterinary Science (Adelaide, Australia), with longitudinal sera from a subset of case and control subjects with 'long' or 'short' illness respectively having quantification of IgG, IgM and IgA phase I and phase II specific immunoglobulin via indirect fluorescent antibody test (IFA), and total phase I or phase II specific immunoglobulin via complement fixation (CF). Antibody titres were quantitative and reflected the serum dilution at which antibody activity was still detectable.

6.2.2 Flow cytometry

To examine the proportions of leucocytes and their activation states in the peripheral blood of subjects over the course of their illness, a standard protocol was used for four colour surface marker staining and data collection via fluorescent activated cell sorting (FACS). Monoclonal antibodies were all obtained from Becton-Dickinson (BD Bioscience). The surface markers included in the analysis of PBMC subsets were: CD3 (T lymphocytes), CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes), CD14, CD16, CD56, and CD19. The activation markers analysed included CD69 and HLA-DR.

Commercial fluorochrome-conjugated antibodies were added to clean 75mm x 12mm plastic tubes (BD-Bioscience, San Diego, CA). The antibodies were titrated to determine

acceptable working concentrations for reliable staining without loss of reasonable fluorescent signal. In general, the quantity of antibody required was dependent on the fluorochrome to which each was conjugated. For all fluorescein (FITC)-, phycoerythrin (PE)- and PerCP®-conjugated antibodies, 10μL was acceptable, but for allophycocyanin (APC)-conjugated antibodies this was reduced to 3μL. Antibodies were added in appropriate combinations as outlined below (Table 6.1, Table 6.2). The antibody panel used for the early stages of the study (Table 6.1) was later expanded to enable more detailed characterization of the circulating leucocytes (Table 6.2).

Table 6.1: Standard antibody panel used for flow cytometric analysis of circulating leucocyte subsets and their activation status

Tube no.	FITC	PE	PerCP	APC
1	IgG1	IgG2a	IgG1	IgG1
2	CD45	CD14	-	-
3	CD3	CD4	CD8	-
4	HLA-DR	CD4	CD8	CD69
5	HLA-DR	CD16	-	CD69
6	HLA-DR	CD14	-	CD69

Table 6.2: Expanded antibody panel used for flow cytometric analysis of circulating leucocyte subsets and their activation status

Tube no.	FITC	PE	PerCP	APC
1	IgG1	IgG2a	IgG1	IgG1
2	CD3	CD8	CD45	CD4
3	CD3	CD16+56	CD45	CD19
4	HLA-DR	CD8	CD4	CD69
5	HLA-DR	CD16	CD4	CD14
6	HLA-DR	CD19	-	CD69
7	CD16	CD11b	CD4	CD69

Stored PBMC were thawed and washed in PBS to remove the RPMI indicator dye. Depending on the number of available cells, 1-5x10^5 PBMC were seeded into each tube as 50µL aliquots, gently mixed, and incubated for 15-30 min in the dark. The cells in each tube were washed twice with 0.5mL PBS with 2% bovine serum albumin (BSA, Sigma Aldrich) and then re-suspended in 2% paraformaldehyde. All tubes were stored in the dark at 4°C until data acquisition. Data were collected on a FACSCalibur® and analysed with Cellquest® software (BD Bioscience). All analysis was compared against irrelevant mouse isotype-matched antibodies.

6.2.3 Stimulation assays – Peripheral blood mononuclear cells

To determine the non-specific cytokine producing capacities of PBMC taken from subjects over the course of their illness, stimulation assays were conducted on a subset of subjects

with ‘short’ or ‘long’ duration of acute Q fever, and in subjects from the control cohorts (EBV and RRV). Thawed PBMC were incubated in RPMI containing 10% FCS in 96-well flat-bottom plates for 24 hours at 37°C in 5% CO_2, with various stimulants. The stimulants were either none (media), Dynabeads® bearing anti-CD3 and anti-CD28 (Dynal Biotech, Oslo, Norway), or lipopolysaccharide (LPS) (Salmonella-derived, Sigma). PBMC (10^5 per well) were incubated in replicates of 6 wells, with the supernatants pooled after cell-free harvest then divided into 3 aliquots for storage at –80°C. Dynabeads were added at a 4 beads: 1 cell ratio (4 x 10^5 beads/well) and LPS at 10ng/mL.

Cultured PBMC supernatants, or serum, were analysed undiluted following the instructions for the multi-cytokine Bio-Rad 10-plex kit (Bio-Rad, Hercules, USA) in a 96-well plate. Standards were made in a dilution series from reconstituted stock following the manufacturer’s recommendations, and run in duplicate. Samples were run in single wells as the bead-based immunoassay format provides multiple replicates within the well. The plate was pre-wetted with assay buffer, before addition to each well of a diluted bead mix, each bead conjugated to a primary antibody specific for one of 10 cytokines. These beads were washed without removal from the plate prior to addition of 50µL of sample or standard to appropriate wells. The plate was incubated with rocking for 30min in the dark, washed, and then the biotinylated secondary antibody mix (containing 10 antibodies) added to all wells. The plate was incubated with rocking in the dark for 30min, washed, and then streptavidin-PE was added to all wells. The plate was incubated with rocking for 10min in the dark, and then washed. A final volume of assay buffer was added to each well and the plate analysed on a Bioplex® reader with Bioplex Manager® software v2.0 or v3.0 (Bio-

Rad). The vacuum manifold used for washing the plates was from Millipore (Millipore, Billerica, USA). The software does not allow calculation of values that lie outside the reliable region of the standard curve, and declares them to be out of range. Previous quality control experiments in our laboratory have revealed that this multiplex method performed well when compared with commercial ELISAs (e.g. Spearman's correlation for IL-6 $p<0.001$, data not shown).

6.2.4 HLA genotyping

Human leucocyte antigen (HLA) alleles determine the ability of an individual to mount specific cellular immune responses to peptide epitopes. Given the importance of a robust cellular immune response in clearing *C. burnetii* infection, an aim of this study was to determine whether specific HLA alleles may be associated with illness severity or course. HLA genotyping was conducted for the entire DIOS cohort using the sequence specific oligonucleotide (SSO) method by collaborators at the Australian Red Cross Blood Service (Kimura and Sasazuki, 1992) from genomic DNA. Genotype was determined at the loci of highest variance in the MHC class I (HLA-A, HLA-B) and MHC class II (HLA-DR) to 2 digit specificity. Briefly, a highly variable region of the gene of interest was amplified by PCR. This PCR product was then blotted to a series of membranes, and each membrane was hybridised to one of a panel of digoxigenin-labelled sequence specific oligonucleotide (SSO) probes. Detection of bound SSO was identical to the methods used for a Southern blot, and made use of the alkaline phosphatase chemiluminescent substrate CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1]decan}-4-yl) phenyl phosphate), followed by exposure to x-ray film to visualise the results. Allele

specificities were determined by comparing the patterns of SSO binding for each subject with the reference binding patterns for each allele.

6.2.5 HLA-restricted peptide design

A significant proportion of subjects within the Q fever cohort possessed the HLA-A2 allele, the HLA-A3 allele, or both HLA-A2 and HLA-A3. These two HLA restrictions were therefore chosen for design of *C. burnetii* derived peptides for analysis of T cell responses. A 60kDA chaperone protein (ORF02702; CBU1718) was chosen as it had been been identified as immunogenic for stimulation of antibody production (Zhang *et al.*, 2004), and was common to both the small and large cell coxiella variants (Coleman *et al.*, 2007). The amino acid sequence (Seshadri *et al.*, 2003) was entered into two *in silico* epitope prediction software programs: ProPred (Singh and Raghava, 2003) and NetCTL (Larsen *et al.*, 2005). These programs delivered ranked lists of potentially immunogenic epitopes for each HLA restriction (9mers). Peptides were chosen based on the relative score assigned by prediction software. Peptides containing cysteine residues were substituted with alternative peptides due to technical difficulties in peptide production. Peptides were obtained at 70% purity, with a standard amine at the N terminus and free acid at the C terminus. Sequences for each peptide are given in Table 6.3.

Table 6.3: Sequences for HLA-A2 and HLA-A3 restricted peptides

Peptide name	Peptide sequence (N-peptide-OH)
HLA-A2-1	K V L K F S H E V
HLA-A2-2	A L H A T R A A V
HLA-A2-3	K L Q E R L A K L
HLA-A2-4	I L V E G I K A V
HLA-A2-5	A M L Q D I A V L
HLA-A3-1	G M N P M D L K R
HLA-A3-2	S L D D L G S A K
HLA-A3-3	G V V K V A A V K
HLA-A3-4	G V A L I R V L K
HLA-A3-5	G T I S A N S D K

All peptides were soluble in PBS, and stocks were stored at 100µg/mL in aliquots at -80°C. Peptides were used in subsequent assays as individual peptides or as two pools (HLA-A2 peptides 1-5 and HLA-A3 peptides 1-5) at a final concentration of 2µg/mL for each peptide.

6.2.6 IFN- γ ELISpot assay

ELISpot assays allow the enumeration of cytokine producing cells within a stimulated cell population, and were used to quantitate IFN-γ producing cells in PBMC cultures stimulated with whole inactived *C. burnetii* or HLA-A2 and HLA-A3- restricted peptides. MULTISCREEN high protein binding Immobilon-P membrane 96-well plates (Millipore, Bedford, USA) were used. Each plate was coated with anti-human interferon gamma (anti-

IFN-γ) antibody (1-DIK clone, Mabtech, Sweden) (2.5µg/mL in 0.1M sterile carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, pH 9.6), 100µL/well) and incubated either overnight at 4°C or for 2h at 37°C/5% CO_2. Plates were washed six times with 200µL/well PBS (Invitrogen) and then blocked for 1 hour at room temperature with 200µl/well complete medium/10% FCS.

Once blocking was complete, the media was removed from the plate. PBMC were added (50µL) to give $2x10^5$ cells/well. Whole formalin-inactivated phase I and phase II antigen (PanBio) were added to triplicate test wells (50µL per well, 2µg/mL final concentration). HLA-A2 and HLA-A3 restricted peptides were tested individually, or as two pools of five peptides each, in triplicate wells (50µL per well, 2µg/mL final concentration of each peptide). Triplicate wells with 50µL phytohaemagglutinin (PHA, Sigma, 2µg/mL) or 50µL LPS (Salmonella derived, Sigma,10ng/mL) were positive controls, CEF peptides (2µg/mL, 50µL per well, final well concentration of 2µg/mL) were a positive control for antigen specific responses, and 50µL complete medium/10% FCS was added to triplicate negative control wells. The underside of each plate was covered loosely with aluminium foil (per manufacturer's instructions) and then incubated for 18-24 hours (37°C/5% CO_2).

Cells were removed from the plate, and the plate was then washed six times (200µL/well, PBS) and blotted on paper towel to remove excess liquid. Biotin-conjugated detector antibody (clone 7-B6-1-Biotin, 1mg/mL, Mabtech) diluted 1:500 in PBS supplemented with 0.5% bovine serum albumin (BSA) was added (100µL/well) and the plate incubated

for 2 hours at room temperature. Detection antibody was removed from the plate, then the plate was washed six times (200µL/well PBS) and blotted. Streptavidin-alkaline phosphatase (ALP) (Mabtech, diluted 1:1000 in PBS) was added (100µL/well) and incubated for 1 hour at room temperature. The streptavidin-ALP was removed, then the plate was washed six times (200µL/well, PBS). One 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet (BCIP/NBT) (Sigma) was dissolved in 10mL distilled water, the resulting mixture filtered using a 0.45µm filter and added to each well (100µL/well). After spots had developed (20-30min), the plate was washed six times under running tap water and allowed to dry.

The number of spots per well was counted using a digital imaging system (Autoimmun Diagnostika Gmbh AID Elispot system and ELISPOT software, Strassburg, Germany), the mean of triplicate wells taken and converted to spot forming cells (SFC) per million PBMC. Each value was adjusted for background by subtracting the mean response to the appropriate control antigen.

6.3 Results

6.3.1 The humoral immune response

Longitudinal sera samples from a subset of subjects with prolonged illness (cases, n= 6) and matched subjects whose Q fever illness resolved promptly (controls, n=15) were screened for phase I and phase II-specific antibody, including assessment of the

contribution made to the total antibody titre of antibodies with IgM, IgG or IgA isotype. Antibody titres were then collated by "days post symptom onset" across the case and control subjects (as the interval between symptom onset and enrolment varied between subjects), and mean values at each time point were compared (Figure 6.1). In general, early phase II-specific immunoglobulin responses were of higher titre than phase I-specific responses. In the weeks and months following the onset of illness, total phase II-specific responses gradually declined in titre, while total phase I-specific titres increased, such that phase-specific differences in antibody titre were less apparent late in infection (12 months post symptom onset).

Acute illness was associated with an early peak in phase II-specific IgM, followed by a rise in phase I-specific IgM over the first months of infection. IgM titres specific for either antigenic phase then declined over the first 12 months after illness onset. Phase II-specific IgG antibody titres were generally already of high titre at the first sampling point for each subject, and remained high over the first 12 months of infection. By contrast, phase I-specific IgG titres demonstrated a gradual increase over this time frame. In a minority of subjects (n=7), phase I and phase II-specific IgA titres became detectable in later time points (data not shown).

When case and control subjects were compared, no significant differences were noted in antibody titre at any time point (Figure 6.1). High inter-subject variance was apparent in antibody titres for subjects from either group, and therefore differences that were observable between case and control subjects were not statistically significant (Mann-

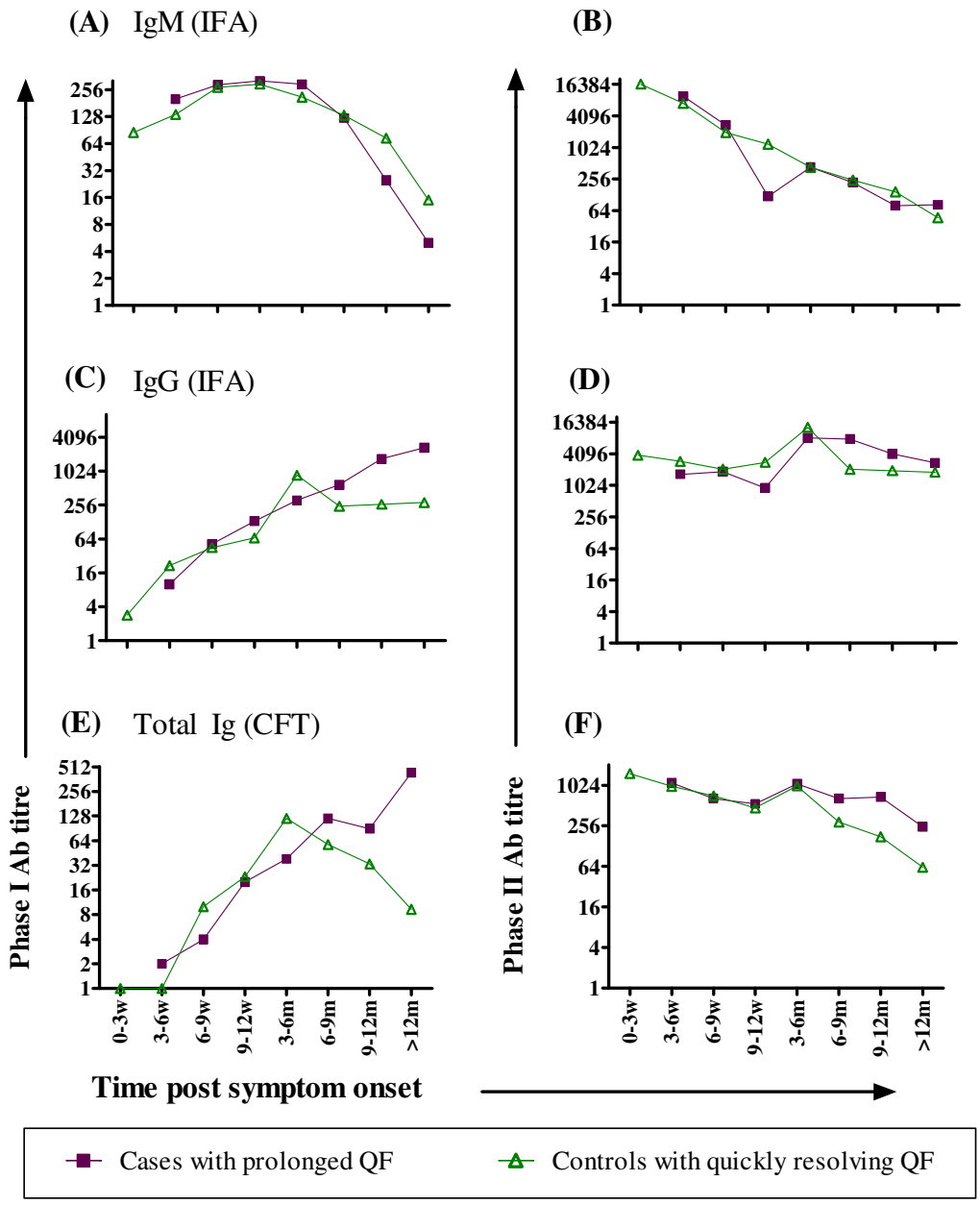

Figure 6.1: Mean antibody titres in subjects with varied Q fever illness duration. Q fever-specific antibody was quantitated in longitudinal sera samples from subjects with prolonged illness (n=6, ■) or quickly resolving Q fever (n=15, Δ). Phase I-specific (A, C, E) and phase II-specific (B, D, F) antibody was assessed by immunofluorescence assay (IFA) for IgM (A, B) and IgG (C, D) or complement fixation test (CTF) for total Ig (E, F).

Whitney U test, $p>0.05$). For example, the apparently divergent antibody production kinetics that occur at late time points (> 12 months) when examining the total phase I-specific immunoglobulin titres by CFT were skewed by a single high value data point within only three data points available for subjects with prolonged symptoms following acute Q fever. The presence of IgA antibody was not associated with the course of infection as subjects in both the case (n=2) and control (n=5) groups were identified with detectable *C. burnetii*-specific IgA titres at later time points. Thus, it does not seem likely that varied illness duration occurs as a result of altered patterns of phase I or phase II-specific antibody induction, nor do particular antibody isotypes appear to be associated with prolonged illness following acute Q fever.

6.3.2 Circulating leucocytes

The host adaptive immune response to any infection is dependent on its ability to activate, and induce proliferation of, appropriate immune effector cells. It was hypothesised that differences in illness duration might vary in subjects with acute Q fever due to impaired immune activation, resulting in delayed pathogen clearance, or due to prolonged cellular activation following clearance of *C. burnetii*, resulting in immunopathology in the absence of infection. To examine these possibilities, PBMC from the case-control series were examined by FACS, to quantitate the relative proportions of leucocytes within the circulation, and to examine their activation state.

Samples collected longitudinally over the first 12 months or more after symptom onset were assessed for the expression of cell surface markers indicative of B cells (CD19), T

cells (CD3, CD4, CD8), monocytes (CD14, CD16), and NK cells (CD16, CD56). Markers of activation were assessed (HLA-DR, CD69) in combination with the above markers, to track changes in cellular activation states over the course of the Q fever illness.

No significant time-dependent changes in the proportion of circulating B cells, monocytes or NK cells were noted in longitudinally collected PBMC samples (Figure 6.2). In the control subjects there was high inter-subject variation in the proportion of CD14+ monocytes as a percentage of the lymphocyte gate, however, the proportion of monocytes present in each subject's peripheral blood remained constant throughout the illness course. Despite the presence of two subjects in the control cohort who had a high proportion of monocytes, the mean percentages of monocytes were broadly comparable across the two groups. Furthermore, the activation state of each subject's monocytes did not vary significantly over the 12 months following illness onset, suggesting that early activation of monocytes does not decrease following the resolution of illness.

Inclusion of CD56 and CD19 as markers in the FACS panel occurred late in the course of the study and therefore it was not possible to compare NK cell or B cell proportions present in PBMC from case and control subjects. When FACS data were pooled for all subjects for whom PBMC were available, no temporal changes in NK cell or B cell proportions were noted (Figure 6.2).

Analysis of the changes in CD4+ and CD8+ T cells following the onset of acute Q fever demonstrated that although the proportions of total CD4+ or CD8+ T cells did not change

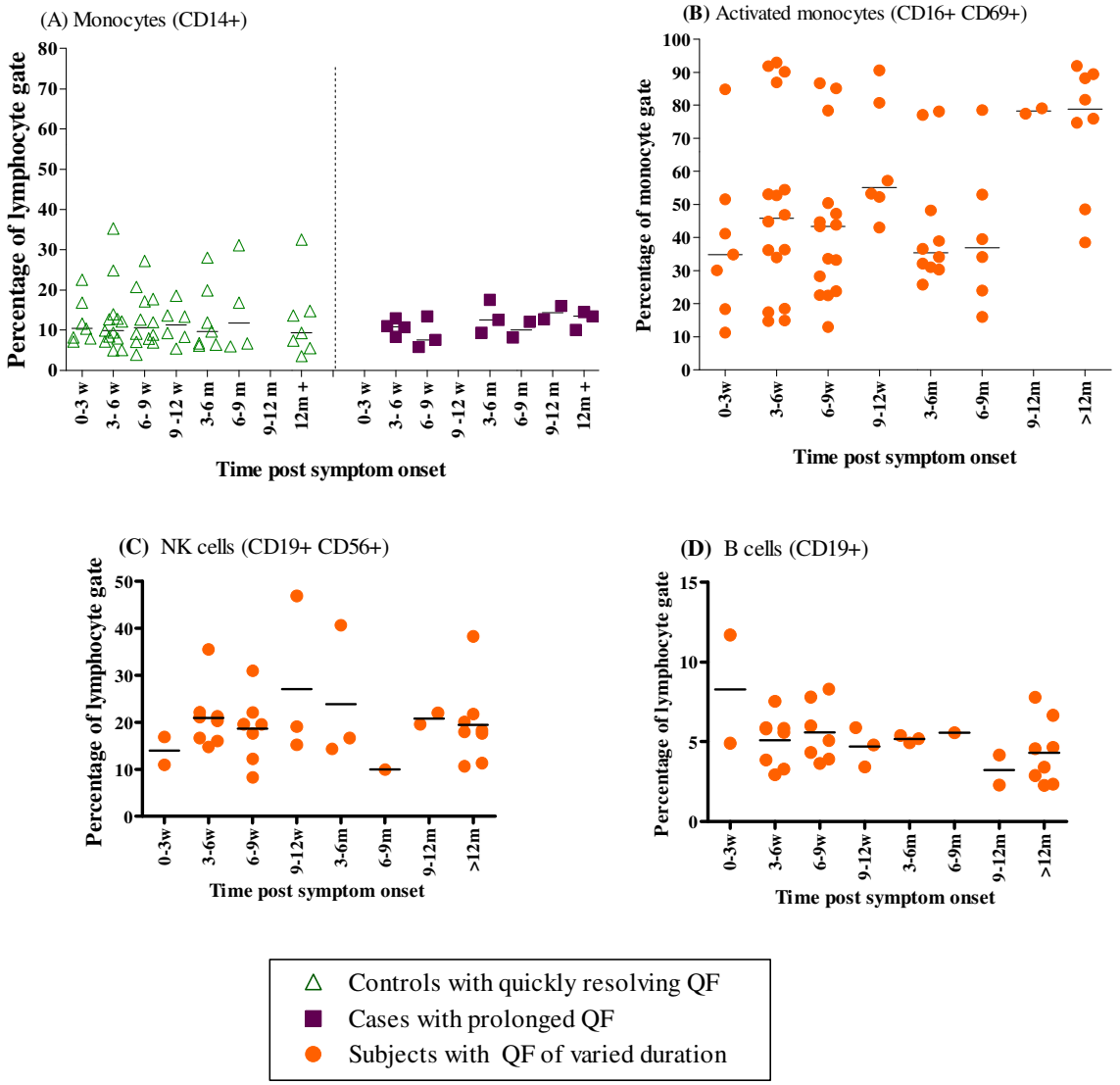

Figure 6.2: Longitudinal changes in leukocyte proportions and activation states (1). Flow cytometry was used to quantitate proportions of monocytes (A) within the lymphocyte gate in cases with prolonged Q fever illness (▪) and control subjects with quickly resolving illness (Δ). In subjects with varied Q fever illness course (●), the proportions of activated monocytes (B), NK cells (C) and B cells (D) were also determined. Data points are individual samples, lines represent the mean of all samples for each time point.

over time, there was an increase in activated (HLA-DR+) CD8+ T cells early in infection that gradually declined as the disease progresses (Figure 6.3). This trend was evident both in subjects with prolonged illness and in those subjects whose Q fever illness resolved quickly. No other patterns relating to changes in T cell proportions or their activation state were evident when comparing case and control subjects.

In this case-control series, acute Q fever was accompanied by an early increase of activated CD8+ T cells, but not with a general increase in total CD8+ T cells. No temporal changes were observed in the proportion of B cells, T cells, monocytes or NK cells that were present in the periphery over the 12 months following symptom onset. Furthermore, no significant differences were apparent when the proportions and activation state of circulating leucocytes from subjects with prolonged symptoms following acute Q fever were compared with with those present in subjects who had promptly resolving illness.

6.3.3 HLA genotyping

All subjects in DIOS with available DNA were genotyped at HLA-A, HLA-B and HLA-DR, including subjects with serologically confirmed Q fever (n=60), EBV (n=66) or RRV (n=61). The allele frequencies were collated for subjects with Q fever and compared with those found in the EBV and RRV subjects for HLA-A (Table 6.4), HLA-B (Table 6.5), and HLA-DRB1 (Table 6.6). Alleles that were over-represented or under-represented in the Q fever cohort relative to the control cohorts were determined by Chi-square test (or Fisher's exact test when cell number were <5), and significance levels (*p* values) reported in the tables.

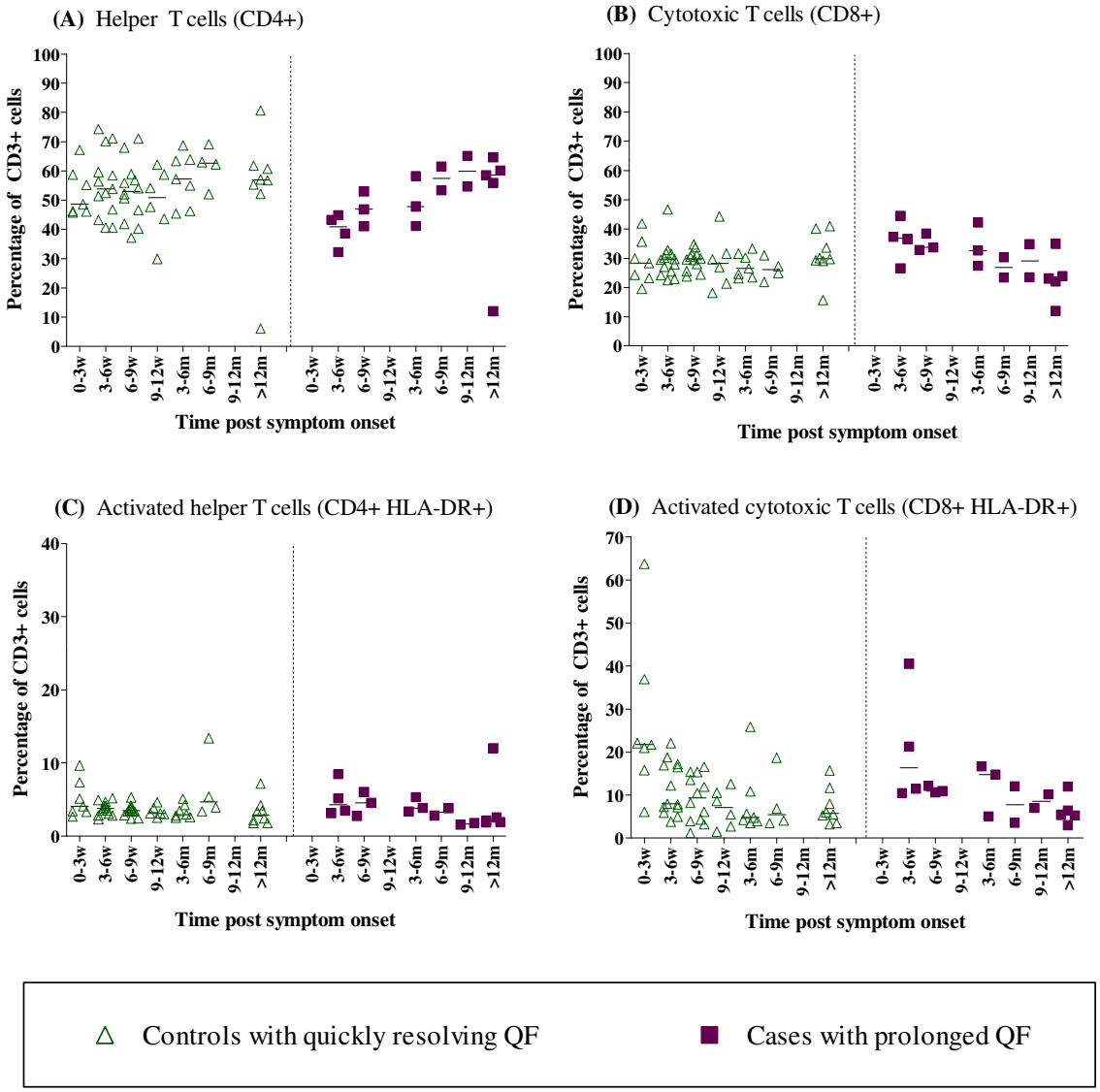

Figure 6.3: Longitudinal changes in leukocyte proportions and activation states (2). Flow cytometry was used to quantitate proportions of helper T cells (A), cytotoxic T cells (B), activated helper T cells (C) and activated cytotoxic T cells (D) with respect to total CD3+ cells in cases with prolonged Q fever illness (■) and control subjects with quickly resolving illness (Δ).

Table 6.4: HLA-A allele frequencies for subjects with Q fever in comparison with control subjects with EBV or RRV infections.

Allele	Copies in Q fever cohort (proportion of n=120 alleles)	Copies in EBV/RRV control cohort (proportion of n=254 alleles)
A1	23 (0.19)[#]	47 (0.19)
A2	38 (0.32)	67 (0.26)
A3	29 (0.24)	39 (0.15) (p=0.039)[*]
A11	2 (0.02)	17 (0.07) (p=0.043)[^]
A23	0	4 (0.02)
A24	5 (0.04)	18 (0.07)
A25	1 (0.01)	7 (0.03)
A26	4 (0.03)	12 (0.05)
A29	6 (0.05)	16 (0.06)
A30	3 (0.03)	4 (0.02)
A31	1 (0.01)	3 (0.01)
A32	3 (0.03)	7 (0.03)
A33	1 (0.01)	0 (0.00)
A34	2 (0.02)	0 (0.00)
A66	1 (0.01)	0 (0.00)
A68	1 (0.01)	13 (0.05) (p=0.042)

number (proportion)

[*] *p* value determined by Chi-square test

[^] *p* value determined by Fisher's exact test

Table 6.5: HLA-B allele frequencies for subjects with Q fever in comparison with control subjects with EBV or RRV infections.

Allele	Copies in Q fever cohort (proportion of n=120 alleles)	Copies in EBV/RRV control cohort (proportion of n=254 alleles)
B7	25 (0.21)[#]	40 (0.16)
B8	19 (0.16)	39 (0.15)
B13	3 (0.03)	5 (0.02)
B14	9 (0.08)	4 (0.02) (p=0.0058)[^]
B15	8 (0.07)	16 (0.06)
B18	2 (0.02)	5 (0.02)
B27	5 (0.04)	7 (0.03)
B35	14 (0.12)	25 (0.10)
B37	0 (0.00)	5 (0.02)
B38	1 (0.01)	6 (0.02)
B39	1 (0.01)	3 (0.01)
B40	7 (0.06)	8 (0.03)
B41	1 (0.01)	1 (0.00)
B44	11 (0.09)	42 (0.17) (p=0.037)[*]
B45	2 (0.02)	2 (0.01)
B47	1 (0.01)	0
B49	2 (0.02)	4 (0.02)
B50	0 (0.00)	1 (0.00)
B51	1 (0.01)	19 (0.07) (p=0.0058)[^]

Allele	Copies in Q fever cohort (proportion of n=120 alleles)	Copies in EBV/RRV control cohort (proportion of n=254 alleles)
B55	2 (0.02)	4 (0.02)
B57	6 (0.05)	15 (0.06)
B58	0 (0.00)	1 (0.00)

number (proportion)

* *p* value determined by Chi-square test

^ *p* value determined by Fisher's exact test

Table 6.6: HLA-DRB1 allele frequencies for subjects with Q fever in comparison with control subjects with EBV or RRV infections.

Allele	Copies in Q fever cohort (proportion of n=120 alleles)	Copies in EBV/RRV cohort (proportion of n=254 alleles)
DRB1*1	15 (0.13)#	28 (0.11)
DRB1*3	14 (0.12)	39 (0.15)
DRB1*4	21 (0.18)	45 (0.18)
DRB1*7	20 (0.17)	40 (0.16)
DRB1*8	2 (0.02)	7 (0.03)
DRB1*9	0 (0.00)	4 (0.02)
DRB1*10	1 (0.01)	1 (0.00)
DRB1*11	10 (0.08)	16 (0.06)
DRB1*12	0 (0.00)	1 (0.00)
DRB1*13	13 (0.11)	28 (0.11)

Allele	Copies in Q fever cohort (proportion of n=120 alleles)	Copies in EBV/RRV cohort (proportion of n=254 alleles)
DRB1*14	1 (0.01)	5 (0.02)
DRB1*15	21 (0.18)	34 (0.14)
DRB1*16	2 (0.02)	4 (0.02)

number (proportion)

Allele frequencies in the Q fever subjects that were significantly different from those observed in the control cohort were found only in the HLA class I. Alleles that were under-represented in the Q fever cohort included HLA-A11, HLA-A68, HLA-B44 and HLA-B51 (Chi-square test or Fisher's exact test, $p<0.05$). This may suggest that these class I alleles confer protection from infection, or from symptomatic Q fever illness. Conversely, HLA-A3 and HLA-B14 were over-represented in the Q fever cohort in comparison with the control cohorts (Chi-square test or Fisher's exact test, $p<0.05$). These alleles may be associated with increased susceptibility to Q fever, or with increased tendency to develop symptomatic illness following *C. burnetii* exposure. However, correction (Bonferroni) for the number of alleles tested at each locus (n=17 for HLA-A; n=22 for HLA-B) would not leave any significant associations. No significant differences in allele frequency were found when comparing HLA-DRB1 allele frequencies in Q fever and control subjects.

Having identified alleles which may play a role in susceptibility to, or protection from, symptomatic Q fever, a subsequent aim was to determine whether the presence of any of these seven provisionally associated HLA alleles might be associated with severe or

prolonged Q fever illness. Measures of severity including the PCA-derived severity index and the SOMA score at baseline were not significantly different when comparing subjects who carry or do not carry each allele of interest (students t test, $p>0.05$). Furthermore, none of these alleles was associated with illness duration (students t test, $p>0.05$). Given the highly polymorphic nature of the HLA alleles, and subsequently the generally low numbers of subjects carrying each allele, expanding the cohort may enable the detection of allele-specific effects on Q fever severity or duration.

Previous reports suggested that carriage of the HLA-DRB1*11 was associated with susceptibility to post Q fever fatigue syndrome (Helbig *et al.*, 2005). A total of eight subjects in the Q fever cohort were identified to carry the HLA-DRB1*11 allele. These subjects did not report significantly longer duration of fatigue following acute Q fever in comparison to subjects who did not carry HLA-DRB1*11 (mean duration 204 days vs 143 days, student's t test, $p>0.05$). Thus, there was no evidence to suggest an association between HLA-DRB1*11 allele and post Q fever fatigue in this cohort.

6.3.4 The cellular immune response

Attempts to quantitate *C. burnetii*-specific T cell responses to inactivated whole cell antigen were complicated by the non-specific effect of LPS present in the *C. burnetii* outer cell membrane. A typical antigen dose-response curve should show a plateau at high concentrations where addition of extra antigen has no effect due to saturation of all antigen-specific T cells (Hesse *et al.*, 2001). However, antigen titration within an IFN-γ ELISpot assay failed to identify a plateau for either phase I or phase II antigen within the range of

0.025-10μg/mL (Figure 6.4). This suggested antigen-non-specific activation via the immunostimulatory effect of LPS within the whole inactivated *C. burnetii* antigens. The responsiveness to *C. burnetii* LPS varied widely across Q fever subjects. For instance, subject B had very few IFN-γ-secreting cells in response to either phase I or phase II antigen, even at high dose concentrations, while subject D demonstrated a high number of IFN-γ-producing cells in response to either phase even at relatively low stimuli dose (Figure 6.4). For each donor, the number of phase II-responsive cells was always greater than the number of phase I-responsive cells when the stimuli were used at equal doses, which mirrors data obtained in monocytes and suggests a higher cytokine-stimulating potential of the phase II antigen.

In order to overcome the problem of identifying antigen-specific responses in cultures contaminated by *C. burnetii*-derived LPS, ten potentially immunogenic peptides were designed and synthesised to screen for *C. burnetii*-specific responses. HLA-A2 and HLA-A3 restrictions were chosen for the peptide design, as one or both of these alleles were expressed across the majority of subjects for whom PBMC samples were available across multiple time points. In the absence of any previously demonstrated *C. burnetii* proteins known to induce T cell responses, the design relied on use of the amino acid sequence of the *C. burnetii* HspB heat shock protein, which has previously been demonstrated to have T and B cell antigenicity in mice (Vodkin and Williams, 1988; Li *et al.*, 2005; Coleman *et al.*, 2007). Use of epitope prediction software yielded a list of peptides ordered by relative score, from which peptides were chosen for synthesis. Peptides containing cysteine residues were substituted for alternative peptides due to technical difficulties in their

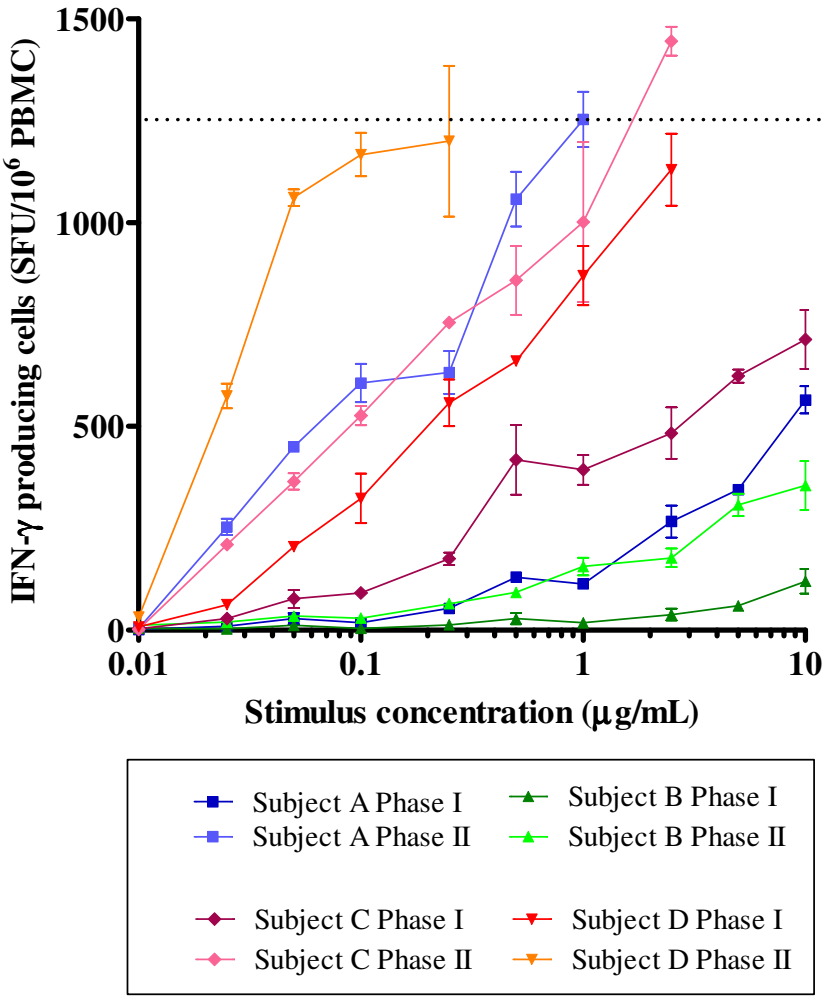

Figure 6.4: Enumeration of IFN-γ-producing cells within *C. burnetii*-stimulated PBMC cultures. Antigen titration experiments were conducted to determine the optimal concentration of whole inactivated *C. burnetii* antigens for use in ELISpot assays. PBMC samples ($2x10^5$/well) from four Q fever subjects were cultured in triplicate wells in the presence of *C. burnetii* phase I or phase II antigens and IFN-γ-producing cells were quantitated by ELISpot. Spot forming units (SFU) were counted in each well via an automated plate counter. Across a minimum of three replicate wells, unstimulated PBMC had a mean of 25SFU/10^6 PBMC or less, and PHA stimulated PBMC had a mean of 1250SFU/10^6 PBMC or more. Technical difficulties prevented the accurate enumeration of wells with greater than 250SFU/well (1250SFU/10^6 PBMC, dotted line). Data are presented as mean (error bars are standard error) of replicate wells.

synthesis. These peptides were utilised as stimuli for IFN-γ ELISpot assays to detect peptide-specific responses in PBMC samples taken from Q fever subjects at two or more time points.

A total of 45 PBMC samples from 24 patients were screened for HLA-A2 (n=19 subjects) and HLA-A3 (n=15 subjects) peptide-specific responses via IFN-γ ELISpot assay. Of these subjects, ten carried both HLA-A2 and HLA-A3. In only one subject, an assay of PBMC from a single time point generated spot counts in HLA-A3 stimulated wells that were suggestive of peptide-specific responses (Figure 6.5). A mean of 16 spots per well (compared to a mean of six spots in media control wells) was observed. Repetition of the assay using PBMC from the same time point failed to generate comparable results, as did an assay using PBMC from an alternative time point. In both cases, spot counts in media wells exceeded those obtained in peptide-stimulated wells. Thus, it is not likely that antigen-specific responses had been successfully identified in this subject.

6.4 Discussion

The adaptive immune responses play a critical role in *C. burnetii* clearance with both humoral and cellular responses shown to be generated *in vivo* (Jerrells *et al.*, 1975; Murphy and Magro, 1980; Embil *et al.*, 1990) and in vaccinated individuals who are protected from infection (Worswick and Marmion, 1985; Marmion *et al.*, 1990). Immune sera have been shown to confer protection against infection in immunocompetent animals (Abinanti and Marmion, 1957), and humoral responses remain detectable in naturally infected human

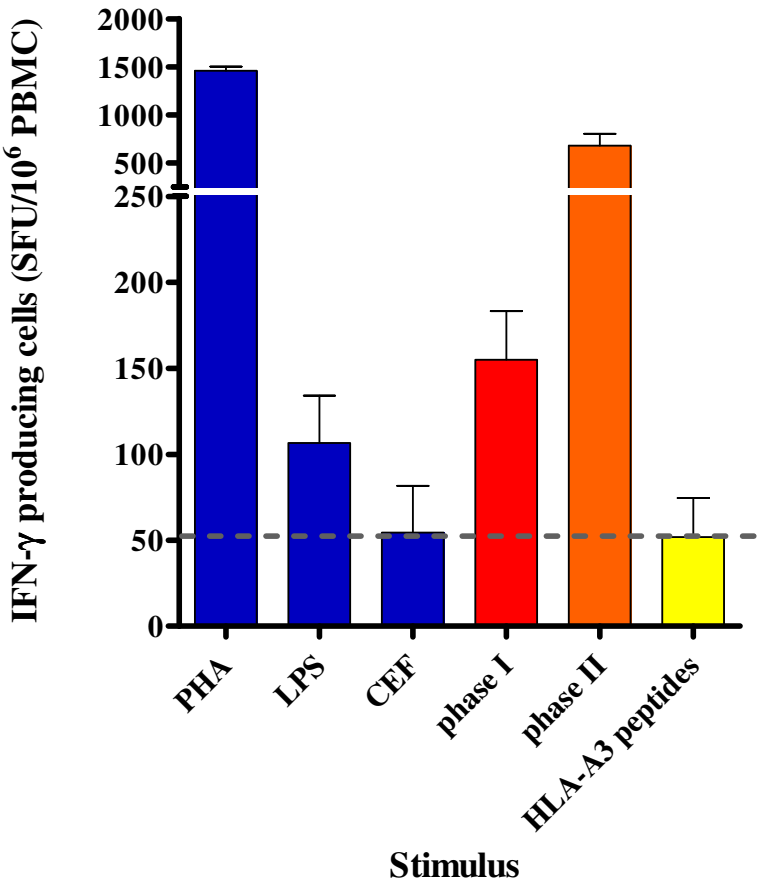

Figure 6.5: Detection of HLA-A3-restricted antigen-specific responses to *C. burnetii*-derived peptides. PBMC from a single Q fever subject were stimulated in triplicate wells with HLA-A3-restricted peptides (yellow bars), whole inactivated phase I (red bars) and phase II (orange bars) antigens, or control stimuli including phytohaemaglutinin (PHA), LPS (derived from *E. coli*), and a control peptide pool containing known epitopes from cytomegalovirus, Epstein-Barr virus and influenza (CEF) (all blue bars). IFN-γ-producing cells were enumerated by ELISpot assay. Grey dotted line shows the mean SFU/well for unstimulated control wells. All data are corrected by subtracting values in unstimulated control wells and presented as mean and standard error of 3 replicate wells.

subjects for years after initial infection (Jerrells *et al.*, 1975). However, in the absence of cellular responses, specific antibody is not sufficient for protection against *C. burnetii* infection (Zhang *et al.*, 2007), and an absence of T cells is associated with uncontrolled bacterial replication in murine models (Andoh *et al.*, 2007). Thus, antibodies may contribute to the *C. burnetii*-specific immune response, but are not thought to be sufficient for clearance or protection against subsequent infection.

The role of antibody responses in the protective host response in acute and chronic Q fever is poorly understood, with most emphasis placed on antibody measurement as a diagnostic tool rather than a determinate of disease outcome. IgG and IgM antibodies directed against phase II organisms have been documented to appear early in infection and to be maintained for several months following acute Q fever (Dupont *et al.*, 1994) (Guigno *et al.*, 1992). By contrast, IgA and IgG responses directed against phase I organisms are considered diagnostic of chronic localised Q fever (Soriano *et al.*, 1993), although some dispute this (Dupont *et al.*, 1994). In this cohort, antibody production profiles followed typical patterns with early high titre IgM responses and more delayed IgG responses of lower magnitude, with phase II-specific responses occurring earlier and at higher magnitude than phase I-specific responses. IgA was detected at later time points in a subset of subjects, despite the fact that none had evidence of chronic, localised Q fever. The presence of this IgA response was not consistently associated with post Q fever fatigue.

Cellular responses are critical in the host response to *C. burnetii* infection, with many studies demonstrating that an absence of T cell immunity in mice leads to uncontrolled

bacterial replication and an inability to clear the microorganism (Andoh *et al.*, 2007; Zhang *et al.*, 2007). Furthermore, vaccine-induced immunity occurs in association with specific T cell responses that remain detectable for five years or more after vaccination, in contrast with declining titres of specific antibody over a comparable time frame (Marmion *et al.*, 1990). In this cohort, acute Q fever was accompanied by an early increase in the proportion of activated CD8+ T cells. These T cells are key producers of the cytokine, IFN-γ, and are therefore likely to be critical in driving microbicidal activity in infected monocytes and macrophages (Ghigo *et al.*, 2002).

Increased activation of CD8+ T cells early in the Q fever illness was not accompanied by a comparable activation of CD4+ T cells. CD8+ T cells typically have cytolytic activity, and presumably assist in *C. burnetii* clearance by lysing infected host cells as has been shown in other intracellular bacterial infections (Kaufmann, 1988). The host response to *C. burnetii* would ideally result in high production of IFN-γ, which is known to be important in activating microbicidal activity in infected monocytes and macrophages (Ghigo *et al.*, 2002). CD4+ T cells are more effective producers of cytokines than CD8+ T cells (Kelso *et al.*, 1991) therefore it is perhaps surprising to find that activation of these cells was not detectable over the course of infection. Furthermore, induction of IFN-γ by CD8+ T cells would be expected to drive the proliferation of CD4+ T cells (Gajewski *et al.*, 1989), however no change in relative CD4+ T cell number was observed in response to infection. This outcome may reflect the fact that the first sampling point for all subjects in the cohort was several weeks after the onset of illness.

It was hypothesised that prolonged fatigue following acute Q fever may result from defects in immune activation (resulting in an inability to clear *C. burnetii*), or from an inappropriately prolonged immune response characterised by immune cell activation in the absence of persistent *C. burnetii* infection. There was no evidence to suggest that the proportion of leucocyte subsets (such as T cells, B cells, NK cells or monocytes) differed between subjects with prolonged, or promptly resolving, illness. Furthermore, the activation state of immune cells did not differ between case and control subjects, suggesting that deficits in immune activation, or excessively prolonged immune activation, are not responsible for the ongoing symptoms of fatigue seen in some subjects. Unfortunately, analysis of this kind provides no information about the specificity of activated cells or about their activity *in vivo* and relied on the use of cryopreserved cells rather than fresh whole blood.

The study examined the role of polymorphisms in HLA alleles in susceptibility to, or outcome of, acute Q fever. HLA present pathogen-derived peptides to trigger T cell activation and cytokine production, and the HLA alleles that are present in an individual will determine their ability to mount an adaptive immune response to a particular pathogen. Information about the adaptive immune response to *C. burnetii* might therefore be inferred from determining whether specific HLA alleles might be associated with susceptibility to, or outcome from, acute Q fever.

Comparisons of this kind require knowledge of the HLA allele frequencies within the population, and in the absence of a population-wide screen, allele frequencies in HLA-A,

HLA-B and HLA-DRB1 were compared between subjects with acute Q fever and subjects in the control cohorts (RRV and EBV). In doing this comparison, an assumption was made that all subjects enroled into DIOS shared an equal exposure risk to *C. burnetii*. *C. burnetii* is known to survive in harsh environmental conditions, and although human cases of infection are usually the result of exposure to infected livestock or their contaminated waste products, wind-borne infection has also been documented (Embil *et al.*, 1990; Smith *et al.*, 1993; Manfredi Selvaggi *et al.*, 1996; Hawker *et al.*, 1998; Carrieri *et al.*, 2002; van Woerden *et al.*, 2004). It therefore seems reasonably to assume that even individuals residing within rural townships in the study region might be exposed to *C. burnetii*. The younger mean age of the EBV sub-cohort, however, is likely to imply a lower cumulative risk of exposure to *C. burnetii* in this group.

The HLA alleles are highly polymorphic, and more than 50 alleles were identified in DIOS subjects across the HLA-A, HLA-B and HLA-DRB1 loci that were examined in this study. A number of HLA class I alleles were somewhat over-represented or under-represented in the Q fever cohort in comparison to the control subjects although statistical correction for the number of alleles tested would negate these associations. Although HLA class I allele carriage varied significantly between the subjects with acute Q fever and subjects of the control cohort, none of the differentially carried alleles were associated with measures of illness severity or duration. The previous reports of an HLA-DRB1*11 association with post Q fever fatigue syndrome (Helbig *et al.*, 2005) are in contrast with suggestions that HLA-DRB1*11 is carried at lower frequency in patients with chronic fatigue syndrome (albeit with unknown triggering infection or other initiating factors) (Smith *et al.*, 2005),

and were not supported by the present study. Very few subjects in the Q fever cohort carried the HLA-DRB1*11 allele, and these subjects did not report a longer duration of fatigue following acute Q fever. The biological significance of polymorphisms in the HLA loci therefore remains unclear in the context of acute Q fever. Expanding the cohort may improve the detection of HLA-specific effects on Q fever susceptibility or illness outcome.

Attempts to identify antigen-specific T cell responses to whole inactivated *C. burnetii* antigens instead highlighted the antigen-non-specific effects of *C. burnetii* LPS. PBMC from subjects with current or previous Q fever infection generated increasing numbers of IFN-γ-producing cells in response to increasing concentrations of whole inactivated organisms. In general, antigen-specific responses would be expected to reach a plateau at high antigen concentration as there are a finite number of antigen-specific cells within a sample, and antigen-specific cells will not proliferate over the short time-frame of an ELISpot assay (Hesse *et al.*, 2001). Furthermore, antigen-non-specific effects were evident when phase I and phase II antigen stimulation resulted in the induction of IFN-γ-secreting cells in healthy donors, who had no evidence of prior exposure to *C. burnetii* (DTH skin test negative and antibody negative, data not shown). This suggested that the detection of antigen-specific responses was not possible when using whole inactivated organisms as stimuli – due to the presence of LPS in the outer cell wall.

The importance of phase specificity in the host response to infection is apparent when comparing the protective effect of phase I vaccinations with the non-protective immune responses generated in response to phase II preparations (Ormsbee *et al.*, 1964; Genig,

1965; Genig *et al.*, 1965; Ormsbee and Marmion, 1990). Phase-specific differences were evident in the *in vitro* cellular response to *C. burnetii*: phase II preparations consistently induced greater numbers of IFN-γ-producing cells than phase I preparations. The mitogenic effects of bacterial LPS are believed to be indirect: activated monocytes cause polyclonal activation of T cells (Mattern *et al.*, 1998). Comparable phase-specific differences were observed in the host monocyte response to stimulation (Chapter 5). It therefore seems likely that the increased activation of IFN-γ-producing T cells in response to phase II organisms relates to the enhanced ability of phase II to activate monocyte responses rather than direct effects of phase II LPS on T cell activity. Phase-specific differences in antigen-specific cellular responses also seem likely to exist, given the known protective effect of phase I-specific cellular responses following vaccination.

Very little research has focused on the identification of antigen-specific responses in the context of Q fever. The size and complexity of *C. burnetii* make it technically difficult and very expensive to screen the entire organism for immunogenic epitopes. Instead, PBMC from individuals with prior exposure to *C. burnetii* can be used to screen for proteins that may also elicit cellular responses *in vivo*. The work presented here focused on heat shock protein B, which has high identity across *C. burnetii* isolates (Fernandes *et al.*, 2009) and is cross-reactive with sera from immune individuals (Coleman *et al.*, 2007). Importantly, HspB is thought to be induced in response to environmental stressors in the phagolysosome of host macrophages (Coleman *et al.*, 2007), making it likely to be abundantly expressed during natural infection and therefore a probable target of the immune system.

Given the early expansion of activated CD8+ T cells in acute Q fever, the primary goal was to detect HspB-specific CD8+ T cell responses in the Q fever cohort. In addition to the HLA class I-restricted nature of the predicted peptide epitopes, the short length of each peptide ensured that only CD8+ T cell responses would be detected. Optimal CD8+ epitope length is traditionally considered to be 8-10 amino acids, although it now seems probable that this is a result of the length of available peptides that remain after antigen processing rather than due to longer peptides being unable to bind MHC class I (Bell *et al.*, 2009). When CD8+ T cell responses are of primary interest, 9-mer peptides have been shown to be more effective than longer peptides (15-mer) in restimulating antigen-specific responses (Kiecker *et al.*, 2004). Thus, the peptides used in this study were an ideal length for the task of characterising CD8+ T cell responses.

Prediction of HLA-restricted epitopes requires knowledge about the types of amino acid sequences that are likely to be bound by the HLA allele of interest, and peptide motifs have been identified for many HLA alleles (Rammensee *et al.*, 1993). Such peptide motifs are only of interest if they may also be expected to be generated, intact, by proteosome-mediated degradation of the protein in which the target sequences occur (Niedermann *et al.*, 1999). Antigen processing is further dependent on transporter associated with antigen processing (TAP) transport efficiency (Tenzer *et al.*, 2005). A number of *in silico* epitope prediction tools exist that make use of existing data regarding peptide-HLA binding specificities, TAP transporter efficiencies and proteosome-mediated polypeptide processing to generate probable HLA-restricted peptides within an input amino acid sequence, including ProPred and NetCTL (Singh and Raghava, 2003; Larsen *et al.*, 2005). When the

HspB amino acid sequence was analysed by ProPred or NetCTL for HLA-A2 or HLA-A3-restricted epitopes, the peptide sequences that were identified as probable epitopes were comparable.

Despite screening a large number of subjects across multiple time points, it was not possible to reliably identify HLA-A2 or HLA-A3 restricted epitopes within the HspB protein of *C. burnetii*. The IFN-γ ELISpot assay is a highly sensitive technique that is capable of detecting low levels of antigen-specific cells (Karlsson *et al.*, 2003) and is often used for detecting vaccine-induced T cell responses or for epitope identification (Mashishi and Gray, 2002). Failure to identify peptide-specific responses is therefore unlikely to result from limitations in the ELISpot assay itself. The HspB protein has been shown to be immunogenic in mice and to generate protective immunity when provided as a fusion protein vaccine (Vodkin and Williams, 1988; Li *et al.*, 2005), and is cross-reactive with sera from immune humans (Coleman *et al.*, 2007). Further work could involve the construction of overlapping peptides spanning the entire protein sequence, to ensure that epitope prediction was not overlooking non-predicted antigenic regions, and comparably examination of other *C. burnetii* proteins. An alternative approach may be to elute antigenic peptides from the T cells of patients with vigorous T cell activation responses against *C. burnetii* followed by high performance liquid chromatography (HPLC), although this would require large numbers of fresh cells (Demotz *et al.*, 1989).

6.5 Conclusions

The host response to *C. burnetii* infection (or vaccination) is known to occur in association with humoral and cellular immune responses. Phase-specific variance is reported to be critical in the generation of protective immunity, with phase I-specific responses being protective and phase II-specific responses offering limited protection. The kinetics and magnitude of the host response to infection, including humoral and cellular components, were compared in subjects with prolonged fatigue following acute Q fever and in subjects who had promptly resolving illness. No significant differences were found when comparing phase I or phase II-specific antibody responses of IgM, IgG or IgA isotype, nor were total antibody responses different between the two subject groups.

Exposure to *C. burnetii* was accompanied by an early expansion of activated CD8+ T cells in some subjects, however no temporal changes were observed in the total CD8+ T cell population as a proportion of all leucocytes, and nor were there changes in the activation or proportion of CD4+ T cells. Attempts to identify antigen-specific cellular responses via IFN-γ ELISpot assay were unsuccessful due to an indirect mitogenic effect of LPS acting on monocytes. This effect varied in magnitude across different subjects, and was always more pronounced in response to phase II LPS than that seen in response to phase I LPS. This mirrors findings in monocytes that phase II organisms are more potent inducers of cytokine production, and was not an unexpected finding.

Indirect assessment of antigen-specific responses was attempted by comparing HLA allele carriage in the Q fever cohort in comparison to subjects in the control cohorts. Although

some HLA class I alleles were over-represented or under-represented within the subjects who had Q fever, none of these alleles were associated with illness severity or duration, and the study failed to recapitulate previous data suggesting a role for HLA-DRB1*11 in the subsequent development of Q fever syndrome following the acute illness. Having identified a large number of subjects who carry the HLA-A2 and/or HLA-A3 allele, potential epitopes with HLA-A2 or HLA-A3 restriction were mapped in HspB, a protein with known immunogenicity in humans and mice. Screening of PBMC samples from multiple subjects and multiple time points throughout the Q fever illness course failed to identify peptide-specific IFN-γ-specific responses.

7. Implications and further research

Although serological screening suggests that *C. burnetii* infection may often be asymptomatic, the data obtained in this cohort suggest that when symptomatic, acute Q fever is often a severe and debilitating illness. With the exception of fever, symptoms of both a 'physical' (e.g. sweats) and a 'psychological' (e.g. irritability) nature remained prevalent in a significant minority of patients for weeks or months following the onset of the acute illness. A consistent feature of the ongoing illness in this subset was disabling fatigue, which frequently prevented participation in usual daily activities.

The symptoms of acute Q fever are comparable with those reported for a wide range of unrelated acute infections. The stereotyped pattern of symptoms that are evident during acute infections has been termed the "acute sickness response" (Vollmer-Conna, 2001). This symptom complex is also evident in the responses of birds and animals to acute infections (Johnson *et al.*, 1993; Johnson and von Borell, 1994). These symptoms may also be elicited by the administration of LPS and thus relicating micro-organisms are not a requisite for the acute sickness response (Cheng *et al.*, 2004). It is evident therefore that pathogen-specific phenomena do not cause these symptoms of acute illness, but instead that immune activation drives the response. A growing body of data suggests that the symptoms of an acute sickness response are associated with the production of cytokines (Konsman *et al.*, 2008; Vollmer-Conna *et al.*, 2008) and that psychological stressors may act synergistically with immune stressors to enhance the cytokine and acute sickness responses (Brydon *et al.*, 2009).

While some studies suggest a role for cytokines in promoting chronic, localised infection following acute Q fever (Capo *et al.*, 1996) in which replicating organisms may ultimately be isolated from the heart valves or other sites, it is uncertain whether the post Q fever fatigue syndrome results from ongoing cytokine production. PBMC from patients with post Q fever fatigue syndrome have been reported to show an aberrant cytokine production profile when restimulated with *C. burnetii* antigens *in vitro*, including increased production of the pro-inflammatory cytokine, IL-6 (Penttila *et al.*, 1998), however in the absence of conclusive demonstration of persisting antigenic stimulation in these subjects it is unknown how relevant these cytokine-producing capacities are *in vivo*. More interestingly, spontaneous *ex vivo* production of IL-6 and IL-1β by PBMC correlate strongly with the symptoms of the acute sickness response (Vollmer-Conna *et al.*, 2004). However, in a study completed and reported in parallel with those described in this thesis, no evidence for ongoing cytokine production (in the same assays system utilised for the acute infection study) was identified in subjects with post Q fever fatigue syndrome and matched control subjects who recovered promptly after acute Q fever (Vollmer-Conna *et al.*, 2007). Nevertheless, the finding in DIOS that post-infective fatigue syndromes strongly correlate with the severity of the acute illness (Hickie *et al.*, 2006) suggest that increased cytokine production (and hence more severe illness manifestations) early in the acute phase result in a tendency to suffer prolonged fatigue following infection – through mechanisms other than ongoing cytokine production detected in the peripheral blood.

A large number of publications point to the interaction of peripheral inflammatory responses with the central nervous system. Cytokines convey a message to the brain that infection has occurred in the periphery, acting via the endocrine route, or directly signalling via the afferent vagus nerve. The brain is then able to direct the host's behaviour towards facilitation of recovery. Thus, the symptoms of any infection, including fatigue, fever, withdrawal from social activities and sleepiness, are directed by the brain to prioritise recovery above other activities (reviewed in Dantzer and Kelley, 2007). It is plausible that the host response to acute infection induces permanent changes in the brain such that symptoms of the acute sickness response remain beyond the duration of infection. One possible mechanism for such an effect is via sustained activation of microglial cells, which are derived from circulating monocytes, are continuously recruited at a low rate into the brain, and undergo macrophage differentiation *in situ*. These cells are central to immunological surveillance in the brain. Microglia are critical int eh central nervous system (CNS) response to both peripheral and CNS central infections, and other inflammatory disorders. These cells are members of the 'quadpartite synapse' which included the presynaptic and postsynaptic neurons, astrocytes and microglia (Bennett, 2007). By release of pro-inflammatory cytokines and other mediators they influence neural transmissions. This bridging role of microglial activation suggests a plausible pathway from infection to neurobehavioural disorders such as post-infective fatigue states. Of particular interest is the observation that microglial activation may be triggered by a single exposure to LPS-induced TNF-α production and that this stimulus was associated with neuronal injury, which was then sustained for ten months in the absence of systemic inflammation (Qin *et al.*, 2007).

A long held theory of the cause of chronic fatigue syndrome is that ongoing symptoms are a result of persisting pathogen (for example, herpesviruses) following an acute infection. Persisting organisms are proposed to drive ongoing immune responses, which in turn drive symptoms of fatigue. These theories remain popular despite a growing body of evidence to suggest that subjects with chronic fatigue do not have higher rates of seropositivitiy for the viruses that are proposed to drive their symptoms, nor do they have evidence of active viral infection (Soto and Straus, 2000).

Mycobacterium tuberculosis is one example of an intracellular, macrophage tropic, bacterial pathogen that is known to persist within an asymptomatic host, where factors such as immune suppression may cause reactivation of infection. Thus, active tuberculosis is problematic in HIV-1 positive individuals with acquired immunodeficiency syndrome (AIDS) (Gold, 1988), or in patients receiving immune suppressive therapies (Gomez-Reino *et al.*, 2007; Hernandez *et al.*, 2008). Other bacterial infections have been proposed to generate latent infections which may later be reactivated, including *M. leprae* (Vilela Lopes *et al.*, 2009), although latent infections are more commonly noted for viral infections such as *Herpes simplex virus* type 1 (Freeman *et al.*, 2007), BK virus (a polyomavirus) (Sessa *et al.*, 2008), *Varicella zoster virus* (Mueller *et al.*, 2008) and HIV-1 (Lifson *et al.*, 1988).

C. burnetii has certainly been demonstrated to persist in some subjects in the form of chronic, organ-specific infection such as endocarditis (Stein and Raoult, 1995). However, the role of persisting organisms in post Q fever fatigue syndrome remains unclear.

Evidence of persisting *C. burnetii* genomes has been reported in a subset of subjects regardless of clinical outcome, with detection occurring more commonly in bone marrow aspirates than in blood samples, leading to the suggestion that it is the presence of *C. burnetii* in the periphery that results in chronic symptoms of fatigue (Marmion *et al.*, 2005). It is noteworthy that successful culture of *C. burnetii* from PCR-positive samples was not possible, and therefore there was no evidence that persisting *C. burnetii* genomes were associated with the presence of viable or replicating organisms (Marmion *et al.*, 2005). The use of antibiotic therapy to clear any residual *C. burnetii* has been reported to be effective in improving the symptoms of post Q fever fatigue syndrome, however such reports are not the result of randomised, placebo-controlled trials and must be interpreted with caution (Ledina *et al.*, 2007).

The notion that *C. burnetii* persists in subjects with no evidence of chronicity is supported by reports of infection reactivation during states of immune suppression. Q fever seropositivity is associated with adverse pregnancy outcomes (Langley *et al.*, 2003), and there are reports that women who contract Q fever during pregnancy may experience reactivation of their infection during subsequent pregnancies (Stein and Raoult, 1998). However, it is surprising that such cases of reactivation have not been reported to occur in the context of HIV-1 infection, or in cases of immune suppression such as following organ transplantation. Instead, repeated outbreaks of Q fever among a group of individuals with HIV-1 were specifically reported to lack cases of relapsed *C. burnetii* infection (Boschini *et al.*, 1999). Thus, it is unclear why Q fever during pregnancy is particularly associated with

a tendency for later reactivation of infection, and whether the persistence of organisms that presumably occurs in these women might also occur in other cases of acute infection.

In this study, the variable detection of *C. burnetii* in longitudinally collected samples of a single patient, and the generally high cycle threshold values obtained for "positive" samples, suggests that if *C. burnetii* persists following acute infection, it does so at very low levels in the circulation. The assays used in this study were variably able to detect such low levels of *C. burnetii*, and thus it was not possible to conclude whether persistence of *C. burnetii* was truly associated with ongoing fatigue. Future work may include the sampling of alternative tissues, or the development of more sensitive assays for the detection of *C. burnetii* genomes. Ideally, evidence that such genomes are associated with ongoing replication of viable bacteria would also be sought.

Given the proposed importance of the host response to infection in driving symptomatic illness, a major goal of this work was to characterise the host response to *C. burnetii*. Work performed in the monocytic cell line, THP-1, provided evidence for the interaction of *C. burnetii* with TLR-2, although it remains possible that TLR-4 may also play a role in the host response to infection *in vivo*. This work confirms and extends previous findings in murine models that TLR-2, but not TLR-4, is involved in the activation of immune cells and the stimulation of cytokine production in response to *C. burnetii* (Zamboni *et al.*, 2004). The inability of *C. burnetii* to activate responses via TLR-4 likely relates to the structure of the lipid A moiety of its LPS, which has very different biological activity to lipid A moieties isolated from other bacteria such as *E. coli*. Lipid A derived from *C. burnetii* LPS

does not activate murine monocytes, while lipid A from *E. coli* clearly induces CD25 expression. In fact, *C. burnetii*-derived lipid A can inhibit monocyte responsiveness to *E. coli* lipid A in a dose dependent and TLR-4 dependent manner (Zamboni *et al.*, 2004).

The biological relevance of receptor utilisation lies in the intracellular signalling that occurs after TLR-2 or TLR-4 engagement. These receptors act via shared and unique pathways (Zhang *et al.*, 1999; Arbibe *et al.*, 2000; Kawai *et al.*, 2001), and thus subtle differences are believed to exist in the host response to TLR-2 and TLR-4 ligands. Stimuli acting via TLR-2 or TLR-4 both induce pro-inflammatory cytokines via NF-κB-directed gene transcription (MyD88-dependent pathway). However, interaction via TLR-4 may also induce IFN-β expression via IRF-3 directed gene transcription (MyD88-independent pathway). IFN-β may then drive activation of the Stat1 transcription factor and subsequent expression of genes such as inducible nitric oxide synthase (iNOS) (Ohmori and Hamilton, 2001). This IFN-β/Stat1 pathway is not active in response to TLR-2 ligation (Toshchakov *et al.*, 2002).

In the work reported here, IFN-β and iNOS expression were not induced in *C. burnetii*-stimulated THP-1 cells. This provides further evidence that *C. burnetii* interacts with host innate immune cells via TLR-2 and not TLR-4. The lack of iNOS induction may represent a critical flaw in the host response to infection, given the known importance of nitric oxide production in the clearance of murine *C. burnetii* infection (Brennan *et al.*, 2004). Engagement via TLR-2 or TLR-4 has also been shown to drive polarised T helper type responses: engagement of TLR-2 will generally cause DCs to drive Th2-type responses,

while Th1-type responses occur following DC TLR-4 engagement (Pulendran *et al.*, 2001). The potential interaction of *C. burnetii* with TLR-2 therefore has significant impact on the quality of the resultant adaptive immune response in addition to shaping innate immune responses. Furthermore, it is plausible that the virulence of *C. burnetii* relates in part to the lack of integration of multiple TLR pathways in the host response to infection.

It remains to be seen whether there is interaction between *C. burnetii* and TLR-2 in primary cells. Although THP-1 cells are frequently used as models of primary monocyte function, it is unclear how comparable they are in terms of surface receptor expression and function with the monocytes or macrophage sub-population which are targeted by *C. burnetii*. The THP-1 cell line displayed cell surface receptor expression of both CD14 and CD16, a pattern that parallels the receptor expression of the "inflammatory" monocyte subset that is preferentially expanded during infection (Weber *et al.*, 2000) – whether this expansion is also found in acute Q fever warrants further investigation. THP-1 cells also strongly expressed TLR-2, and produced large amounts of TNF-α following TLR ligation, mirroring observations of the CD14+ CD16+ primary monocyte subset (Belge *et al.*, 2002). *C. burnetii* is believed to trigger an atypical M2 polarised activation profile of host macrophages (Benoit *et al.*, 2008a), however M1 polarised macrophages are believed to be more efficient clearers of bacterial infections (Benoit *et al.*, 2008b). It was not possible to identify iNOS induction in stimulated THP-1 cells, suggesting they may phenotypically resemble the M2 polarised macrophage. Further characteriation of THP-1 cells, including analysis of their cell surface receptor expression and biological activity, would be required to confirm whether these cells represent the best model for the study of *C. burnetii*.

Further work to characterise the involvement of TLRs in the host response to *C. burnetii* should involve primary monocytes, differentiated monocytic cell lines and differentiated primary monocytes. Differentiating agents such as phorbol myristate acetate (PMA) and vitamin D3 are known to generate cells of varying phenotype and therefore different macrophage-like cell types may be examined *in vitro* (Biskobing and Rubin, 1993). If TLR-specific antibodies prove to be ineffective in blocking signalling via TLR-2 and TLR-4 in primary cells, the use of alternative blocking strategies such as small inhibitory RNA (siRNA) may be feasible. Inhibitory RNA techniques have been successfully used in THP-1 cells to study TLR function via knockdown of MyD88 or other molecules involved in the downstream signalling pathways (Ahmad *et al.*, 2008; Dennis *et al.*, 2009), and in primary monocyte-derived macrophages to knockdown the function of other molecules involved in intracellular signalling (Cheung *et al.*, 2008). Use of inhibitory RNA techniques in primary monocytes, although technically challenging, would therefore be expected to be feasible in the study of TLR function in response to *C. burnetii*.

Putative functional polymorphisms have been identified in TLR-2 and TLR-4, and have been found to be associated with infectious disease susceptibility or severity (reviewed in Schroder and Schumann, 2005). In this study, individuals carrying the TLR-4 Asp299Gly or TLR-2 Arg753Gln polymorphism were identified within the Q fever cohort, but were not associated with susceptibility, severity, or illness duration of acute Q fever. The available power to detect a disease association in the DIOS cohort of a similar magnitude to previously published findings in comparable infectious diseases was calculated. With alpha

set at 0.05, there was 81% power to replicate the TLR-2 Arg753Gln association with pulmonary tuberculosis (Ogus *et al.*, 2004), and 83% power to detect the association between the TLR-4 Asp299Gly and Gram negative sepsis (Lorenz *et al.*, 2002). The lack of association between TLR polymorphisms and acute Q fever parameters reported here was therefore unlikely to be a result of insufficient sample size.

The TLR-4 Asp299Gly polymorphism occurs in the extracellular domain of TLR-4, and thus the presence of this polymorphism may alter the LPS binding capacity of TLR-4, or inhibit the formation of the LPS receptor complex. LPS has been shown to interact with all components of the TLR-4/MD-2/CD14 complex, although the first one hundred amino acids of TLR-4 are believed to be the most critical for LPS binding (da Silva Correia *et al.*, 2001). It is uncertain how the substitution of glycine for asparagine at amino acid number 299 may alter TLR-4 function, particularly as it relates to its potential interaction with *C. burnetii* LPS. By contrast, the TLR-2 Arg753Gln polymorphism occurs in the intracellular TIR domain that is responsible for signal transduction following TLR ligation. If this polymorphism is truly functional *in vivo*, its effects would be expected to be uniform across a variety of TLR-2 ligands, as only downstream signalling events are likely to be affected. A lack of association between TLR-2 polymorphisms and Q fever incidence, severity or duration cannot be explained by the possibility that *C. burnetii* LPS and TLR-2 interact via a unique molecular mechanism that circumvents any functional defect conferred by the polymorphism. It therefore seems most likely that absence of a significant association between TLR polymorphisms and Q fever susceptibility, severity or course relates to an

absence of functional defect in heterozygous carriers of each TLR polymorphism (von Aulock *et al.*, 2003; von Aulock *et al.*, 2004).

Adaptive immune responses were investigated as potential correlates of illness persistence following acute Q fever. No association was found between patterns of Q fever-specific immunoglobulin production and prolonged fatigue following the acute illness. Other studies that have investigated a role for Q fever-specific immunoglobulin in the persistence of fatigue following acute infection have failed to find significant differences in the antibody responses of subjects who had promptly resolving illness in comparison to those with post Q fever fatigue syndrome (Marmion *et al.*, 2005).

There is a growing body of evidence arguing against an association between antibody titres and chronic fatigue states. There are reports that CFS is associated with an increased in prevalence or titre of IgM or IgG specific for EBV, HHV-6, Herpes simplex viruses (HSV)-1 and 2, or Coxsackievirus B1 and B4 (Manian, 1994; Lerner *et al.*, 2004; Kogelnik *et al.*, 2006). In these studies, no single antibody specificity or isotype was identified to occur in all subjects with fatigue, and comparable antibody profiles were often evident, albeit at lower frequency, amongst the control cohorts. Subtle differences in the early host response to EBV were noted in humoral responses for subjects from DIOS who developed post-infective fatigue following infectious mononucleosis due to EBV, but no ongoing differences in immune activation were present beyond the acute phase of infection (Cameron *et al.*, 2006). The balance of evidence argues strongly against an association between post-infective fatigue states and altered pathogen-specific antibody responses.

Investigation of the antigen-specific cellular response to Q fever was made difficult by the presence of LPS within the whole, inactivated *C. burnetii* preparations that were available as recall antigens. It may be argued that the biological effects of *C. burnetii* LPS are relevant *in vivo*, and that there is no need to dissect the specific contributions made by antigen-specific and antigen-non-specific effects. While bulk cellular responses do not seem to correlate with post Q fever fatigue syndrome, antigen-specific responses may determine an individual's susceptibility to chronic symptoms of fatigue following the acute illness. Alternatively, the identification of antigen-specific cellular responses may direct the development of a vaccine with fewer side effects such that pre-sensitised individuals may be safely vaccinated. Hence, a concerted effort was made in the studies presented in this thesis to identify antigen-specific cellular responses against *C. burnetii* – without success.

Use of CD8+ T cell epitope prediction platforms can be a useful tool for the identification of potentially immunogenic regions of a protein of interest. However, the reliability of such algorithms depends heavily on the input data upon which they were built. In general, information relating to peptide-HLA binding affinities derived from the SYFPEITHI database (Rammensee *et al.*, 1999) and data regarding antigen processing factors such as proteosome cleavage and TAP transporter function (Petrovsky and Brusic, 2004) are combined to predict whether short peptide sequences within a larger protein are likely to be bound to a particular HLA type. For example, the dependence on TAP transporter function varies by HLA type, with HLA-A3-binding peptides shown to have high affinity for TAP

while HLA-A2-binding peptides have low affinity for TAP (Brusic *et al.*, 1999) hence prediction algorithms must take these factors into account.

The choice of HLA-A2 and HLA-A3 for study within the Q fever cohort was primarily associated with the prevalence of these alleles within the subjects for whom PBMC samples were available. However, these two HLA types also occur within two different HLA supertypes, and are the archetypal examples of the binding patterns that are evident in all the HLA alleles that occur in each supertype (Sette and Sidney, 1999). Validation of epitope prediction platforms have indicated a success rate of approximately 80% in the context of the identification of potential CTL epitopes within the genome of the virus identified to cause severe acute respiratory syndrome (SARS) (Sylvester-Hvid *et al.*, 2004). Thus, the platforms used to identify potential epitopes within heat shock protein B were likely to have been successful in predicting high affinity HLA-A2 or HLA-A3-binding sequences for approximately 80% of the peptides, which would equate to four peptides for each HLA allele.

The algorithms by which epitope prediction platforms operate have been designed to incorporate all the information that is known about the antigen processing and presentation process to identify peptides that may bind a specific HLA allele with high affinity. Such estimates do not calculate whether the resultant HLA-bound peptide will be immunogenic. High HLA class I binding affinity has been found to correlate highly with the immunogenicity of potential cytotoxic T cell epitopes (Sette *et al.*, 1994). It is noteworthy that despite this association, not all high affinity binding peptides are immunogenic. One

study screened for CTL responses to a total of 43 peptides with high or intermediate binding affinities for HLA-A2 in patients with acute hepatitis B virus infection, and found that only 12 elicited cytotoxic effects in at least one of twenty-five HLA-A2 carrying subjects (Sette *et al.*, 1994). Given comparable numbers of subjects with Q fever who carried HLA-A2 and/or HLA-A3, and assuming comparable rates of identification of immunogenic peptides from those with binding affinity, the limited peptides that were chosen in the work reported here would have been estimated to include only one peptide for each HLA type that recalled antigen-specific responses *in vitro*. Expanding this peptide pool, and drawing on a larger number of *C. burnetii* protein sequences, is likely to enhance detection of antigen-specific responses in the Q fever cohort.

In this study, immunogenicity was tested by only one method, the IFNγ ELISpot assay. There are a number of different assays that may be used to detect cellular activity. These approaches may be used to identify immunogenic peptides via the detection of antigen-specific responses, or having identified immunogenic peptides, to characterise the immune response of each subject to a particular peptide or pool of peptides. Peptides may firstly be used to stimulate antigen-specific T cells via the inclusion of monocytes or B cells as antigen presenting cells in each cell culture system. Then, T cell responses may be characterised by the detection of perforin formation within tetramer (antigen)-specific CD8+ T cells by flow cytometry (Appay and Rowland-Jones, 2002), cytokine responses may be determined by ELISA, or by a multiplex-bead based detection system that allows the quantitation of multiple cytokines in a single sample (Khan *et al.*, 2004), and proliferation may be quantitated by conventional tritiated thymidine incorporation assay or

the newly reported RT-qPCR assay that detects polymerase delta interacting protein 38 (PDIP-38) gene expression in association with cellular proliferation (Klaile *et al.*, 2008). These techniques may be useful in the identification of immunogenic epitopes for the inclusion in novel vaccines, or may help to characterise the T cell response to *C. burnetii* and its potential role in Q fever severity or course.

There were areas of the host immune response that were not covered by the work in this thesis. Given that *C. burnetii* infection is thought to occur predominantly via inhalation, an investigation of the early stages of the host immune response to the pathogen, including aspects of mucosal immunity, would add significantly to our understanding of the pathogenesis of this illness. The role of antimicrobial peptides, which are selectively toxic to bacterial cells but not host mammalian cells (Brogden, 2005), also remains to be elucidated in the case of *C. burnetii* infection. There are significant gaps in our understanding of the host response to *C. burnetii*, and therefore these immune response factors are just a few examples of areas that warrant further attention.

Q fever is an acute illness caused by *C. burnetii* infection. The outcome of infection varies widely from asymptomatic seroconversion to an acute febrile illness that may require hospitalisation. A subset of patients remains unwell with an illness featuring fatigue that persists for months or years after the acute phase of Q fever, however it is unknown what factors predispose an individual to develop Q fever fatigue syndrome, or conversely to suffer an illness that resolves promptly. The immune response to infection has been proposed to drive the symptoms of post Q fever fatigue syndrome, however it has not been

possible to demonstrate any difference in the immune responses seen in subjects with this syndrome in comparison to subjects with quickly resolved illness. Further studies to improve our knowledge of the host response to *C. burnetii* infection, including investigation of the interaction between the pathogen and the innate immune system, and the subsequent generation of adaptive immune responses, are warranted. Additionally, studies of the interaction between inflammatory responses in the periphery during acute infection and any resulting change in function within the CNS are likely to be informative in elucidating the pathogenesis of post Q fever fatigue syndrome.

Appendix 1: SPHERE questionnaire

Office Use Only D U B B O

Time point - Site number - Dr surname - Dr initial - Patient number

SPHERE

We would like to know about your general health. For ALL questions, please tick, cross or colour the circle which most closely matches your response. There are no right or wrong answers. Please answer all questions.

Over the **past few weeks** have you been troubled by:

	never or some of the time	a good part of the time	most of the time
1. Headaches?	○	○	○
2. Feeling irritable or cranky?	○	○	○
3. Poor memory?	○	○	○
4. Pains in your arms or legs?	○	○	○
5. Feeling nervous or tense?	○	○	○
6. Waking up tired?	○	○	○
7. Rapidly changing moods?	○	○	○
8. Fainting spells?	○	○	○
9. Nausea?	○	○	○
10. Arms or legs feeling heavy?	○	○	○
11. Feeling unhappy & depressed?	○	○	○
12. Gas or bloating?	○	○	○
13. Fevers?	○	○	○
14. Back pain?	○	○	○

	never or some of the time	a good part of the time	most of the time
15. Sore throat?	○	○	○
16. Numb or tingling sensations?	○	○	○
17. Feeling constantly under strain?	○	○	○
18. Joint pain?	○	○	○
19. Weak muscles?	○	○	○
20. Feeling frustrated?	○	○	○
21. Diarrhoea or constipation?	○	○	○
22. Getting annoyed easily?	○	○	○
23. Everything getting on top of you?	○	○	○
24. Dizziness?	○	○	○
25. Feeling tired after rest or relaxation?	○	○	○
26. Feeling lost for words?	○	○	○
27. Losing confidence?	○	○	○
28. Being unable to overcome difficulties?	○	○	○

Have you recently:	No	Yes
29. thought that you should cut down on alcohol or addictive drugs?	○	○
30. had a friend, relative or doctor suggest that you should cut down on alcohol or addictive drugs?	○	○

31. When did you first notice symptoms of this illness? ☐☐ / ☐☐☐☐ (month/year)

32. Do you still have symptoms? ○ No ○ Yes

Today's date ☐☐ / ☐☐ / ☐☐☐☐

Appendix 2: PSC questionnaire

Office Use Only D U B B O

Time point - Site number - Dr surname - Dr initial - Patient number

PSC

This questionnaire asks about your physical health over the past few weeks. For each question, please tick, cross or colour the circle that most closely matches your response. There are no right or wrong answers. Please answer all questions.

Over the past few weeks have you:	Never or some of the time	A good part of the time	Most of the time
1. Generally felt unwell?	○	○	○
2. Lost your appetite?	○	○	○
3. Had 'chills' or shivers?	○	○	○
4. Had a sore throat?	○	○	○
5. Had a fever?	○	○	○
6. Had tender glands in your neck or elsewhere?	○	○	○
7. Had a headache?	○	○	○
8. Had any abdominal or stomach pains?	○	○	○
9. Had pains in your joints?	○	○	○
10. Had muscle aches and pains?	○	○	○
11. Had a cough?	○	○	○
12. Been short of breath or had difficulty breathing?	○	○	○
13. Had problems with being in bright light?	○	○	○
14. Had pains in your chest?	○	○	○
15. Felt excessively tired or exhausted?	○	○	○
16. Felt dizzy or lightheaded?	○	○	○
17. Had a rash?	○	○	○
18. Been sweating more than usual?	○	○	○

Do you have any further comments concerning your illness?

Today's date / /

Appendix 3: BDQ questionnaire

Draft

Office Use Only D U B B O

Time point - Site number - Dr surname - Dr initial - Patient number

BDQ

The following questions concern some daily activities that are sometimes affected by your health. We would like to know whether your health problems have recently limited you in any of these areas. For each question, please tick, cross or colour the circle that most closely matches your response. There are no right or wrong answers. Please answer all questions.

During the past few weeks:	No, not at all	Yes, sometimes or a little	Yes, moderately or definitely
1. Have your health problems limited you in any of the following activities:			
a. The kinds or amount of vigorous activity you can do, like lifting heavy objects, running or sports?	○	○	○
b. Walking long distances (that is, 1-2 km)?	○	○	○
c. The kinds or amount of moderate activity you can do, like moving a table, carrying groceries or goods?	○	○	○
d. Climbing stairs or walking uphill?	○	○	○
e. Bending, lifting or stooping?	○	○	○
f. Eating, dressing, bathing or using the toilet?	○	○	○
2. Have you had to cut down or stop any activity you used to do, such as hobbies, because of some illness or injury?	○	○	○
3. Have you not been able to do something that your family (or household) expected from you as part of the daily routine?	○	○	○
4. Have your personal problems decreased your motivation for work?	○	○	○
5. Have your personal problems decreased your personal efficiency at home, school or work?	○	○	○
6. Has there been a deterioration in your social relations with friends, workmates or other persons?	○	○	○

7. During the last month, how many days in total were you unable to carry out your usual daily activities fully?	☐☐ days
8. During the last month, how many days in total did you stay in bed all or most of the day because of illness or injury?	☐☐ days

Today's date ☐☐ / ☐☐ / ☐☐☐☐

Appendix 4: SOMA questionnaire

Over the **past few weeks** have you been troubled by:	never or some of the time	a good part of the time	most of the time
1. Muscle pain after activity?	○	○	○
2. Needing to sleep longer?	○	○	○
3. Prolonged tiredness after activity?	○	○	○
4. Poor sleep?	○	○	○
5. Poor concentration?	○	○	○
6. Tired muscles after activity?	○	○	○

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