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Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

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Declaration

I declare that the work presented in this thesis has been composed and completed by myself, except where stated otherwise in the text and acknowledgements. This work has not previously been submitted for any other degree or personal qualification.

Laura Doull

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List of Abbreviations

AB	Aberrant body
ANOVA	Analysis of variance
ASC	Apoptosis-associated speck-like protein containing CARD
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CARD	Caspase activation recruitment domain
CD	Cluster of differentiation
CFT	Complement fixation test
Chsp60	Chlamydial heat shock protein 60
CHO	Chinese hamster ovary
CLR	C-type lectin receptor
ConA	Concanavalin A
CTR	Cluster tandem repeat
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DE	Differentially expressed
DNA	Deoxyribonucleic acid
EAE	Enzootic abortion of ewes
EB	Elementary body
EHEC	Enterohemorrhagic <i>E. coli</i>

ELISA	Enzyme linked immunosorbent assays
FBS	Fetal bovine serum
FDR	False discovery rate
GLMM	General linear mixed model
GM-CSF	Granulocyte-macrophage colony stimulating factor
IDO	Indoleamine 2, 3-dioxygenase
FITC	Fluorescein isothiocyanate
IFN- α	Interferon-alpha
IFN- γ	Interferon-gamma
IFU	Inclusion forming units
inc	Inclusion membrane protein
IMDM	Iscove's modified Dulbecco's medium
IL	Interleukin
IRF	Interferon regulatory factory
ISG	Interferon stimulated gene
KO	Knockout
LB	Lysogeny broth
LGV	Lymphogranuloma venereum (LGV)
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
mAb	Monoclonal antibody
MDDC	Monocyte derived dendritic cell
MDM	Monocyte derived macrophage
MHC I	Major histocompatiblity complex class one
MHC II	Major histocompatibility complex class two

MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MRI	Moredun Research Institute
MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NLR	NOD-like receptor
NLRP3	NLR pyrin domain containing 3
NO	Nitric oxide
NOD	Nucleotide binding and oligomerization domain
OEA	Ovine Enzootic Abortion
pAb	Polyclonal antibody
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
Pmp	Polymorphic membrane protein
PRR	Pathogen recognition receptor
PZ	Plasticity zone
RB	Reticulate body
RIP2	Receptor interacting protein 2
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute

ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Real-time PCR
SD	Standard deviation
S-PCR	Standard PCR
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
STD	Sexually transmitted disease
T3SS	Type 3 secretion system
TARP	Translocated actin recruiting protein
Th1	T-helper type 1
Th2	T-helper type 2
TLR	Toll-like receptor
UV	Ultraviolet radiation

Abstract

Chlamydia abortus is the most common known infectious cause of ovine abortion worldwide but is rarely linked with bovine abortion. The reasons for this differential pathogenesis are unknown but may involve differences in innate immune recognition and immune responsiveness. This is supported by the observation that chlamydial abortion in sheep is associated with an inflammatory cytokine/chemokine cascade that is not commonly observed in cattle. Studies with other *Chlamydia* species have demonstrated that innate inflammatory pathways including inflammasome activation contribute to both pathogen clearance and pathology. Pattern recognition receptors (PRRs) activate these innate immune signalling pathways but are relatively poorly characterized in ruminants. We hypothesize that the ruminant hosts differ in their ability to innately sense *C. abortus* infection and activate the inflammasome. The main aims of this project were to: analyse PRR expression in innate immune cells; assess cytokine production from innate immune cells in response to *C. abortus*; investigate the role of PRRs in the induction of innate immune responses to *C. abortus*; and, conduct RNA-seq analysis on macrophages following infection with *C. abortus* to identify important immune signalling pathways.

Ruminant oro-nasal turbinate cells, monocyte derived dendritic cells (MDDCs) and monocyte derived macrophages (MDMs) express the cell-surface PRRs TLR2 and TLR4 and also the intracellular PRRs NOD 1 and NLRP3. Oro-nasal turbinate cells produce CXCL8 late into the chlamydial developmental cycle independent of IL-1 β . In contrast, ruminant MDMs and MDDCs secrete early IL-1 β in response to *C. abortus* infection. In MDMs and MDDCs, live and UV-inactivated *C. abortus* induced TNF- α and CXCL8 but live infection was required for IL-1 β secretion. Therefore, intracellular *C. abortus* multiplication is necessary to stimulate the IL-1 β processing pathway within these cells. In order to determine PRR function, NOD1 and NLRP3 were knocked down in ruminant MDMs using siRNA. In both ovine and bovine MDMs, NOD1 was identified as a factor in *C. abortus* mediated IL-1 β production. NLRP3 knockdown in bovine but not ovine MDMs also reduced IL-1 β production, indicating species-specific differences in *C. abortus* recognition. The

RNA-seq analysis of ruminant MDMs identified novel pathways of immune activation by *C. abortus* and potentially important species-specific differences. An improved understanding of the innate immune pathways activated in susceptible and resistant hosts following *C. abortus* infection will inform on disease pathogenesis and could contribute to novel chlamydial vaccine design.

Lay summary

Chlamydia abortus is the most common known infectious cause of ovine abortion and therefore is of considerable importance to the farming industry. *C. abortus* rarely causes abortion in cattle which is interesting considering that sheep and cattle are often farmed together. In sheep, the bacterium activates the immune system and induces inflammation at the placenta which results in abortion. The bacteria multiply inside the host cell and the way that the host immune system recognises and responds to *C. abortus* is not well understood. Receptors found on the surface and inside the host immune cells are responsible for recognising foreign molecules and activating response pathways. Response pathways often result in the production of signalling molecules which aid communication between host immune cells. Important signalling molecules include CXCL8, IL-1 β , IL-6 and TNF- α . We hypothesize that cattle and sheep differ in the way that they recognise and respond to the bacteria, which results in the different outcomes of infection. The main aims of this project were to: assess receptor expression on immune cells; assess the production of signalling molecules by immune cells in response to *C. abortus*; assess the role of receptors in the activation of response pathways to *C. abortus*; and, assess the expression of genes activated in macrophages (immune cell) in response to *C. abortus*.

Receptors which were expressed inside (NOD1 and NLRP3) and on the surface of the cell (TLR2 and TLR4) were identified on various immune cells including: cells which line the oro-nasal cavity, macrophages and dendritic cells. Oro-nasal cells produced CXCL8 but not IL-1 β unlike macrophages and DCs which were capable of both CXCL8 and IL-1 β production. Using technology to reduce the expression of receptors we identified an important role for NOD1 in the production of IL-1 β in both sheep and cattle macrophages. NLRP3 was involved in IL-1 β production in cattle but not sheep macrophages indicating differences in the recognition of *C. abortus* between species. The analysis of gene expression following *C. abortus* infection in macrophages has identified the type of response pathways activated and differences in the pathways activated between sheep and cattle. Identifying

differences in the way that sheep and cattle recognise and respond to *C. abortus* will improve our understanding of how abortion is mediated by the immune system and could contribute to the design of improved chlamydial vaccines.

Chapter 1: General Introduction

1.1 Chlamydial diversity and host tropism

1.1.1 Classification

The phylum *Chlamydiae* encompasses the *Chlamydiaceae* and *Chlamydia*-like organisms. The discovery of *Chlamydia*-like organisms (environmental *Chlamydiae*) in 1990 challenged the perception of chlamydial diversity, which was previously based on just the *Chlamydiaceae* family but now includes 8 families (Horn, 2008). Research into the *Chlamydia*-like organisms is expanding; however, the *Chlamydiaceae* family is the focus of this review.

Previously the *Chlamydiaceae* were classified into two genera: *Chlamydia* (*Chlamydia trachomatis*, *C. suis* and *C. muridarum*) and *Chlamydophila* (*Chlamydophila abortus*, *C. psittaci*, *C. caviae*, *C. felis*, *C. pecorum* and *C. pneumoniae*). This division was based on genetic differences in the 16S and 23S ribosomal RNA in a variety of isolates from multiple hosts (Everett et al., 1999). This division was not widely accepted at the time, and although it was used extensively by veterinary chlamydiologists, it was perhaps most notably not adopted by groups working on the human pathogen *C. pneumoniae* (Stephens et al., 2009). The criterion for species to fall within a particular genus was set at 95% identity. However, species from different genera were over 94% identical suggesting that the separation was based on sequence differences that may not be biologically meaningful (Schachter et al., 2001). Growing evidence of genetic similarity between the two genera has meant that the single *Chlamydia* genus classification has been re-adopted (section 1.1.3) until the genomic data have been completely reviewed (Entrican et al., 2012; Greub, 2010; Sachse et al., 2015; Stephens et al., 2009). Genome sequences for most chlamydial species have recently become available, which has allowed for more thorough investigation into the genetics behind species diversity (see section 1.1.4) (Clarke, 2011).

1.1.2 Chlamydial developmental cycle

All *Chlamydiae* share a characteristic biphasic intracellular developmental cycle. The bacterium exists extracellularly as an infectious but metabolically inactive elementary body (EB) (Abdelrahman and Belland, 2005). The bacteria attach to the host cell before becoming internalized after complex host cell-pathogen interactions (Dautry-Varsat et al., 2005). The EBs transform into metabolically active reticulate bodies (RBs) within a protective intracellular niche termed an inclusion. Within the inclusion the RBs multiply by binary fission and then transform back into EBs (Abdelrahman and Belland, 2005), before being released via host cell lysis or by extrusion to infect other cells and begin the cycle once again (Hybiske and Stephens, 2007) (Figure 1.1).

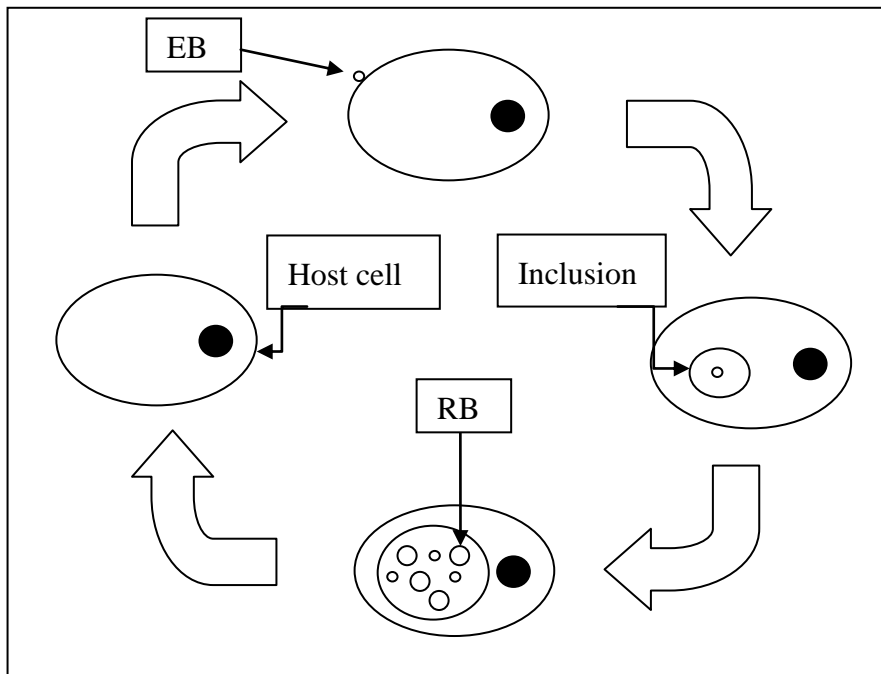


Figure 1.1: The chlamydial developmental cycle. The infectious but metabolically inactive elementary body (EB) attaches to the host cell. Once internalised, the EB transforms into a metabolically active reticulate body (RB) and begins to multiply within the cell. RB multiplication and the transformation back to EBs is asynchronous, before the EBs are released to infect further host cells (adapted from Abdelrahman and Belland, 2005).

1.1.3 Host and tissue tropism

The *Chlamydia* genus is currently composed of 12 species, namely *C. trachomatis*, *C. muridarum*, *C. suis*, *C. pneumoniae*, *C. felis*, *C. pecorum*, *C. caviae*, *C. abortus*,

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C. psittaci, *C. avium*, *C. gallinacea* and *C. ibidis* (Sachse et al., 2015). These bacteria can cause a spectrum of diseases in a range of hosts (Table 1.1). The primary sites of chlamydial infection are mucosal surfaces and in some cases pathology is limited to these sites. However, some species, and biovars within species, can invade further to cause pathology at distal sites (Meeusen et al., 2004) .

Table 1.1: The host tropism and disease spectrum of the *Chlamydia* genus.

Chlamydial species	Typical host (s)	Serovars	Example associated diseases	References
<i>C. trachomatis</i>	Humans	D, E, F, G, H, I, J and K	Genital tract disease	Nunes and Gomes, 2014
		A, B, Ba and C	Trachoma	
		L1, L2 and L3	Lymphogranuloma venereum (LGV)	
<i>C. pneumoniae</i>	Humans, horses and amphibians		Respiratory disease	Nunes and Gomes, 2014
<i>C. muridarum</i>	Mice		Respiratory and urogenital disease	Schoborg, 2011
<i>C. suis</i>	Swine		Enteritis and urogenital disease	Schoborg, 2011
<i>C. felis</i>	Cats		Conjunctivitis	Nunes and Gomes, 2014
<i>C. pecorum</i>	Ruminants, koalas, swine and horses		Pneumonia, conjunctivitis, polyarthritis, enteritis and mastitis	Bachmann et al., 2014a; Nunes and Gomes, 2014; Schoborg, 2011
<i>C. caviae</i>	Guinea pigs		Ocular disease and urogenital disease	Schoborg, 2011
<i>C. abortus</i>	Ruminants and swine		Abortion	Schoborg, 2011
<i>C. psittaci</i>	Poultry and psittacine birds		Avian chlamydiosis	Knittler et al., 2014
<i>C. avium</i>	Pigeons and psittacine birds		Unknown	Sachse et al., 2014
<i>C. gallinacea</i>	Poultry		Unknown	Sachse et al., 2014
<i>C. ibidis</i>	Feral sacred ibis		Unknown	Vorimore et al., 2013

A number of the *Chlamydia* species are pathogens of clinical importance in humans. *C. trachomatis* is the most common bacterial cause of sexually transmitted disease in humans. Infection is often asymptomatic but can cause pathology including pelvic infertility disease (PID) and infertility. The serovars within the lymphogranuloma

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venereum (LGV) biovar of *C. trachomatis* are capable of disseminating from the genital mucosa to the draining lymph nodes and may cause chronic granulomatous disease (Fehlner-Gardiner et al., 2002). Trachoma caused by serovars within the trachoma biovar of *C. trachomatis* is the leading cause of preventable blindness worldwide (Fehlner-Gardiner et al., 2002). Intravaginal inoculation of mice with *C. muridarum* results in a genital infection that has similar characteristics to *C. trachomatis* infection in women. *C. muridarum* is not a known pathogen of humans but provides a suitable model of disease for studies. *C. pneumoniae* is also an important human pathogen that can cause respiratory disease. Other chlamydial species which have known zoonotic potential include *C. psittaci*, *C. abortus* and *C. felis* (Nunes and Gomes, 2014). *C. abortus* is particularly important because of the severe consequences of infection, including abortion and septicaemia, in pregnant women (Essig and Longbottom, 2015).

1.1.4 Comparative *Chlamydiaceae* genomics

Genome sequencing has dramatically improved our understanding of the host adaptation and basic biology of *Chlamydiaceae*. Comparative analyses of the *Chlamydia* genomes have demonstrated high levels of conservation across species and a common reduced genome size (Knittler et al, 2014). The reduced genome size is likely due to the increased reliance on host-derived molecules that accompanies an obligate intracellular life cycle. Evidence of this in the *Chlamydiaceae* is demonstrated by the loss of a number of genes involved in metabolic pathways, as these molecules could be scavenged from the host (Thomson et al., 2005).

A number of common genes with important functions have been identified within all the *Chlamydiaceae* genomes. Polymorphic membrane proteins (Pmps) and inclusion membrane proteins (inCs) (Gupta and Griffiths, 2006) are thought to function in interactions with the host, including in adhesion and antigen driven immune evasion (Nunes and Gomes, 2014). *Chlamydiaceae* also possess a Type 3 Secretion System (T3SS), which is thought to be important for virulence as it allows the bacterium to insert effectors into the host cell. Translocated actin recruiting protein (TARP) is an example of a T3SS effector found in all sequenced *Chlamydiaceae* genomes, which

is thought to be responsible for the remodelling of host cell actin to facilitate infection (Nunes and Gomes, 2014). The plasticity zone (PZ) has been identified as a hypervariable region present in all *Chlamydiaceae* genomes. A number of genes have been identified within the PZ regions that are thought to contribute to chlamydial pathogenicity and niche tropism (Rockey, 2011; Thomson et al., 2005)

Comparative genomics has been used to compare *Chlamydiaceae* genomes and has revealed interspecies and intraspecies differences which may explain host and tissue tropism (Bachmann et al., 2014b).

1.1.4.1 Interspecies variation

There is a high degree of conservation in both gene order and content despite the varied host range and tissue tropism of the various *Chlamydiaceae* species (Bachmann et al., 2014a). Variability has been identified within the PZ region and *pmp* genes between different species, which may be important for determining host adaptation (Bachmann et al., 2014b).

The PZ region of different species encode a number of genes including a tryptophan (*Trp*) operon, toxin genes and biotin synthesis genes, all of which have been implicated in interspecies variation. The *Trp* operon encodes tryptophan synthase, which is responsible for the synthesis of tryptophan essential for bacterial survival. The genital serovars of *C. trachomatis* encode a functional *Trp* operon which allow the bacteria to synthesise tryptophan from indole found in the genital microflora (Coers et al., 2009). The absence of the *Trp* operon means that the organisms are dependent on host cell tryptophan and are potentially more susceptible to IFN- γ mediated indolamine 2, 3-dioxygenase (IDO) activation, which mediates tryptophan depletion. *C. pneumoniae*, *C. muridarum* and *C. abortus* all lack a functional *Trp* operon, which may explain why these bacteria colonize certain anatomical niches with greater access to host tryptophan or where they have protection from IFN- γ mediated degradation (Thomson et al., 2005). IFN- γ avoidance genes may also have a role in determining host-tropism. *C. muridarum* has developed mechanisms to avoid IFN- γ inducible GTPases that are more important for immune defence against

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Chlamydia in mice than tryptophan depletion (Nelson et al., 2005). IFN- γ is an important cytokine in immune defence to chlamydial infections and this will be discussed in section 1.4.3.

Genes encoding a cytotoxin, similar to the Enterohemorrhagic *E-coli* (EHEC) adherence factor, are present in the PZ region of *C. psittaci*, *C. felis*, *C. caviae*, *C. muridarum*, *C. pecorum* and *C. pneumoniae* but absent in other chlamydial species (Voigt et al., 2012). *C. psittaci*, *C. trachomatis*, *C. pecorum*, *C. felis* and koala *C. pneumoniae* isolates express membrane attack complex/perforin genes (*MACPF*) (Sait et al., 2014; Voigt et al., 2012). The function of the cytotoxin and *MACPF* genes in chlamydial species is not yet known (Voigt et al., 2012). The *guaAB-add* gene cluster encodes accessory virulence genes thought to be involved in purine biosynthesis and is present in *C. pneumoniae*, *C. muridarum*, *C. felis* and *C. psittaci* genomes but not in *C. trachomatis* or *C. abortus* (Read et al., 2013). Biotin synthesis genes have been found in *C. pneumoniae*, *C. abortus*, *C. psittaci*, *C. felis* and *C. pecorum* genomes but were absent in *C. muridarum*, *C. caviae* and *C. trachomatis* (Sait et al., 2014). The presence of biotin synthesis genes within the *C. abortus* genome may be important for the ability of the bacteria to survive in the placenta (Thomson et al., 2005), as pregnancy has been shown to result in a biotin-deficiency in humans (Mock et al., 2002).

The *pmp* genes were first identified on the outer membrane of *C. abortus* (Longbottom et al., 1998) and have since been identified in all other *Chlamydia* species. The different species have varying numbers of *pmp* genes, from nine in *C. trachomatis* and *C. muridarum* to 21 in *C. pneumoniae* and *C. psittaci* (Knittler et al., 2014). *Pmp* genes have been shown to be immunogenic and are thought to be involved in the attachment of EBs to the host cell surface. The *pmp* genes also contribute to antigenic variation facilitating evasion of the host immune system (Longbottom et al., 1998). They are characterized by a high rate of mutation between and within species, possibly indicative of adaptation to different hosts. The increased number of *pmp* genes in some species may be reflective of the ability to infect multiple hosts (Knittler et al., 2014).

Therefore, it is likely that the combination of a number of genetic differences, and not a single gene, are responsible for determining host and niche tropism between species of *Chlamydia*.

1.1.4.2 Intraspecies variation

Chlamydial strains are isolates of the same species that are usually differentiated based on genetic variation, particularly in *ompA* that encodes the major outer membrane protein (MOMP). However, more recent typing studies have moved to a whole genome approach (Nunes and Gomes, 2014). *C. trachomatis* is the only chlamydial species which contains serovars (Table 1.1), which are groups of strains separated based on MOMP differences (Byrne et al, 2010). Different chlamydial serovars/strains can have varied host and tissue tropism (Bachmann et al., 2014b). Studying the genetics of these strains will help identify the specific genes involved in host-pathogen interactions.

Mutations have been identified in the *Trp* operon of the ocular strains of *C. trachomatis*, which indicate they do not encode functional tryptophan synthase. Unlike in the genital tract, indole is not thought to be present (Fehlner-Gardiner et al., 2002) in the relatively limited cultivable microbiota of the conjunctiva (compared to other mucosal surfaces) (Willcox, 2013) and, therefore, the loss of the functional *Trp* operon may contribute to niche tropism of ocular strains (Fehlner-Gardiner et al., 2002). Cytotoxin genes have been identified in ocular and genital strains of *C. trachomatis* but have been lost by the LGV strains. Chlamydial cytotoxin may inactivate GTP-binding proteins, which inhibits the trafficking of vesicles within the cell and may restrict infection to the mucosal site. Therefore, loss of the cytotoxin gene may facilitate dissemination of the LGV strains beyond the genital mucosa (Belland et al., 2001; Thomson et al., 2008). The N-terminal of the cytotoxin gene is complete in genital but not ocular strains of *C. trachomatis*. The N-terminal of the cytotoxin gene encodes a glycosyltransferase domain, which may promote the colonization of the genital tract (Bachmann et al., 2014b). Single nucleotide polymorphisms (SNPs) identified within the *pmp* gene family, particularly in *pmpH*, can distinguish ocular, genital and LGV *C. trachomatis* strains (Stothard et al.,

2003). In addition, SNP distribution in two *pmp* genes can distinguish rectal from cervical *C. trachomatis* strains (Jeffrey et al., 2010). Therefore, the *pmp* genes may also contribute to the tissue tropism of different chlamydial strains.

A study comparing *C. pecorum* strains from koala, sheep and cattle found that the region with the largest number of SNPs was the *pmp* gene region. A different region that includes genes encoding T3SS components and effector proteins also contained a large number of SNPs. A number of pseudogenes, including a toxin gene, were identified in the koala strains that were found to be intact in the ruminant strains. The loss of gene function suggests that these genes were not necessary for infection of the koala host but potentially important for infection of the ruminant hosts (Bachmann et al., 2014b). Three ruminant *C. pecorum* strains that caused different pathological conditions (asymptomatic enteric infection, polyarthritis and metritis) were found to have highly conserved genomes. A higher level of cluster tandem repeat sequences (CTRs) in *ORF663* in the asymptomatic strain was identified compared to the pathogenic strains, which may account for the observed differences in pathology. The product of *ORF663* is thought to be surface expressed and a reduction in CTRs may decrease surface area for antibody binding and increase pathogenicity (Sait et al., 2014). Comparison of the koala *C. pneumoniae* (LPCoLN) strain to 4 human strains of *C. pneumoniae* identified over 2000 SNPs in the *pmp* genes consistent with adaptation to different hosts. There were also polymorphisms detected in the MACPF region and the koala strain was missing the *guAB-add* cluster but contained a plasmid unlike the human strains (Mitchell et al., 2010). Similarly, a study comparing 20 *C. psittaci* strains identified differences in the PZ region, particularly in the *gua-AB-add* and toxin gene, which may reflect adaptation to particular niches (Read et al., 2013).

Genes encoding recombination elements including chlamydiaphages and a conserved plasmid can be found in many of the *Chlamydiaceae* genomes and there is evidence that gene exchange usually occurs between different strains of the same species (Nunes et al., 2013; Nunes and Gomes, 2014). The analysis of multiple *C. trachomatis* strains has identified that recombination alters the sequence of important

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

genes (Joseph et al., 2012) and therefore is likely to be critical in promoting strain diversity. Isolates of *C. trachomatis* that do not possess the plasmid are rare, suggesting that it is important for *C. trachomatis* biology. The plasmid has been shown to encode a gene that facilitates the accumulation of glycogen and normal inclusion morphology (Song et al., 2013). *C. trachomatis* strains that have been cured of the plasmid protect mice against pathology following subsequent challenge with the wild-type strain (O'Connell et al., 2007). Ocular infection of macaques with a plasmid deficient strain was also shown to be non-pathogenic and protective against future challenge with the plasmid-positive strain (Kari et al., 2011). Therefore, *in vivo* the chlamydial plasmid has been identified as an important virulence factor that stimulates the pathological immune response and may potentially activate a TLR2 dependant pathway (Kari et al., 2011; O'Connell et al., 2011).

Cattle and sheep are often grazed together and are therefore likely to be exposed to similar pathogens, including *C. abortus*. *C. abortus* rarely causes abortion in cattle, which is interesting considering it is an important cause of abortion in sheep (see section 1.2.1). Whole genome sequencing of *C. abortus* strains isolated from cattle, sheep and goats revealed that the strains were highly conserved. Little variation was identified in the PZ region suggesting there was minimal intraspecies variation between *C. abortus* strains. Therefore, it is unlikely that genetic differences in the strains that infect cattle and sheep are responsible for the observed differential disease pathogenesis (Longbottom et al, unpublished). However, minimal genetic variation has previously been identified to induce differences in *C. psittaci* pathology (Miyairi et al., 2011), which suggests that the role of genetic variation between *C. abortus* strains in host tropism cannot be completely discounted.

Intraspecies genetic variation has been identified within the PZ region or within *pmp* genes of the *Chlamydiaceae* family. The functional relevance of most of this genetic variation has yet to be fully elucidated. Further analysis with novel genetic transformation systems should help to further identify the role for specific genes in both host and tissue tropism.

1.1.5 Genetic transformation

The development of genetic transformation techniques for *Chlamydiaceae* has been hindered due to its obligate intracellular developmental cycle. The necessity for host cells to facilitate chlamydial growth makes the transformation process complicated, as complex technical steps are required to purify the bacteria and transformants take longer to generate (Beare et al., 2011). Early studies inserted DNA into *C. trachomatis* using electroporation but this method had limitations (Tam et al., 1994). Recently, however, three genetic transformation techniques have been successfully developed to identify gene function in *C. trachomatis*.

A reverse-genetic method involving chemical mutagenesis was used to generate *C. trachomatis trpB* null mutants. The *trpB* mutants could not be rescued from IFN- γ induced tryptophan starvation with the addition of indole unlike the non-mutant strain identifying the known role for *trpB* in the expression of tryptophan synthase (Kari et al., 2011). The endogenous *Chlamydia* plasmid and an *E. coli* plasmid were combined to construct a shuttle vector in another study. The vector was capable of introducing penicillin resistance and GFP into a plasmid-cured strain of *C. trachomatis*. This technology was also used to demonstrate the role for the plasmid in driving glycogen synthesis and accumulation in the chlamydial inclusion (Wang et al., 2011). A different study developed dendrimers (synthetic nanomolecules), which deliver plasmid DNA into *C. pneumoniae* and *C. trachomatis*. Similarly to the previous study, GFP was expressed by *Chlamydia* following dendrimer delivery of the GFP containing plasmid (Gerard et al., 2013; Kannan et al., 2013; Mishra et al., 2012). Dendrimers have also been used for targeted chlamydial gene knock-down (Mishra et al., 2012). Genetic transformation techniques have great potential for further elucidation of the genetic basis of pathogen virulence.

1.1.6 Host species diversity in response to *Chlamydia* infection

Successful pathogens evolve to survive within their host niche; however, genetic differences in the host can determine susceptibility to infection as well as the way the

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

immune system recognises and responds to infection. Mouse models are often used in studies of the immune response to chlamydial infection but these models have limitations. Chlamydial infection in mice does not induce a chronic infection, instead viable *Chlamydia* is only detected within 4 weeks post-infection contrasting to the months (may last over a year) before clearance with chlamydial infection in humans (Miyairi et al., 2010). Similarly, *C. abortus* establishes a persistent infection within the ruminant host but not within mice (Caro et al., 2009). This suggests that there are differences in the way that the host first recognizes the bacteria, which mediates clearance or the induction of persistence.

Not all women infected with *C. trachomatis* will go on to develop upper genital tract hydrosalpinx, which is indicative of differences in disease severity. A study investigating 11 strains of mice from different genetic backgrounds identified differences in host susceptibility to hydrosalpinx development following *C. muridarum* infection (Chen et al., 2014). Diversity in immune response genes may determine differences in how the host recognises and responds to a pathogen (Sironi et al., 2015). SNPs in a number of inflammatory immune genes have been associated with the development of severe trachoma pathology in humans (Atik et al., 2008; Mozatto-Chamay et al., 2001; Natividad et al., 2007). Similarly, the presence of multiple SNPs in specific *PRR* genes has also been associated with increased risk of chlamydial tubal pathology, although this was not found to be significant (den Hartog et al., 2006).

Host species diversity may play an important role in determining the disease pathogenesis of *C. abortus*. Cattle and sheep diverged around 30 million years ago (Hedges et al., 2006), which suggests that they are evolutionarily relatively closely related species. However, differences have been identified in cattle and sheep immune genes that may have functional consequences, including in TLR9 which responds in a species-specific manner to specific ligands (Werling et al., 2009). Therefore, genetic differences between cattle and sheep may impact on innate immune recognition and response, which may influence the differential outcome to *C. abortus* exposure. Although this project focuses on *C. abortus*, there is evidence

for differential disease pathogenesis between cattle and sheep for other intracellular pathogens, most notably the apicomplexan protozoan parasites *Toxoplasma gondii* and *Neospora caninum*. Both can infect cattle and sheep, and whereas *T. gondii* is a major cause of abortion in sheep but not cattle, the converse is true for *N. caninum* (Innes and Mattsson, 2007). This suggests some fundamental differences between these hosts that merit further investigation.

Pathogens and hosts are continually under selective pressure from each other to evolve in order to survive (Sironi et al., 2015). Complex host-pathogen interactions are therefore likely to determine the outcome of pathogen exposure between different hosts. Comparing the innate immune response of two closely related hosts to the same pathogen may reveal species-specific differences that contribute to disease pathogenesis.

1.2 Epidemiology, transmission and pathogenesis of *C. abortus*

1.2.1 Epidemiology

C. abortus is a major cause of lamb loss worldwide, with the exception of Australia and New Zealand (Entrican et al., 2010). Within the UK, *C. abortus* is the single most common diagnosed cause of infectious abortion in sheep (Figure 1.2) and is therefore of great economic and welfare importance. The UK Veterinary Investigation Surveillance Reports 2007-2014 statistics identified *C. abortus* as the cause of over 40% of diagnosed infectious ovine fetopathies but less than 1% of bovine fetopathies (Figure 1.2) (Animal and Plant Health Agency (APHA), 2014).

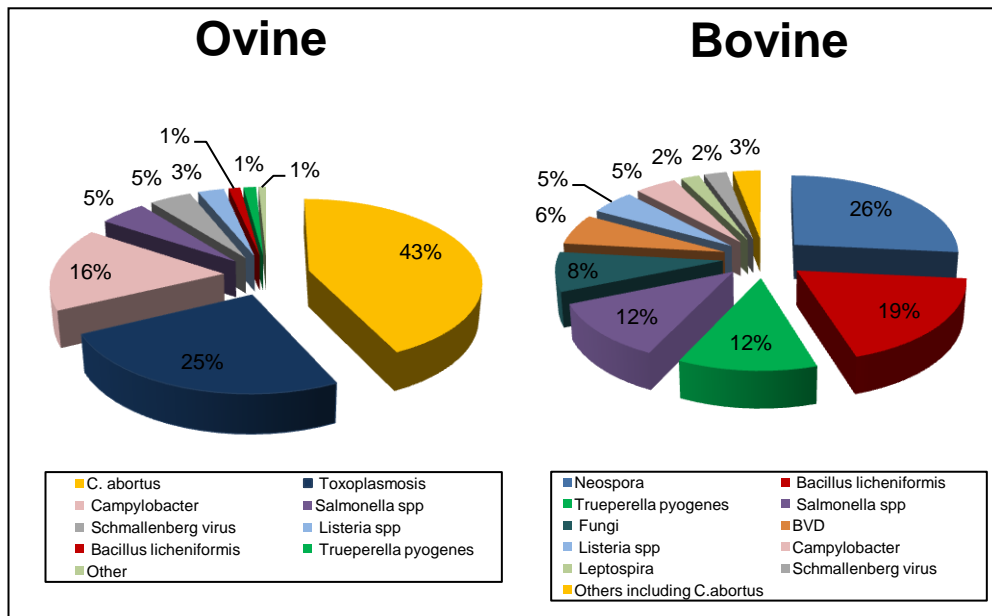


Figure 1.2: Diagnosed infectious causes of ovine and bovine fetopathies 2007-2014. The UK Veterinary Investigation Surveillance reports from 2007-2014 were used to produce pie charts of the diagnosed infectious causes of ruminant fetopathies (Animal and Plant Health Agency (APHA), 2014)

Cattle are susceptible to infection even if they do not, in general, exhibit disease. In cattle *C. abortus* can cause chronic sub-clinical reproductive problems and has been linked with infertility and mastitis (Appino et al., 2007; Kaltenboeck et al., 2005; Reinhold et al., 2011). Much can be learned from the study of hosts that can be infected by the same pathogen but present with different pathologies.

C. abortus is also a zoonotic pathogen, which can cause abortion in pregnant women (Longbottom and Coulter, 2003). The first diagnosed cases of human abortion caused by *C. abortus* were highlighted in a letter to the Veterinary Record in 1986, when 5 pregnant women became seriously ill and lost their children (Eddy and Martin, 1986). In humans, the bacteria can induce septicaemia that results in disseminated intravascular coagulation, which can lead to spontaneous abortion (Forsbach-Birk et al., 2013; Johnson et al., 1985). The extent of human infection, particularly within the high-risk agricultural population, is somewhat unknown due to the lack of clinical signs in non-pregnant individuals. Pregnant women are therefore advised

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against close contact with birthing ruminants and adverse human responses due to *C. abortus* infection are currently rare (Essig and Longbottom, 2015).

There have been few studies on the identification of *C. abortus* in wildlife, but those that have been done have identified *C. abortus* infection in wild boar and deer (Hotzel et al., 2004; Salinas et al., 2009). *C. abortus* was found in animals from fenced estates with no contact with livestock which suggests that wildlife may be an important self-propagating reservoir for disease (Salinas et al., 2009).

1.2.2 Transmission

A major route of transmission of *C. abortus* into a naïve sheep flock is through the introduction of infected replacement ewes (Milne et al., 2009). Infected ewes can shed large numbers of the bacteria in vaginal discharge and aborted material, such as placenta and coats of lambs (Essig and Longbottom, 2015). The bacteria can then remain viable within the environment for several days, which may be prolonged with colder weather facilitating further transmission (Longbottom and Coulter, 2003).

Following oral-nasal ingestion, the bacteria can then persist, at a site as yet unknown, asymptotically within the non-pregnant ewe. In the subsequent pregnancy, *C. abortus* infects the placenta and initiates pathology at around day 90 of gestation (Kerr et al., 2005), which causes abortion usually within the last 2-3 weeks of gestation (Rocchi et al., 2009). Often the first sign of the disease is the discovery of an infected dead lamb, which makes disease control difficult (Essig and Longbottom, 2015). If a ewe is infected after around 110-120 days of gestation, they will generally deliver normally but may still abort during their next pregnancy. Interestingly, not all ewes which are infected with *C. abortus* will go on to abort (Figure 1.3) (Longbottom and Coulter, 2003). Once a ewe has aborted due to *C. abortus* it does not abort again with re-infection. However, the immunity that develops within these ewes is not necessarily sterile, as they have been shown to shed at oestrus and with subsequent lambing facilitating further transmission (Livingstone et al., 2009).

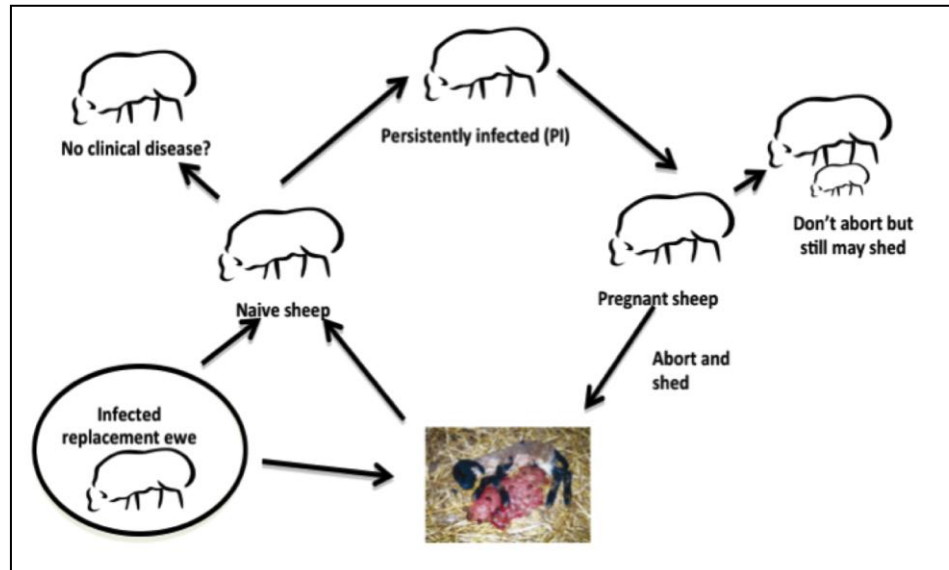


Figure 1.3: The transmission cycle of *C. abortus*. The introduction of an infected replacement ewe into a previously clean flock is a major route of transmission for *C. abortus*. Infected ewes can shed the bacteria in vaginal discharge and aborted material allowing the bacteria to infect naïve sheep via the oro-nasal route. Within the newly infected ewe, *C. abortus* establishes a persistent infection until pregnancy when it can induce abortion. Although not all sheep will go on to abort they may still shed the bacteria also contributing to the transmission cycle (Essig and Longbottom, 2015; Milne et al., 2009).

The *C. abortus* transmission cycle means that after the introduction of a small number of infected ewes into a flock there are usually few abortions within the first year but an abortion storm, which can affect around 30% of the flock, in the second year as more ewes are exposed to infected material (Milne et al., 2009).

1.2.3 Pathogenesis

In order to understand the pathogenesis of *C. abortus* it is important to understand three key stages: persistence and recrudescence, inflammation mediated abortion and the development of protective immunity.

1.2.3.1 Persistence and recrudescence

A characteristic feature of all chlamydial species, including *C. abortus*, is the ability to establish chronic sub-clinical infections. *In vitro* and with certain stressors RBs

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may develop into aberrant bodies (ABs), which allow the bacteria to persist within the host cell until the absence of stressors means they can continue multiplying (Schoborg, 2011). A number of stressors have been identified, including antibiotics and IFN- γ . Some researchers think that AB formation allows the development of these characteristic sub-clinical infections. However, the clinical relevance of AB formation is not known as its existence is yet to be proven *in vivo* (Bavoil, 2014).

C. abortus is usually sub-clinical until pregnancy, when the bacteria can recrudesce from its persistent state and cause abortion. It is not yet known where the bacterium persists, although some studies suggest it may reside in the tonsil when the animals are first infected (Jones and Anderson, 1988; Longbottom et al., 2013b). Therefore, it remains unclear how *C. abortus* can persist sub-clinically *in vivo* or what triggers its recrudescence at pregnancy (Rocchi et al., 2009).

1.2.3.2 Inflammation mediated abortion

Recrudescence from the persistent state allows *C. abortus* to invade the placenta and multiply within trophoblast cells. This results in the initiation of an inflammatory cascade, which is characterized by the production of the pro-inflammatory TNF- α and CXCL8. Increased pro-inflammatory cytokine/chemokine production leads to recruitment of an inflammatory infiltrate, consisting of neutrophils, monocytes/macrophages, T cells and few B cells (Gutierrez et al., 2011; Rocchi et al., 2009). This infiltrate induces cell death and the formation of foetal membrane lesions, which disrupts the chorionic epithelium and also causes vascular thrombosis contributing to abortion (Rocchi et al., 2009). The development of placental lesions usually occurs after 90 days gestation, which coincides with abortion in the later stages of pregnancy (section 1.2.2) (Buxton et al., 1990; Longbottom et al., 2013b). Inflammation is important in the mediation of clearance of bacterial infections; however, the abundant expression of inflammatory cytokines such as TNF- α at the materno-fetal interface is associated with abortion (Buxton et al., 2002). Therefore, the immune response in the placenta may be inappropriately activated in response to *C. abortus* resulting in abortion.

1.2.3.3 Immunity that protects against chlamydial abortion

Protective immunity does develop to *C. abortus* as once a ewe has aborted due to infection it does not abort again with re-infection (Littlejohn, 1950). However, protective immunity does not completely clear the bacteria as the animal can still shed *C. abortus* post-abortion, as described in section 1.2.2 (Livingstone et al., 2005). The development of protective immunity is complex. A recent paper identified that a low dose of *C. abortus* induced a persistent infection that resulted in abortion, whereas a high dose resulted in lower rates of abortion. This is indicative of the development of protective immunity with the larger dose. In the one ewe that did abort with the larger dose, a reduced number of *C. abortus* were detected in vaginal swabs post abortion compared to the mean number from ewes that aborted following challenge with the lower dose. This indicates that a strong immune response that promotes bacterial clearance is induced with the larger dose (Longbottom et al., 2013b).

Ewes produce low levels of antibodies in response to *C. abortus* infection which are rapidly increased at the time of abortion. There was no significant difference in antibody level in sheep following infection that went on to abort compared to those that didn't abort (Livingstone et al., 2005). Sheep which were vaccinated against *C. abortus* and gave birth to healthy lambs also had comparable antibody titres pre-infection challenge to those which went on to abort (Chalmers et al., 1997). These studies suggest that antibodies are not involved in protection against abortion during primary infection of the placenta, but may function in the development of protective immunity following abortion.

Cell-mediated immunity is likely to play a more important role than antibodies in protection to primary *C. abortus* infection due to the bacteria's obligate intracellular nature (Entrican et al., 2004). *In vitro*, IFN- γ has been shown to restrict *C. abortus* growth through tryptophan depletion (Entrican et al., 1998), described in section 1.1.4.1. Increased IFN- γ *in vivo* has been identified in the lymph of immune sheep (sheep which have previously aborted due to *C. abortus*) in response to challenge with *C. abortus*, suggesting a protective role for the cytokine (Graham et al., 1995).

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Peripheral blood mononuclear cells (PBMC) were collected from pregnant ewes experimentally infected with *C. abortus* at 2-week intervals and stimulated *in vitro* with Concanavalin A (ConA). A higher level of IFN- γ was detected in ewes that were infected but did not go on to abort compared to those which did abort. Similarly, PBMC from vaccinated animals produced higher IFN- γ *in vitro* in response to *C. abortus* EBs compared to non-vaccinated control sheep. Collectively, this suggests that early IFN- γ production may be protective against *C. abortus* induced abortion (Rocchi et al., 2009).

Pregnancy complicates the potentially protective role of IFN- γ with *C. abortus* as IFN- γ has been shown to be incompatible with successful pregnancy. Pregnancy is thought to induce a switch in maternal immunity to a Th2 response (Rocchi et al., 2009), and a predominance of IL-4 production has been identified with human pregnancy. However, a study of ovine PBMC found that IL-4, IFN- γ and IL-10 responses to antigen were unaltered with pregnancy, suggesting potentially important differences between sheep and human pregnancy (Wattegedera et al., 2008).

There have been limited studies on the type of host innate immune interactions that induce protective immunity to *C. abortus*, and the primary cellular producer of IFN- γ has not yet been identified *in vivo*. In order to understand more about chlamydial immunity the next two sections will review the better-characterized immune responses of hosts to *C. trachomatis*, *C. muridarum* and *C. pneumoniae*.

1.3 *Chlamydia* and the innate immune system

The immune system is composed of innate and adaptive arms. The innate immune system is the first line of defence and once activated can inform and shape the adaptive immune system.

1.3.1 Cells of the innate immune system

The skin, mucosal linings and bodily fluid act as the first barriers that *Chlamydia* encounters. These barriers are made up of innate immune cells including epithelial

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cells, monocyte/macrophages and DCs. *Chlamydia* are primarily epitheliotropic where the bacteria can stimulate an inflammatory response through the production of pro-inflammatory cytokines, which induce the recruitment of innate immune cells such as neutrophils, natural killer (NK) cells, macrophages and dendritic cells (DCs) (Roan and Starnbach, 2008). Often, the site of chlamydial infection is not the site of pathology and innate immune cells may also play an important role in chlamydial dissemination. The diverse roles of the innate immune cells in relation to chlamydial infection including inflammation, polarization of the adaptive immune system and dissemination will be discussed.

Neutrophils are one of the first cells to arrive at the site of chlamydial infection (Register et al., 1986). They are usually short-lived as undergo spontaneous apoptosis (Zandbergen et al., 2004) and are thought to respond to chlamydial infection through the release of inflammatory mediators (Frazer et al., 2011). Early *in vitro* studies demonstrated that neutrophils can internalize *Chlamydia* and are generally chlamydiacidal, however some viable bacteria were able to persist within these cells (Register et al., 1986). More recent studies have shown that chlamydial infection can prolong the life of neutrophils through the delay of apoptosis, allowing multiplication of the bacteria within the cell (Frazer et al., 2011; Zandbergen et al., 2004). The neutrophils may also function in the dissemination of the bacteria from the site of infection and can be taken up by macrophages for long-term survival (Rupp et al., 2009). Neutrophils were also found to enhance chlamydial replication in epithelial cells (Rodriguez et al., 2005). Studies *in vivo* suggest that increased neutrophil recruitment is associated with the development of chlamydial pathology and increased bacterial load (Frazer et al., 2011; Rodriguez et al., 2005).

NK cells have been shown to be recruited to the site of chlamydial infection, where they are thought to act as an early source of IFN- γ (Tseng and Rank, 1998). A role for NK cells has also been identified in the induction of lysis of *Chlamydia*-infected epithelial cells. *Chlamydia* have been shown to down-regulate MHC I expression on epithelial cells, which allows the bacteria to avoid presentation to cells of the adaptive immune system. However, this down-regulation also makes them targets for

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NK cells as MHC I is required for NK inhibitory receptors (Hook et al., 2004). NK cell depletion in mice infected with *C. muridarum* lead to exacerbated disease with higher bacterial load. In this study, the role identified for NK cells was in the modulation of DC function for the production of a protective Th1 response (Jiao et al., 2011). Transcriptomic profiles of the conjunctiva indicated an up-regulation of genes involved in NK cell activation in individuals with active trachoma compared to non-infected controls (Natividad et al., 2010).

Phagocytes such as macrophages and DCs are thought to be important for bridging the innate and adaptive immune systems as they process chlamydial antigens and present them on MHC I/II complexes to T cells. Early studies revealed that chlamydial EBs are rapidly taken up by macrophages and can survive and replicate within them, although replication is thought to be limited (Rey-Ladino et al., 2007). Survival within macrophages only occurs at low infectious doses as higher doses resulted in ‘immediate cytotoxicity’ (Manor and Sarov, 1986; Wyrick and Brownridge, 1978; Wyrick et al., 1978). In order to survive in macrophages, bacteria must be able to avoid lysosomal enzymes and it has been shown that the fusion of vacuoles containing *Chlamydia* with lysosomes is inhibited (Eissenberg and Wyrick, 1981). Survival within macrophages facilitates their potential role in the dissemination of the bacteria from the site of infection (Gieffers et al., 2004; Moazed et al., 1998). Macrophages infected with *Chlamydia* have also been shown to induce T cell death through apoptosis, indicative of a role in the induction of persistent infections (Jendro et al., 2000). *C. pneumoniae* has been shown to induce the formation of macrophage foam cells, which accumulate in the formation of atherosclerotic plaques contributing to pathology (Kalayoglu and Byrne, 1998).

Dendritic cells are antigen-presenting cells that have an important role in the activation and polarization of T cell immunity. Studies have shown that DCs are recruited to the site of chlamydial infection (Brunham and Rey-Ladino, 2005) and that bacteria can survive and persist within them. This suggests that DCs may also be involved in the maintenance of persistent infections and dissemination (Rey-Ladino et al., 2007). Co-culture of *C. pneumoniae* infected human MDDCs with T cells

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induced type 1/type 17 polarization (Flego et al., 2013). *In vitro*, DCs pulsed with heat-inactivated *C. muridarum* induced the proliferation of type 1 polarized T cells, which produced IFN- γ . The adoptive transfer of these pulsed DCs protected mice from pathology following subsequent genital challenge with *C. muridarum* (Su et al., 1998).

Innate immune cells are critical for the early recognition of chlamydial infection. However, a number of studies have demonstrated the ability of *Chlamydia* to alter the function of innate immune cells to promote self-survival. Increased knowledge on how these innate immune cells recognise and respond to infection could be critical for understanding the balance between the mediation of persistence and the induction of inflammatory responses for chlamydial clearance.

1.3.2 Pathogen recognition receptors

Pathogen recognition receptors (PRRs) are sentinels of the innate immune system. PRRs are found on the surface or within innate immune cells and recognise unique molecular patterns, including pathogen associated microbial patterns (PAMPs) (Janeway, Jr., 1989) and damage-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004). Cells express different complements of PRRs, which once activated induce signaling pathways within the cell. These signaling pathways can result in the production of cytokines and chemokines, activation of the adaptive immune response and induction of cell death (Bortoluci and Medzhitov, 2010). Although PRRs are a critical host defence mechanism, a number of microbes have evolved mechanisms to use receptors to promote self-survival (Matzinger, 2002).

There are four main groups of PRRs, namely Toll-like receptors (TLRs), nucleotide binding and oligomerization domain (NOD) - like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs). TLRs and NLRs respond to pathogenic bacteria independently and collaboratively (Oviedo-Boyso et al., 2014). The specific TLRs and NLRs that have been implicated in the detection of chlamydial infection will be discussed further.

1.3.2.1 Toll-like receptors

TLRs are found on immune cells such as monocytes/macrophages and DCs (Rodriguez et al., 2006), but are less ubiquitous on epithelial cells (McClure and Massari, 2014). They are often found on the host cell surface but also within endosomes. Generally, once activated, TLRs signal through the myeloid differentiation primary response gene 88 (MyD88) adaptor protein that activates the nuclear factor-kappa B (NF- κ B) transcription factor switching on pro-inflammatory gene expression. In humans, ten members of the TLR family (twelve in mice) have been identified which recognise PAMPs derived from a range of different types of microbes (Kumar et al., 2011). Some microbes are thought to be recognised by a single TLR, however others have been shown to be recognised by multiple TLRs (Beutler, 2009).

A number of studies have implicated a role for both TLR2 and TLR4 in the host detection of chlamydial infection. TLR2 has been shown to recognise a number of different PAMPs, including lipoproteins found on Gram-negative bacteria (Philpott and Girardin, 2004). TLR4 recognises lipopolysaccharide (LPS) which is the major cell wall component of Gram-negative bacteria (Kumar et al., 2011). Chlamydial heat shock protein 60 (Chsp60) is a major protein that is produced within the intracellular chlamydial inclusion (Bulut et al., 2009). Both TLR2 and TLR4 have been implicated in binding Chsp60 (Da Costa et al., 2004), however the contribution of each receptor to chlamydial-induced inflammation is controversial. Chsp60 derived from *C. pneumoniae* has been shown to activate pro-inflammatory cytokines predominantly through TLR4 but not TLR2 in knockout (KO) mice (Bulut et al., 2009). However, a different *C. pneumoniae* study identified that double TLR2/TLR4 KO mice failed to recruit polymorphonuclear neutrophils or produce keratinocyte chemokine in response to Chsp60, unlike the single knockouts (Da Costa et al., 2004). Differences identified in studies investigating the same chlamydial species are likely due to experimental design and the fact that receptors play different roles throughout the course of infection.

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Most studies with the whole organism, rather than Chsp60 alone, support a more important role for TLR2 compared to TLR4 in innate immune activation. A number of *in vitro* studies with different cell types have shown that TLR2 is critical for the activation of intracellular signalling pathways and pro-inflammatory cytokine production in response to chlamydial infection (Netea et al., 2002; Prebeck et al., 2001; Rodriguez et al., 2006). TLR3 has also been implicated in the production of innate immune cytokines in oviduct epithelial cells in response to *C. muridarum* infection (Derbigny et al., 2012). The activation of TLR2 signalling pathways may contribute to the development of pathology *in vivo*. TLR2 KO mice had reduced oviduct pathology following challenge with *C. muridarum* compared to the TLR4 KO or wild-type mice (Darville et al., 2003). A plasmid deficient strain of *C. muridarum*, which failed to activate TLR2 dependant cytokine production, did not induce oviduct pathology unlike the virulent *C. muridarum* strain (O'Connell et al., 2007).

The innate immune activation mediated by both TLR2 and TLR4 *in vivo*, has been shown to be beneficial. Combined TLR2/TLR4 KO mice had the highest *C. pneumoniae* burden, cellular infiltrate and biggest weight loss contributing to an increased mortality rate compared to single KO and wild type mice (Rodriguez et al., 2006). Similarly, a different study found that single TLR2 and TLR4 KO mice had no effect on bacterial burden following *C. pneumoniae* infection (Mueller et al., 2004). This suggests that the combined function of TLR2/4 is important in host defence to *C. pneumoniae* infection.

MyD88 is the downstream signalling adaptor protein for all the TLRs, with the exception of TLR3 and TLR4 that can signal through MyD88 independent pathways (Akira and Takeda, 2004). MyD88 KO mice failed to up-regulate early pro-inflammatory cytokine production and exhibited delayed clearance of *C. pneumoniae*, ultimately resulting in increased mortality (Naiki et al., 2005). Similar results of reduced early pro-inflammatory cytokines and delayed bacterial clearance were also identified in MyD88 KO mice challenged with *C. muridarum* (Chen et al., 2010). Therefore, studies support the involvement of the MyD88 signalling pathway

in the innate immune response to chlamydial infection. However, the specific role for single TLRs in disease pathogenesis and whether they are activating a protective or pathogenic response may be dependent on a number of factors.

1.3.2.2 NOD-like receptors

NLRs can be grouped broadly into two groups: receptors which function through NODosomes and receptors which activate inflammasome signalling complexes. NODosome receptors include NLRP10, NOD1, NOD2, NLRC3, NLRC5, NLRX1 and CIITA. Inflammasome receptors include NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4 and NAIP (Barbe et al., 2014). The NODosome receptors contain a caspase activation and recruitment domain (CARD) and the inflammasome receptors contain an Apoptosis-associated Speck-like protein containing a CARD (ASC), which contains a pyrin domain and CARD. Assembly of the NODosome mediates the activation of NF- κ B through RIP 2, whereas inflammasome assembly induces the activation of caspase-1 (Figure 1.4) (Le and Harton, 2013).

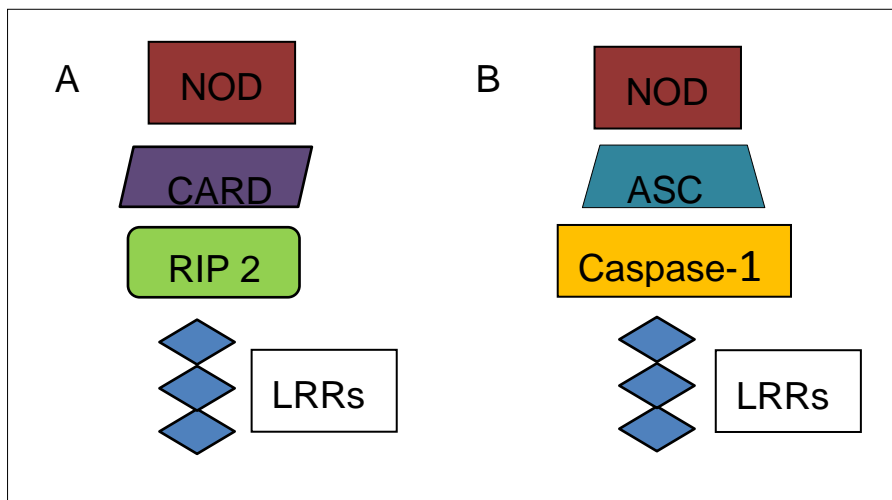


Figure 1.4: NOD-like receptor sub-types can form NODosome (A) and inflammasome (B) signalling complexes. Upon ligation of the NLR, the leucine-rich repeats (LRRs) undergo conformational change which allows CARD dependant recruitment of adaptor proteins, such as receptor interacting protein 2 (RIP2) in the case of the NODosome and caspase-1 in the case of the inflammasome (Adapted from Le and Harton, 2013).

Generally, studies have focussed on the roles of NOD1 and NLR pyrin domain containing-3 (NLRP3) in chlamydial recognition and activation of the innate immune response.

1.3.2.2.1 NOD 1

NOD1 recognises peptidoglycan, which is a component of most bacterial cell walls that contributes to cell division and provides an anchor for transmembrane proteins. *Chlamydiaceae* are sensitive to antibiotics that inhibit cell wall synthesis and encode genes involved in peptidoglycan biosynthesis. However, conventional methods of peptidoglycan detection had not been able to prove its existence within the bacteria (Liechti et al., 2014). This disparity became known as the ‘chlamydial anomaly’ (Jacquier et al., 2014). However, recent advances in peptidoglycan detection techniques have allowed the identification of low quantities within *C. trachomatis* (Liechti et al., 2014). Therefore, NOD1 may function in the recognition of chlamydial peptidoglycan.

Previous *in vitro* studies have identified a role for NOD1 in the activation of NF- κ B signalling pathways and production of pro-inflammatory cytokines and chemokines, including CXCL8, in response to chlamydial infection (Buchholz and Stephens, 2008; Kavathas et al., 2013; Opitz et al., 2005; Welter-Stahl et al., 2006). CXCL8 was not produced in response to heat-inactivated *C. pneumoniae*, implicating NOD1 as a key receptor involved in the detection of the intracellular stage of chlamydial infection (Opitz et al., 2005). Mice deficient of receptor interacting protein 2 (RIP2), the downstream adapter protein of NOD1, exhibited delayed *C. pneumoniae* clearance and greater inflammation resulting in higher levels of mortality than wild-type mice (Shimada et al., 2009).

1.3.2.2.2 NLRP3 Inflammasome

The best-characterized inflammasome is NLRP3, which has a diverse range of activators including bacterial PAMPs, DAMPS and inorganic compounds such as nanoparticles (Abderrazak et al., 2015). Activation of the NLRP3 inflammasome requires two signals. The first signal is a priming signal, which involves TLR

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stimulation leading to the up-regulation of NLRP3 gene expression and the production of the inactive pro-IL-1 β /IL-18. Signal 2 involves NLRP3 receptor ligation inducing assembly of the inflammasome complex and activation of caspase-1, which mediates the cleavage of the IL-1 β /IL-18 into its active form (Figure 1.5) (Franchi et al., 2012).

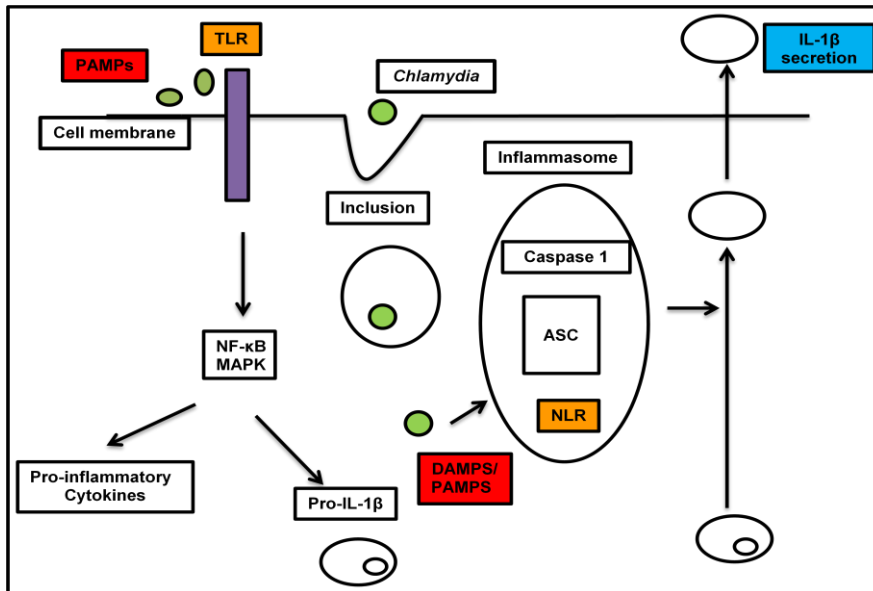


Figure 1.5: Activation of the NLRP3 inflammasome is a two-step process. The first step of NLRP3 activation involves ligation of a PRR, which activates NF- κ B and MAPK pathways priming NLRP3 through up-regulating its expression. The second step involves activation of the intracellular NLRP3 receptor, which induces assembly of the inflammasome complex (adapted from Franchi et al., 2012).

The role of the NLRP3 inflammasome in response to *Chlamydia* infection has been highlighted by a number of studies. Knockdown of NLRP3 in cervical epithelial cells was shown to partially inhibit caspase-1 activation in response to *C. trachomatis* (Abdul-Sater et al., 2009). Bone marrow derived macrophages (BMDMs) from NLRP3 KO or ASC KO mice produced significantly reduced IL-1 β /IL-18 in response to *C. pneumoniae* compared to macrophages from wild-type mice (He et al., 2010; Itoh et al., 2014). A different study identified that *C. pneumoniae* induced IL-1 β production was partially dependant on NLRP3 in human PBMC and murine BMDM (Eitel et al., 2012).

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NLRP3 recognises lysosomal acidification, potassium efflux and reactive oxygen species (ROS), which are induced by a range of stimuli (Abdul-Sater et al., 2010; He et al., 2010). The addition of an antioxidant to deplete ROS and the blockage of the potassium channels in monocytes, resulted in a decrease in caspase-1 production and IL-1 β secretion in response to *C. trachomatis* or *C. muridarum* infection (Abdul-Sater et al., 2009; Abdul-Sater et al., 2010). Chlamydial T3SS may cause lysosomal disruption, potassium efflux and ROS production contributing to NLRP3 activation (Abdul-Sater et al., 2009; He et al., 2010). A T3SS inhibitor has been shown to abrogate caspase-1 activation with chlamydial infection, which supports the potential role of NLRP3 in T3SS detection (Abdul-Sater et al., 2009). Spleen tyrosine kinase (Syk) transmits signals from cell surface receptors and has also been implicated in NLRP3 activation with chlamydial infection (Abdul-Sater et al., 2010).

Studies have also identified redundancy in components of the NLRP3 inflammasome for defence against chlamydial infection. ASC KO mice exhibited significantly increased *C. muridarum* colonization and delayed bacterial clearance, unlike NLRP3 or NLRC4 KO mice. This implicates a role for a different inflammasome receptor in ASC activation in response to *C. muridarum*. IL-1 β levels in genital secretions was found to be independent of NLRP3 or ASC after day 2 post infection, suggesting production of IL-1 β may be through inflammasome independent pathways (Nagarajan et al., 2012).

The role of caspase-1 activation in response to chlamydial infection extends beyond the cleavage of pro-IL-1 β /IL-18. Caspase-1 can also drive pyroptosis, which is a type of cell death considered to be pro-inflammatory due to the rapid breakdown of the cell membrane and release of cell contents. Pyroptosis is thought to be an important innate immune mechanism against intracellular bacteria as it promotes clearance from the host cell niche (Miao et al., 2011). Caspase-1 KO mice infected with *C. pneumoniae* exhibited delayed bacterial clearance, which resulted in increased lung pathology and mortality rate (Shimada et al., 2011). However, in caspase-1 KO mice infected with *C. muridarum* there was an observed decrease in urogenital tract pathology with reduced inflammatory infiltrate after initial infection. This suggests

that caspase-1 may be involved in the development of pathology rather than resolution of infection (Cheng et al., 2008). Recently, a role for caspase-1 in facilitating chlamydial growth in macrophages and epithelial cells has also been demonstrated. The authors suggested that caspase-1 functions in lipid utilization for membrane biogenesis, which is hijacked by *Chlamydia* to facilitate inclusion development (Abdul-Sater et al., 2009; Itoh et al., 2014).

Therefore, studies have identified a role for the NLRP3 inflammasome in IL-1 β production in response to chlamydial infection. However, a number of different pathways are also likely to contribute. The different components of the inflammasome may also have a number of roles beyond IL-1 β production that are involved in determining chlamydial infection outcome.

1.3.3 Cytokines and chemokines

Cytokines and chemokines, which are produced with innate immune recognition, play a number of important roles in host immune defense to pathogens. Critically, they are involved in the recruitment and activation of further innate immune cells and the stimulation of the adaptive immune response. Depending on both the timing and location of production, innate immune cytokines have been shown to have protective and/or pathogenic roles with chlamydial infection. The innate immune cytokines are often differentially expressed following heat-killing or UV-inactivation of the *Chlamydia*. This may indicate that a live intracellular infection is necessary for the activation of cytokine-producing signaling pathways. Studies have identified the up-regulation of a number of innate immune cytokines and chemokines, including CXCL8, TNF- α , IL-6 and IL-1 β from a broad range of innate immune cells in response to live chlamydial infection (Table 1.2).

Table 1.2: Production of innate cytokines/chemokines by different cell types in response to chlamydial infection (adapted from Rusconi and Greub, 2011).

Cytokine/Chemokine	Cellular producers
CXCL8	Epithelial cells and monocytes
TNF- α	PBMC, monocytes, macrophages and oviduct epithelial cells
IL-6	Epithelial cells, monocytes and macrophages
IL-1 β	PBMC, monocytes and macrophages

These innate immune cytokines and chemokines are pleiotropic and their specific roles in chlamydial infection have been identified by a number of studies. CXCL8 binds to CXCR1 and CXCR2 that are expressed by various cell types, including neutrophils, inducing chemotaxis (Russo et al., 2014). The chemokine also functions in the activation of neutrophils, basophils and T cells (Buchholz and Stephens, 2006). CXCL8 has been shown to be involved in the recruitment of neutrophils to the site of *C. pneumoniae* infection (Molestina et al., 1999) and also inhibits spontaneous neutrophil apoptosis (Zandbergen et al., 2004). IL-6 functions in the differentiation of B cells into antibody producing plasma cells and also as an activator of T cells. IL-6 KO mice displayed a decreased Th1 response with reduced IFN- γ production following chlamydial lung challenge compared to the wild type control (Williams et al., 1998).

TNF- α is a powerful cytokine that induces vasodilation, recruits leukocytes to the site of infection (Bradley, 2008) and kills intracellular microbes (Kamalakaran et al., 2013). Antibody mediated inhibition of early local TNF- α production decreased the level of apoptosis in inflammatory cells, but had no effect on chlamydial clearance in the genital tract (Darville et al., 2000). A recent study demonstrated that TNF- α KO mice had reduced upper genital tract pathology, but no reduction in bacterial clearance following primary and secondary chlamydial infection implicating a pathogenic role for the cytokine (Kamalakaran et al., 2013; Murthy et al., 2011). Similarly, TNF- α was detected in the tears of scarring trachoma patients more often than in non-infected control patients. An increased percentage of scarring trachoma patients possessed certain polymorphisms in the *TNF- α* gene promoter, supporting a pathogenic role for the cytokine (Conway et al., 1997). However, studies of

chlamydial infection in the lung have identified a role for TNF- α in chlamydial clearance and host survival (Williams et al., 1990).

IL-1 β is a potent pro-inflammatory cytokine that has been identified as an important mediator of bacterial infection associated pre-term birth (Romero et al., 1992). IL-1 β KO mice challenged intra-vaginally with *C. muridarum* presented with reduced oviduct pathology and bacterial clearance compared to the challenged wild type mice (Prantner et al., 2009). IL-1R deficient mice presented with increased lung fibrosis compared to wild-type mice challenged intra-nasally with *C. pneumoniae*, suggesting a potential role for IL-1 β and IL-1 α in the mediation of protective immunity (He et al., 2010). The critical role for IL-1 β in *C. pneumoniae* lung infection was demonstrated using caspase-1 KO mice. The addition of recombinant IL-1 β early post-infection rescued caspase-1 KO mice from a higher mortality rate and impaired bacterial clearance (Shimada et al., 2011).

Innate immune cytokines can contribute to the inflammatory cascade by stimulating the production of other pro-inflammatory cytokines, for example, IL-1 β can bind to the IL-1 receptor and stimulate the release of TNF- α and IL-6 (Netea et al., 2010). Considering this and KO experiment results, it is unlikely that a single cytokine will be responsible for determining chlamydial pathogenesis. Instead, the host niche as well as timing and quantity of each cytokine that contributes to the inflammatory milieu is likely to be critical.

1.4 *Chlamydia* and the adaptive immune system

1.4.1 Evidence for the development of protective chlamydial immunity

The development of protective immunity to chlamydial infection has been mostly characterized in mice and guinea pigs (Batteiger et al., 2010). Rodents are able to resolve a primary infection naturally within a few weeks (Brunham and Rey-Ladino, 2005) and are resistant to infection in a short period after the resolution of a primary infection (1-3 months), however after around 75 days they become susceptible to re-

infection (Rank and Whittum-Hudson, 2010). Partial immunity is thought to remain for an extended period of time as mice have reduced bacterial load and shortened duration of infection following subsequent challenge (Rank and Whittum-Hudson, 2010). Due to ethical issues, there have been limited studies into the duration of untreated chlamydial infection in humans. Studies have shown that the infection usually lasts for many months to years before being spontaneously cleared in the absence of antibiotics (Batteiger et al., 2010; Geisler et al., 2013). The prevalence of ocular and genital chlamydial infection decreases with age (Bailey et al., 1999), which suggests that protective immunity does develop over time. Studies in STD clinic patients suggest that those that have had repeated infections had lower bacterial load, which is indicative of the development of partial immunity (Batteiger et al., 2010). The different components of the adaptive immune system involved in clearance of a chlamydial infection and their role in protection from re-infection will be discussed.

1.4.2 Cell mediated immunity

Early experiments found that cell mediated immunity was critical for the resolution of chlamydial infection. Most of these early experiments used nude mice, which lack a thymus resulting in greatly reduced numbers of functional T cells. Wild-type mice were found to clear *C. muridarum* infection 20 days post intra-vaginal infection; however, nude mice were unable to clear the pathogen, which resulted in the development of a chronic infection. Nude mice also had reduced antibody levels and therefore further experiments were required to identify the specific role of cell mediated and humoral immunity (Rank et al., 1985). A follow-on study adoptively transferred T cells from wild type mice immunized with *C. muridarum* into nude mice, which were infected with *C. muridarum* pre-transfer. Mice which received the adoptive transfer were capable of resolving the infection, unlike the control nude mice (Ramsey and Rank, 1991).

CD4⁺ T cells are recruited to the genital tract following chlamydial infection (Gondek et al., 2012; Johansson et al., 1997b). CD4⁺ KO mice have demonstrated

that CD4⁺ T cells are important for the control of bacterial shedding following a primary *C. trachomatis* infection and in protection from re-infection (Johansson et al., 1997b). A recent study, using a more relevant transcervical chlamydial inoculation model in mice, demonstrated that depletion of CD4⁺ T cells was sufficient to abrogate the protective immune response to secondary *C. muridarum* or *C. trachomatis* challenge (Gondek et al., 2012). The CD4⁺ T cells involved in protective immunity to *C. trachomatis* were identified to be mainly Th1-type as they produced IFN- γ rather than the characteristic Th2-type cytokine IL-4 (Cain and Rank, 1995; Van Voorhis et al., 1996). IL-4 KO mice were able to clear a primary *C. trachomatis* infection, unlike IFN- γ KO mice, which provides further evidence that protective immunity is mediated by a Th1 rather than a Th2 response (Johansson et al., 1997b). The level of IFN- γ produced by CD4⁺ T cells alone has been shown to be sufficient for the clearance of *C. muridarum* from the genital tract of mice (Li et al., 2008). Th2 responses have instead been associated with chlamydial persistence and more severe disease (Mascellino et al., 2011).

The role for CD8⁺ T cells in immunity to *Chlamydia* is less clear but some studies suggest they may contribute to the protective immune response. CD8⁺ T cells have been shown to have a cytolytic effect on *C. trachomatis* infected cell lines (Beatty and Stephens, 1994). Studies have also identified CD8⁺ T cell involvement in mice in bacterial clearance following secondary challenge with *C. abortus* and *C. pneumoniae* (Buzoni-Gatel et al., 1992; Penttila et al., 1999). A recent study, demonstrated that intranasal priming with *C. trachomatis* activates both CD4⁺ and CD8⁺ T cells to protect mice against transcervical challenge with *C. trachomatis* (Nogueira et al., 2015). A role for CD8⁺ T cells in IFN- γ production in response to *C. muridarum* infection has also been identified *in vivo*. However, in the same study CD8⁺ T cell depletion did not lead to significant increases in mortality, unlike CD4⁺ T cell depletion (Magee et al., 1995). Similarly, CD8⁺ T cell KO mice did not display any significant defects in controlling bacterial shedding, compared to the wild-type, in response to primary or secondary challenge with *C. trachomatis* (Johansson et al., 1997b). In macaques, the *in vivo* depletion of CD8⁺ T-cells identified that the cells were critical for the protection mediated by a plasmid-deficient live attenuated

trachoma vaccine (described in section 1.1.4.2) (Olivares-Zavaleta et al., 2014). The detection of MOMP specific CD8⁺ T cells was associated with the detectable presence of ocular *C. trachomatis* infection but not clinical signs of disease in humans. This suggests that the CD8⁺ T cells may play a role during sub-clinical *C. trachomatis* infection (Holland et al., 2006). The normal functioning of CD8⁺ T cells requires CD4⁺ T cells, which means the role of CD8⁺ T cells in chlamydial infection may be underappreciated and not yet fully understood (Wizel et al., 2008).

Recently, Th17 cells have also been identified as potentially important immune mediators in relation to chlamydial infection. Th17 cells are characterized by the production of IL-17A (Andrew et al., 2013), and have been identified along with Th1 cells in the draining iliac lymph nodes of mice following genital infection with *C. muridarum* (Scurlock et al., 2011). The neutralization of IL-17A in mice following intranasal *C. muridarum* challenge resulted in impaired bacterial clearance and increased pathology (Bai et al., 2009). IL-17 receptor knockout mice displayed no difference in rate of bacterial clearance or pathology following genital *C. muridarum* infection (Scurlock et al., 2011). However, a more recent study using IL-17A KO mice, infected with genital *C. muridarum*, identified increased bacterial clearance and reduced oviduct pathology (Andrew et al., 2013). The contrasting results observed between the genital tract studies were thought to be due to the different KO models used (IL-17 receptor and IL-17A) (Andrew et al., 2013). Studies that interfered with IL-17A signaling consistently identified decreased Th1 responses (Andrew et al., 2013; Bai et al., 2009; Scurlock et al., 2011), which was attributed to an altered DC phenotype in IL-17A neutralized mice (Bai et al., 2009).

Antigen-specific CD4⁺ Th1-type T cells appear to be critical in the mediation of protective immunity to both primary and secondary chlamydial challenge. However, the contribution of other T cell subsets may depend on the model of chlamydial infection used.

1.4.3 Cytokines

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A number of inflammatory cytokines, including IFN- γ , IL-12 and IL-17 are produced in response to chlamydial infection. *In vitro* studies have demonstrated a role for IFN- γ in the restriction of intracellular chlamydial growth, which is important for host defense but may also induce a persistent infection important for disease pathogenesis (Mascellino et al., 2011). The mechanism of action of IFN- γ is through tryptophan depletion, which is described in section 1.1.4.1. Early studies assessed the effect of IFN- γ on mouse fibroblast cells and found that IFN- γ treatment before and after (up to four hours) *C. trachomatis* infection significantly reduced the number of cells with detectable inclusions (Rothermel et al., 1983). This type of *in vitro* study has been repeated with the same results in a number of different cell types with different chlamydial strains.

IFN- γ receptor KO mice suffered a more severe prolonged primary infection with *C. trachomatis*, supporting a role for IFN- γ in protective immunity (Johansson et al., 1997a). Similarly, IFN- γ KO mice presented with delayed resolution and increased dissemination of *C. muridarum* primary infection compared to the wild-type mice. The response of IFN- γ KO mice to secondary infection was similar to the wild-type mice, suggesting that IFN- γ may not be essential for resistance to re-infection (Cotter et al., 1997). Mice treated with anti-IFN- γ and then infected with *C. muridarum* developed a chronic infection. The addition of recombinant IFN- γ into nude mice infected with *C. muridarum* was able to induce resolution in some animals, suggesting that IFN- γ does play an important role in determining the outcome of infection (Rank et al., 1992). These studies indicate an important role for IFN- γ in the development of protective host immunity for the resolution of a primary chlamydial infection.

However, IFN- γ has also been shown to mediate persistent chlamydial infections and therefore may be involved in the promotion of chronic chlamydial disease. A study has associated elevated levels of IFN- γ to women with *Chlamydia*-associated fertility disorders compared to those infected but with no complications (Agrawal et al., 2007). Therefore, the balance of IFN- γ including the timing and quantity of production may be critical in determining the outcome of chlamydial infection.

IL-12 is an important cytokine that functions in the promotion of Th1-type responses. The neutralization of IL-12 *in vivo* led to delayed clearance of *C. muridarum* from the genital tract compared to the wild type controls (Perry et al., 1997). IL-12 consists of a p35 and a p40 subunit of which p40 is shared with IL-23, an inflammatory cytokine that promotes Th17 responses. Mice deficient in p35 or p40 displayed delayed clearance of primary *C. muridarum* infection, although were able to clear re-infection at a similar rate to the wild-type mice. IL-12p40 KO mice did display increased dissemination to the kidneys compared to the IL-12p35 KO mice or wild type mice, which suggests a role for IL-23 in controlling spread of chlamydial organisms (Chen et al., 2013a).

There are six IL-17 family members: IL-17A (also known as IL-17), IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. The best characterized members of the family are IL-17A and IL-17F, which are produced by Th17 cells but also $\gamma\delta$ T cells and macrophages. The main mode of action of IL-17A and IL-17F is in the induction of pro-inflammatory cytokine production from cell types which express the IL-17 receptor, including epithelial cells, macrophages and neutrophils (Jin and Dong, 2013). A role for IL-17A has recently been identified in response to chlamydial infections. The cytokine has been shown to enhance the TH1 response and neutrophil recruitment in *C. muridarum* genital tract infection (Scurlock et al., 2011). The neutralization of IL-17A resulted in impaired bacterial clearance (Bai et al., 2009), increased dissemination (Zhang et al., 2009b) and more severe pathology following intranasal challenge with *C. muridarum* (Bai et al., 2009; Zhang et al., 2009b).

Therefore, the balance of adaptive immune cytokine production is likely to be important in determining whether the inflammatory response will be protective or pathogenic.

1.4.4 Humoral immunity

Generally, the role for antibodies/B cells in protective immunity to primary infection is thought to be limited. It is well documented that the host produces antibodies in response to primary chlamydial infection. However, mice which were deficient in B

cells have been shown to resolve a primary infection with *C. muridarum* as effectively as the wild-type mice (Su et al., 1997).

A role for antibodies in protection from re-infection has been demonstrated by a number of studies. The depletion of both CD4⁺ and CD8⁺ T cells in immune mice had no effect on their ability to resolve a secondary challenge with *C. muridarum* compared to the wild-type (Morrison and Morrison, 2001). However, mice that lack mature B cells are more susceptible to colonization with a secondary infection with *C. muridarum* and shed higher levels of the bacteria (Morrison et al., 2000; Su et al., 1997). B-cell deficient mice do develop a degree of protective immunity, as present a shorter duration of secondary infection (Morrison et al., 2000). Similar experiments depleting B cell deficient immune mice of CD4⁺ T cells made mice susceptible to reinfection with *C. muridarum*; however, susceptibility to reinfection could be negated by the addition of immune serum. Interestingly, the addition of immune serum had no effect on primary infection of CD4⁺ T cell depleted mice (Morrison and Morrison, 2005).

Therefore, studies have identified a redundancy for humoral immunity in the protective immune response to primary chlamydial infection. However, a contributory role along with CD4⁺ T cells has been identified in protection from secondary challenge.

1.5 Diagnosis and Treatment of *C. abortus* infection

1.5.1 Clinical signs

Following infection, *C. abortus* usually establishes an asymptomatic infection within ewes. Occasionally, behavioural changes and vulval discharge can be detected for up to 48 hours before abortion, however often the first sign of infection is abortion in the last few weeks of gestation. Lambs may be weak or stillborn despite a normal appearance or present with a 'pot-bellied' appearance (Essig and Longbottom, 2015). Some infected ewes can give birth to healthy lambs or in the case of multiple births, one healthy and one dead lamb. Thickened placental membranes that are inflamed

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and often have yellow exudate on their surface are characteristic of *C. abortus* infection (Longbottom and Coulter, 2003). Ewes that have aborted due to *C. abortus* usually go on to make a full recovery.

1.5.2 Diagnostic tests

Flock history of disease and characteristic placental pathology are usually indicative of *C. abortus* infection, however confirmation requires laboratory tests. The gold standard test is isolation and propagation of the bacteria, which allows further characterization using molecular and biochemical techniques. However, this is time consuming and lab-intensive so more rapid tests have been developed (Essig and Longbottom, 2015).

Smears and tissue sections can be taken from potentially infected placental membranes and stained to reveal chlamydial infection. *Chlamydia* can be detected in placental smears using various stains, including Giemsa or Ziehl-Neelsen stains (Essig and Longbottom, 2015). Immunohistochemistry can be used to detect chlamydial antigens (such as MOMP or LPS) within tissue sections. Serological diagnosis in the form of the complement fixation test (CFT) is the most commonly used method for *C. abortus* detection. However, the CFT is cross-reactive as the antibodies bind to LPS commonly expressed by all *Chlamydiaceae*. Other more specific methods of *C. abortus* antibody detection include ELISAs, which detect antibodies to specific antigens (MOMP and Pmps). There are a number of commercialized serological tests available; however, a weakness of the current serological tests is that they cannot differentiate between naturally infected and vaccinated animals (Sachse et al., 2009).

The detection of chlamydial DNA by conventional PCR is rapid; however, the technology does not allow quantification of the target. Real-time PCR and DNA microarrays using the ArrayTube platform is an area of expanding development, which is favoured because of the rapidity and sensitivity of results. A real-time PCR that detects the *ompA* gene has been shown to be a specific method for *C. abortus* detection (Sachse et al., 2009). The recent advent of a PCR coupled with restriction

fragment length polymorphism (PCR-RFLP), which identifies specific SNPs unique to the vaccine strain, has allowed the differentiation of vaccinated and naturally infected animals (Laroucau et al., 2010; Wheelhouse et al., 2010).

1.5.3 Control measures

Antibiotics such as oxytetracycline can be used effectively to limit disease severity (Entrican et al., 2001) and reduce further losses during abortion storms. However, antibiotics are not guaranteed to prevent abortion or shedding of the bacteria and due to the absence of clinical signs with *C. abortus* they are not an effective control strategy. The development of antibiotic resistance is also a potential risk and resistance has been detected in *C. suis* strains isolated from pigs (Entrican et al., 2012).

Following chlamydial abortion, the bacteria are shed into the environment and therefore measures should be taken to limit contamination by isolating the ewe. The area should then be decontaminated and contaminated material safely destroyed (Essig and Longbottom, 2015). The importance of good biosecurity was emphasized in a recent survey, which identified that the majority of *C. abortus* was detected in flocks where other abortifacients (i.e. *Toxoplasma* and *Campylobacter*) were present (Longbottom et al., 2013a).

The introduction of infected replacement animals is the major route of transmission between farms, as described in section 1.2.2. Maintaining closed flocks or only buying replacements from enzootic abortion of ewes (EAE) accredited flocks is the best way of preventing the introduction of disease (Longbottom et al., 2013a). The EAE accreditation scheme involves the submission of annual blood tests, which following testing of two consecutive negative samples allows the EAE accredited status to be awarded (Premium sheep and goat health scheme, 2008). Often, keeping closed flocks is not plausible and in these cases animals should be vaccinated (section 1.6).

1.6 Vaccination

The ideal vaccine against any disease would induce sterile immunity and prevent infection. However, the more realistic aims for a chlamydial vaccine are to reduce pathology and limit shedding of infectious organisms (Beagley et al., 2009b). Inactivated and live attenuated chlamydial vaccines have been used with some success for animal chlamydioses. Commercially-available chlamydial vaccines include *C. abortus* vaccines and an inactivated *C. felis* vaccine (Longbottom, 2003). There are currently no commercially available vaccines to prevent chlamydial infections in humans. Live attenuated vaccines were tested against *C. trachomatis*, but they had major limitations. The vaccines exhibited only a short duration of immunity and increased immunopathology was observed in vaccinated non-human primates following subsequent exposure to a different chlamydial strain. Therefore, the focus for improved chlamydial vaccines has shifted to safer rational based design, particularly recombinant subunit vaccines involving MOMP (Brunham and Rappuoli, 2013).

1.6.1 Current *C. abortus* vaccines

There are currently three vaccines that are commercially available to prevent OEA: Enzovax[®], CEVAC Chlamydia[®] and Mydiavac[®]. Enzovax[®] and CEVAC Chlamydia[®] are based on the live-attenuated 1B strain of *C. abortus*, which is a temperature sensitive mutant that can grow at 35°C but not 39.5°C (the body temperature of sheep) (Entrican et al., 2012; Longbottom and Livingstone, 2006). Mydiavac[®] is composed of inactivated whole organism with a mineral oil adjuvant and has only recently been reintroduced by Benchmark Animal Health (Essig and Longbottom, 2015). Enzovax[®] and CEVAC Chlamydia[®] should not be administered to pregnant sheep or handled by pregnant women (Entrican and Lunney, 2012), whereas an advantage of Mydiavac[®] is that it can be used in pregnant ewes (Longbottom and Livingstone, 2006). Both the live attenuated and inactivated vaccines have been successfully used to prevent OEA (Essig and Longbottom, 2015; Longbottom and Coulter, 2003), but they have a number of limitations with use.

The vaccines are expensive to produce due to the need for bulk *Chlamydia* growth and isolation (Longbottom and Coulter, 2003). They also do not induce sterile immunity as vaccinated ewes can still shed the bacteria at lambing, facilitating transmission to naïve ewes (Essig and Longbottom, 2015). Recently, the novel PCR-RFLP that can differentiate vaccinated from naturally infected ewes, as described in section 1.5.2, has been used to implicate the 1B vaccine strain as a causal factor of *C. abortus* induced abortion (Laroucau et al., 2010; Wheelhouse et al., 2010). This causal link has since been reinforced by more recent studies and raises safety concerns (Livingstone et al., 2014; Sargison et al., 2015). Although the molecular detection technique has been recently developed, archived samples from the 1990s also tested positive for the vaccine strain. This suggests that the apparent link to the 1B vaccine strain is unlikely to be down to a recent breakdown in the vaccine (Wheelhouse et al., 2010). Nonetheless, vaccination is still the most effective method of controlling *C. abortus* and the current benefits outweigh the risks of use (Essig and Longbottom, 2015).

1.6.2 Towards an improved *C. abortus* vaccine

A number of factors need to be considered for the design of a new improved vaccine, including safety, cost and efficacy. Critically, a new *C. abortus* vaccine must not be able to revert to virulence and preferably would induce sterile protective immunity (Entrican et al., 2012). Therefore, studies have focused on developing new recombinant subunit or DNA vaccines, but also on novel routes of administration and selection of improved immune-stimulatory adjuvants.

Recombinant subunit vaccines offer the opportunity to select protective antigens whilst omitting antigens that are pathogenic. Many early studies focused on recombinant protein vaccines using MOMP, which had little success in efficacy trials. This has led researchers to identify new antigens, such as Pmps and T3SS effector proteins, for the design of a multi-component subunit vaccine (Longbottom and Livingstone, 2006). Subunit vaccines often don't induce sufficient levels of cell-mediated immunity, and therefore they are used in conjunction with delivery systems

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and adjuvants. One recent study compared the immune-stimulatory potential of the *Vibrio cholerae* ghost (VCG) platform with the CpG + Fms-like tyrosine kinase 3 ligand adjuvant. The adjuvants were used in conjunction with *C. abortus* polymorphic membrane protein 18D (Pmp18D). VCG was found to be superior for activating DCs and reducing shedding in the mouse model after *C. abortus* challenge, but has not been evaluated in sheep (Pan et al., 2015).

DNA vaccines involve using a plasmid that encodes a gene of interest from the pathogen. The plasmid is taken up by host cells and the gene of interest expressed and recognized within the host, inducing a protective immune response. This technology is attractive due to its inexpensive generation and stability, which omits the need for cold chain storage (Schautteet et al., 2011). DNA vaccination was shown to be effective at reducing incidence of avian chlamydiosis in turkeys (Longbottom and Coulter, 2003). Similarly, they were shown to effectively induce protective immunity to *C. abortus* in smaller mammals such as mice; however, they have not had the same success in larger mammals such as sheep (Ou et al., 2013). A recent study identified that the addition of a lambda bacteriophage delivery system to *ompA* DNA vaccines, increased the early level of MOMP specific IgG and lymphocyte proliferative responses compared to the commercial 1B vaccine in piglets (Ou et al., 2013). Improved plasmid delivery and expression of the antigen is hoped to increase the efficacy of DNA vaccination for future *C. abortus* vaccine design (Longbottom and Livingstone, 2006).

As *C. abortus* is transmitted oral-nasally, mucosal immunity may be important in determining disease outcome (Entrican et al., 2012). The route of administration has previously been shown to determine the efficacy of recombinant chlamydial vaccines. Combining systemic and mucosal vaccination was shown to be more effective at preventing infection with *C. muridarum* than individual administration (Schautteet et al., 2011). Inducing protective mucosal immunity could be critical for an effective *C. abortus* vaccine as it may limit entry and dissemination from the site of infection, which may prevent the establishment of a latent infection.

Further studies are required to assess how the innate immune system educates the adaptive immune system in order to stimulate a protective immune response or induce a latent infection (Longbottom et al., 2013b). This knowledge could be used in combination with novel vaccine technology to design improved targeted vaccines. Identifying immune correlates of protection in ruminants has been hindered by a lack of veterinary immunology reagents (Entrican et al., 2012). However, the recent advent of veterinary immunology toolkit projects, which develop and commercialize novel veterinary reagents, means that more studies can now be conducted (Entrican and Lunney, 2012).

1.7 General hypothesis and aims

The sequencing of *Chlamydia* genomes has revealed a number of genes that may be associated with disease pathogenesis and tissue tropism within and between species. However, the *C. abortus* genome has revealed little that could explain the differences in pathology between cattle and sheep. Analyzing the early host immune response to the pathogen will help to expose differences that may explain disease pathogenesis. Studies with human and mouse *Chlamydia* strains have demonstrated that innate inflammatory pathways and particularly inflammasome activation contribute to both pathogen clearance and pathology. A better understanding of how *C. abortus* is recognized and activates the innate immune response will contribute to the design of improved novel vaccines for sheep.

The underlying hypothesis of this thesis is that **ruminant hosts differ in their ability to innately sense *C. abortus* infection and activate the inflammasome**. In order to address this hypothesis, I had a number of overarching aims:

- Analyse the expression of pattern recognition receptors (PRRs) known to be important for sensing chlamydial infections (TLR2, TLR4, NOD1 and NLRP3) in innate immune cells of cattle and sheep.
- Compare the inflammatory response of these cells following infection with *C. abortus* by analyzing expression of IL-1 β , IL-6, CXCL8, and TNF- α .

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- Investigate the roles PRRs play in the induction of innate immunity to *C. abortus* through interference of signaling using siRNA.
- Conduct RNA-seq analyses on cattle and sheep macrophages infected with *C. abortus* to identify differential pathways of immune activation in cattle and sheep.

The research I conducted towards these aims is described and discussed in the following chapters.

Chapter 2: Material and Methods

These are the general protocols used throughout the study. Chapter-specific protocols are supplied within each chapter.

2.1 Tissue culture with adherent cell lines

2.1.1 Resuscitation of cryopreserved cell lines

Cells were removed from liquid nitrogen and defrosted rapidly in a 37°C water bath before the addition of cold Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAA, Hanniger, Austria). Cells were centrifuged at 300g for 10 minutes at 4°C and resuspended in culture medium (IMDM supplemented with 10% heat-inactivated FBS). A viability count was performed by diluting 20µl cells in 20µl of 1% nigrosin before adding to a haemocytometer. The total cells were calculated to determine the appropriate flask size for cell line propagation.

2.1.2 Propagation of cell lines

Cells were routinely cultured in vented tissue culture flasks (Corning Costar, High Wycombe, UK) in culture medium until confluence. The adherent cells were washed twice with warm 1x phosphate buffered saline (PBS). The cells were then passaged using trypsin-versene (0.25% w/v trypsin and 20% v/v versene) and re-seeded into new flasks with fresh culture medium.

2.1.3 Cryopreservation of cell lines

Cells were centrifuged at 300g for 10 minutes at 4°C and a viability count conducted as described in section 2.1.1. Cells were washed and centrifuged before re-suspension in freezing mix (40% culture medium, 50% heat-inactivated FBS and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Dorset, UK)). Cells were adjusted to a concentration of between 1×10^6 /ml and 5×10^6 /ml in ice-cold freezing mix. Cells were then added to cryovials (Nunc, Roskilde, Denmark) in 1ml volumes. The

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cryovials were put in proprietary freezing tubs (Nalgene Mr. Frosty freezing container; Thermofisher Scientific, Rochester, NY, USA) containing isopropanol, placed in a -80°C freezer and allowed to freeze slowly overnight before being transferred to liquid nitrogen for long-term storage.

2.2 Preparation of cells from whole blood

2.2.1 Animals

All animal experimental procedures were approved by Ethics Committees at the Roslin Institute (RI) and Moredun Research Institute (MRI) and performed to Home Office Guidelines under Project Licences (PPL 60/4394 and PPL 60/3854 respectively).

2.2.1.1 Sheep

Eight healthy, Texel-Greyface cross female sheep were housed indoors at Moredun Research Institute with access to food and water *ad libitum*. The sheep were between 2-4 years old for the duration of study.

2.2.1.2 Cattle

Eight healthy, Holstein-Friesian female cattle were housed indoors at the Roslin Institute with access to food and water *ad libitum*. The cattle were between 2-4 years old for the duration of study.

2.2.2 Generation of peripheral blood mononuclear cells

Blood was collected aseptically from the jugular vein into blood collection bags containing the anticoagulant Citrate Phosphate Dextrose Adenosine -1. Bag sizes were 350ml (Henry Schein, Melville, UK) for sheep blood and 500ml (Starstedt, Leicester, UK) for cattle blood. Buffy coat layers composed of white blood cells and platelets were collected by centrifugation of whole blood, in 50 ml volumes, at 1200g for 15 minutes. Buffy coats that were aspirated from two blood tubes were pooled into 50ml 1xPBS, before splitting and layering onto two tubes with 25ml lymphoprep (Axis Shield, Dundee, UK) for density gradient centrifugation at 1200g for 25

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minutes. The interface containing mononuclear cells was collected and resuspended in 1xPBS for centrifugation at 350g for 10 minutes. Contaminating red cells were removed using Ammonium-Chloride-Potassium (ACK) lysis buffer (Life Technologies) and washed three times in 1xPBS with centrifugation at 300g for 10 minutes.

2.2.3 Generation of CD14⁺ monocytes and monocyte-derived dendritic cells

PBMCs were generated as described in section 2.2.2. The cells were centrifuged at 300g for 10 minutes before resuspension in 50mls MACs buffer (1xPBS, 0.5% bovine serum albumin (BSA) and 2 mM EDTA) at 4°C followed by the addition of 20µl of CD14 MicroBeads (Miltenyi Biotech, Surrey, UK) per 1×10^7 cells. The cells were incubated at 4°C for 15 minutes before washing in MACs buffer with centrifugation at 300g for 10 minutes. The cell pellet was resuspended in MACs buffer before magnetic separation through an LS column (Miltenyi Biotech) on a QuadroMACS™ separator (Miltenyi Biotech) following the manufacturer's instructions. Briefly, the cells were resuspended in 500µl MACs buffer for every 1×10^8 cells before adding to the column. The column was washed three times with 3mls of MACs buffer before adding 5mls and detaching the cells with the plunger. CD14 expression was measured by flow cytometry (section 2.11) in PBMCs before separation, non-magnetically labeled cells and magnetically labeled cells. The CD14⁺ cells were centrifuged at 300g for 10 minutes and resuspended to a concentration of 1×10^6 cells/ml, in ovine or bovine DC kit (Bio-Rad AbD Serotec, Kidlington, UK) diluted in IMDM supplemented with 10% heat inactivated FBS, 50 µg/ml gentamycin (Roussel Laboratories Ltd, West Malling, UK) and 50µM β-mercaptoethanol (Sigma-Aldrich). These kits contain recombinant ovine or bovine IL-4 and GM-CSF respectively. The CD14⁺ cells were then cultured at 3×10^6 cells per well in six well plates (Nunc) at 37°C for either 3 days (cattle) or 6 days (sheep).

2.2.4 Generation of monocyte-derived macrophages

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PBMCs were isolated as described in section 2.2.2. The bovine monocyte-derived macrophages (MDMs) were generated using a previously published protocol (Jensen et al., 2014) as follows. Briefly, bovine PBMC were adjusted to 5×10^6 cells/ml in Roswell Park Memorial Institute (RPMI)-1640 (Life Technologies) without serum and 20mls of the PBMC were incubated for 2 hours at 37°C in 75cm² tissue culture flasks. The RPMI containing non-adherent cells was then aspirated and replaced with bovine macrophage culture medium (RPMI-1640 supplemented with 20% FBS (Life Technologies), 4mM L-glutamine, 50µM β-mercaptoethanol and 50 µg/ml gentamycin) and the adherent cells cultured at 37°C for 14 days with the medium changed on day 4 and day 10.

The ovine MDMs were generated by adjusting the PBMC to 1×10^7 cells per ml in ovine macrophage culture medium (IMDM supplemented with 20% autologous serum, 50µM β-mercaptoethanol and 50 µg/ml gentamycin) before adding 20mls of the cell suspension to 75cm² tissue culture flasks. The cells were cultured for 7 days at 37°C which included washing vigorously 3 times with 1xPBS at day 3, to remove non-adherent cells, and replacing with fresh medium.

The differentiated bovine and ovine MDMs displayed the expected morphological macrophage characteristics, which included increased cytoplasm with a vacuolar appearance. The MDMs were washed 3 times with 1xPBS before dissociating the cells with the recombinant enzyme TrypLE™ Express (Life Technologies). Cell purity was assessed using flow cytometry (section 2.11) that consistently showed that over 90% of adherent cells expressed the molecule SIRP-α, consistent with them being of the monocyte/macrophage lineage. The MDMs were then plated out at 6×10^5 cells per well in six well plates and rested for 24 hours before further experimentation.

2.2.5 Generation of ovine autologous serum

Blood was collected aseptically from the jugular vein into red top vacutainers (Becton Dickinson, Cambridge, UK) and incubated at 37°C for 3-4 hours. The vacutainers were then incubated at 4°C for 30 minutes and the serum transferred to

universals before centrifugation at 1300g for 10 minutes. The clear serum was collected avoiding the small contaminating red blood cell pellet.

2.3 Preparation of *C. abortus* stocks

2.3.1 Strain

The S26/3 *C. abortus* strain was previously isolated at the Moredun Research Institute (MRI) from a ewe vaccinated with the 1956 single isolate (A22) vaccine but which subsequently aborted (Anderson, 1986). The isolate was then grown in the yolk sac of fertile chicken eggs that were homogenized and the bacterial suspensions diluted in sucrose-phosphate-glutamate (SPG) (sucrose based medium containing FBS, antibiotics and fungicides) and stored at -80°C (Graham et al., 1995).

2.3.2 Propagation of *C. abortus*

Sub-confluent 225cm² flasks of HeLa cells (ATCC, Middlesex, UK) were washed with wash buffer (500ml Hank's balanced salt solution (HBSS) supplemented with 2.5 mls 8% sodium hydrogen carbonate) before adding infection buffer (wash buffer supplemented with 30µg/ml diethylaminoethyl (DEAE)-dextran) for 20 minutes. The infection buffer was removed and replaced with 1 vial of S26/3 seed stock in 5mls propagation buffer (IMDM supplemented with 5% heat-inactivated FBS, 1µg/ml cyclohexamide and 50µg/ml gentamycin) and incubated for 3-4 hours at 37°C. The inoculum was replaced with 18mls fresh propagation buffer and flasks incubated at 37° C for 72 hours or until lysis was observed.

2.3.3 Harvesting of *C. abortus* and preparation of seed stocks

After 72 hours of culture, the HeLa cell monolayer was disrupted using glass beads and collected together with the supernatant and centrifuged at 900g for 10 minutes. The cell pellet was resuspended in supernatant and sonicated to release intracellular *C. abortus*. The suspension was spun again at 900g for 10 minutes. The supernatant was pooled and sub-aliquoted into eppendorfs in 1ml volumes for final centrifugation in a microfuge (1-15K, Sigma-Aldrich) at 10000g for 10 minutes at 4°C. The pellets

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were then resuspended in SPG and stored at -80°C. These seed stocks were titrated (section 2.3.4) and used throughout the project in the various infection protocols.

2.3.4 Titration of the *C. abortus* stock

HeLa cells were grown to sub-confluence in 8 well chamber slides (Nunc) before being infected with seven log₁₀ sequential dilutions of a *C. abortus* seed stock along with an uninfected medium control. Chamber slides were incubated for 72 hours at 37° C before the supernatant was removed, the cells fixed in cold 90% acetone/10% methanol and slides stored at -20°C. Slides were rehydrated in 1xPBS before 50µl Image-iT® FX signal enhancer (Life Technologies) was added to each well for 30 minutes in a humidity chamber protected from light. The slides were washed and labelled with 100µl per well of 1:10000 dilution of ascites mouse IgG1 monoclonal anti-*C. abortus* LPS antibody (clone 13/4), which was produced at the MRI (Graham et al., 1995), in 1xPBS and incubated for 30 minutes. Following three washes with 1xPBS, 100µl per well of 1:10000 dilution of Alexafluor 488 goat anti-mouse IgG1 secondary polyclonal antibody (pAb) (Life Technologies) in 1xPBS was added for 30 minutes. The slides were washed before they were mounted with ProLong Gold anti-fade reagent containing DAPI (Life Technologies), which stained the nuclei.

2.4 Infection protocols

Infection protocols varied between the different experiments so exact protocols will be specified in each experimental chapter. Generally, the cells were challenged with live and in some cases UV-inactivated *C. abortus* S26/3. *C. abortus* was inactivated by exposure to 2000 Joules of UV in a UVlink cross-linker (Uvitec, Cambridge) and a challenge dose equivalent to multiplicity of infection (MOI) 10 used. Following challenge, supernatants and cell lysates (following addition of RLT lysis buffer discussed in section 2.7) were collected and stored at -80°C for further analyses.

2.5 Immunofluorescence

Cells were grown to sub-confluence and fixed as described in 2.3.4. The slides were washed and 100µl per well of 1:1000 anti-*C.abortus* major outer membrane protein (MOMP) mouse IgG2b monoclonal antibody (mAb) (4/11) (Insight Biotechnology Ltd, Wembley, UK) diluted in 1xPBS was added to the slides for 30 minutes. After washing, labelling was visualized by adding 100µl per well of 1:2000 Alexafluor 488 goat anti-mouse IgG (H+L) secondary polyclonal antibody (pAb) (Life Technologies) diluted in 1xPBS. Slides were mounted using ProLong Gold anti-fade reagent containing DAPI.

2.6 Cytokine sandwich ELISAs

Details of the coating buffers, antibodies and standards used in the cytokine sandwich ELISAs are supplied in Table 2.1. Briefly, high binding 96 well microplates (Greiner bio-one, Stonehouse, UK) were coated with 50µl (100µL for TNF- α) primary antibody diluted in carbonate buffer and incubated overnight at 4°C. The plates were washed six times with wash buffer (1xPBS with 0.05% Tween20; Sigma-Aldrich) before adding 100µl of the blocking buffer and incubating at room temperature (RT) for 1 hour. The plates were washed twice with wash buffer before adding the standards and samples in 50µl volumes for 1hour incubation at RT. The plates were washed six times before adding the 50µl (100µl for anti-TNF- α) secondary antibody and incubating at RT for 1 hour. The plates were washed six times and 50µl goat anti-rabbit HRP (Dako, Cambridgeshire, UK) or 100µl Streptavidin HRP (Dako), both at 2µg/ml, added for 1 hour before repeating the 6 wash step. Peroxidase activity was detected by the addition of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) (KPL, Maryland, USA) substrate and the reaction stopped by adding 0.2 M sulphuric acid. Optical density (OD) readings were taken at 450nm by a microplate reader (M-RX II, Dynex Technologies, Worthing, UK). Standard curves were obtained using commercial or recombinant cytokines (Table 2.1) generated in house by expression in Chinese hamster ovary (CHO) cells using a

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standard protocol (Wattegedera et al., 2004). Cytokine concentrations in the samples were calculated from the standard curves and expressed as pg/ml.

Table 2.1: Components of the cytokine sandwich ELISAs used for the detection of ruminant innate immune cytokines.

Cytokine	Blocking buffer	Primary antibody (Concentration) (Supplier)	Secondary antibody (Concentration) (Supplier)	Recombinant standards (Concentration) (Supplier)
IL-1β	3% bovine serum albumin (BSA) in wash buffer	Mouse anti-sheep mAb (5 μ g/ml) (Bio-Rad AbD Serotec)	Rabbit anti sheep pAb (2 μ g/ml) (Bio-Rad AbD Serotec)	Bovine IL-1 β (20ug/ml) (Bio-Rad AbD Serotec)
CXCL8	4% marvel in wash buffer	Mouse anti-sheep mAb (5 μ g/ml) (Bio-Rad AbD serotec)	Rabbit anti sheep pAb (2 μ g/ml) (Bio-Rad AbD serotec)	Ovine CXCL8 (41ng/ml) (In house)
TNF-α	3% BSA in wash buffer	Anti-bovine TNF-alpha pAb (10 μ g/ml) (Thermofisher Scientific)	Anti-bovine TNF-alpha biotin-conjugated pAb (1 μ g/ml) (Thermofisher Scientific)	Bovine TNF- α (20ug/ml) (Bio-Rad AbD Serotec)
IL-6	3% BSA in wash buffer	Mouse anti-sheep mAb (5 μ g/ml) (Bio-Rad AbD serotec)	Rabbit anti sheep pAb (2 μ g/ml) (Bio-Rad AbD serotec)	Ovine IL-6 (Unknown)* (In house)

* Concentration of ovine IL-6 could not be calculated (discussed in Chapter 3).

mAb- monoclonal antibody, pAb- polyclonal antibody

2.7 Generation of RNA and cDNA production

Total RNA was extracted using the RNeasy® Mini kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. The protocol included the QIAshredder (Qiagen) homogenisation pre-step and DNase (Qiagen) for the removal of genomic DNA. All centrifugation steps were completed at RT in a microfuge. Briefly, cell supernatant was removed and cells were lysed within culture wells, of up to 5×10^6 cells, using 350µl RLT lysis buffer containing β-mercaptoethanol. The samples were then homogenised in the QIAshredder column with centrifugation at maximum speed for 2 minutes. The next step was the addition of 350µl of 70% ethanol to the lysate, which was mixed before transferral to the RNeasy spin column for centrifugation at 9000g for 15 seconds. The column was washed with 350µl of buffer RW1 and centrifugation at 9000g for 15 seconds. The removal of genomic DNA involved the addition of 80µl DNase (10µl of DNase I stock solution mixed with 70µl of buffer RDD) for incubation on the membrane for 15 minutes. The column was washed with 350µl buffer RW1 and centrifuged at 9000g for 15 seconds. This was followed by a further two wash steps with 500µl of buffer RPE and centrifugation at 9000g for 15 seconds and 2 minutes respectively. The column membrane was dried by centrifugation at maximum speed for 1 minute. RNA was eluted with addition of 30µl of RNase-free water and centrifugation for 1 minute at 9000g. The RNA concentration was increased by re-eluting the RNA onto the membrane for further centrifugation for 1 minute at 9000g. The concentration of RNA was assessed using a nanodrop spectrophotometer (Thermofisher Scientific) and only RNA samples with a 260/280 ratio > 1.8 were used for reverse transcription.

RNA was reverse transcribed into cDNA using Taqman® reverse transcription reagents (Invitrogen). Briefly, a mastermix was prepared comprising 10x reverse transcription buffer, 25mM MgCl₂, 10mM dNTP mix, 50µM Random hexamers, Mutiscribe™ Reverse Transcriptase (20U/µL), RNase inhibitor (20U/µL) and molecular grade dH₂O for a final volume of 30µl. 20µl (1µg) of RNA was added and the reaction incubated at 25°C for 10 minutes, 48°C for 60 minutes before

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inactivation at 95°C for 5 minutes. The RNA and cDNA produced was stored at -80°C.

2.8 Standard PCR

Standard PCR (S-PCR) was used to detect the expression of NOD1, NLRP3, TLR2 and TLR4 in cDNA. Primers were designed using primer 3 (<http://primer3.sourceforge.net/>) which considers a number of factors in design including primer length, melting temperature and GC content (Rozen and Skaletsky, 2000). The specificity was assessed using Blast Local Alignment Sequencing Tool (www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1990). The sequences are shown in Table 2.2. All PCR primers were synthesised by MWG (Eurofins MWG Operon, Wolverhampton, UK). PCR reactions contained 12.5µl Biomix™ (Bioline, London, UK), 1µM of each primer, 2.5µl cDNA and made up to 25µl with 5µl of molecular grade dH₂O. Cycling conditions were 95° C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, the appropriate annealing temperature (Table 2.2) for 30 seconds, 72°C for 1 minute and a final extension of 72° C for 5 minutes. PCR products were run along with a 100bp ladder (New England Biolabs, Hitchin, UK), to assess if products were of the correct size, on a 1% agarose gel containing Gel Red™ (Biotum, Hayward, CA, USA) and visualized using a UV illuminator. Positive controls were generated by stimulating PBMC with Concanavalin A (Con A; ICN Biochemical, Cleveland, OH, USA) at 10µg/ml for 24 hours and negative controls generated by excluding reverse transcriptase in cDNA preparation. PBMC stimulated with ConA were used as a positive control because conA activates T cells, which in turn can activate the other cells in culture up-regulating PRR expression.

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Table 2.2: Standard PCR primer sequences for the detection of PRRs

Target	Accession number	Forward Primer (5'-3')	Reverse Primer (5'-3')	Expected product size (base pairs)	Annealing temperature
Ovine NOD1	XM_004007930.1	CCATCTTTGAAGTCGGGATG	CGGGCTTTATCAAGTTTCCA	378	50°C
Ovine NLRP3	XM_004008740.1	AATCCCTTCCCTGAGGTTG	GGGAATGGTTGGTGCTTAGA	531	52°C
Ovine TLR2	NM_001048231.1	GCTCCTGTGACTTCCTGTCC	AGTGGGAGAAGTCCAGCTCA	555	52°C
Ovine TLR4	NM_001135930.1	GGACAACCAACCTGAAGCAT	TGAGGTTCCCTTGGCAAATTC	525	50°C
Bovine NOD1	NM_001256563.1	GCCTTCTTTGCTGCCCTTTTTTC	CCCCACCTTTTCACTCTGTGTC	395	52°C
Bovine NLRP3	NM_001102219.1	TCAAGCTGTCCACCAGAATG	CAGAATTCACCAACCCAGT	711	52°C
Bovine TLR2	NM_174197.2	GGGGATGACAGAGGATGAGA	GGCAGAAAGCTGATGGAAAG	404	52°C
Bovine TLR4	NM_174198.6	GACCCTTGCGTACAGGTTGT	GGTTGAGTAGGGGCATTTGA	538	52°C

2.9 Taqman real-time PCR

Primers and probes were either designed by primer express (PE, Life Technologies) to meet Taqman requirements or had been previously published as detailed in Table 2.3 and Table 2.4. All primers and probes were synthesised by MWG. The specificity was assessed using BLAST and primers were designed to span exon-exon junctions to avoid amplifying genomic DNA. The 25µl reactions contained 12.5µl of Taqman Universal PCR Mastermix (Life Technologies), 3.75µl of molecular grade dH₂O, 2.5µl of primers/probes and 1.25µl cDNA. Reactions were analysed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions; 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute. Samples were run in triplicate and a non template control included in each assay.

The newly designed primers/probes were assessed using a 10-fold dilution series of cDNA from PBMC stimulated with ConA or plasmids containing the genes of interest (generated as described in section 2.10). The efficiencies of the assays were calculated by plotting the average cycle threshold (Ct) against log cDNA concentration. The slope values were consistently between -3.32 and -3.22 which, using $E = 10^{(-1/\text{slope})} - 1$, is between 100 and 104% efficiency and the correlation coefficient $R_2 > 0.99$. Gene expression was normalized to eukaryotic 18s rRNA endogenous control (VIC/TAMRA™, Life Technologies) to correct for inherent variability between samples. The stability of 18s rRNA was evaluated in turbinates cells (Chapter 3) and was found to be stable in MDMs (Chapter 4). The fold change was calculated using the $2^{-\Delta\Delta CT}$ method relative to the medium only control at each time point. Samples that were undetectable due to extremely low expression levels were assigned a Ct of 40 to allow the calculation of fold change for statistical comparison.

Table 2.3: Taqman RT-PCR primers and probes used to detect ovine targets.

Target	Primer and probe Sequences (5'-3')	Genebank accession number/ reference
CXCL8	F CACTGCGAAAATTCAGAAATCATTGTTA R CTTCAAAAATGCCTGCACAACCTTC Pr CCAACGGAAAAGAGGTGTGCTTAGACCCC	NM_001009401
IL-1β	F CGAACATGTCTTCCGTGATG R TCTCTGTCCTGGAGTTTGCAT Pr TGTGATGCAGCCGTGCAGTCA	(Budhia et al., 2006)
TNF-α	F GAACCAGAGGCCTGTTGA R GGTGCCTCAGCCTCTTCT Pr TGGTTGCAGGAGCCACCACG	(Budhia et al., 2006)
IL-6	F TCAGCTTATTTTCTGCCAGTCTCT R TCATTAAGCACATCGTCGACAAA PrCGTCATTCTTCTCACATATCTCCTTTCTTATTGCAGAG	(Leutenegger et al., 2000)
TLR2	F CTGCTGGAGCCCATTGACA R TATTGCAGCTCTCAAATTC AACCA Pr AGGCCGTTCCCCAGCGCTTCT	(Thonur et al., 2012)
TLR4	F ACTCTTGTCATTGGATACATTTCTTTATG R GTAAGATTTAGCCAAGTGAGGTTCCCT Pr TGCAGTTTCAACCGTATCACGGCCT	(Thonur et al., 2012)
NOD1	F AATCGGTGATGAAGGAGCAAA R GCCGTTGAATGCAAGACTCA Pr CTCTGAGGAACCACCCCAGCTTGACC	XM_004007930
NLRP3	F CGCCATGTGGAATCCCTTT R GGTCCATATGAGAGTGTCTAACTTCCT Pr AGGTTGCTCCATAACTCGCCCAAGGA	XM_004008740

F-forward primer; R-reverse primer; Pr-probe dual labelled with 5'-FAM and 3'-TAMRA

Table 2.4: Taqman RT-PCR primers and probes used to detect bovine targets.

Target	Primer and probe Sequences (5'-3')	Genebank accession number/reference
CXCL8	F CACTGTGAAAAATTCAGAAATCATTGTTA R CTTACCAAATACCTGCACAACCTTC Pr AATGGAAACGAGGTCTGCTTAAACCCAAG	(Leutenegger et al., 2000)
IL-1β	F AGCATCCTTTCATTCATCTTTGAAG R GGGTGCATCACACAGAACTC Pr CCTGTCATCTTCGAAACGTCTCCGA	(Galvao et al., 2012)
TNF-α	F TCTTCTCAAGCCTCAAGTAACAAGT R CCATGAGGGCATTGGCATA Pr AGCCCACGTTGTAGCCGACATCAACTC	(Leutenegger et al., 2000)
IL-6	F TCAGCTTATTTTCTGCCAGTCTCT R TCATTAAGCACATCGTCG Pr CGTCATTCTTCTCACATATCTCCTTTCTTATTGCAGAG	(Leutenegger et al., 2000)
TLR2	F ACGACGCCTTCGTGTCCTAC R GCTCCTGGACCATGAGGTTTC Pr CGAGCGGGATTCTACTGGGTGG	(Werling et al., 2004)
TLR4	F TGGAGGACATGCCAGTGCT R CACCGACACACTGATGATCGT Pr AGTTTCAGGAACGCCACTTGTGAGCTG	(Werling et al., 2004)
NOD1	F GACCCAGTGAGCGGATACAG R AGCTCCTCCTTCTGGGCATA Pr CTGCAACAGCAACTGGGCCGGGA	NM_001256563
NLRP3	F GCCAAGTGGATTTTTGCTGC R GCATTCTCCATTCTGGCTC Pr CAGGAGAGACCTTTATGAGAAAGCTAAGAGGGA	NM_001102219

F-forward primer; R-reverse primer, Pr-probe dual labelled with 5'-FAM and 3'-TAMRA

2.10 Generation of plasmid clones

Plasmid clones were generated for the validation and optimisation of ovine NOD1, NLRP3 and bovine NOD1 RT-PCR. S-PCR was conducted using PBMC stimulated

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with ConA and the products fractionated using electrophoresis (section 2.8) using primers (Table 2.5), which covered the sequence amplified by RT-PCR primers/probe.

Table 2.5: Standard PCR primer sequences used for the generation of plasmid clones.

	Forward Primer (5'-3')	Reverse Primer (5'-3')
Ovine NOD 1	CCATCTTTGAAGTCGGGATG	CGGGCTTTATCAAGTTTCCA
Ovine NLRP3	CATGGGCCATTTCCCAAAA	GGAATGGTTGGTGCTTAGA
Bovine NOD1	TGGACCTGGTACAGAGCAAG	TCTTGAAGCAGCTGAGGGTG

2.10.1 Purification of PCR products

The DNA band of interest was excised and purified using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Briefly, the band was excised from the gel and placed into sterile eppendorfs for weighing. Three volumes of buffer QG was added to one volume of gel for incubation at 50° C, vortexing every 2-3 minutes until the gel had melted. One volume of isopropanol was added and the sample mixed before adding to the Qiaquick spin column for centrifugation at 10000g for 1 minute. The column was washed with 750µl of buffer PE and centrifuged at 10000g for 1 minute. Following a repeated centrifugation step the column was placed in a sterile eppendorf before the addition of 30 µl buffer EB. A final centrifugation step produced purified DNA, which was quantified using the nanodrop.

2.10.2 Gene cloning

The DNA was ligated into a pGEM®-T easy vector (Promega, Southampton, UK) following the manufacturer's instructions. The DNA (1µl) was added to a sterile eppendorf with 1µl pGEM®-T easy vector, 5µl 2x rapid ligation buffer, 1µl T4 DNA ligase and 2µl nuclease-free water. A positive control with control insert DNA and a background control with no DNA were also included. Following overnight ligation at 4°C, 2 µl of the ligation reaction was added to 50µl of JM109 competent *E. coli* cells (Promega). The transformation reaction was incubated on ice for 20 minutes before

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the cells were heat-shocked at 42°C for 50 seconds. The tube were returned to ice for 2 minutes before the addition of 950µl of SOC medium for 1.5 hour incubation with shaking at 150rpm at 37°C. The cells were pelleted at 900g for 10 minutes before resuspension in 200µl SOC medium. 100µl of transformation reaction was plated onto ampicillin/IPTG/X-Gal lysogeny broth (LB) agar plates (100µg/ml ampicillin (Sigma-Aldrich) with LB agar spread with 20µl of 50mg/ml X-Gal (Promega) and 100µl of 100mM IPTG (Promega)) for the detection of transformed colonies based on colour (non-transformed colonies are blue, transformed colonies are white). The plates were incubated overnight at 37°C before the selection of colonies for screening by standard PCR (section 2.8).

2.10.3 Plasmid purification

The culture was expanded by inoculating 10mls of LB (containing 100µg/ml ampicillin) with the S-PCR positive colonies. The cultures were incubated at 37°C with 200rpm shaking overnight. The plasmids were purified using the Qiaprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. Briefly, 5 mls of bacterial culture was centrifuged at 4000g for 3 minutes before the pellet was resuspended in 250µl buffer P1. The alkaline lysis of the bacterial cells involved the addition of 250µl of buffer R2 followed by inversion to mix. The reaction was neutralized through the addition of 350µl buffer N3 with further inversions. The solution was centrifuged at 10000g for 10 minutes and the supernatant applied to Qiaprep spin column and centrifuged at 10000g for 30 seconds. Sequential wash steps with buffer PB and buffer PE followed. The DNA was eluted off the column with 50µl buffer EB and centrifugation at 10000g for 1 minute. The DNA was quantified using the nanodrop.

2.10.4 Sequencing

Plasmid DNA was sent to MWG for sequencing with the vector specific M13 forward and reverse primers using the ABI 3730xl sequencer (Applied Biosystems). The sequences were trimmed from the pGEM®-T easy vector and the sequences were analysed for known similarity with other sequences using BLAST(Altschul et

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al., 1990). The sequences were aligned to the predicted sequences using CLUSTAL W v 1.83 multiple sequence alignment programme (Thompson et al., 1994).

2.11 Flow cytometry

Cells were suspended in 1xPBS before the addition of LIVE/DEAD® Fixable violet stain (Life Technologies) (1µl per 1×10^6 cells) and incubated for 30 minutes protected from light. The cells were washed with 1xPBS and centrifuged at 300g for 10 minutes. Cells were resuspended in 20% normal goat serum (Merck Millipore, Watford, UK) in 1xPBS and blocked for 30 minutes in round bottom 96well plates. The plates were washed with 100µl FACs buffer (5% FBS, 0.05% sodium azide in 1xPBS) and spun at 900g for 2 minutes before the addition of 50µl of the primary antibodies or the equivalent isotype control mAb (Table 2.6). Following incubation for 30 minutes at 4°C the cells were centrifuged at 900g and washed twice with FACs buffer. The secondary antibodies were added in 50µl volumes and the plates incubated for a further 30 minutes. The plates were washed with FACs buffer and the final wash step performed with 1xPBS. The cells were then fixed in 1% paraformaldehyde in 1xPBS and analysed using the MACSQuant Analyser (Miltenyi Biotec, Surrey, UK).

Table 2.6: Antibodies and isotypes used in flow cytometry.

Antibody	Clone	Isotype	Dilution	Supplier	Catalogue number
Isotype controls					
IgG1 isotype*	VPM21	IgG1	1:500	In house	
IgG2b isotype*	VPM22	IgG2b	1:500	In house	
Primary mAb					
Mouse anti-sheep CD14 antibody**	VPM65	IgG1	1:1000	In house	
Mouse anti-bovine signal regulatory protein α (SIRP α) antibody**	IL-A24	IgG1	1:2000	In house	
Rat anti-sheep MHC class II antibody (DR, DQ, DP)***	SW73.2	IgG2b	1:500	In house	
Mouse anti-bovine CD11b antibody	CC126	IgG2b	1:500	Bio-Rad AbD serotec	MCA1425GA
Mouse anti-bovine CD1w2 antibody	CC14	IgG1	1:500	Bio-Rad AbD serotec	MCA831G
Mouse anti-bovine CD80 antibody	IL-A159	IgG1	1:500	Bio-Rad AbD serotec	MCA2436GA
Mouse anti-bovine CD205 antibody	CC98	IgG2b	1:500	Bio-Rad AbD serotec	MCA1651GA
Secondary pAb					
Goat anti-mouse IgG2b:RPE antibody			1:500	Bio-Rad AbD serotec	STAR134PE
AlexaFluor® 488 goat anti-mouse IgG1 antibody			1:2000	Life technologies	A21121
AlexaFluor® 488 goat anti-rat IgG (H+L) antibody			1:2000	Life technologies	A1106

* Ammonium sulphate concentrated supernatant **Ascites ***Supernatant
mAb- monoclonal antibody, pAb- polyclonal antibody

Chapter 3: Characterizing the innate immune response of ruminant oro-nasal turbinate cells to *C. abortus* infection¹

3.1 Introduction

There are several routes by which *C. abortus* can be delivered to sheep under experimental conditions to induce abortion and mimic disease. Early studies identified that oral as well as subcutaneous, intravenous and intradermal inoculation of *C. abortus* could induce abortion in pregnant ewes (McEwen et al., 1951). These *in vivo* infection models have been very valuable for vaccine and pathogenesis studies, but of these, the oral route is the most likely to represent a natural route of infection. The inoculation of *C. abortus* over the tonsillar crypts in pregnant ewes resulted in similar abortion rates to subcutaneous inoculation, although intra-ruminal inoculation failed to induce abortion (Jones and Anderson, 1988). More recent studies identified that oral and intranasal inoculation prior to pregnancy also induced abortion (Gutierrez et al., 2011; Longbottom et al., 2013b). Therefore, the natural route of infection for *C. abortus* is likely to be oro-nasal, which means that cells of the oro-nasal mucosa may be the primary targets for infection.

An early serial kill study utilising oro-nasal inoculation with *C. abortus* in the non-pregnant ewe attempted to identify the initial stages of *C. abortus* infection. Chlamydial LPS was detected in blood and tissues using the commercial IDEIA chlamydial ELISA kit and immunofluorescence, and Chsp60 detected by immunohistochemistry. *Chlamydia* was detected in the tonsil, suprapharyngeal lymph node, abomasum and jejunum at 27 hours post infection; followed by the

¹ The work described in this chapter has provided the basis of a peer-reviewed publication (see appendix 9.3).

Doull L, Wattedegera SR, Longbottom D, Mwangi D, Nath M, Glass EJ, Entrican G., 2015. Late production of CXCL8 in ruminant oro-nasal turbinate cells in response to *Chlamydia abortus* infection. *Vet Immunol Immunopathol.* In press, corrected proof.

lung, spleen and mandibular lymph node at 48 hours. There was no chlamydial antigen detected at 25 days post-inoculation or in the non-pregnant uterus at any time-point (Amin and Wilsmore, 1995). It therefore appears that following oro-nasal transmission the bacterium initially infects cells of the oral-pharyngeal tonsil area before wider dissemination into the lymph nodes. The bacterium then resides in an unknown location until late stage pregnancy when an optimal environment allows recrudescence and invasion of the placenta to induce pathology.

The oro-nasal mucosa is a first line of defence against inhaled pathogens. The mucosa consists of epithelial cells, which act as a barrier to pathogens (Kweon, 2014). Epithelial cells are capable of recognising pathogens through PRRs and express immune molecules, including cytokines and chemokines in response to challenge with bacteria, viruses and fungi (Kato and Schleimer, 2007). *Chlamydiae* are epitheliotropic (Igietseme et al., 2001) and are thought to primarily infect their hosts via the epithelial cells of the mucous membranes (Lu et al., 2000). The cellular paradigm of chlamydial pathogenesis identifies that *Chlamydiae* infected epithelial cells produce pro-inflammatory cytokines and chemokines that recruit neutrophils, T cells, B cells and macrophages. These immune cells can become activated within the pro-inflammatory environment to produce further inflammatory mediators inducing an inflammatory cascade. The inflammatory environment could lead to chlamydial clearance and healing or contribute to pathology and scarring (Stephens, 2003).

The oro-nasal transmission of *C. abortus* suggests that turbinate cells, which line the oral-nasal mucosa, are likely to be among the first cells that the bacterium encounters. Cells which line mucosal surfaces have been shown to play critical roles in the pathogenesis of other chlamydial species (Rasmussen et al., 1997; Stephens, 2003). Therefore, the role for ruminant turbinate cells in response to *C. abortus* was investigated. The expression of important chlamydial PRRs and pro-inflammatory cytokines (as described in Chapter 1) were assessed. One of the difficulties with working in ruminant immunology is the lack of commercially available reagents (Entrican et al., 2012). While there are antibodies (monoclonal and polyclonal) that can be used in pairs to detect ruminant cytokines and chemokines there are very few

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commercially available kits that are sold with a recombinant ovine/bovine standard. In the case of ruminant CXCL8, IL-1 β , TNF- α or IL-6 the antibody pairs have been produced against one species (Table 2.1) but are not optimised or marketed for comparative studies between sheep and cattle. It was therefore necessary to evaluate these pairs using recombinant target cytokine for both cattle and sheep. The aims of chapter were to:

- Optimise and validate cytokine ELISAs for the detection of both ovine and bovine CXCL8, IL-1 β , TNF- α and IL-6.
- Determine the expression of TLR2, TLR4, NOD1 and NLRP3 by ruminant turbinate cells.
- Assess the susceptibility of ruminant turbinate cells to *C. abortus* infection.
- Assess the production of CXCL8, TNF- α , IL-1 β and IL-6 by ruminant turbinate cells in response to *C. abortus* infection.

3.2 Materials and methods

3.2.1 Oro-nasal turbinate cell lines

Ruminant turbinate cells were generated at post-mortem by harvesting foetal turbinate tissues, which were then trypsinised to make a single cell suspension and cultured as previously reported (McClurkin et al., 1974). The primary cell lines used in all experiments represent different passages derived from the same foetal material. After resuscitation, cells were adjusted to 1×10^5 cells/ml and grown to sub-confluence by adding 1 ml/well in 24 well plates (Corning Costar, High Wycombe, UK) or 500µl/well in 8 well chamber slides.

3.2.2 Cross-species ELISA assessment

Serial dilutions of both the ovine and bovine recombinant cytokines and chemokines (Table 3.1) were run on commercial antibody ELISA pairs (Table 2.1). The ovine recombinant cytokines were CHO supernatants generated in house as described in section 2.6.

Table 3.1: Ovine and bovine recombinant cytokines/chemokine used for the generation of ELISA standard curves.

Cytokine/Chemokine	Ovine recombinant standards (Concentration) (Supplier)	Bovine recombinant standards (Concentration) (Supplier)
IL-1β	Ovine IL-1 β (28.5ng/ml)* (In house)	Bovine IL-1 β (20 μ g/ml) (Bio-Rad AbD Serotec)
CXCL8	Ovine CXCL8 (41ng/ml)* (In house)	Bovine CXCL8 (25 μ g/ml) (Thermoscientific)
TNF-α	Ovine TNF- α (Unknown concentration)** (In house)	Bovine TNF- α (20 μ g/ml) (Bio-Rad AbD Serotec)
IL-6	Ovine IL-6 (Unknown concentration)*** (In house)	Bovine IL-6 (5 μ g/ml) (Kingfisher Biotech, Saint Paul, USA)

* Estimated concentrations based on standard curve of affinity purified ovine recombinant standard (CSIRO, Australia) measured using the ELISA as described in Section 2.6.

** Unknown concentration but contains 56 biological units/ml as evaluated by the L929 mouse fibroblast killing assay (Preston et al., 1993).

***Concentration not quantified but known to be detected by the ELISA (discussed in section 3.3.1).

3.2.3 Detection of PRR expression

The expression of NOD1, NLPR3, TLR2 and TLR4 by ruminant turbinate cells was assessed using standard PCR (as described in section 2.8) on cDNA (generated as described in section 2.7).

3.2.4 Infection of ruminant turbinate cells

Ovine and bovine turbinate cells were challenged with an MOI of 0.1, 1 and 10 live *C. abortus*, UV-inactivated *C. abortus* (equivalent to MOI 10) and medium only

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controls. The cell supernatant and lysate was harvested at 24, 48 and 72 hours and stored as described in section 2.4. The experiments were repeated on three separate occasions.

3.2.5 Immunofluorescent detection of *C. abortus*

The chamber slides were fixed and stained for the detection of *C. abortus* as described in section 2.5.

3.2.6 Detection of innate immune cytokine/chemokine protein production

The production of CXCL8, IL6, TNF- α and IL-1 β protein by ruminant turbinate cells was assessed using cytokine sandwich ELISAs, as described in section 2.6, on the harvested supernatant.

3.2.7 Comparison of normalizing genes for RT-PCR

RNA was extracted from ruminant turbinate cells and cDNA produced according to the protocols detailed in section 2.7. The expression stability of the commonly used reference genes GAPDH, β -actin (Table 3.2) and 18s rRNA (VIC/TAMRATM, Life Technologies) was assessed in bovine turbinate cell samples across different time and treatment groups. The two most stable reference genes, GAPDH and 18s rRNA, were also assessed in ovine turbinate cell samples across different time and treatment groups.

Table 3.2: Taqman RT-PCR primers and probes used to detect ovine and bovine reference genes.

Normalizing gene	Primer and probes Sequences (5'-3')	Genebank accession number/reference
Bovine GAPDH	F CAGGTTGTCTCCTGCGACTT R TGTCGTACCAGGAAATGAGCTT Pr CGATGCTGGGGCTGGCATTGCC	NM_001034034
Bovine β-actin	F AGATCAAGATCATCGCGCCC R GCCTAGAAGCATTGCGGTG Pr TGGCGGCTCCATCCTGGCCTCGC	NM_173979
Ovine GAPDH	F GCATCGTGGAGGGACTTATGA R GCCATCACGCCACAGCTT Pr CACTGTCCACGCCATCACTGCCA	(Wattegedera et al., 2010)

F-forward primer; R-reverse primer, Pr-probe dual labelled with 5'-FAM and 3'-TAMRA

18s rRNA was determined to be the most stably expressed reference gene within the turbinate cells as had the smallest standard deviation (SD) from the mean across samples (Table 3.3).

Table 3.3: Comparison of 18s rRNA, β -actin and GAPDH stability across ovine and bovine turbinate cell treatment groups.

Species	Normalizing gene	Mean (Ct)	Standard deviation
Bovine	18s rRNA	13.38	0.76
Bovine	β -actin	20.65	1.25
Bovine	GAPDH	22.93	0.80
Ovine	18s rRNA	12.81	1.03
Ovine	GAPDH	23.05	1.26

3.2.8 Detection of innate immune cytokine/chemokine mRNA

The production of CXCL8, IL6, TNF- α and IL-1 β mRNA by ruminant turbinate cells was assessed using RT-PCR, as described in section 2.9, on cDNA generated from lysates as described in section 2.7.

3.2.9 Statistical analysis

The fold change mRNA was calculated using the comparative $2^{-\Delta\Delta CT}$ method, which included calibrating the expression from the treatment groups to the medium control for each time point. Both mRNA and protein data showed increased variability with increased mean, therefore the data was transformed. The mRNA data were transformed by the logarithmic (after adding a constant value of 1) and the protein data by square root transformation. Univariate analysis of variance (ANOVA) was used to assess the main effects of the independent variables (species, treatment group and time) as well as possible two and three-way interaction effects for the dependant variable (CXCL8 mRNA or protein). The blocking structure included the cell line, cell line interaction with treatment group and cell line interaction with time. The overall statistical significance of the interaction effects were assessed using the *F*-statistic. If a significant effect was identified ($p < 0.05$) post-hoc analysis was conducted, which involved calculating the two-sided probabilities of comparisons of means of treatment groups at each time point. Probabilities were adjusted using a False Discovery Rate (FDR) approach (Benjamini and Hochberg, 1995) to take into account the multiple comparisons of means. Statistical analyses were performed using GenStat (GenStat version 16.2) and R (R Core Team, version 3.1).

3.3 Results

3.3.1 Comparison of ruminant ELISA standard curves

As described in Table 3.1, the concentrations of CHO-expressed recombinant ovine CXCL8 and recombinant ovine IL-1 β had previously been calculated by titration against affinity purified ovine recombinant standards expressed in bacteria (Seow et al., 1994a; Seow et al., 1994b). In both cases, the ELISAs for these mediators are based on a mAb capture and pAb detector (Table 2.1) raised to the bacterially-expressed recombinants (Caswell et al., 1998; Rothel et al., 1997). These ELISAs both detect the CHO-expressed recombinant products and the commercially-available recombinant respective bovine orthologue (Figure 3.1A, B). The commercially-available recombinant bovine CXCL8 and IL-1 β are sold as defined concentrations. In the case of CXCL8, the ovine and bovine standards match well. In the case of IL-1 β the ELISA appears to be more sensitive for the bovine standard, but this may be due to the way the standards have been calculated.

The anti-ovine IL-6 ELISA was developed in a similar manner to the CXCL8 and IL-1 β ELISAs, comprising a mAb capture and pAb detector (Table 2.1) both raised against bacterially-expressed recombinant ovine IL-6 (McWaters et al., 2000). This ELISA also detects the in-house CHO-expressed recombinant ovine IL-6, but unlike the CXCL8 and IL-1 β , there was no existing purified recombinant ovine standard for comparison. Hence, the values are expressed as dilutions of the CHO recombinant ovine standard. In this case, the ovine ELISA appears to be relatively poor at detecting the commercially-available recombinant bovine orthologue as shown by the low optical densities (ODs) given that the starting concentration of this material was 1000ng/ml (Figure 3.1C).

The TNF- α ELISA consisted of a pAb in both capture and detection phases raised against recombinant bovine TNF- α (Table 2.1). In this case it was necessary to test the ELISA for the ability to detect the ovine orthologue. Again, this was done using CHO-expressed recombinant cytokine. As for the IL-6, there was no purified recombinant ovine TNF- α available to use as a comparator for determination of

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concentration so values are again expressed as dilutions of the CHO-expressed TNF- α . This ELISA does detect the ovine orthologue, but appears to have low sensitivity as the curve is relatively flat. Additionally, the starting concentration of the bovine standard was 1640 ng/ml, again indicative of low sensitivity even for the bovine cytokine as concentrations in excess of 1ng/ml give very low ODs (Figure 3.1D). In conclusion, from these profiles it is clear that the four ELISAs detect the respective bovine and ovine orthologues, but not with equal sensitivity.

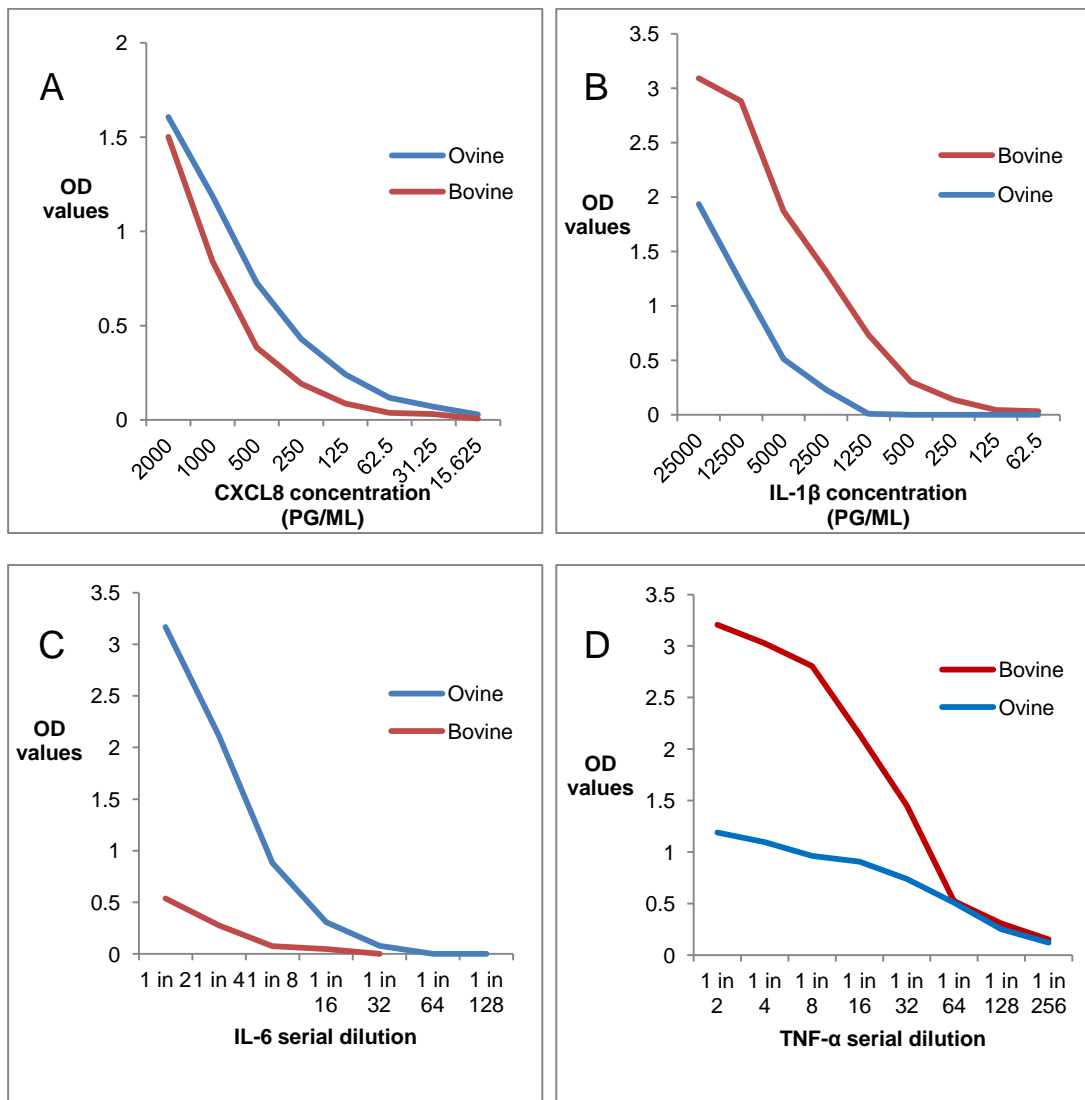


Figure 3.1: Comparison of CXCL8, IL-1 β , TNF- α and IL-6 ELISA standard curves generated using recombinant ovine and bovine cytokines/chemokines.

3.3.2 Expression of TLR2, TLR4, NOD1 and NLRP3 by ruminant turbinate cells

Standard PCR was used to identify that bovine (Figure 3.2A) and ovine (Figure 3.2B) turbinate cells expressed NLRP3, NOD1, TLR2 and TLR4. There were no bands detected in the negative control and bands of the correct size identified in the positive control (ConA stimulated PBMC) and turbinate cell samples.

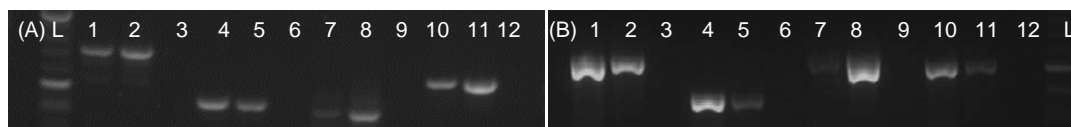


Figure 3.2: Expression of NLRP3, NOD1, TLR2 and TLR4 by bovine (A) and ovine (B) turbinate cells. Total RNA was extracted from ruminant turbinate cells and Con A stimulated PBMC, which was then reverse transcribed and PRRs amplified with specific primers using S-PCR. No reverse transcriptase controls were used as negative controls. Lanes 1, 4, 7 and 10: turbinate cells; Lanes 2, 5, 8 and 11: PBMC; Lanes 3, 6, 9 and 12: no reverse transcriptase control. Lanes 1-3: NLRP3; Lanes 4-6: NOD1; Lanes 7-9: TLR2; Lanes 10-12: TLR4. L: 100bp ladder. Products obtained were of the expected molecular weight (Table 2.2).

3.3.3 *C. abortus* inclusion formation in ruminant turbinate cells

Ovine and bovine turbinate cells were challenged with an MOI of 10, 1 and 0.1 live *C. abortus*, UV-inactivated *C. abortus* (MOI10 equivalent) and medium alone. Challenge with live *C. abortus* resulted in the formation of large intracellular inclusions in ruminant turbinate cells (Figure 3.3A, Figure 3.4A) at 72 hours post-infection. A decrease in the number of inclusions was observed with the lower MOI of 1 and 0.1 (Figure 3.3B, C; Figure 3.4B, C). Inclusions were not visible in cells exposed to UV-inactivated *C. abortus*. However, visible fluorescent specks were present (Figure 3.3D, Figure 3.4D) that were not detected in the medium alone controls (Figure 3.3E, Figure 3.4E) and may represent inactivated bacteria adhering to the cells or in the cell cytosol.

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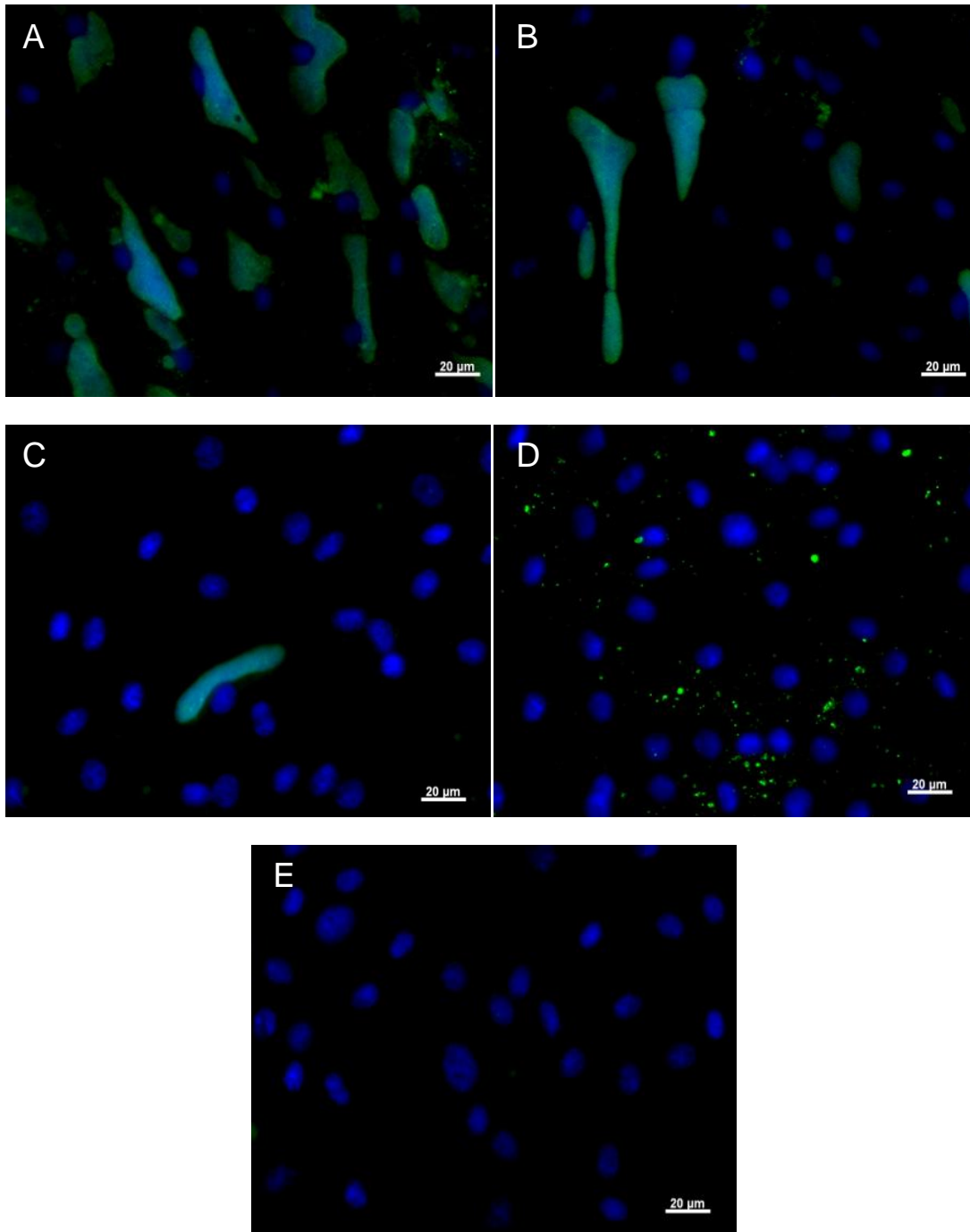


Figure 3.3: Immunofluorescent images of bovine turbinate cells demonstrating susceptibility to *C. abortus* infection. *C. abortus* was labelled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody. Host cell nuclei are stained blue with DAPI. Bovine turbinate cells challenged with MOI 10 live *C. abortus* (A), MOI 1 live *C. abortus* (B), MOI 0.1 live *C. abortus* (C), UV-inactivated *C. abortus* (MOI 10 equivalent) (D) and medium alone (E) for 72 hours. The scale bars correspond to 20µm.

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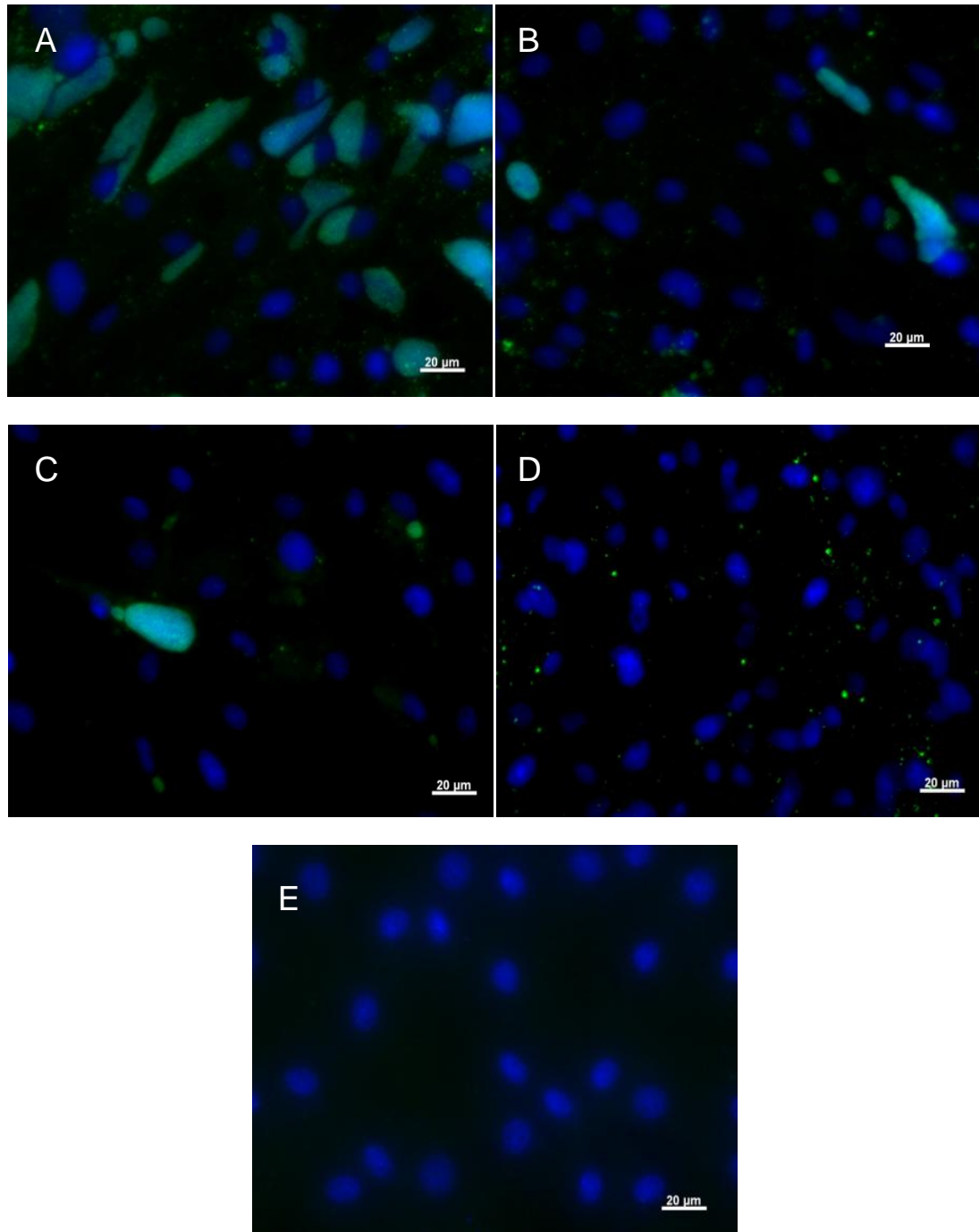
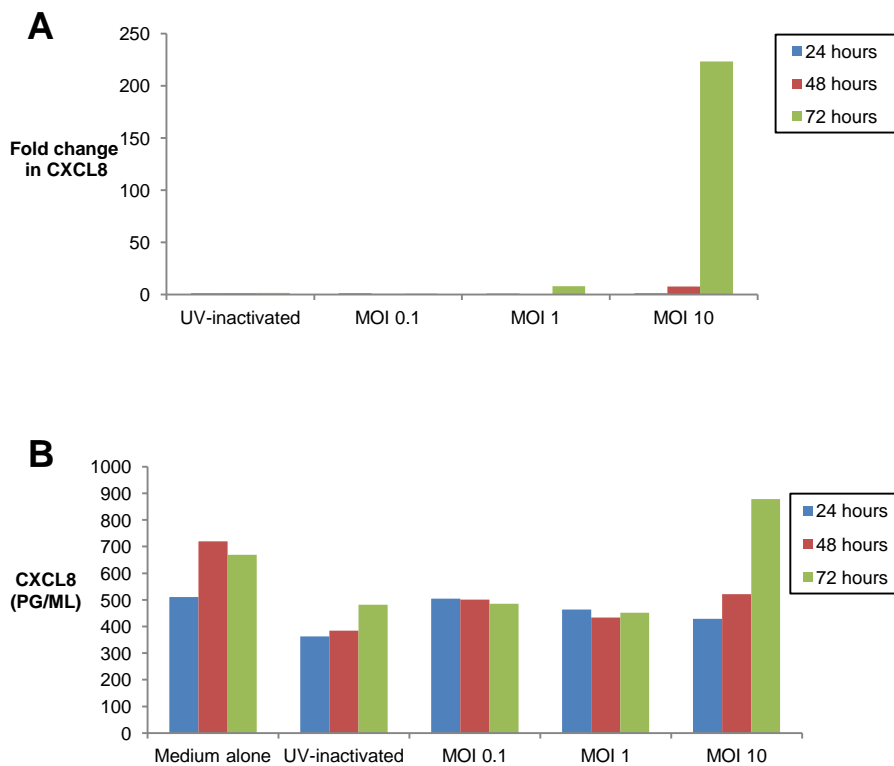


Figure 3.4: Immunofluorescent images of ovine turbinate cells demonstrating susceptibility to *C. abortus* infection. *C. abortus* was labelled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody. Host cell nuclei are stained blue with DAPI. Ovine turbinate cells were challenged with MOI 10 live *C. abortus* (A), MOI 1 live *C. abortus* (B), MOI 0.1 live *C. abortus* (C), UV-inactivated *C. abortus* (MOI 10 equivalent) (D) and medium alone (E) for 72 hours. The scale bars correspond to 20µm.

3.3.4 Innate immune cytokine/chemokine mRNA and protein production by ruminant turbinate cells in response to *C. abortus* infection

There was no IL-1 β or TNF- α mRNA or protein production detected in ovine or bovine turbinate cells at any time point or dose of *C. abortus* (data not shown). There was also no IL-6 protein detected but IL-6 mRNA analysis was not completed. Increased production of CXCL8 mRNA and protein was observed at 72 hours with an MOI 10 but not MOI 0.1 or MOI 1 live *C. abortus* infection in both ovine and bovine turbinate cells (Figure 3.5).



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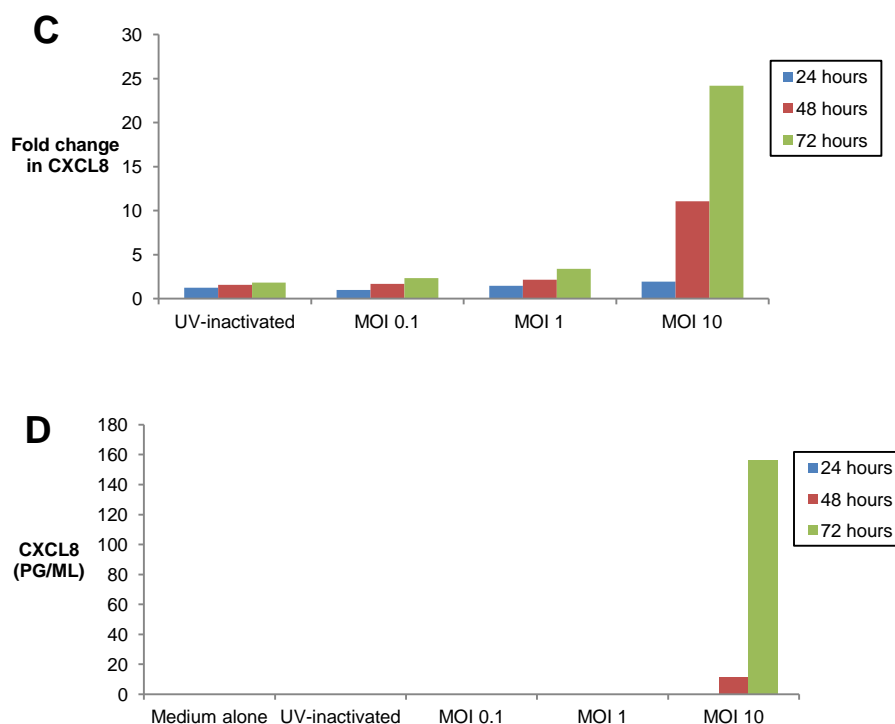
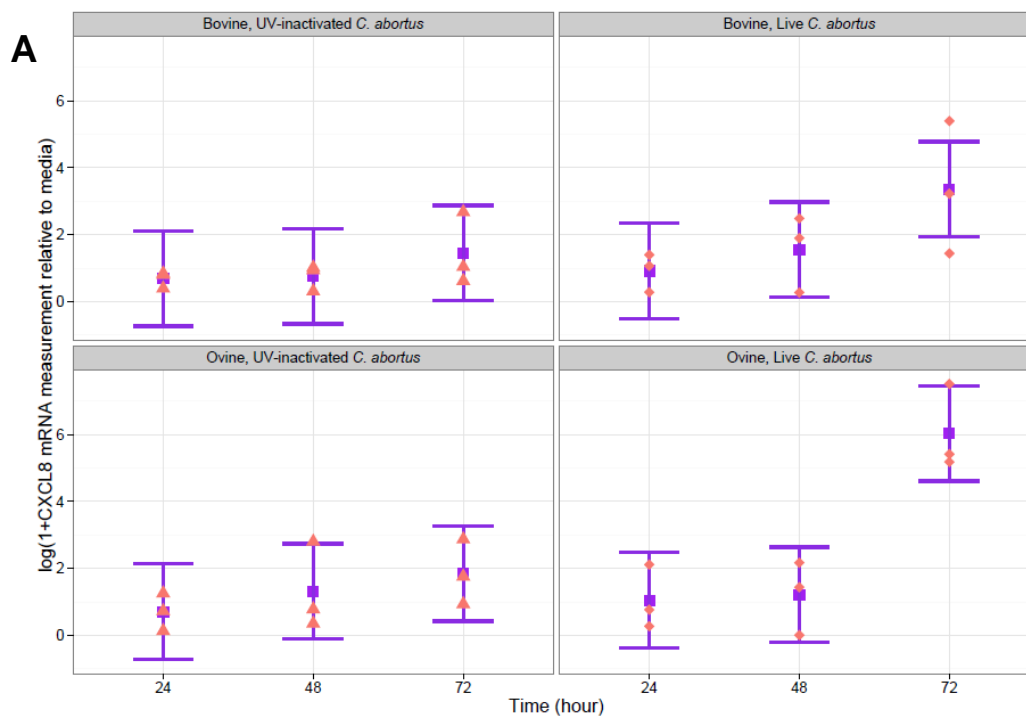


Figure 3.5: The pattern of expression of CXCL8 production from ovine and bovine turbinate cells in response to *C. abortus*. CXCL8 mRNA (A, C) and protein (B, D) was measured in ovine (A, B) and bovine (C, D) turbinate cells. CXCL8 mRNA production was measured in 4 treatment groups (UV-inactivated *C. abortus*, MOI 0.1, MOI 1, MOI 10 live *C. abortus*) calculated relative to the medium alone control at each time point. CXCL8 protein production was measured in 5 treatment groups (medium alone, UV-inactivated *C. abortus*, MOI 0.1, MOI 1, MOI 10 live *C. abortus*). The graphs are representative of results from the three passages of a primary cell line.

As there was no observable increase in CXCL8 production in response to an MOI 0.1 or MOI 1 statistical analysis was only conducted on the CXCL8 expression in response to live *C. abortus* (MOI 10) infection compared to medium alone and UV-inactivated *C. abortus*. A significant time- and treatment-effect was detected in the CXCL8 mRNA ($p < 0.05$) and protein ($p < 0.001$) in the ovine and bovine turbinate cells. The production of CXCL8 mRNA was significantly higher ($p < 0.001$) at 72 hours post-infection with live *C. abortus* compared to the UV-inactivated treatment group in both ovine and bovine turbinate cells (Figure 3.6A). The production of CXCL8 protein was also significantly higher in response to live *C. abortus* compared

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with the UV-inactivated *C. abortus* group at 72 hours post infection in both ovine ($p < 0.001$) and bovine ($p < 0.05$) turbinate cells. Significantly higher CXCL8 protein was produced in response to live *C. abortus* compared with the medium only group at 72 hours post infection in both ovine ($p < 0.001$) and bovine ($p < 0.05$) turbinate cells (Figure 3.6B). The mean CXCL8 protein production did not differ significantly ($p > 0.05$) between the medium only group and the UV-inactivated *C. abortus* challenged group at any time point in either species. There was no significant interaction effect observed between species and treatment group, and between species and time for the expression of mRNA ($p > 0.12$) and protein ($p > 0.77$). Therefore, the production of CXCL8 followed a similar trend across treatment groups and time points in both ovine and bovine turbinate cells



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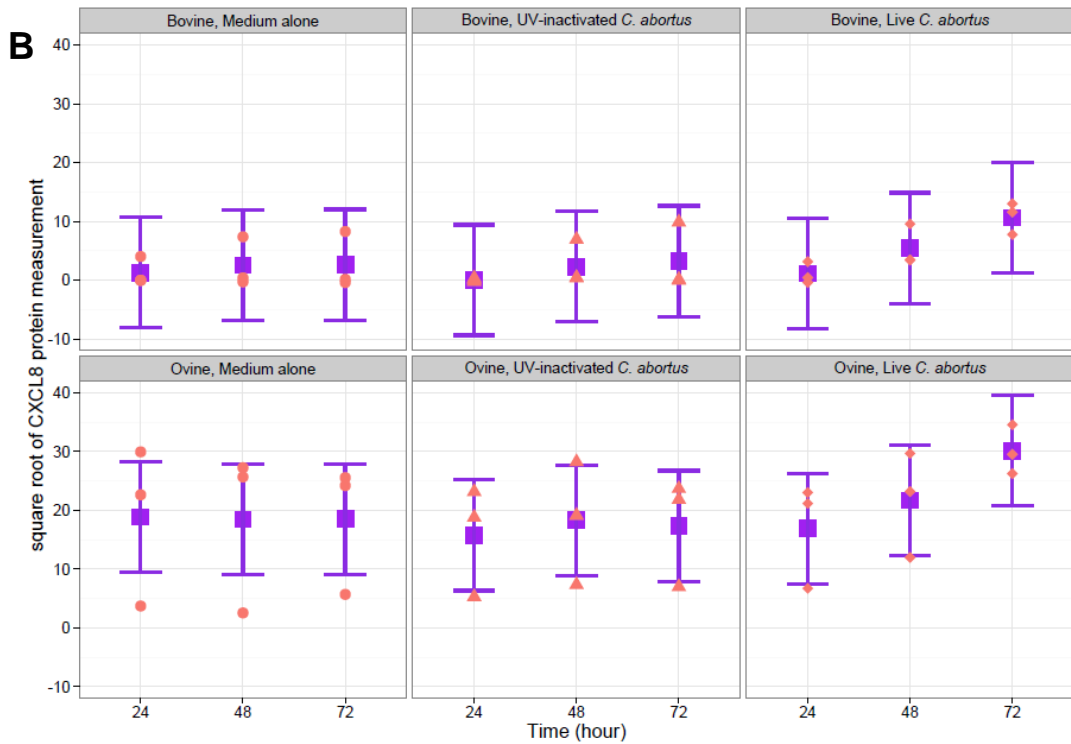


Figure 3.6: CXCL8 mRNA (A) and protein (B) production by ruminant turbinates cells in response to *C. abortus*. The expression of CXCL8 mRNA (on the logarithmic scale after adding a constant of one), measured in two treatment groups (UV-inactivated *C. abortus* and live *C. abortus*) relative to medium alone at each time point and protein (on the square root scale) measured in three treatment groups (medium alone, UV-inactivated *C. abortus* and live *C. abortus*) at three different time points (24, 48 and 72 h) in bovine and ovine turbinates cells. The plots show mRNA or protein data for each treatment group in separate panels; with shapes (circle, triangle and diamond) representing each of the three treatment groups. The mean expression is represented by a solid square with corresponding 95% confidence intervals (error bar).

3.4 Discussion

The limited availability of species-specific reagents is a major hurdle in the veterinary immunology field. An early objective was to find reagents that could be used to detect both sheep and cattle cytokines. The ELISAs for CXCL8, IL-1 β , TNF- α and IL-6 were found to be cross-reactive for both sheep and cattle, but with apparent differences in sensitivity. These differences in sensitivity may be due to the affinity of the mAbs/pAbs in the ELISAs for the two species or due to differences in the estimated concentration of the recombinant standards given that it is currently not possible to reliably measure the concentrations of CHO-expressed ovine cytokines/chemokines. This is because the culture supernatants contain other proteins (including low levels of FBS and cell-derived products) and the proteins are not tagged, making purification difficult. Validated commercially-available cytokine/chemokine ELISA kits that have standards for both cattle and sheep would be ideal and preferable for species-comparison studies, however the veterinary immunological toolkit still contains many gaps in capability (Entrican and Lunney, 2012). Hence, reproducible standard curves were generated with known concentrations of either ovine or bovine recombinant products and used to quantify cytokine/chemokine production in all further experiments.

As turbinate cells are a component of the oro-nasal mucosa, they are likely to be important sentinels of the immune system. The human nasal mucosa expresses TLR2, TLR4 (Vandermeer et al., 2004), NOD1 and NLRP3 (Bogefors et al., 2010), and this is the first study to demonstrate that turbinate cells of the ruminant oro-nasal mucosa also express these receptors. A varied complement of PRRs would be beneficial in the detection of a broad range of pathogens and induce the production of appropriate host-defence mediators. The expression of both intracellular and extracellular PRRs also suggests that ruminant turbinate cells have the capability to respond to all developmental stages of *C. abortus* infection.

Live *C. abortus* formed large inclusions within the turbinate cells. Chlamydial entry into the host cell is induced by the EB, which interacts with the plasma membrane to

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induce endocytosis (Moore and Ouellette, 2014). Inclusion size was originally thought to reflect bacterial replication; however, recent studies have identified that *C. trachomatis* inclusions expand in the absence of replication (Engstrom et al., 2015). UV-inactivated bacteria did not form inclusions within the turbinate cells. UV-inactivation damages chlamydial DNA and renders them unable to multiply (Byrne and Moulder, 1978). However, UV-inactivation does not damage components on the bacterial surface, unlike heat-killing, and the uptake into a mouse fibroblast cell line was shown not to be affected (Byrne, 1976). The fluorescent specks of bacteria that were visible following challenge with UV-inactivated *C. abortus* may be indicative of clumps of inactivated bacteria on the turbinate cell surface or within the host cell cytosol.

Previous studies using epithelial cells from mucosal surfaces have identified that they are immunoreactive to chlamydial infection. *C. pneumoniae* induced NF- κ B activation leading to CXCL8 production in human airway epithelial cells (Jahn et al, 2000). Primary endocervical epithelial cells infected with *C. trachomatis* have been shown to produce increased pro-inflammatory cytokines, including CXCL8, IL-6 and IL-1 α (Rasmussen et al., 1997). In contrast, a more recent study using polarized endocervical epithelial cells did not identify a significant up-regulation in CXCL8, IL-6 or TNF- α (Buckner et al., 2013). The ruminant turbinate cells produced CXCL8 but not IL-6, TNF- α or IL-1 β in response to *C. abortus* infection. CXCL8 is a pro-inflammatory chemokine that can function in chemotaxis driving the recruitment of immune cells including neutrophils to the site of infection. Neutrophils have been identified as potential carriers of *C. pneumoniae*, which may perpetuate the survival and dissemination of the bacteria (Rupp et al., 2009; Zandbergen et al., 2004). Therefore, the production of CXCL8 by ruminant turbinate cells may aid in the dissemination of *C. abortus* from the site of infection. Further experiments using LPS are required to determine whether the lack of production of NF- κ B dependant IL-6, TNF- α or IL-1 β is specific to *C. abortus* or an indication that turbinate cells do not respond to traditional TLR stimulation.

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Significant CXCL8 protein and mRNA production was only detected at 72 hours post *C. abortus* infection, which is late in the chlamydial developmental cycle (Longbottom and Coulter, 2003). The late production of CXCL8 has previously been identified as a feature of *C. trachomatis* infection of epithelial cells (Buchholz and Stephens, 2006; Rasmussen et al., 1997). Late CXCL8 production at 72 hours was also identified in trophoblast cells in response to live *C. abortus* (Wheelhouse et al., 2009). There are a number of potential explanations for late CXCL8 production which include: inhibition of CXCL8 production by *C. abortus* during early developmental stages, the cell PAMP threshold is not reached until the bacteria has multiplied or cell lysis is necessary for CXCL8 production (Buchholz and Stephens, 2008; O'Connell et al., 2006).

An active *C. abortus* infection was necessary for the stimulation of CXCL8 expression as no significant production was detected in response to UV-inactivated *C. abortus* compared to the medium alone control. This is consistent with findings from a previous study that demonstrated that CXCL8 production in ovine trophoblasts is dependent on challenge with live compared to UV-inactivated *C. abortus* (Wheelhouse et al., 2009). Similarly, CXCL8 production in an epithelial cell line was identified to be dependent on a live *C. trachomatis* infection. In this study the production of CXCL8 was inhibited with UV-inactivation of the *C. trachomatis* or following treatment with chlamydiacidal antibiotics (O'Connell et al., 2006). Therefore, an active replicative developmental cycle is required for the activation of CXCL8 producing signalling pathways. Previous studies have identified that the MAPK and NF- κ B signalling pathways regulated the induction of CXCL8 production following chlamydial infection (Buchholz and Stephens, 2006; Buchholz and Stephens, 2008; Rasmussen et al., 1997; Welter-Stahl et al., 2006). TLR2 and MyD88 co-localise intracellularly with the chlamydial inclusion and were identified to be necessary for CXCL8 production from an epithelial cell line challenged with *C. trachomatis* (O'Connell et al., 2006). Intracellular NOD 1 mediated production of CXCL8 in a different epithelial cell line challenged with *C. trachomatis* (Buchholz and Stephens, 2008). The ruminant turbinate cells were found to express both TLR2

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and NOD1, which indicates that *C. abortus* may activate similar signalling pathways for the production of CXCL8.

The same trend in CXCL8 production was identified within both ovine and bovine turbinate cells. There were no species specific differences identified in the production of CXCL8, IL-1 β , TNF- α or IL-6, which is interesting as it suggests that the initial course of *in vivo* infection may be similar. *C. abortus* infection has commonly been identified in cattle; however, the role for *C. abortus* in clinical disease is not fully understood. *C. abortus* infection in cattle is thought to be primarily sub-clinical, but has been associated with infertility and mastitis (Reinhold et al, 2011). Therefore, the early course of infection involving the establishment of a sub-clinical *C. abortus* infection may be similar between sheep and cattle. Following this, the turbinate cells are unlikely to play a critical role in determining the differential disease pathogenesis between sheep and cattle.

Understanding how the pathogen is first recognised by the host is important for deciphering early disease pathogenesis. In this study, the first aim to optimise and validate innate cytokine ELISAs was completed. The ELISAs were shown to be cross-reactive to both cattle and sheep, but differences in sensitivity were demonstrated. To address the second aim, the ruminant oro-nasal turbinate cells were shown to express both intracellular and extracellular PRRs, which may be important in chlamydial detection. Thirdly, *C. abortus* was shown to be capable of infecting both ovine and bovine turbinate cells to produce large intracellular inclusions. The final aim identified similar trends in cytokine and chemokine production in cattle and sheep turbinate cells in response to *C. abortus* infection: production of CXCL8 late in the chlamydial developmental cycle and undetectable levels of IL-1 β , IL-6 and TNF- α . Therefore, the production of CXCL8, IL-6, TNF- α and IL-1 β by turbinate cells in response to *C. abortus* is not a discriminating factor for disease pathogenesis between cattle and sheep.

Chapter 4: Characterizing the innate immune response of ruminant monocyte-derived cells to *C. abortus* infection

4.1 Introduction

Monocytes, DCs and macrophages are important innate immune cells that compose the mononuclear phagocyte system (MPS) (Guilliams et al., 2014). Monocytes circulate in blood and were originally thought to primarily function as precursors for mononuclear phagocytes that are resident in tissues (reviewed by Ginhoux and Jung, 2014). However, recent studies suggest that DCs can be derived from distinct DC precursor cells and most tissue macrophages have embryonic origin and can proliferate to maintain tissue-resident populations (Guilliams et al., 2014). Monocytes are recruited to sites of inflammation where they can differentiate into monocyte-derived macrophages (MDMs) or monocyte-derived DCs (MDDCs) depending on environmental stimuli (Ginhoux and Jung, 2014). Cells with dendritic-like and macrophage-like functions can be differentiated from blood monocytes *in vitro* (Guilliams et al., 2014). Monocytes differentiate into MDDCs following culture with IL-4 and GM-CSF (Peters et al., 1996) and into MDMs through adherence (Jensen et al., 2014). The ability to differentiate MDM and MDDC *in vitro* overcame the difficulties associated with *ex vivo* isolation of these cells from tissues. Consequently, these *in vitro*-differentiated cells are often used for functional experimental studies (Haniffa et al., 2015).

Monocytes, macrophages and DCs are extremely heterogeneous and this can make discrimination between them difficult (Geissmann et al., 2010). Generally, cells of the MPS can be characterized based on their cellular origin, morphology, phenotype and specialized functions. DCs were first defined by Ralph Steinman in the 1970s as cells of the mouse spleen with dendrite processes, which give them a characteristic star shape (Steinman and Cohn, 1973). The main functions of DCs are to sample the environment, sense damage and danger, and present antigens to prime naïve T cells. Macrophages are large cells with a vacuolated cytoplasm that phagocytose cell-

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derived debris and pathogens within their local environment and promote inflammation (Guilliams et al., 2014). Macrophages also have anti-inflammatory functions that may contribute to the restoration of tissue homeostasis (Murray and Wynn, 2011).

The advent of flow cytometry has enabled the phenotyping of cells based on their cell surface markers, which has been invaluable for characterizing cell subsets (Guilliams et al., 2014). Combinations of different cell surface markers have been used to discriminate between the cells of the MPS in both humans and mice (Vu Manh et al., 2015a). However, the availability of antibodies for cell surface marker detection in ruminants is more limited. We can combine mouse and human phenotyping data with studies to date in cattle, to build a likely representative picture of the ruminant MPS.

CD14 is part of the LPS receptor complex and is expressed on bovine monocytes and macrophages (Berthon and Hopkins, 1996), but is down-regulated on human MDDCs compared to monocyte precursors (Elkord et al., 2005). CD172- α is a member of the signal regulatory protein (SIRP) family and its expression is restricted to myeloid cells, including bovine monocytes, macrophages and populations of DCs (Ellis et al., 1988; Hall et al., 1993). CD11b is a β_2 -integrin involved in cell adhesion and is expressed by bovine monocytes, DCs and macrophages (Ammon et al., 2000; Howard and Naessens, 1993). The co-stimulatory markers CD80 (and CD86) and the major histocompatibility complex Class II molecules (MHCII) are expressed on antigen presenting cells, which includes macrophages and dendritic cells (Elkord et al., 2005; Santin et al., 1999). CD1w2 is a homologue of the human CD1b surface marker and is expressed by bovine cortical thymocytes and DC subsets but not monocytes. CD205 is a c-type lectin that is part of the mannose receptor family (Butler et al., 2007) and is found on bovine cortical thymocytes and DC subsets (Gliddon et al., 2004; Howard and Naessens, 1993). CD205 is considered a “gold standard” DC marker in mice (Kato et al., 2006). To summarize, the predicted expression of cell surface markers in the ruminant MPS are presented in Table 4.1.

Table 4.1: Cell surface markers that can be used to differentiate monocytes, MDMs and MDDCs. Marker expression information was collated from studies in humans, mice and cattle.

	CD14	CD172	CD11b	CD80	MHC II	CD1w2	CD205
Monocytes	+	+	+	-	-	-	-
DCs	-	+	+	+	+	+	+
Macrophages	+	+	+	+	+	-	-

Monocytes are often recruited to the site of bacterial, viral, protozoan or fungal infection. Monocyte recruitment is an important immune defence mechanism contributing to pathogen clearance but may also contribute to the induction of immunopathology if not appropriately controlled. At the site of infection, monocyte-derived cells can secrete pro-inflammatory cytokines, chemokines and antimicrobial products. They can also carry antigen to the draining lymph node for activation of the adaptive immune response (Shi and Pamer, 2011).

Monocytes are among the first immune cells to be recruited to sites of chlamydial infection where they contribute to the *in vivo* inflammatory milieu (Darville and Hiltke, 2010). *In vitro*, *Chlamydiae* have been shown to be capable of infecting mononuclear phagocytes (Datta et al., 2014; Flego et al., 2013; Jendro et al., 2000), suggesting that these cells may play a role in disease pathogenesis and host immunity. Although *Chlamydiae* infect their hosts via mucosal surfaces, they have also been implicated in disease at non-mucosal sites, for example reactive arthritis in joints (*C. trachomatis*) and coronary artery disease (*C. pneumoniae*), indicative of systemic dissemination (Beagley et al., 2009a). In the case of *C. abortus* infection in sheep, the organism is thought to infect via the oronasal route, primarily infecting epithelial cells and then accessing lymphoid tissue via the tonsil. The bacteria may then systemically disseminate through blood to the lymphoid organs, where it may reside sub-clinically until pregnancy (Kerr et al., 2005). The mechanism of dissemination of invasive species of *Chlamydiae* remains unclear (Beagley et al., 2009a). Free organisms have not been detected in blood plasma and therefore cellular

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vectors such as mononuclear phagocytes are thought to be necessary for facilitating dissemination from the site of infection. *C. pneumoniae* has been detected *in vivo* in blood monocytes following intratracheal *C. pneumoniae* infection of rabbits (Gieffers et al., 2004).

A previous study has identified that ovine alveolar macrophages produce pro-inflammatory cytokines in response to *C. abortus* (Entrican et al., 1999). However, the effects of *C. abortus* infection in different monocyte-derived cell subsets in ruminants have never been investigated. The innate immune response of these cells to *C. abortus* may be important for the differential pathogenesis of the infection in sheep and cattle. With this in mind, the objectives of this chapter were to:

- Characterize ruminant monocytes, MDDCs and MDMs.
- Determine the expression of TLR2, TLR4, NOD1 and NLRP3 by ruminant MDDCs/MDMs.
- Assess TLR2, TLR4, NOD1 and NLRP3 expression in response to *C. abortus* infection.
- Assess innate immune cytokine/chemokine production in response to *C. abortus* infection.

4.2 Materials and methods

4.2.1 CD14⁺ monocytes, MDMs and MDDCs

Ruminant monocytes, MDMs and MDDCs were generated as described in section 2.2.

4.2.2 Kwik-Diff™ staining

MDMs (1×10^5) were deposited onto glass slides using the Shandon Cytospin 3 (Thermoscientific) at 700rpm for 10 minutes. The Kwik-Diff™ kit (Thermoscientific) was used to fix and stain the cells with eosin and methylene blue following the manufacturer's instructions. Slides were mounted with DPX mountant (Sigma-Aldrich).

4.2.3 Phagocytosis assay

MDMs were seeded in 8 well chamberslides at 1×10^5 cells per well and incubated for 24 hours at 37°C. The Zymosan A (*S. cerevisiae*) Bioparticles™ labeled with Fluorescein (Life Technologies) were then added at 100 beads per cell and incubated for 1 hour at 37°C. Ice-cold 1xPBS was added to stop phagocytosis and the cells were washed 4 times with the 1xPBS to remove extracellular beads. The cells were fixed in 4% PFA before they were mounted using ProLong Gold anti-fade reagent containing DAPI.

4.2.4 Non-specific esterase staining

Non-specific esterase (NSE) staining was detected in MDMs using the α -Naphthyl Acetate kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cells were cytopun onto slides as described in section 4.2.2 and fixed in Citrate-Acetone-Formaldehyde for 30 seconds before rinsing with deionized water. The slides were incubated for 30 minutes at 37°C in a solution containing Sodium Nitrite solution, Fast Blue BB Base solution, TRIZMAL™ 7.6 buffer concentrate and α -Naphthyl Acetate solution. The slides were rinsed in deionized water and counterstained in Haematoxylin solution, Gill No. 3 for 2 minutes. The non-specific

esterase kit forms black deposits if the α -naphthyl esterase is hydrolysed by non-specific esterases, which are primarily found in monocytes, macrophages and histocytes.

4.2.5 Phenotyping ruminant monocytes, MDDCs and MDMs

The ruminant monocytes, MDDCs and MDMs were phenotyped using the flow cytometry protocol described in section 2.11 and the data analysed as described in section 4.2.6.

4.2.6 Gating strategy for flow cytometry

The cells were first gated on forward and side scatter to exclude debris to generate population 1 (P1) for further gating (Figure 4.1A). The cells within P1 were then gated on live cells by analysing the live/dead cell vioblue fluorescent stain to generate population 2 (P1/P2) (Figure 4.1B). The P1/P2 cells were then gated to exclude doublets using forward scatter area (FSC-A) and forward scatter height (FSC-H) to generate population 3 (P1/P2/P3) (Figure 4.1C). The level of FITC or PE fluorescence from the cells within the P1/P2/P3 gate was assessed. Quadrants were set on the isotype-matched controls so that cells were gated on FITC/PE expression to generate population 4 (P1/P2/P3/UL4) (Figure 4.1D) to assess the percentage positive cells. The isotype-matched control was overlaid onto the stained population and presented in histogram format (Figure 4.1E). Median fluorescent intensities (MFIs) were calculated by subtracting the median fluorescence of the P1/P2/P3 isotype control population from the P1/P2/P3 marker population.

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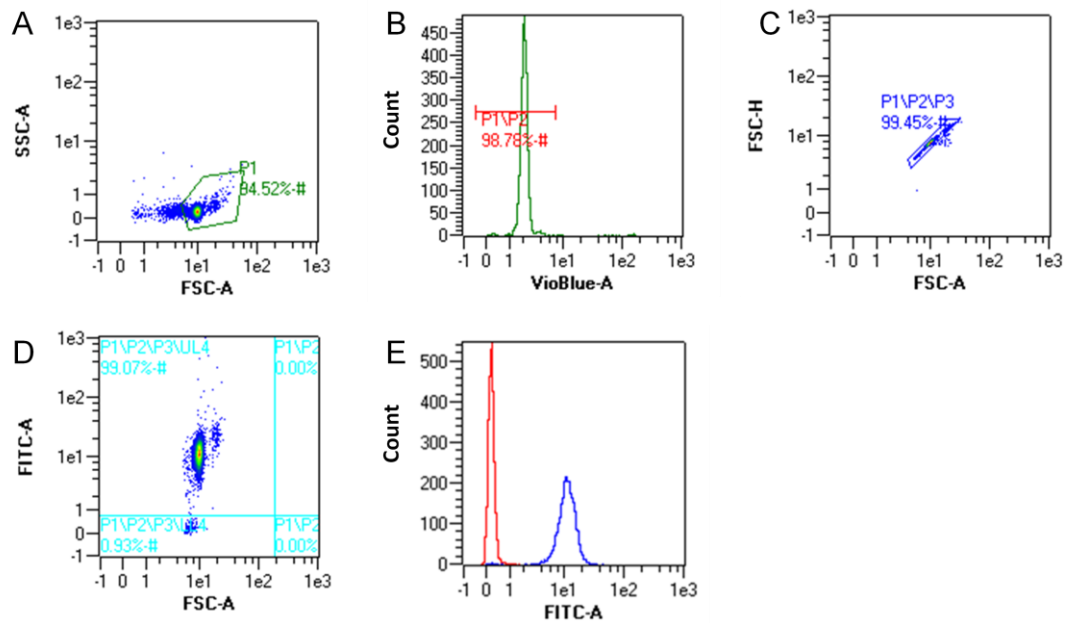


Figure 4.1: The gating strategy used for the phenotyping of monocytes, MDDCs and MDMs. The cells were gated based on forward and side scatter (A), live cells (B), single cells (C) and FITC or PE fluorescence with quadrants based on the isotype matched control (D). The fluorescence of the marker population (blue histogram) was overlaid with the isotype control (red histogram) (E). The figure is an example of the gating strategy on CD14⁺ ovine monocytes.

4.2.7 Infection of ruminant MDMs and MDDCs

Ruminant MDMs and MDDCs were infected with *C. abortus* S26/3 at an MOI 10, 1 or 0.1 diluted in culture medium. Cells were also exposed to UV-inactivated *C. abortus* (equivalent to MOI10) and culture medium alone. Supernatants and cell lysates were collected at 6, 24, 48 and 72 hours post infection and stored at -80°C for further analyses (described in section 2.4). The experiments were repeated on four separate occasions.

4.2.8 Immunofluorescent detection of *C. abortus*

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The MDMs were seeded at 1×10^5 and MDDCs at 5×10^5 in chamber slides and infected according to section 4.2.7 before fixing and staining using the protocol detailed in section 2.5.

4.2.9 Detection of innate immune cytokine/chemokine protein

The production of CXCL8, IL6, TNF- α and IL-1 β protein by ruminant MDMs and MDDCs was assessed using cytokine sandwich ELISAs, as described in section 2.6, on the harvested supernatant.

4.2.10 Detection of PRR expression using S-PCR

The expression of NOD1, NLPR3, TLR2 and TLR4 by ruminant MDMs and MDDCs was assessed using standard PCR (as described in section 2.8) on cDNA (generated as described in section 2.7).

4.2.11 Detection of PRRs and innate immune cytokine/chemokine mRNA using RT-PCR

The expression of TLR2, TLR4, NOD1, NLRP3, CXCL8, IL-6, TNF- α and IL-1 β was detected in ruminant MDM 6 hour post-infection samples using RT-PCR (described in section 2.9) on cDNA (generated as described in section 2.7). 18s rRNA was used as a reference gene as it was expressed at a stable level across treatment groups and animal replicates with the average SD of 0.18 in ovine MDMs and 0.27 in bovine MDMs.

4.2.12 Statistical analyses

The MFIs were assessed for normality using the Anderson-Darling test. Non-normal data were log-transformed. The difference between the cell types was assessed using a one way ANOVA. For the cattle, CD11b staining could not be transformed into normal distributions and therefore a non-parametric Kruskal-Wallis test was conducted followed by pairwise Mann-Whitney U tests. The difference between the species was assessed using two-sample T-tests following log-transformation. The CD205 monocytes and CD11b DCs cross-species comparison could not be

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transformed into normal distributions and therefore comparisons were made with the Mann-Whitney U test.

The fold change RT-PCR data were tested for normality using the Anderson-Darling test. As the data were non-normally distributed they were rank transformed (Beasley et al., 2009). The different treatment groups were analysed using a one-way ANOVA and comparisons between treatments made using Tukey's pairwise comparison test which resulted in adjusted (controlling for the family error rate for multiple comparisons) p-values. The cross-species comparison was completed using a general linear model with species, treatment and species*treatment fixed factors.

The cytokine/chemokine protein data were tested for normality using the Anderson-Darling test. As the data were not normally distributed they were rank transformed. Linear mixed models were used to assess the fixed effects: time, treatment and time*treatment with animal set as a random effect. Dr Javier Palarea used zero inflated general linear mixed models to assess two of the data sets (ovine MDDC IL-1 β and bovine MDDC IL-1 β) which had inflated numbers of zeros. Zero-inflated modelling could not be applied to three of the data sets (bovine MDM TNF- α , ovine MDDC TNF- α and ovine macrophage IL-1 β) due to the fact that all of the medium-alone values were zero. Removing the medium-alone group allowed the data (bovine MDM TNF- α and ovine MDDC TNF- α) to be analysed using the LMM. If there was a significant F-statistic ($p < 0.05$) from the linear mixed models, two-sided probabilities of comparisons of means were obtained. The probabilities were adjusted using a FDR approach (Benjamini and Hochberg, 1995). However, the one remaining data set (ovine MDM IL-1 β) had a number of zeros in other treatment groups so could not be analysed using a mixed model approach and therefore summary measures (Matthews et al., 1990), which involves calculating area under the curve, was used. The Kruskal-Wallis test was used to assess the effect of treatment and the pairwise comparisons completed using the Mann-Whitney U test.

The requirement for different statistical models for the cytokine/chemokine protein data sets meant that the cross-species comparison could not be incorporated into a

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single model. Quantitative differences were assessed using the area under the curve of cytokine production from the MOI10 treatment group and compared between species using a two-sample T-test. The MOI10 treatment group was chosen because there was production of cytokine across all data sets and it is the treatment extreme likely to have the largest response.

Statistical analysis for the RT-PCR and flow cytometry data was conducted using Minitab v. 17. The linear mixed models were conducted using GenStat (GenStat version 16.2) and the zero inflated GLMM using R (R Core Team, version 3.1).

4.3 Results

4.3.1 Ruminant MDDC morphology

The ruminant MDDCs developed dendrite like protrusions but appeared to develop differently between cattle and sheep. The bovine MDDCs developed in clusters and many became non-adherent (Figure 4.2 A, B), whereas the ovine MDDCs were largely adherent and confluent (Figure 4.2 C, D).

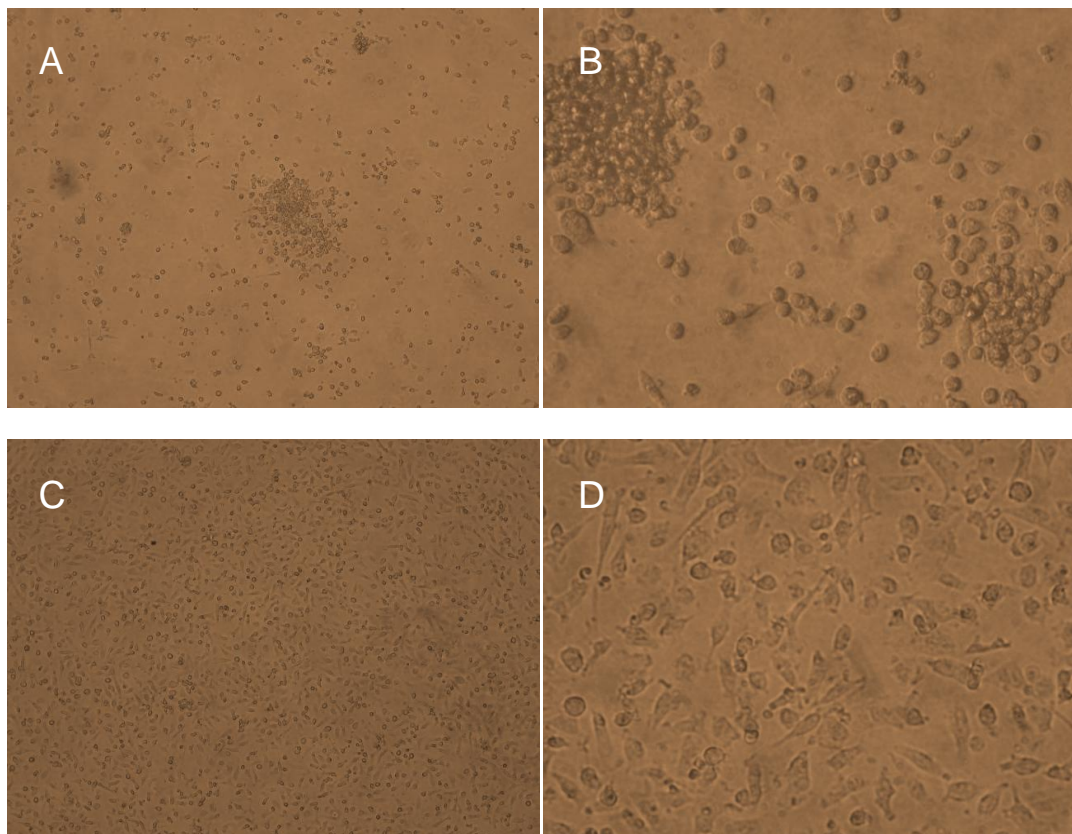


Figure 4.2: Comparison of bovine (A, B) and ovine (C, D) MDDCs following 3 and 6 days in culture respectively. The MDDCs were visualized using light microscopy at x10 (A, C) and x40 (B, D) magnification.

4.3.2 Ovine MDM morphology and function

The production of ovine MDMs required optimisation as the published protocol for the generation of bovine MDMs (Jensen et al., 2014) was not effective for sheep. The replacement of the FBS with autologous serum in the protocol resulted in the

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generation of macrophage-like cells from ovine PBMC. The ovine cells were characteristically large and vacuolated with irregular nuclei (Figure 4.3A, B). The ovine cells also expressed non-specific esterases and were capable of phagocytosis (Figure 4.3C, D), characteristic of macrophages.

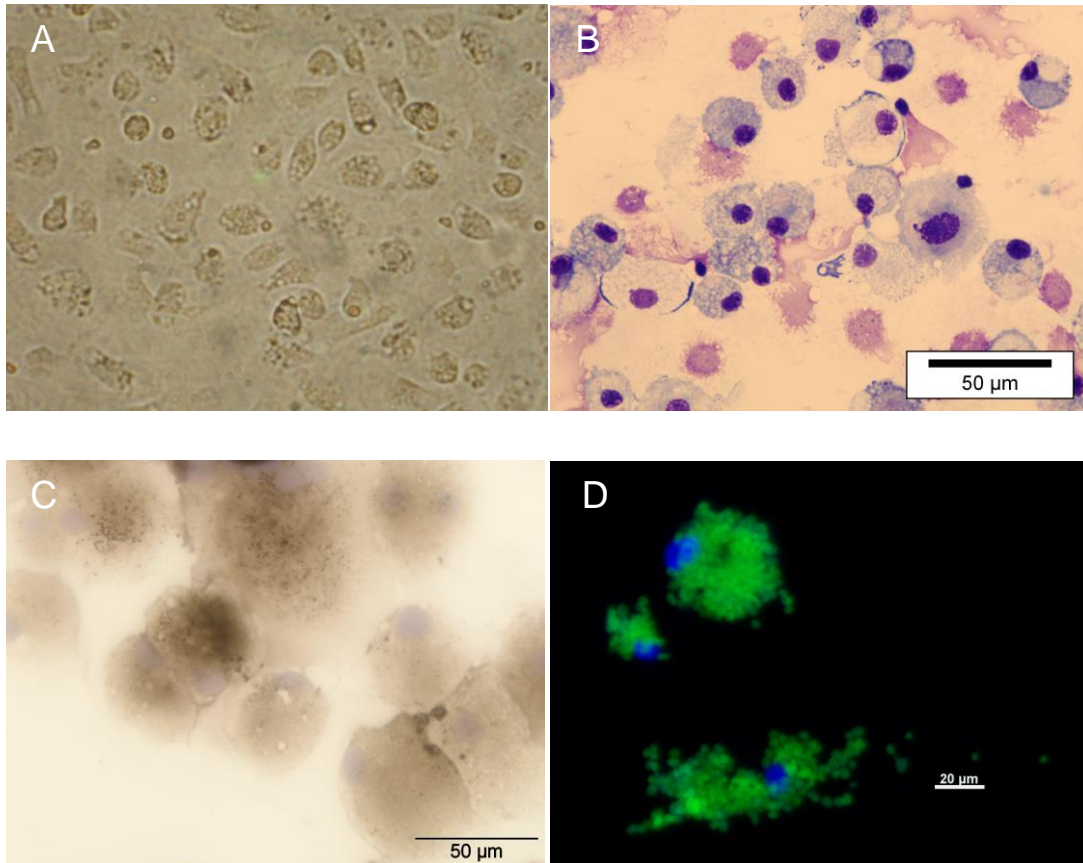


Figure 4.3: Characterisation of ovine MDMs. The cells were analysed microscopically (x40 magnification) (A), using a Kwik-Diff stain (B), using non-specific esterase staining (C) and using fluorescein labelled phagocytic beads (D).

4.3.3 Phenotypic analysis of ruminant MPS cells

Flow cytometric analysis of bovine (Figure 4.4) and ovine (Figure 4.5) monocytes demonstrated that they expressed CD172- α , MHCII, CD11b, CD14 and CD80 but not CD1w2 or CD205.

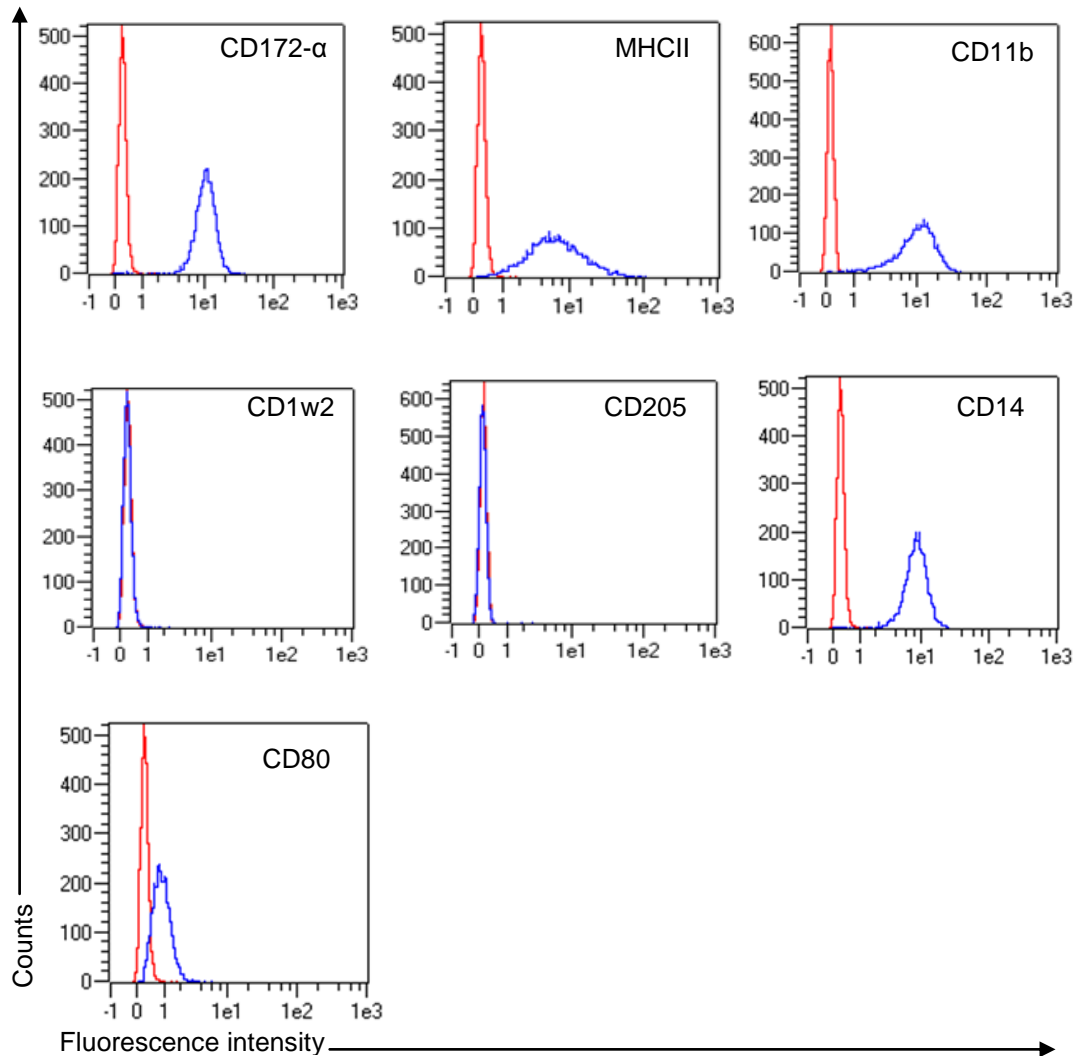


Figure 4.4: Expression of cell surface markers by bovine monocytes. The red histograms represent the isotype-matched controls and the blue histograms represent cell surface markers (CD172- α , MHCII, CD11b, CD1w2, CD205, CD14 and CD80). The histograms are from one animal but are representative of the profiles observed in four animals.

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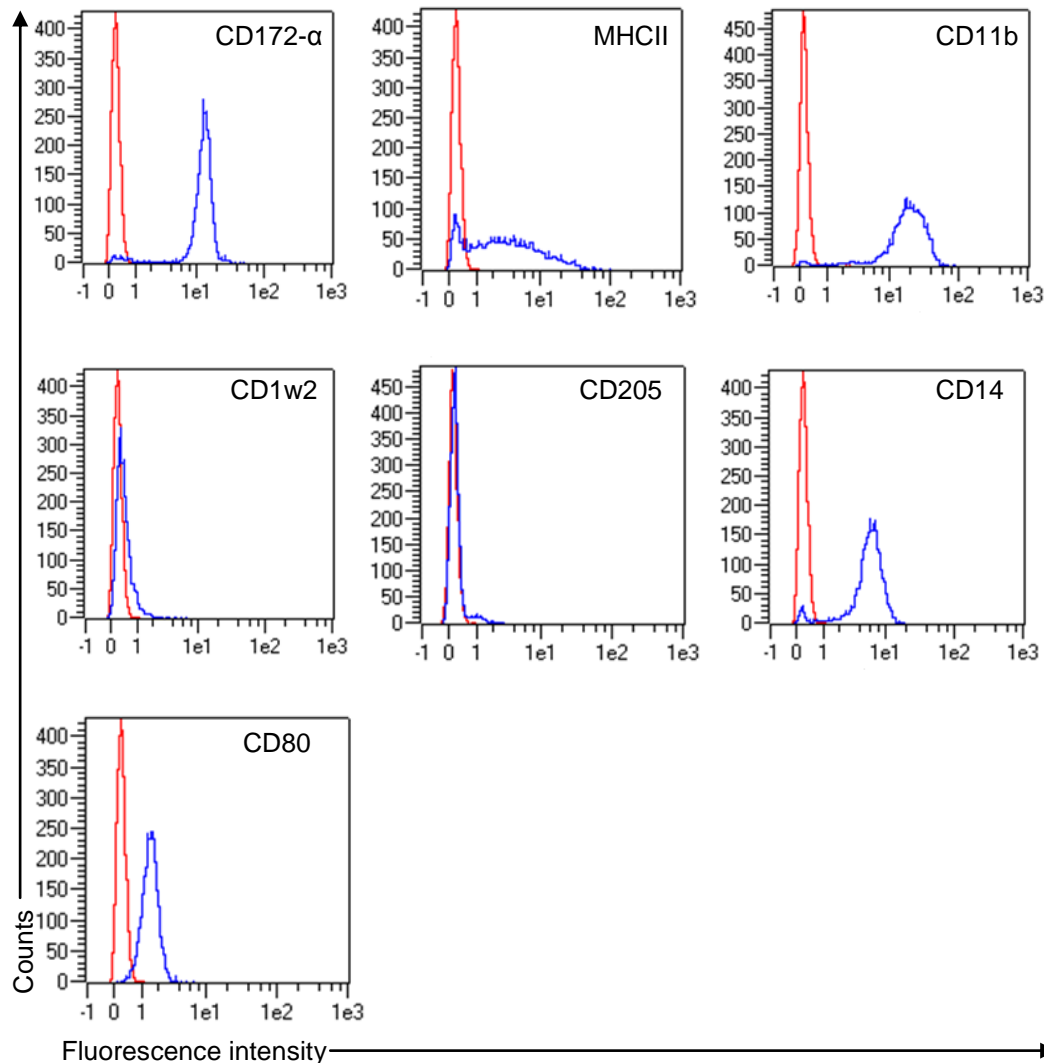


Figure 4.5: Expression of cell surface markers by ovine monocytes. The red histograms represent the isotype-matched controls and the blue histograms represent cell surface markers (CD172- α , MHCII, CD11b, CD1w2, CD205, CD14 and CD80). The histograms are from one animal but are representative of the profiles observed in four animals.

Bovine MDDCs expressed CD172- α , CD11b, CD80, MHCII and CD14 but not CD205 or CD1w2 (Figure 4.6).

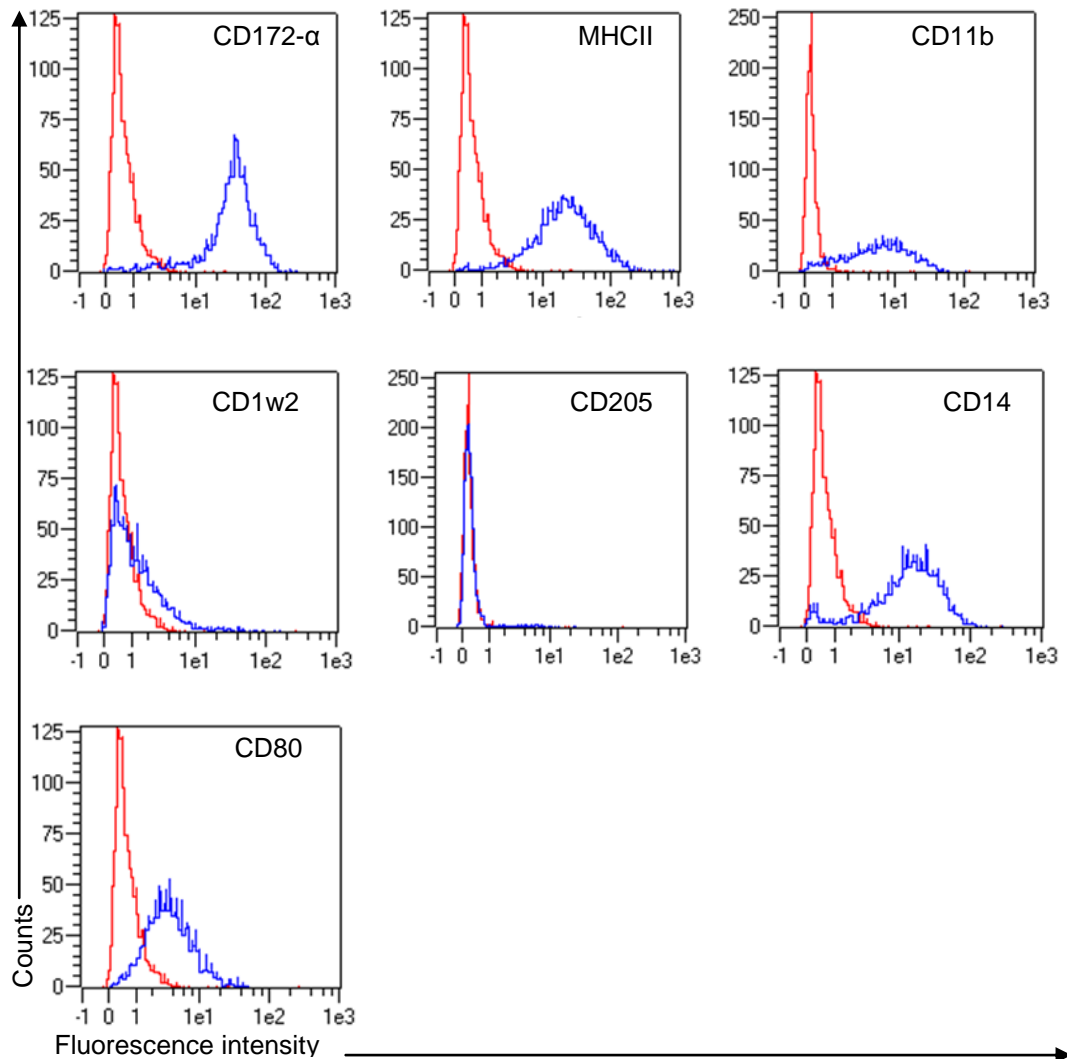


Figure 4.6: Expression of cell surface markers by bovine MDDCs. The red histograms represent the isotype-matched controls and the blue histograms represent cell surface markers (CD172- α , MHCII, CD11b, CD1w2, CD205, CD14 and CD80). The histograms are from one animal but are representative of the profiles observed in four animals.

Ovine MDDCs expressed CD11b, CD80 and CD172- α ; however, two populations of cells with different levels of expression for some of the markers (MHCII, CD11b and CD14) were apparent (Figure 4.7).

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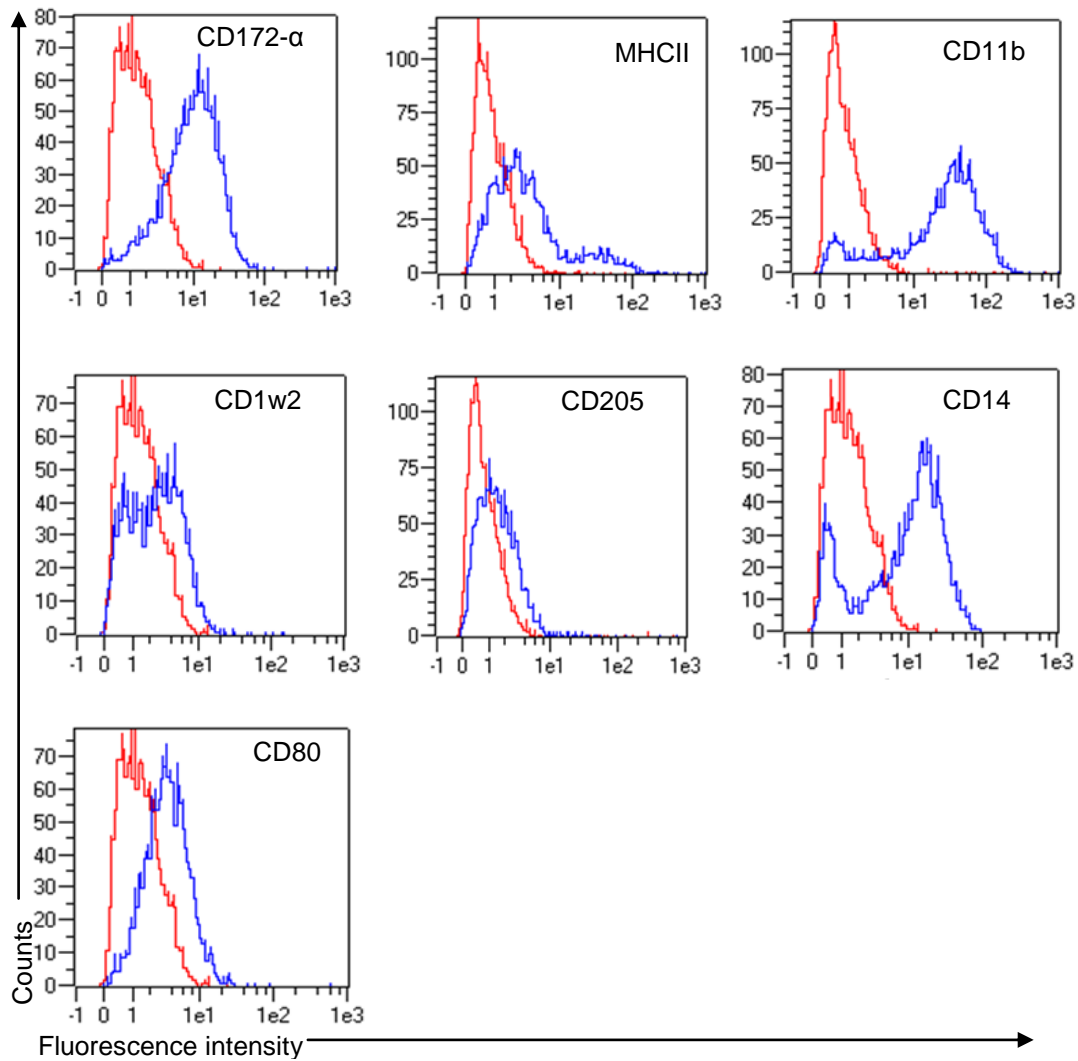


Figure 4.7: Expression of cell surface markers by ovine MDCs. The red histograms represent the isotype-matched controls and the blue histograms represent cell surface markers (CD172- α , MHCII, CD11b, CD1w2, CD205, CD14 and CD80). The histograms are from one animal but are representative of the profiles observed in four animals.

Bovine (Figure 4.8) and ovine (Figure 4.9) MDMs expressed CD172- α , MHCII, CD11b, CD1w2, CD14 and CD80. The ovine MDMs also expressed CD205 unlike the bovine MDMs.

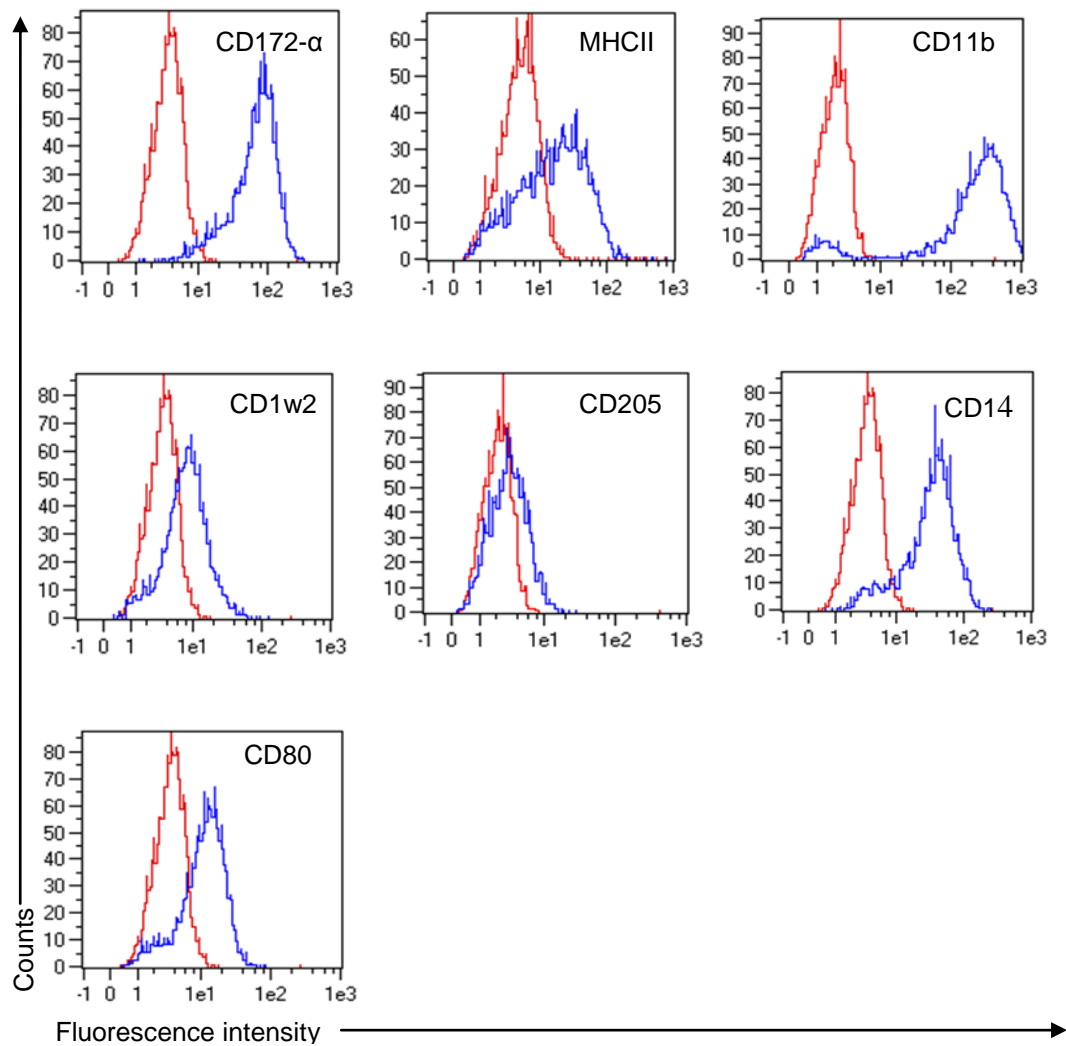


Figure 4.8: Expression of cell surface markers by bovine MDMs. The red histograms represent the isotype-matched controls and the blue histograms represent cell surface markers (CD172- α , MHCII, CD11b, CD1w2, CD205, CD14 and CD80). The histograms are from one animal but are representative of the profiles observed in four animals.

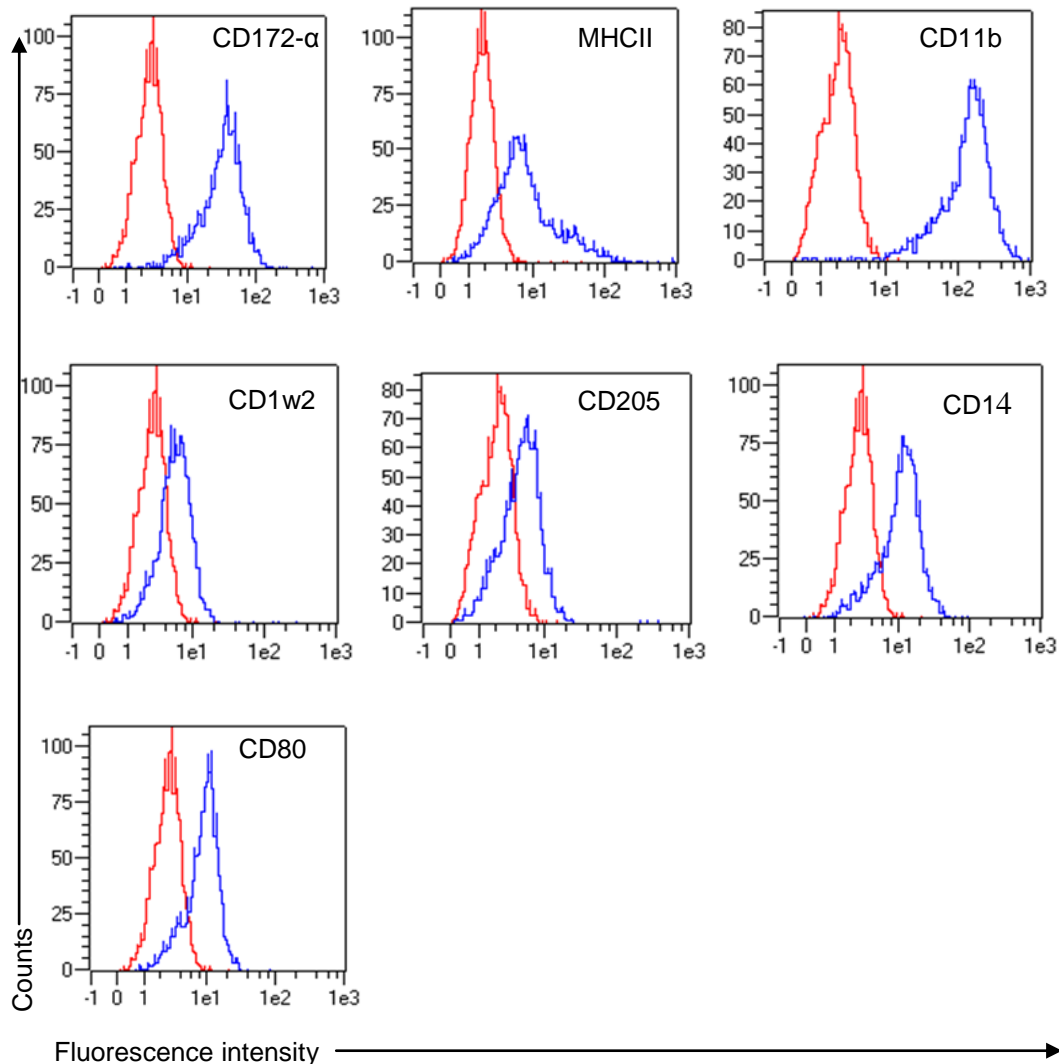


Figure 4.9: Expression of cell surface markers by ovine MDMs. The red histograms represent the isotype-matched controls and the blue histograms represent cell surface markers (CD172- α , MHCII, CD11b, CD1w2, CD205, CD14 and CD80). The histograms are from one animal but are representative of the profiles observed in four animals.

4.3.3.1 Comparison of the percentage positivites and median fluorescent intensities of monocytes, MDDCs and MDMs

The percentage positivites of markers for the different cell types were calculated in sheep (Table 4.2) and cattle (Table 4.3). High percentages of all of the MPS cells expressed CD14, CD11b and CD172 α . Most of the ovine and bovine monocytes and MDMs expressed MHC II and CD80. The percentage positivity of CD80 in ovine and bovine MDDCs was reduced relative to the monocytes. The percentage positivity of MHCII in ovine MDDCs but not bovine MDDCs was reduced

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compared to the monocytes. The percentage of cells expressing CD1w2 and CD205 was low in monocytes but increased with differentiation into MDDCs and MDMs in both cattle and sheep.

The median fluorescent intensities (MFIs) were calculated for the markers for the different cell types in sheep (Table 4.4) and cattle (Table 4.5). There was no significant difference detected in CD14 comparing monocytes to MDDCs or MDMs ($p>0.05$). The MFI of CD11b was significantly up-regulated in both the ovine and bovine MDMs and MDDCs compared to the monocytes ($p<0.05$). The expression of CD11b was significantly higher in MDMs than MDDCs ($p<0.05$). There was a significant up-regulation of MHCII in ovine MDMs compared to MDDCs ($p<0.05$) but no significant differences between the other cell subsets or between bovine cells ($p>0.05$). The MFI of CD172- α was significantly up-regulated in MDMs compared to monocytes and MDDCs in both cattle and sheep ($p<0.05$). CD80 was significantly up-regulated in MDMs compared to monocytes in both cattle and sheep ($p<0.01$) and also in MDDCs compared to monocytes in cattle ($p<0.01$). CD205 expression was significantly up-regulated in ovine and bovine MDMs compared to monocytes and MDDCs ($p<0.01$). The expression of CD205 was up-regulated in ovine MDDCs compared to monocytes ($p<0.01$) but not in bovine MDDCs ($p>0.05$). CD1w2 expression was significantly up-regulated in ovine and bovine MDMs and also ovine MDDCs compared to monocytes ($p<0.05$). There was a significant up-regulation in CD1w2 expression in MDMs compared to MDDCs in ovine ($p<0.01$) but not bovine cells.

The comparison of MFIs between cattle and sheep (Table 4.6) revealed significantly increased expression of MHC II in all bovine compared to ovine mononuclear cells ($p<0.05$). CD11b expression was significantly increased in ovine compared to bovine MDDCs ($p<0.05$) but increased in bovine compared to ovine MDMs ($p<0.01$). CD205 expression was significantly higher in ovine compared to bovine MDDCs ($p<0.05$) and MDMs ($p<0.01$). The expression of CD1w2 was significantly higher in ovine compared to bovine monocytes and MDDCs ($p<0.05$). There was no

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significant difference between cattle and sheep in CD80, CD172- α or CD14 MFIs
($p>0.05$).

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Table 4.2: Percentage positivity of cell surface markers on ovine MPS cells. The percentage positivity means \pm SE.

	CD14	CD1w2	CD172- α	CD80	CD11b	CD205	MHC II
Ovine monocytes	92.63 \pm 2.23	13.69 \pm 3.11	96.3 \pm 0.84	70.11 \pm 16.18	98.40 \pm 0.34	4.30 \pm 0.39	70.66 \pm 3.33
Ovine MDDCs	64.41 \pm 8.36	16.07 \pm 4.67	70.79 \pm 7.04	37.05 \pm 13.30	78.09 \pm 5.96	19.54 \pm 6.47	22.25 \pm 4.06
Ovine MDMs	85.36 \pm 12.17	28.04 \pm 8.92	93.86 \pm 1.28	72.88 \pm 9.48	99.25 \pm 0.44	63.29 \pm 24.24	55.36 \pm 8.69

Table 4.3: Percentage positivity of cell surface markers on bovine MPS cells. The percentage positivity means \pm SE.

	CD14	CD1w2	CD172- α	CD80	CD11b	CD205	MHC II
Bovine monocytes	99.00 \pm 0.15	1.68 \pm 0.27	99.04 \pm 0.15	64.28 \pm 4.36	99.07 \pm 0.16	1.49 \pm 0.24	97.96 \pm 1.05
Bovine MDDCs	86.57 \pm 2.35	9.18 \pm 1.33	89.84 \pm 3.26	45.77 \pm 3.71	87.93 \pm 1.02	3.03 \pm 0.34	93.28 \pm 2.33
Bovine MDMs	84.37 \pm 0.90	20.81 \pm 3.32	95.19 \pm 1.05	55.17 \pm 5.98	95.05 \pm 1.47	7.19 \pm 3.3	57.37 \pm 10.09

Table 4.4: MFIs and p-values of surface markers on ovine MPS cells. The MFIs \pm SE. Green values represent significant differences ($p < 0.05$).

	CD14	CD1w2	CD172- α	CD80	CD11b	CD205	MHC II
MFIs							
Ovine monocytes	5.92 \pm 0.77	0.16 \pm 0.03	11.71 \pm 0.86	1.06 \pm 0.34	15.81 \pm 2.05	0.01 \pm 0.01	1.84 \pm 0.30
Ovine MDDCs	9.75 \pm 2.22	1.56 \pm 0.26	10.87 \pm 1.09	4.04 \pm 0.99	31.36 \pm 3.91	1.53 \pm 0.36	1.73 \pm 0.62
Ovine MDMs	16.45 \pm 5.02	1.94 \pm 0.48	20.78 \pm 3.84	6.60 \pm 0.99	136.39 \pm 11.93	3.27 \pm 0.24	3.41 \pm 0.19
P-values							
Monocytes - MDDCs	0.510	0.030	0.900	0.075	0.008	0.005	0.981
Monocytes - MDMs	0.080	0.008	0.048	0.003	<0.001	<0.001	0.057
MDDCs - MDMs	0.411	0.675	0.024	0.130	<0.001	0.002	0.043

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Table 4.5: MFIs and p-values of surface markers on bovine MPS cells. The MFIs \pm SE. The values highlighted in green represent significant differences ($p < 0.05$).

	CD14	CD1w2	CD172- α	CD80	CD11b	CD205	MHC II
MFIs							
Bovine monocytes	8.59 \pm 0.77	0.03 \pm 0.01	17.95 \pm 4.74	0.77 \pm 0.07	12.42 \pm 0.85	0.02 \pm 0.01	9.08 \pm 1.91
Bovine MDDCs	16.02 \pm 1.33	0.40 \pm 0.08	16.79 \pm 6.02	2.59 \pm 0.26	5.34 \pm 0.15	0.08 \pm 0.07	16.1 \pm 1.69
Bovine MDMs	20.09 \pm 5.54	2.87 \pm 0.70	45.49 \pm 9.19	6.61 \pm 1.66	225.99 \pm 15.38	0.59 \pm 0.15	14.47 \pm 2.82
P-values							
Monocytes - MDDCs	0.301	0.106	0.992	0.004	0.0304	0.749	0.114
Monocytes - MDMs	0.085	<0.001	0.024	<0.001	0.0304	0.002	0.245
MDDCs - MDMs	0.673	0.002	0.040	0.032	0.0304	0.005	0.861

Table 4.6: The p-values of the comparison of ovine and bovine cell surface marker MFIs from MPS cells. The values highlighted in green represent significant differences ($p < 0.05$).

	CD14	CD1w2	CD172- α	CD80	CD11b	CD205	MHC II
Monocytes	0.057	0.017	0.332	0.468	0.202	0.6198	0.033
MDDCs	0.073	0.025	0.564	0.251	0.0304	0.028	0.004
MDMs	0.648	0.325	0.068	0.997	0.006	0.001	0.030

4.3.4 Expression of TLR2, TLR4, NOD1 and NLRP3 by ruminant monocyte-derived cells

The ruminant MDDCs (Figure 4.10) and MDMs (Figure 4.11) expressed TLR2, TLR4, NOD1 and NLRP3 as shown using S-PCR.

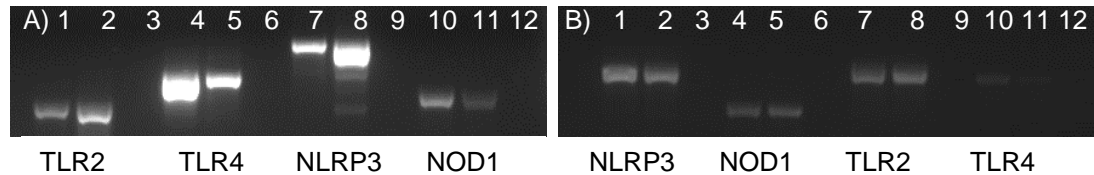


Figure 4.10: Expression of NLRP3, NOD1, TLR2 and TLR4 by bovine (A) and ovine (B) MDDCs. Total RNA was extracted from ruminant MDDCs and Con A stimulated PBMC, which was then reverse transcribed and PRRs amplified with specific primers using S-PCR. No reverse transcriptase controls were used as negative controls. Lanes 1, 4, 7 and 10: DCs; Lanes 2, 5, 8 and 11: PBMC; Lanes 3, 6, 9 and 12: no reverse transcriptase control. Products obtained were of the expected molecular weight (Table 2.2).

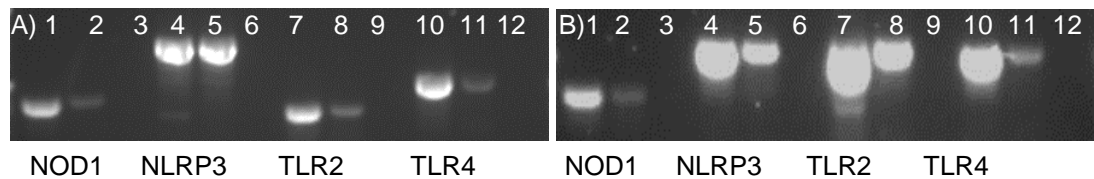


Figure 4.11: Expression of NLRP3, NOD1, TLR2 and TLR4 by bovine (A) and ovine (B) MDMs. Total RNA was extracted from ruminant MDMs and Con A stimulated PBMC, which was then reverse transcribed and PRRs amplified with specific primers using S-PCR. No reverse transcriptase controls were used as negative controls. Lanes 1, 4, 7 and 10: MDMs; Lanes 2, 5, 8 and 11: PBMC; Lanes 3, 6, 9 and 12: no reverse transcriptase control. Products obtained were of the expected molecular weight (Table 2.2).

4.3.5 Immunofluorescent images of *C. abortus*-infected monocyte-derived cells

The bovine and ovine MDDCs were susceptible to *C. abortus* infection, shown by the multiple small inclusions of varying fluorescent intensity. Green fluorescent specks were also visible in the UV-inactivated control but not in the medium alone control (Figure 4.12 and Figure 4.13).

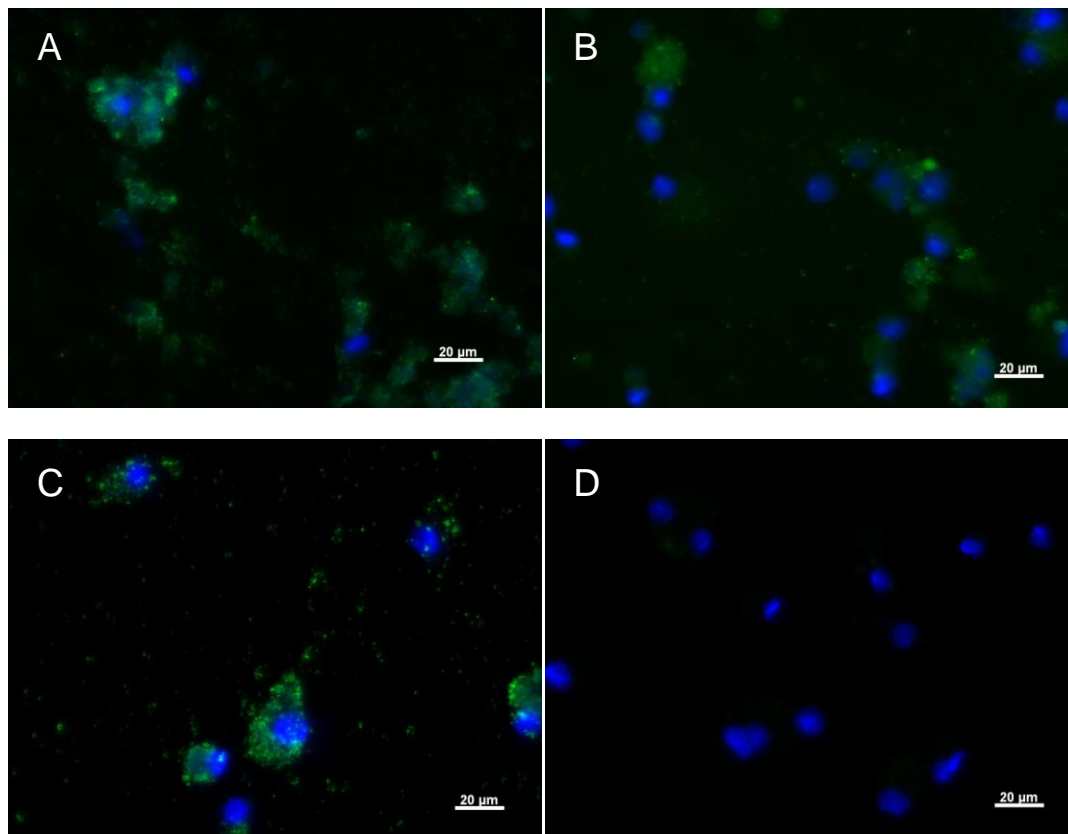


Figure 4.12: Immunofluorescent images of bovine MDDCs demonstrating susceptibility to *C. abortus* infection. *C. abortus* was labelled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody. The host cell nuclei were stained blue with DAPI. Bovine MDDCs were exposed to MOI 10 live *C. abortus* (A), MOI 1 live *C. abortus* (B), UV-inactivated *C. abortus* (MOI 10 equivalent) (C) and medium alone (D) for 48 hours. The scale bars correspond to 20µm.

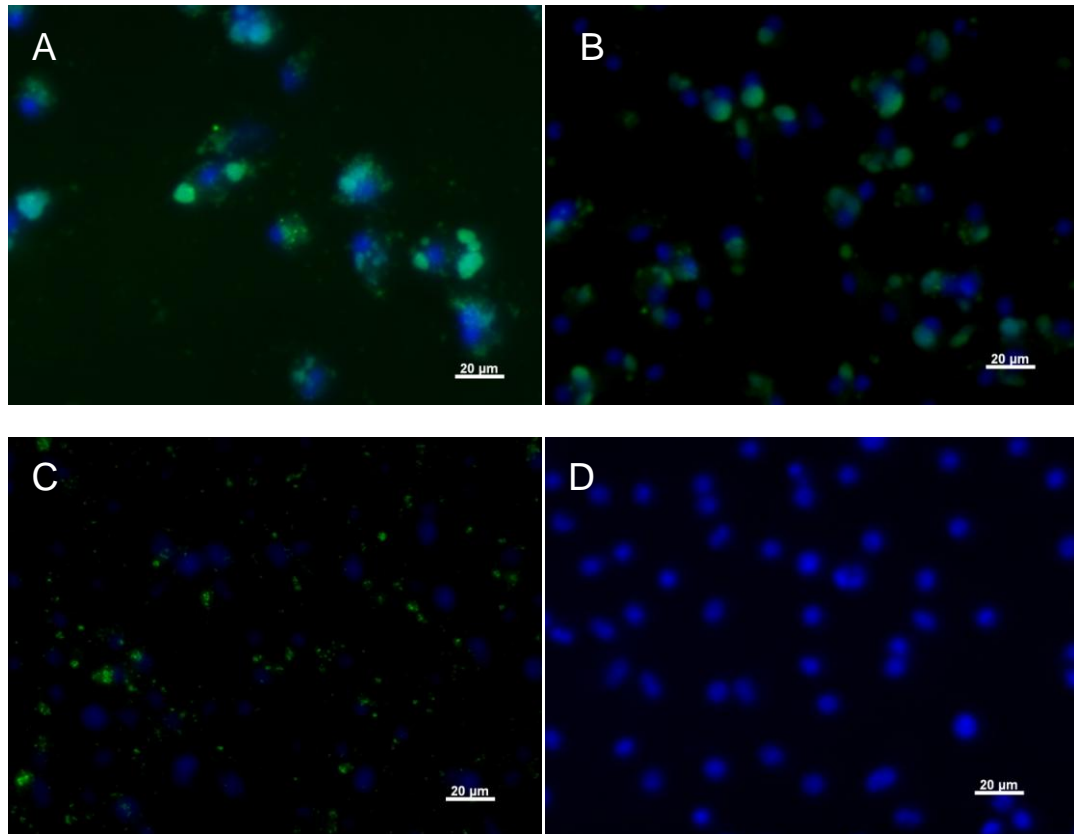


Figure 4.13: Immunofluorescent images of ovine MDDCs demonstrating susceptibility to *C. abortus* infection. *C. abortus* was labelled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody. The host cell nuclei were stained blue with DAPI. Ovine MDDCs were exposed to MOI 10 live *C. abortus* (A), MOI 1 live *C. abortus* (B), UV-inactivated *C. abortus* (MOI 10 equivalent) (C) and medium alone (D) for 48 hours. The scale bars correspond to 20µm.

The bovine and ovine MDMs were also susceptible to *C. abortus* infection with multiple small inclusions visible following infection with live *C. abortus*. Green fluorescent specks were visible in the UV-inactivated control but not in the medium alone control (Figure 4.14 and Figure 4.15).

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

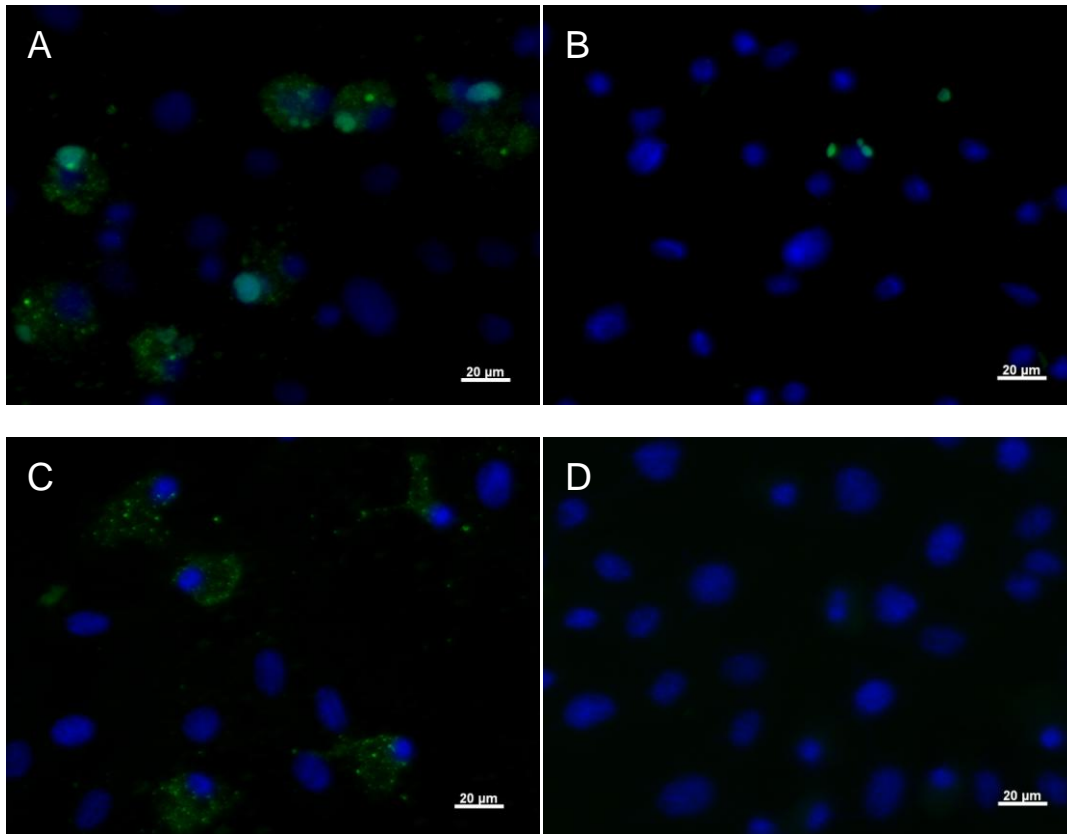


Figure 4.14: Immunofluorescent images of bovine MDMs demonstrating susceptibility to *C. abortus* infection. *C. abortus* was labelled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody. The host cell nuclei were stained blue with DAPI. Bovine MDMs were exposed to MOI 10 live *C. abortus* (A), MOI 1 live *C. abortus* (B), UV-inactivated *C. abortus* (MOI 10 equivalent) (C) and medium alone (D) for 48 hours. The scale bars correspond to 20µm.

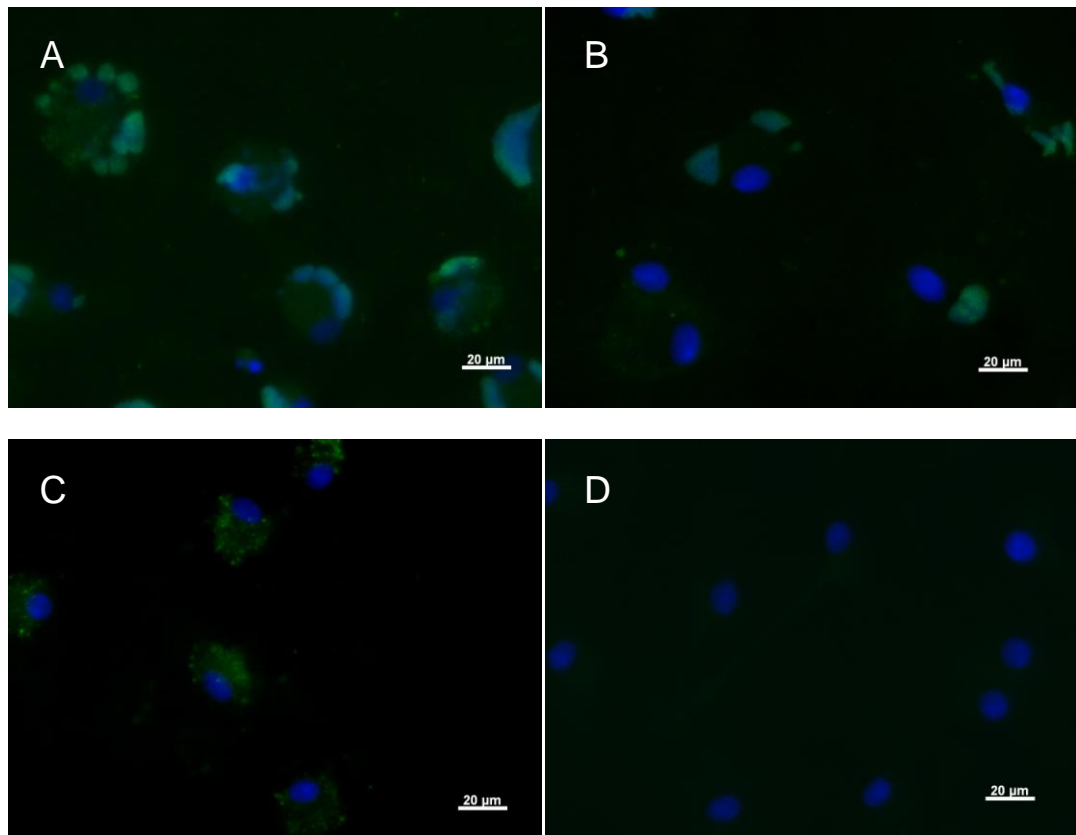


Figure 4.15: Immunofluorescent images of ovine MDMs demonstrating susceptibility to *C. abortus* infection. *C. abortus* was labelled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody. The host cell nuclei were stained blue with DAPI. Ovine MDMs were exposed to MOI 10 live *C. abortus* (A), MOI 1 live *C. abortus* (B), UV-inactivated *C. abortus* (MOI 10 equivalent) (C) and medium alone (D) for 48 hours. The scale bars correspond to 20µm.

4.3.6 PRR and cytokine/chemokine mRNA expression by ruminant MDMs following *C. abortus* infection

The mRNA expression of IL-1 β , IL-6, CXCL8, TNF- α , NOD1, NLRP3, TLR2 and TLR4 was assessed in bovine MDMs in response to medium alone, MOI 0.1, 1, 10 and UV-inactivated *C. abortus* at 6 hours post-infection by RT-PCR. The data are presented as fold change relative to the medium alone control. There was an up-regulation of all of the mRNA targets in response to the different treatments except from NOD1, which appears to be down-regulated with MOI10 and UV-inactivated organisms. There was animal to animal variation in the quantity of production as shown by the large standard errors (Table 4.7).

Table 4.7: Cytokine/chemokine and PRR mRNA fold changes from bovine MDMs treated with medium alone, MOI 0.1, MOI 1, MOI 10 and UV-inactivated *C. abortus*. The results are presented as mean fold change \pm standard error compared to the medium alone control. The experiment was repeated with MDMs from 4 different animals with the exception of a missing MOI 0.1 sample.

	Medium alone	MOI 0.1	MOI 1	MOI 10	UV-inactivated
IL-1β	1	7.17 \pm 3.69	302.96 \pm 249.24	3262.11 \pm 2675.43	6402.99 \pm 5493.23
IL-6	1	1.07 \pm 0.12	3.50 \pm 1.36	29.21 \pm 23.83	11.93 \pm 5.96
CXCL8	1	1.81 \pm 0.66	9.46 \pm 4.42	83.37 \pm 62.78	63.32 \pm 38.87
TNF-α	1	4.11 \pm 1.41	40.77 \pm 30.87	77.33 \pm 26.28	137.24 \pm 84.98
NOD1	1	1.09 \pm 0.06	1.11 \pm 0.07	0.68 \pm 0.17	0.61 \pm 0.14
NLRP3	1	1.80 \pm 0.08	4.05 \pm 0.72	8.01 \pm 1.32	13.51 \pm 3.64
TLR2	1	0.92 \pm 0.10	1.58 \pm 0.14	1.87 \pm 0.25	1.85 \pm 0.28
TLR4	1	1.02 \pm 0.21	1.41 \pm 0.17	1.94 \pm 0.52	1.55 \pm 0.56

The bovine MDM fold change values were not normally distributed and therefore the values were rank-transformed before they were graphed on interval plots (Figure 4.16) and statistically analysed (Table 4.8).

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

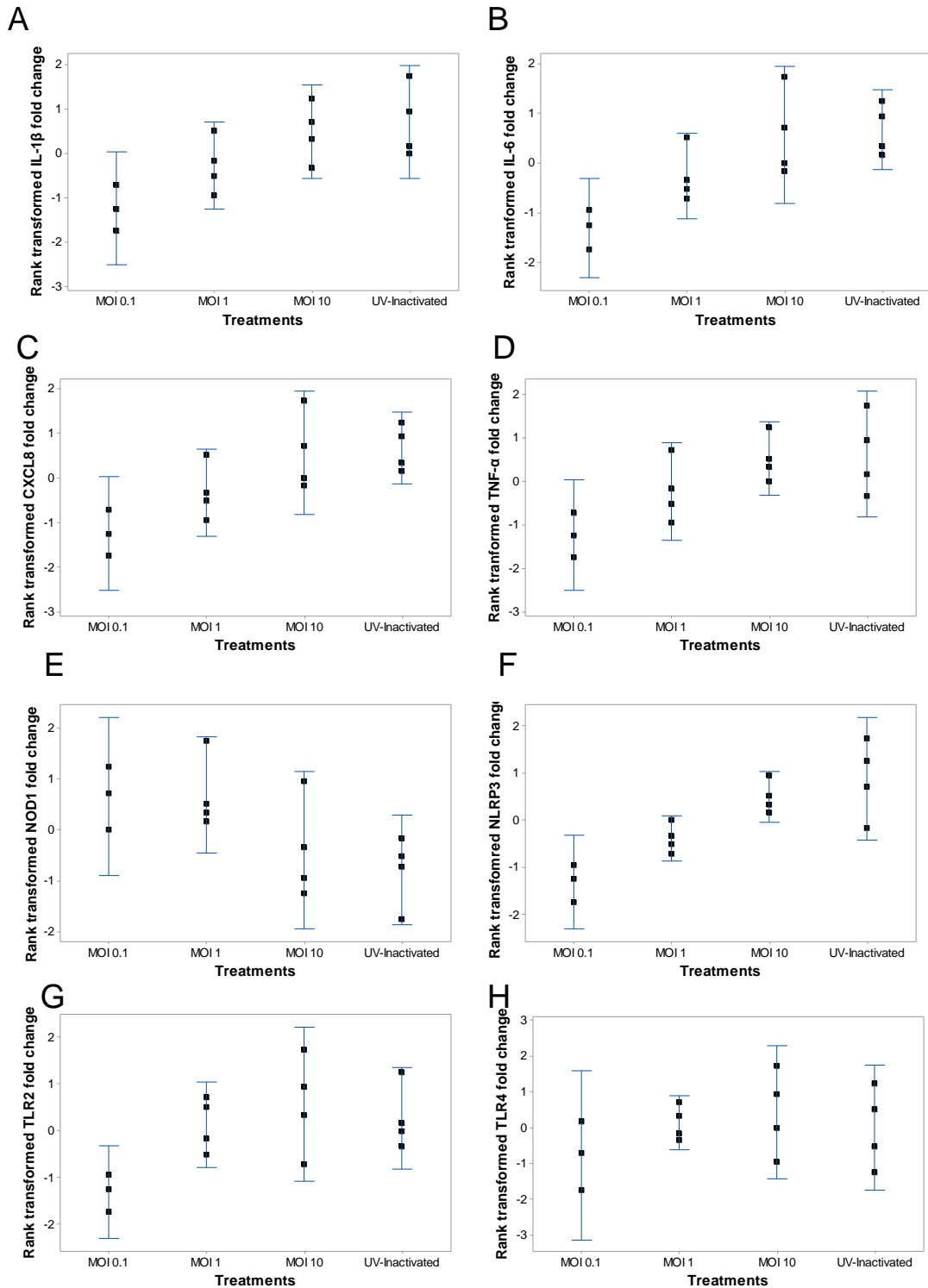


Figure 4.16: The mRNA expression of IL-1 β (A), IL-6 (B), CXCL8 (C), TNF- α (D), NOD1 (E), NLRP3 (F), TLR2 (G) and TLR4 (H) following treatment of bovine MDMs with MOI 0.1, 1 and 10 or UV-inactivated *C. abortus* at the equivalent of MOI 10 for 6 hours. The fold change was calculated by comparing expression to the medium alone control. The

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

results are presented as rank transformed fold change (black squares) \pm 95% confidence intervals (blue error bars). The results are representative of repeat experiments using MDMs from 4 different animals with the exception of a missing MOI 0.1 sample.

There was a significant fold increase in IL-1 β , IL-6, CXCL8 and TNF- α mRNA expression in response to the MOI10 compared to the MOI 0.1 (Table 4.8, all with $p < 0.05$). There was a significant increase in NLRP3 and TLR2 expression with MOI 10 compared to MOI 0.1 (Table 4.8, both with $p < 0.05$). The UV-inactivated organisms compared to the MOI 0.1 produced significantly higher IL-1 β , IL-6, CXCL8, TNF- α and NLRP3 mRNA expression (Table 4.8, all with $p < 0.05$). There was no significant difference between UV-inactivated organisms and the MOI 10 in any of the mRNA targets. There was no significant difference in TLR4 or NOD1 mRNA expression between any of the treatment groups (Table 4.8, all with $p > 0.05$).

Table 4.8: The p-values from the statistical comparison of fold changes between the different treatments in bovine MDMs. Data were analysed using a one-way ANOVA and comparisons between treatments made using Tukey's pairwise comparison. The values highlighted in green represent significant differences ($p < 0.05$).

Comparisons	IL-1 β	IL-6	CXCL8	TNF- α	NOD1	NLRP3	TLR2	TLR4
MOI1 – MOI 0.1	0.294	0.179	0.310	0.286	1	0.342	0.101	0.625
MOI 10 – MOI 0.1	0.027	0.010	0.018	0.030	0.331	0.001	0.028	0.403
UV-inactivated – MOI 0.1	0.013	0.007	0.013	0.022	0.126	0.003	0.069	0.732
MOI 10 – MOI 1	0.405	0.281	0.269	0.454	0.249	0.027	0.831	0.971
UV-inactivated – MOI1	0.214	0.202	0.196	0.349	0.083	0.062	0.994	0.997
UV-inactivated – MOI 10	0.964	0.995	0.996	0.996	0.890	0.963	0.932	0.917

The mRNA expression of IL-1 β , IL-6, CXCL8, TNF- α , NOD1, NLRP3, TLR2 and TLR4 was also assessed in ovine MDMs at 6 hours in response to the same treatment groups. There is an up-regulation of the expression of all the mRNA targets in response to the different treatments (Table 4.9).

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Table 4.9: Cytokine/chemokine and PRR mRNA fold change from ovine MDMs treated with medium alone, MOI 0.1, MOI 1, MOI 10 and UV inactivated *C. abortus*. The results are presented as mean fold change \pm standard error compared to the medium alone control. The results are representative of repeat experiments using MDMs from four different animals.

	Medium alone	MOI 0.1	MOI 1	MOI 10	UV-inactivated
IL-1β	1	4.35 \pm 1.95	98.58 \pm 76.49	2385.83 \pm 1175.68	2195.60 \pm 1018.83
IL-6	1	1.24 \pm 0.13	28.24 \pm 21.88	128.33 \pm 39.28	537.08 \pm 240.03
CXCL8	1	2.77 \pm 0.71	7.47 \pm 4.40	28.71 \pm 17.19	29.06 \pm 17.18
TNF-α	1	2.22 \pm 0.68	8.42 \pm 4.50	33.08 \pm 6.97	36.57 \pm 11.97
NOD1	1	1.32 \pm 0.11	1.19 \pm 0.15	1.41 \pm 0.26	1.55 \pm 0.30
NLRP3	1	1.19 \pm 0.21	1.82 \pm 0.26	5.10 \pm 0.71	4.87 \pm 0.59
TLR2	1	2.02 \pm 0.50	3.57 \pm 1.12	5.08 \pm 0.93	6.28 \pm 0.84
TLR4	1	1.34 \pm 0.12	1.32 \pm 0.18	1.72 \pm 0.21	1.83 \pm 0.18

The ovine MDM fold change values were not normally distributed and therefore the values were rank-transformed before they were graphed on interval plots (Figure 4.17) and statistically analysed (Table 4.10).

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

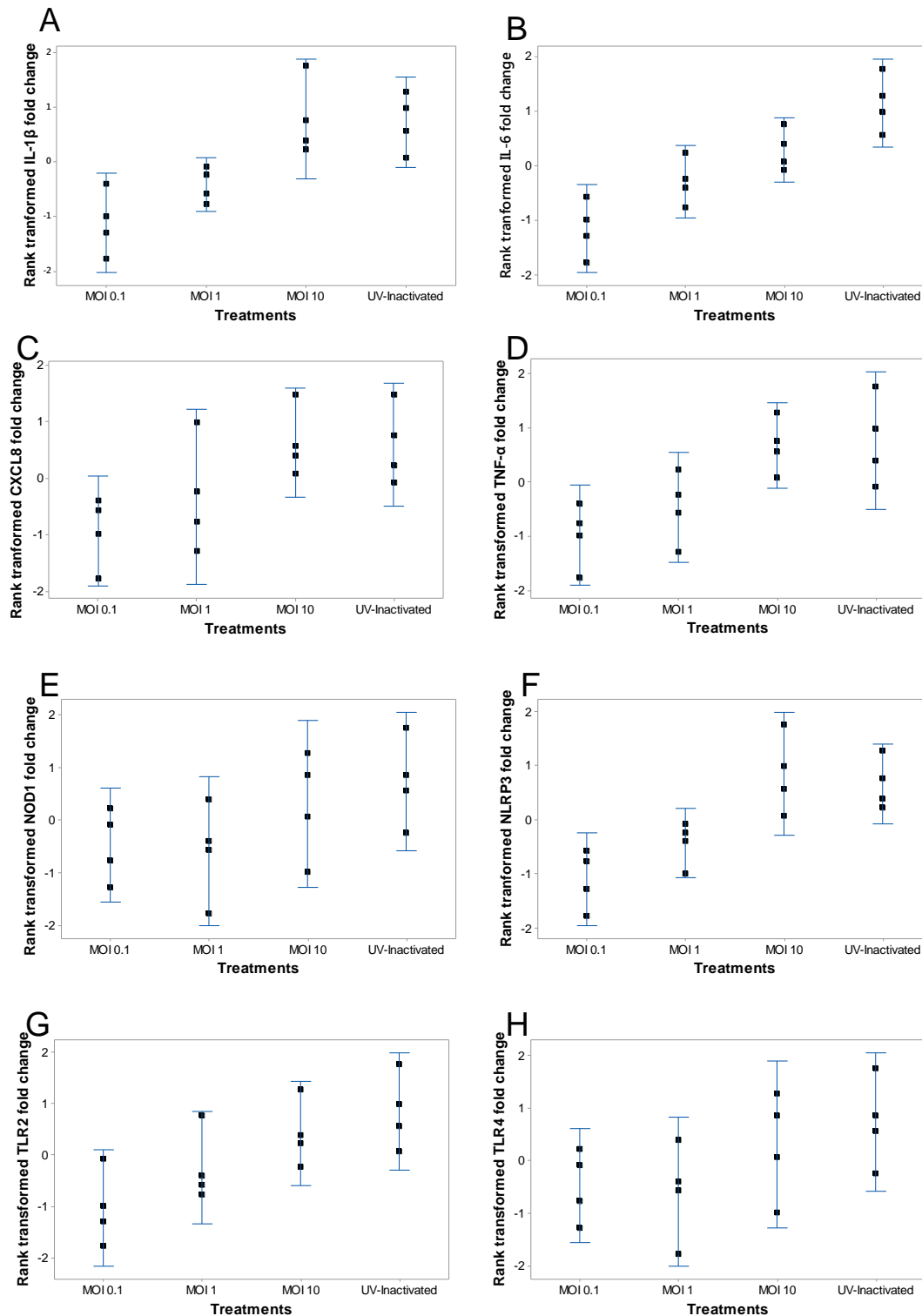


Figure 4.17: The mRNA expression of IL-1 β (A), IL-6 (B), CXCL8 (C), TNF- α (D), NOD1 (E), NLRP3 (F), TLR2 (G) and TLR4 (H) following treatment of ovine MDMs with MOI 0.1, 1 and 10 or UV-inactivated *C. abortus* at the equivalent of MOI 10 for 6 hours. The fold change was calculated by comparing expression to the medium alone control. The

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results are presented as rank transformed fold change (black squares) \pm 95% confidence intervals (blue error bars). The results are representative of repeat experiments using MDMs from four different animals.

There was a significant increase in IL-1 β , IL-6, CXCL8 and TNF- α mRNA expression in response to an MOI 10 compared to an MOI 0.1 *C. abortus* (Table 4.10, all with $p < 0.05$), indicating a dose dependant increase in cytokine production. There was a significant increase in NLRP3 and TLR2 expression in response to the MOI 10 compared to MOI 0.1 (Table 4.10, both with $p < 0.05$). NLRP3 and TLR2 expression was also significantly up-regulated in response to UV-inactivated *C. abortus* compared to the MOI 0.1 (Table 4.10, both with $p < 0.05$). There was no significant difference between UV-inactivated organisms and the MOI 10 in any of the mRNA targets. There was no significant difference in NOD1 or TLR4 expression between any of the different treatment groups (Table 4.10, all with $p > 0.05$).

Table 4.10: The p-values from the statistical comparison of fold change between the different treatments in ovine MDMs. Data were analysed using a one-way ANOVA and comparisons between treatments made using Tukey's pairwise comparison. The values highlighted in green represent significant differences ($p < 0.05$).

Comparisons	IL-1 β	IL-6	CXCL8	TNF- α	NOD1	NLRP3	TLR2	TLR4
MOI1 – MOI 0.1	0.311	0.079	0.654	0.671	0.998	0.149	0.404	0.998
MOI 10 – MOI 0.1	0.002	0.003	0.046	0.015	0.587	0.004	0.049	0.587
UV – 0.1	0.002	0	0.051	0.010	0.240	0.001	0.010	0.240
10 - 1	0.038	0.315	0.303	0.107	0.481	0.131	0.546	0.481
UV-1	0.050	0.003	0.329	0.075	0.182	0.022	0.166	0.182
UV – 10	0.998	0.079	1	0.996	0.890	0.709	0.811	0.890

4.3.6.1 Cross-species mRNA comparison

The level of mRNA expression was compared between sheep and cattle to assess whether there was a difference between species (Table 4.11). Bovine MDMs expressed significantly higher TNF- α (Table 4.11, $p < 0.05$) and NLRP3 (Table 4.11, $p < 0.01$), whereas ovine MDMs expressed significantly higher IL-6, TLR2 and

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NOD1 (Table 4.11, $p < 0.01$). There was no significant difference between species in expression of IL-1 β or CXCL8. The only significant interaction effect of treatment and species was with NOD1 (Table 4.11, $p < 0.05$), which appeared to be down-regulated in bovine MDMs but up-regulated in ovine MDMs although the treatment effect was not significant.

Table 4.11: The p-values from the statistical comparison of sheep and cattle mRNA fold changes. The values highlighted in green represent significant differences ($p < 0.05$).

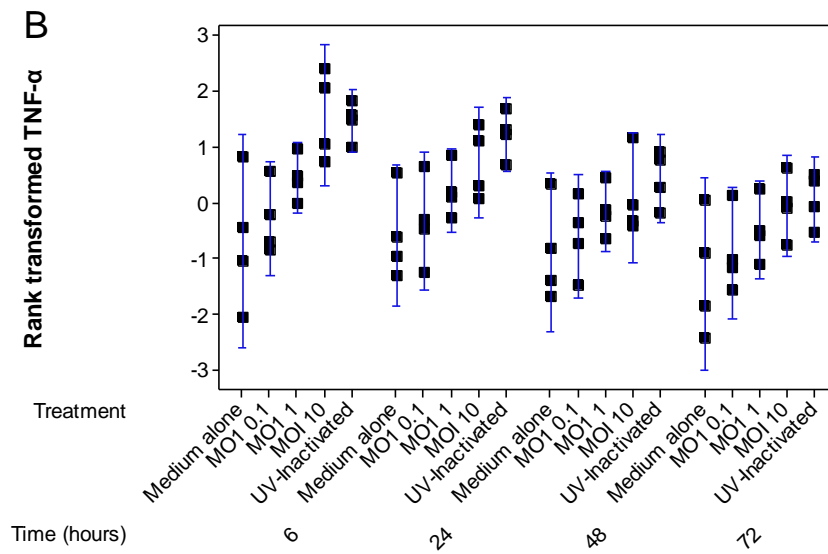
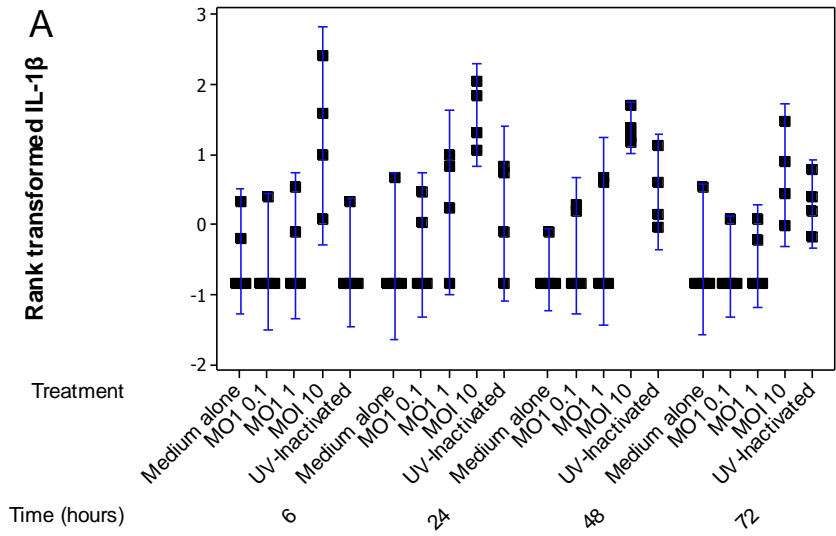
	IL-1 β	IL-6	CXCL8	TNF- α	NOD1	NLRP3	TLR2	TLR4
Treatment	0	0	0	0	0.997	0	0	0.163
Species	0.762	0	0.469	0.043	0	0	0	0.339
Treatment * Species	0.809	0.094	0.404	0.983	0.004	0.819	0.518	0.564

4.3.7 Innate immune cytokine/chemokine protein production by ruminant monocyte-derived cells in response to *C. abortus* infection

The production of CXCL8, IL-6, TNF- α and IL-1 β protein in response to medium alone, MOI 0.1, 1, 10 and UV-inactivated *C. abortus* was assessed in ruminant MDM and MDDC supernatant by ELISA. There was no IL-6 production detected from any of the cell types. The production of CXCL8, TNF- α and IL-1 β in response to medium alone, MOI 0.1, 1, 10 and UV-inactivated *C. abortus* data was rank transformed and graphed using interval plots.

The bovine MDDCs produce IL-1 β , TNF- α and CXCL8 in response to the different treatments (Figure 4.18).

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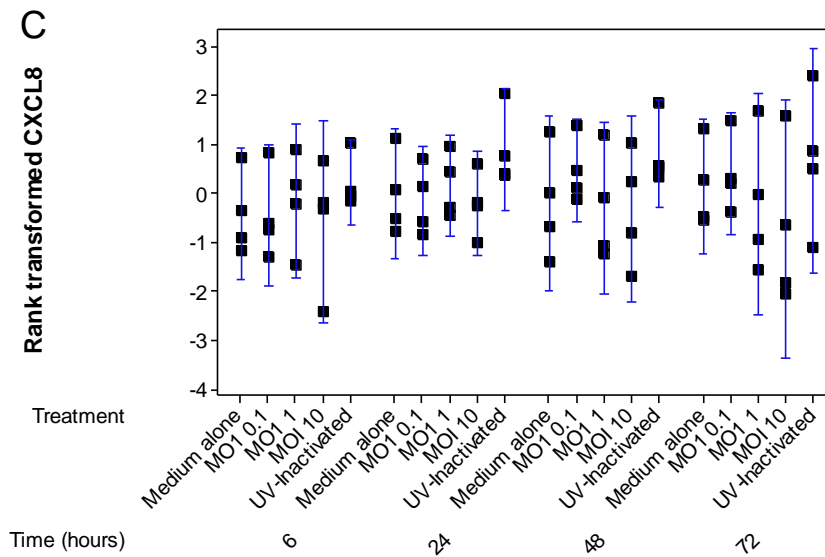


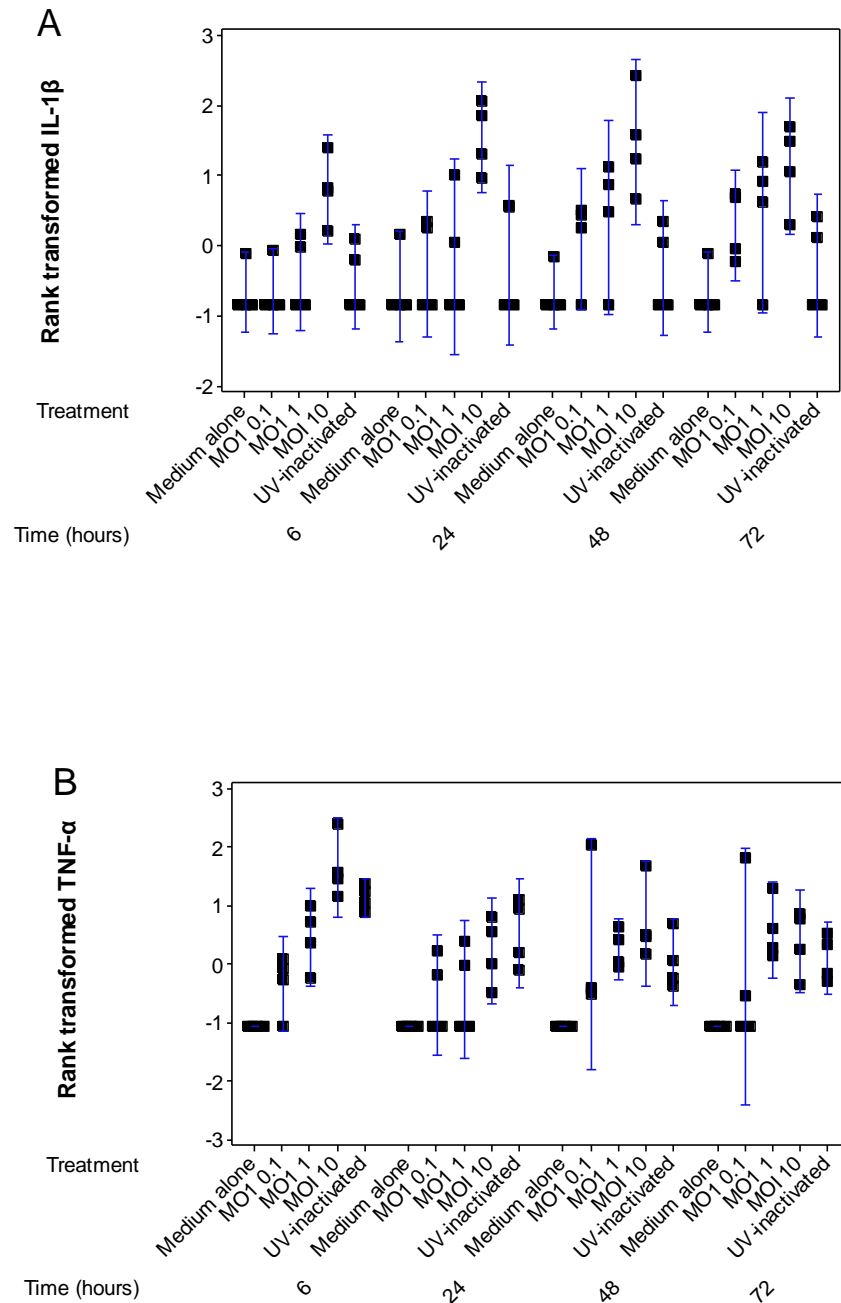
Figure 4.18: Production of IL-1 β (A), TNF- α (B) and CXCL8 (C) protein by bovine MDDCs in response to medium alone, MOI 0.1,1,10 and UV-inactivated *C. abortus*. The rank transformed fold changes from each animal were plotted (black squares) \pm 95% confidence intervals (blue error bars). The results are representative of repeat experiments using MDDCs from four different animals.

There was a significant treatment effect detected for IL-1 β , CXCL8 and TNF- α ($p < 0.05$) in bovine MDDCs. Following pairwise comparison of the treatment groups, MOI 10 *C. abortus* was found to produce significantly higher levels of IL-1 β and TNF- α than the medium alone control ($p < 0.01$). The production of IL-1 β and TNF- α was dose dependant as MOI 10 produced significantly higher levels of cytokine/chemokine than the MOI 0.1 treatment group ($p < 0.05$). There was no significant difference between the UV-inactivated and MOI 10 treatment group for TNF- α production, however there was a significant difference in IL-1 β production ($p < 0.05$). There is also no significant difference between the UV-inactivated and control group with IL-1 β production ($p > 0.05$). There was a significant time effect with TNF- α production ($p < 0.05$) but not with IL-1 β production. The production of TNF- α was significantly reduced at 48 and 72 hours indicating a peak in production at 6 and 24 hours, which then declines. There was no significant difference in CXCL8 production between the unchallenged control group and MOI 10. The

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production of CXCL8 was significantly higher ($p < 0.05$) in the UV-inactivated treatment group compared to MOI 10 or the unchallenged control.

The ovine MDDCs produce IL-1 β , TNF- α and CXCL8 in response to the different treatment groups (Figure 4.19).



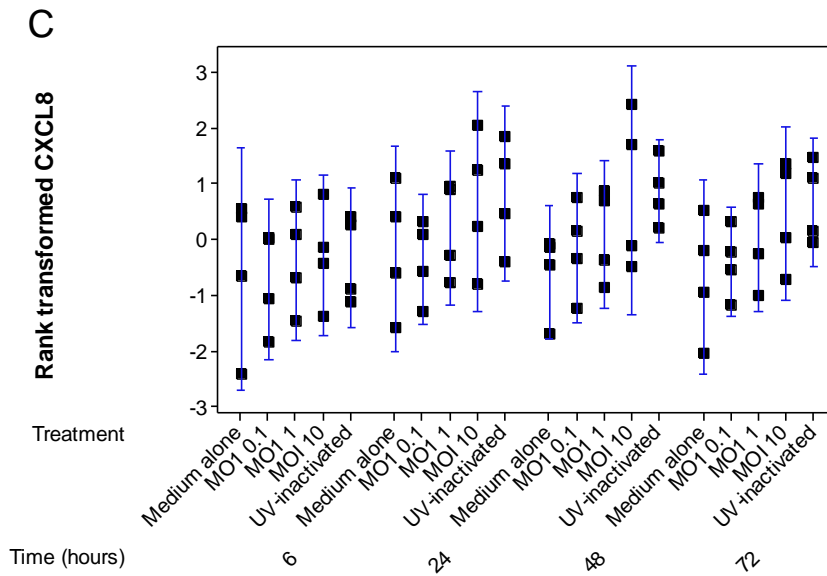


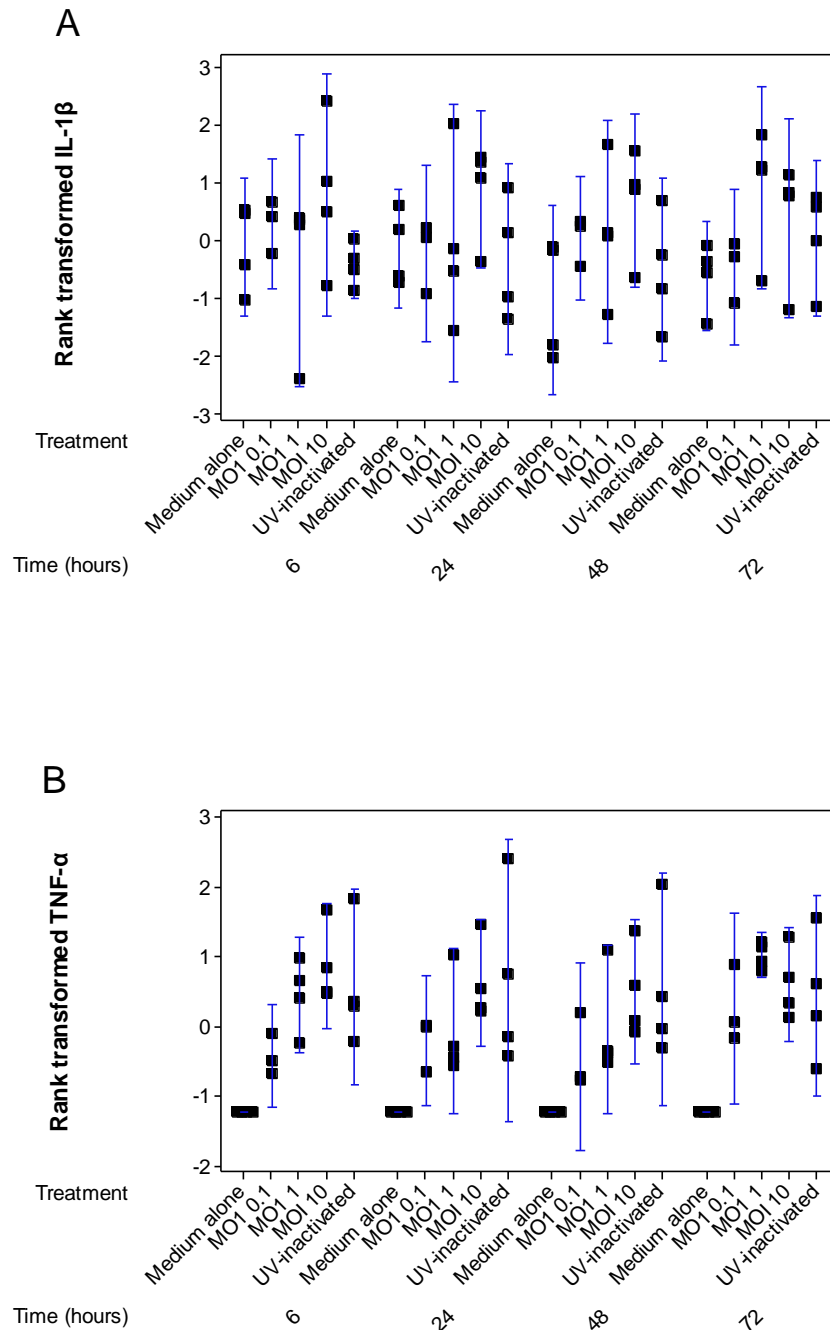
Figure 4.19: Production of IL-1 β (A), TNF- α (B) and CXCL8 (C) protein by ovine MDDCs in response to medium alone, MOI 0.1,1,10 and UV-inactivated *C. abortus*. The rank transformed fold changes from each animal were plotted (black squares) \pm 95% confidence intervals (blue error bars). The results are representative of repeat experiments using MDDCs from four different animals.

There was a significant treatment effect detected for IL-1 β , TNF- α and CXCL8 in ovine MDDCs ($p < 0.01$). CXCL8 and IL-1 β were significantly increased with the MOI 10 compared to the medium alone control ($p < 0.05$). TNF- α production was increased with MOI 10 compared to the medium alone, which produced no TNF- α . The production of CXCL8 and IL-1 β was found to be dose-dependent with significantly higher production with MOI 10 relative to MOI 0.1 ($p < 0.05$). There was no significant difference in CXCL8 or TNF- α production in the MOI 10 compared to the UV-inactivated treatment groups ($p > 0.05$). There was a significant increase in IL-1 β production from the MOI 10 compared to the UV-inactivated treatment groups ($p < 0.05$). CXCL8 is significantly elevated at 24 hours compared to 6 hours ($p < 0.05$) and production remains elevated. There was significant interaction of time and treatment with IL-1 β production ($P < 0.05$). A peak in IL-1 β production is observed at

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24 hours in the MOI 10 treatment group which then decreases whereas production in the MOI 1 and MOI 0.1 continue to increase more gradually.

The bovine MDMs produce CXCL8, TNF- α and IL-1 β protein in response to the different treatment groups (Figure 4.20).



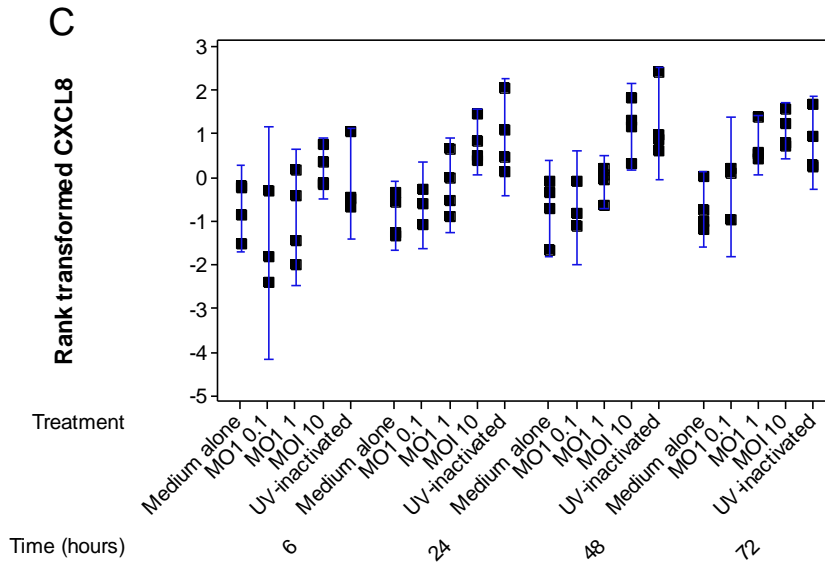


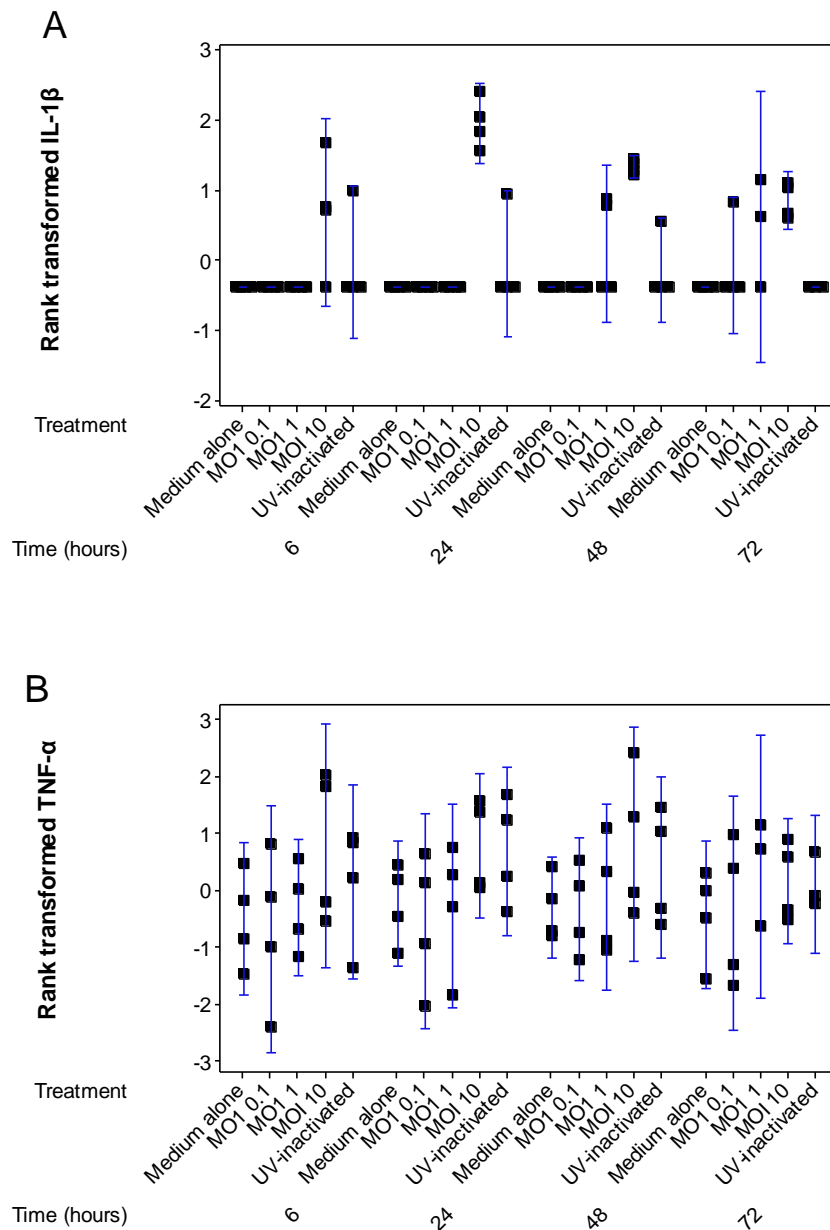
Figure 4.20: Production of IL-1 β (A), TNF- α (B) and CXCL8 (C) protein by bovine MDMs in response to medium alone, MOI 0.1,1,10 and UV-inactivated *C. abortus*. The rank transformed fold changes from each animal were plotted (black squares) \pm 95% confidence intervals (blue error bars). The results are representative of repeat experiments using MDMs from four different animals with the exception of a missing MOI 0.1 sample across all time points from one animal.

There was a significant treatment effect detected for IL-1 β , CXCL8 and TNF- α ($p < 0.05$). Following pairwise comparison of the treatment groups the MOI 10 was found to produce significantly higher levels of IL-1 β and CXCL8 than the medium alone control ($p < 0.05$). TNF- α production was increased with MOI 10 compared to the medium alone, in which no TNF- α was detected. Production of CXCL8, IL-1 β and TNF- α was dose dependant as MOI 10 produced significantly higher levels of cytokine/chemokine than the MOI 0.1 treatment group ($p < 0.05$). There was no significant difference between the UV-inactivated and MOI 10 treatment group for CXCL8 and TNF- α production ($p > 0.05$), however there was significantly lower IL-1 β production in the UV-inactivated treatment group ($p < 0.05$). There was no significant difference between the UV-inactivated and control group with IL-1 β production ($p > 0.05$). CXCL8 was significantly increased at 24 hours compared to 6 hours ($p < 0.05$) and remained elevated at 48 and 72 hours. There was a significant

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interaction of time and treatment with IL-1 β production ($p < 0.05$). An early peak of IL-1 β production in the MOI 10 treatment group is evident which then decreases, whereas production in the MOI 1 treatment group shows a gradual increase with higher production than MOI 10 at 72 hours.

The ovine MDMs produce CXCL8, TNF- α and IL-1 β protein in response to the different treatment groups (Figure 4.21).



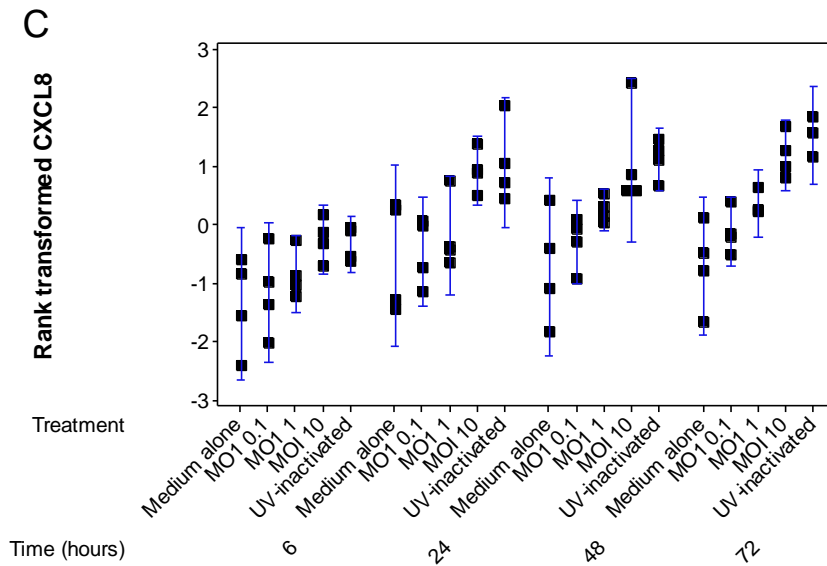


Figure 4.21: Production of IL-1 β (A), TNF- α (B) and CXCL8 (C) protein by ovine MDMs in response to medium alone, MOI 0.1,1,10 and UV-inactivated *C. abortus*. The rank transformed fold changes from each animal were plotted (black squares) \pm 95% confidence intervals (blue error bars). The results are representative of repeat experiments using MDMs from four different animals with the exception of single missing MOI 1 and UV 72 hour samples from 2 different animals.

There was a significant treatment effect detected for CXCL8, IL-1 β and TNF- α ($p < 0.05$). The MOI 10 treatment group produced significantly higher levels of CXCL8 and TNF- α than the medium alone control ($p < 0.01$). The production of IL-1 β was increased with MOI 10 compared to the medium alone, in which no IL-1 β was detected. Production of all cytokines/chemokines was dose dependant as MOI 10 produced significantly higher levels than the MOI 0.1 treatment group ($p < 0.05$). There was no significant difference between the UV-inactivated and MOI 10 treatment group for CXCL8 and TNF- α production. There was a significant increase in IL-1 β production in the MOI 10 treatment group compared to the UV-inactivated treatment group ($p < 0.05$). There was a significant time effect with CXCL8 production, with significantly increased CXCL8 production detected at 24 hours following treatment ($p < 0.05$) that remained high at 48 and 72 hours.

4.3.7.1 Cross-species protein comparison

Comparison between the different species revealed quantitative differences in the production of cytokine/chemokines in the MOI 10 treatment group. There was significantly increased production of TNF- α in bovine compared to ovine MDDCs ($p < 0.01$), but no significant difference in CXCL8 or IL-1 β production between species ($p > 0.05$). There was significantly increased production of CXCL8 and TNF- α by ovine compared to bovine MDMs ($p < 0.05$), but no significant difference in the production of IL-1 β between species ($p > 0.05$).

4.4 Discussion

Ruminant MDDCs and MDMs were generated using conventional protocols and their responses to *C. abortus* assessed. The addition of IL-4 and GM-CSF was used to generate MDDCs and an adherence protocol in the presence of high levels of serum used for the generation of MDMs. The generation of ovine MDMs required optimisation and the addition of autologous serum instead of commercial FBS, which suggests that important components of the FBS were not cross-reactive.

Morphologically, the ruminant MDDCs and MDMs were found to be distinct. The MDDCs developed dendrite like-processes whereas the MDMs developed into large cells with a vacuolated cytoplasm. There was a difference between the ovine and bovine MDDCs in terms of morphology with the ovine MDDCs remaining largely adherent. The ovine MDMs were also confirmed to have macrophage-like functions, including phagocytosis and non-specific esterase activity.

Flow cytometric analysis of cell surface markers was used to characterize the monocytes, MDDCs and MDMs. All ruminant mononuclear phagocytes expressed high levels of the myeloid markers CD11b and CD172- α . There was no significant difference in CD14 expression between monocytes and MDDCs or MDMs. This is consistent with some human MDM studies, although other studies have identified up-regulated/down-regulated expression (Ziegler-Heitbrock and Ulevitch, 1993). In contrast, human MDDCs have previously been identified to down-regulate CD14 expression with the differentiation of monocytes into MDDCs (Elkord et al., 2005). The down-regulation of CD14 in human MDDCs compared to monocytes is thought to be a consequence of IL-4 down-regulating the expression of CD14 mRNA (Lauener et al., 1990). Immature human MDDCs have been shown to retain CD14 expression, although at a lower level relative to the monocytes, with expression disappearing with DC maturation (Elkord et al., 2005). DC maturation occurs following the addition of LPS, viral products, cytokines (including TNF- α) and binding of receptors on the cell surface. However, CD14 expression has also been

identified on porcine MDDCs, which was increased with maturation (Carrasco et al., 2001).

There was no significant up-regulation in MHC II expression with differentiation from monocytes into MDDCs or MDMs. The lack of up-regulation may be explained by the immaturity of the cells as human MDDC maturation has been shown to lead to increased expression of MHC II (Elkord et al., 2005). The absence of CD1w2 and CD205 staining on ruminant monocytes has been previously identified in other studies (Gliddon et al., 2004; Howard and Naessens, 1993; McKeever et al., 1991). In contrast, CD205 expression has been identified on human monocytes, which potentially indicates species-specific differences in marker expression (Butler et al., 2007; Kato et al., 2006). The absence of staining of CD205 and CD1w2 on MDDCs also contrasts with studies that have identified expression in human MDDCs (Butler et al., 2007; Giuliani et al., 1997). The cell-surface expression of CD205 is up-regulated on mature human MDDC compared to immature MDDCs as it is thought to be primarily intracellular until maturation (Butler et al., 2007), which may explain the lack of expression on ruminant immature MDDCs. There was no CD1w2 expression detected on ruminant MDDCs, which is consistent with a previous study which demonstrated no or weak expression in ovine MDDCs (Chan et al., 2002). Therefore, the phenotype of ruminant mononuclear phagocytes differs from the characteristic human phenotypes. Similarly, the phenotype of porcine and equine MDDCs have been shown to differ (Carrasco et al., 2001; Mauel et al., 2006), indicating potentially important species-specific differences. Future work should compare the phenotype of these ruminant cells following maturation as a number of markers are affected by maturation state.

The pattern of cell surface marker expression was similar between cattle and sheep, which indicates that the MPS cells are differentiating in the same way in both species. There was increased MHC II expression in bovine compared to ovine cells as well as increased CD205 and CD1w2 expression in ovine compared to bovine cells, but this may reflect differences in antibody sensitivity. The MHCII antibody

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was raised against sheep and the CD205 and CD1w2 against cattle so if these results are down to sensitivity issues this is somewhat surprising.

Gene expression profiling studies have helped to define human and mouse monocytes, DCs and macrophages (Haniffa et al., 2015). Specific genes have been shown to be differentially expressed in both human and mouse subsets, which can define subsets across the two species (McGovern et al., 2014), however species-specific differences have also been identified (Haniffa et al., 2012). Cattle and sheep monocytes, MDDCs and MDMs have been analysed using RNA-sequencing and will be discussed in more detail in Chapter 6.

Cattle and sheep MDDCs and MDMs expressed TLR2, TLR4, NOD1 and NLRP3. Similarly, human peripheral blood cells express a range of TLRs and CD14⁺ monocytes expressed the highest levels of TLR2, TLR4 and TLR8 compared to B and T cell populations (Zarembek and Godowski, 2002). NLRP3 expression has been identified in mouse myeloid (CD11b⁺) cells and was highly expressed in monocytes and cDCs from the spleen but not in lymphoid cells (Guarda et al., 2011). Human MDDCs have also previously been shown to express NOD1 (Tada et al., 2005). The expression of TLR2, TLR4, NOD1 and NLRP3 by MDMs and MDDCs is likely to be important for their role as innate immune sentinels within inflamed tissues and will aid in the detection of a broad range of microbes. PRR expression on immune cells contributes not only to the production of cytokines/chemokines but also to the induction of phagocytosis, activation, antigen presentation and apoptosis (Zhang et al., 2009a).

Previous studies have identified that TLRs are differentially expressed between monocyte-derived cells. The expression of TLR4 is increased in human MDMs compared to monocytes unlike TLR2, which had similar levels of expression (O'Mahony et al., 2008). Bovine monocytes were found to express higher levels of TLR2 and TLR4 than MDDCs but lower levels than MDMs (Werling et al., 2004). The differential expression of PRRs may reflect the specialized roles of the monocyte-derived cells in innate immune recognition.

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The ruminant MDDCs and MDMs were shown to be susceptible to *C. abortus* infection using immunofluorescent staining. *C. abortus* formed multiple small inclusions within the cells which were distinct from the large inclusions that were observed in the turbinate cells. Previous immunofluorescence studies have also identified that multiple *C. pneumoniae* inclusions are formed within mononuclear phagocytes, which range in size and staining intensity (Airenne et al., 1999). Similarly, the infection of human monocytes and MDDCs with *C. trachomatis* also resulted in the formation of small inclusions of varied staining intensity (Datta et al., 2014). Fluorescent specks were visible in the UV-inactivated *C. abortus* treated ruminant MDDCs and MDMs but no inclusion structures were formed. Further studies using confocal microscopy are necessary to confirm that the UV-inactivated *C. abortus* are inside the cell. A previous study identified that heat-killed *C. psittaci* is internalized by bone marrow derived DCs at a similar rate to live *C. psittaci* (Ojcius et al., 1998). UV-inactivated *C. muridarum* has also been shown to be internalized by MDDCs (Prantner et al., 2009; Rey-Ladino et al., 2005) and within phagocytes is thought to reside in the phagocytic vacuoles (Prantner et al., 2009).

Further work is necessary to assess whether *C. abortus* is capable of producing infectious EBs following infection of mononuclear phagocytes. The outcome of *C. trachomatis* infection of human mononuclear phagocytes was shown to be dependent on strain. The L2 *C. trachomatis* serovar was able to survive and remained infectious following infection of monocytes and MDDCs. However, the *C. trachomatis* Ba and D serovars did not form inclusions in HeLa cells following infection of monocytes and MDDCs and instead became persistent (Datta et al., 2014). Similarly, *C. pneumoniae* was unable to form inclusions in HeLa cells following infection of human monocytes, which indicates that the development of infectious EBs is not supported (Airenne et al., 1999). Following culture in human MDMs the ability of *C. pneumoniae* to form inclusions was reduced but not completely inhibited in Hep-2 cells (Gaydos et al., 1996), which indicates cell-type specific differences.

The critical role for PRRs in a diverse range of innate immune processes suggests that the up-regulation of PRR expression in MDMs increases the cells responsiveness

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to infection. The expression of TLR2 and TLR4 in response to live and UV-inactivated *C. abortus* was assessed. The up-regulation of TLR2 expression was dose-dependent with a significantly increased expression in response to MOI 10 *C. abortus* compared to MOI 0.1 *C. abortus*. There was no significant difference comparing the UV-inactivated to the MOI10 treatment group indicating that intracellular multiplication is not necessary for TLR2 up-regulation. There was no significant difference in TLR4 expression between the different treatment groups. Previous studies have shown that the expression level of TLR2 and TLR4 in human monocytes can be modulated with the addition of bacterial LPS and pro-inflammatory cytokines including IL-1 β , TNF- α , GM-CSF and IFN- γ (Muzio et al., 2000; O'Mahony et al., 2008). Interestingly, the expression of TLR2 and TLR4 in bovine MDMs and MDDCs was not affected following the addition of recombinant bovine IFN- γ (Werling et al., 2004).

There was no significant difference in NOD1 mRNA expression in response to live or UV-inactivated *C. abortus* in ruminant MDMs. A previous study identified that the stimulation of human MDMs with the NOD1 ligand L-Ala- γ -D-Glu-meso-diaminopimelic acid (Tri-DAP) did not significantly increase NOD1 expression (Juarez et al., 2014), which suggests that the MDMs do not up-regulate NOD1 expression in response to stimuli. However, there was a significant dose-dependent increase in NLRP3 expression in the ruminant MDMs. There was no significant difference comparing the UV-inactivated to the MOI10 treatment group, which indicates that the up-regulation of NLRP3 does not require a live infection. A different study identified that the stimulation of mouse macrophages with LPS dose-dependently increased NLRP3 mRNA and protein expression, which was mediated through NF- κ B activation. The up-regulation of NLRP3 expression is likely the first step necessary for NLRP3 inflammasome activation, although up-regulation alone is not sufficient for NLRP3 activation and a second signal is required (described in section 1.3.2.2.2) (Bauernfeind et al., 2009).

Increased PRR expression by cells may enhance cellular responses to PAMPs and be an important host defence mechanism to pathogens. However the up-regulation of

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PRRs has also been implicated in a number of inflammatory diseases, including in type 1 diabetes (Devaraj et al., 2008) and inflammatory bowel disease (Szebeni et al., 2008). Therefore, the up-regulation of TLR2 and NLRP3 in response to *C. abortus* infection may be a protective host-defence mechanism or induce excessive inflammation. The pattern of PRR expression between species was similar; however, there were significant differences in the quantity of up-regulation of TLR2, NOD1 and NLRP3 expression between species. The relevance of PRR expression levels in *C. abortus* disease pathogenesis requires further investigation.

A dose-dependent increase in CXCL8, TNF- α , IL-6 and IL-1 β mRNA was observed at 6 hours post *C. abortus* infection in ruminant MDMs. CXCL8, TNF- α and IL-1 β protein was significantly increased in ovine and bovine MDMs and MDDCs infected with *C. abortus*, which in most cases was dose dependant. The only exception was the production of CXCL8 protein from bovine MDDCs that was not significantly higher in the treated groups relative to the medium alone control, which may be due to the high background CXCL8 levels in these cells. The production of cytokines/chemokine peaked at 24 hours, which is early post-infection compared to the turbinate cells. Therefore, mononuclear phagocytes rapidly respond to chlamydial infection through the up-regulation of pro-inflammatory cytokines/chemokines, which may be due to their specialised roles in the innate immune defence. Similarly, the infection of MDDCs with *C. trachomatis* resulted in the induction of CXCL8, IL-1 β and TNF- α protein at 24 hours post-infection (Datta et al., 2014; Gervassi et al., 2004). Ovine alveolar macrophages challenged with *C. abortus* also produced early IL-1 β and cumulative CXCL8 production over 72 hours post infection (Entrican et al., 1999).

The production of CXCL8, IL-6, TNF- α and IL-1 β mRNA was similar between the UV-inactivated and MOI10 *C. abortus* treatment groups in ruminant MDMs. The production of CXCL8 and TNF- α protein was similar with UV-inactivated and live *C. abortus* in ruminant MDDCs and MDMs. However, the production of IL-1 β protein was dependent on a live infection as it was not induced in response to UV-inactivated *C. abortus*. The production of IL-1 β protein by ovine alveolar

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macrophages was also shown to be dependent on a live *C. abortus* infection compared to heat-killed *C. abortus* (Entrican et al., 1999). Similar results were found in human MDDCs and MDMs where the production of CXCL8 and TNF- α was induced in response to both live and heat-killed *C. trachomatis*, but IL-1 β was dependant on live *C. trachomatis* (Gervassi et al., 2004). Therefore, UV-inactivated/heat-killed *Chlamydia* is sufficient to stimulate the production of CXCL8 and TNF- α but not the production of IL-1 β . The differences in the response of bone marrow derived DCs (BMDCs) to live compared to UV-inactivated *C. muridarum* was assessed by microarray, which identified that a number of genes were differentially up-regulated. These genes included pro-inflammatory cytokine genes (including IL-1 β), chemokine genes involved in neutrophil recruitment and interferon response genes (Zaharik et al., 2007). The difference in response of cells to live and inactivated bacteria may have important implications for vaccine design. The inactivated *C. abortus* vaccine has been shown to be effective in controlling flock outbreaks and has recently been re-introduced onto the market (Essig and Longbottom, 2015). Therefore, this may indicate that IL-1 β is not participating in the mediation of protection induced by the vaccine or that the adjuvant is functioning in the activation of an IL-1 β response.

The production of IL-1 β mRNA in response to UV-inactivated *C. abortus* does not mirror the IL-1 β protein profile and is up-regulated. This indicates that pro-IL-1 β may be up-regulated in response to UV-inactivated *C. abortus* but not cleaved or extracellularly secreted. Therefore, a live infection may be necessary to provide the second signal for cleavage. Similar results have been found in peritoneal macrophages, which were challenged *in vitro* with live and UV-inactivated *C. muridarum*. The UV-inactivated *C. muridarum* did not induce IL-1 β protein although increased IL-1 β mRNA at 3 hours post infection (Prantner et al., 2009). Live and heat-killed *C. trachomatis* could also induce pro-IL-1 β production in MDDCs, but mature IL-1 β production was dependant on a live infection that activated caspase-1 (Gervassi et al., 2004). The production of pro-IL-1 β is controlled by NF- κ B (Cogswell et al., 1994) and therefore UV-inactivated *Chlamydia* is likely to provide a

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sufficient signal for NF- κ B activation. However, pro-IL-1 β cleavage requires a second signal thought to be mediated through intracellular stress signals, including potassium efflux and the generation of ROS (Tschopp and Schroder, 2010).

Considering that UV-inactivated *Chlamydia* does not form inclusions and multiply within the host cell, it follows that it does not activate the same intracellular stress pathways as a live infection.

Quantitative differences were identified in the up-regulation of cytokine/chemokine mRNA and protein produced by ovine and bovine MDDCs and MDMs in response to *C. abortus* infection. However, differences in protein are complicated by sensitivities of the ELISA antibodies. Further investigation into the particular roles of different cytokines *in vivo* following *C. abortus* pathogenesis will identify the importance of the quantitative differences between cattle and sheep.

To conclude, the outcomes of the chapter have been summarized in relation to the four stated objectives. Ruminant monocytes, MDDCs and MDMs were characterized using flow cytometry for the first time, however defining the profile of different subsets is complicated by maturation states and species-specific differences. The MDDCs and MDMs were susceptible to *C. abortus* infection and expressed both intracellular and extracellular PRRs, which are likely to be important for chlamydial detection. The MDMs are capable of responding rapidly to *C. abortus* through the up-regulation of NLRP3 and TLR2 expression. Both MDMs and MDDCs respond to *C. abortus* through the production of CXCL8, TNF- α and IL-1 β . UV-inactivating *C. abortus* does not affect the production of CXCL8 or TNF- α ; however, the production of IL-1 β is inhibited highlighting the strict control mechanisms required for its production. *In vivo*, pro-inflammatory cytokine/chemokine production by mononuclear cells is likely to be involved in the recruitment of innate immune cells and in T cell polarization. A previous study identified that MDDCs infected with *C. pneumoniae* induced the polarization of a Type 1/ Type17 T cell response (Flego et al., 2013). Therefore, the response of ruminant MDDCs and MDMs to *C. abortus* may be critical in the induction of an adaptive immune response and have important implications for vaccine design.

Chapter 5: Identifying the role of NOD1 and NLRP3 in ruminant MDMs in response to *C. abortus* infection

5.1 Introduction

RNA interference (RNAi) is a conserved gene silencing process that was first identified in animal cells using *C. elegans* (Fire et al., 1998), and later in mammalian cells (Elbashir et al., 2001). The RNAi pathway involves cleavage of double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs) by the Dicer enzyme. The siRNA is taken up by a RNA-induced silencing complex (RISC) and unwinds to guide RISC to target mRNA for cleavage, by Argonaute-2, inducing degradation (reviewed by Dykxhoorn and Lieberman, 2005). The RNAi pathway is a natural cellular mechanism that is involved in controlling gene expression for a number of biological processes, including cell growth and proliferation. Dysregulation of the RNAi pathway is linked with diseases, including cancer, which emphasizes its importance in maintaining homeostasis (Wilson and Doudna, 2013).

The RNAi pathway can be exploited to experimentally silence genes in mammalian cells. siRNA and short hairpin RNA (shRNA) can be chemically synthesized and delivered into the cell to directly mediate mRNA degradation. Once inside the cell, shRNA can be cleaved by DICER for the generation of siRNA and facilitate longer-term knockdown of the gene, whereas siRNA only mediates transient gene knockdown. siRNA is often the preferred method for transient gene knockdown as reagents are affordable and easy to use (Miller et al., 2013). Gene knockdown enables functional analysis of defined gene products *in vitro* and *in vivo* and has also been evaluated for potential clinical applications, including in the treatment of viral and genetic diseases (Ambesajir et al., 2012). This technology is especially useful for non-traditional biomedical models, such as sheep and cattle, as there are limited reagents and resources to characterize molecular function compared to what is available for human and mouse studies (Jensen et al., 2014).

SiRNA can be designed to target any gene of interest for knockdown if the gene sequence is known (Dykxhoorn et al., 2006). The siRNA design process is complex as it involves the consideration of a number of factors, including molecule thermodynamics and potential off-target effects. Off-target effects include activating a type-1 interferon response, participating in the microRNA pathway and knocking down non-target genes due to high levels of sequence complementarity. Computer programmes, that incorporate multiple design rules and formulas, are available to help produce siRNA with high levels of gene knockdown but minimal off-target effects (Peek and Behlke, 2007). The design of siRNAs for most studies is now often completed by commercial companies using these computer programmes, including Applied Biosystems[®] and Sigma-Aldrich[®] (Shan, 2010). However, it is not fully understood what makes a perfect siRNA and therefore a number of controls are also required when setting up a siRNA experiment. In order to control for potential off-target effects, ideally two or three siRNAs that target different areas of the gene should be evaluated and shown to deliver the same effect (Peek and Behlke, 2007). A number of other controls which are important include non-transfected cells, transfection reagent only cells, transfection efficiency controls and scrambled or non-target siRNA (Ponthan et al., 2015; Shan, 2010).

The use of siRNA technology in cells of the immune system has proven challenging (Mao et al., 2007). The delivery of siRNA into macrophages is particularly difficult. Macrophages are susceptible to toxicity mediated by transfection protocols and also possess enzymes that can degrade nucleic acids (Zhang et al., 2009a). Immune cells can also respond to siRNA through the production of an interferon response and activation of TLR mediated signaling pathways. However, siRNA technology has successfully been used to investigate gene function of both innate and adaptive immune cells. SiRNA studies have identified the role of specific genes in immune cell activation, differentiation and effector mechanisms (Mao et al., 2007). The specific role of immune cell genes in response to pathogens has also been elucidated using this technology, including the role of PRRs in response to bacterial infections. For example, the role of NOD1 in the recognition of *Campylobacter jejuni* was demonstrated in human epithelial cells (Zilbauer et al., 2007) and the role of NLRP3

inflammasome in the recognition of *Propionibacterium acnes* demonstrated in human monocytes (Qin et al., 2014).

There are a number of different methods that have been used to deliver siRNA into mammalian cells including microinjection, chemical transfection reagents containing cationic lipids, viral vectors and electroporation (Ponthan et al., 2015). Different cell types show varying responses to transfection protocols and therefore it is often necessary to compare a number of different methods (Mocellin and Provenzano, 2004). Recently, an optimised protocol for transfection of siRNA into bovine MDMs was published. Electroporation and various transfection reagents were compared in terms of gene knockdown, toxicity and up-regulation of type 1 interferon response genes. The study identified that an optimised Lipofectamine[®] RNAiMAX protocol resulted in effective gene knockdown, reduced type 1 interferon response gene expression and reduced toxicity, compared to electroporation and other transfection reagents (Jensen et al., 2014). The protocol provides a method for the investigation of the role of specific PRRs in response to stimuli in non-model species such as cattle and sheep.

The intracellular PRRs NLRP3 and NOD1 have important roles in the recognition of human and mouse chlamydial species, and activate the production of pro-inflammatory cytokines, including IL-1 β and CXCL8 (as discussed in detail in Chapter 1). Macrophages have been identified as a major source of IL-1 β *in vivo* in the genital tract following intravaginal infection with *C. muridarum* (Prantner et al., 2009). Ruminant MDMs express a range of PRRs, including NOD1 and NLRP3, and respond to *C. abortus* through the production of pro-inflammatory cytokines including IL-1 β (see Chapter 4). The role of intracellular NOD1 and NLRP3 in ruminant MDMs in response to *C. abortus* infection was assessed using the previously described published siRNA protocol (Jensen et al., 2014). The aims of this chapter were to:

- Establish the siRNA procedure in ovine MDMs.

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- Assess siRNA knockdown of NOD1 and NLRP3 in ruminant MDMs.
- Identify the role (if any) of NOD1 and NLRP3 in IL-1 β production by *C. abortus* in ruminant MDMs.
- Assess the effect of *C. abortus* infection and siRNA knockdown on the different components of the IL- β pathway, including caspase-1 and pro-IL-1 β .
- Identify the role (if any) of NOD1 and NLRP3 in the production of other pro-inflammatory cytokine/chemokines.

5.2 Materials and methods

5.2.1 Ruminant MDMs

Ruminant MDMs were generated as described in section 2.2.4.

5.2.2 Transfection of ruminant MDMs with siRNA

MDMs were plated out at a density of 6×10^5 cells per well in six well plates and rested and allowed to adhere for 24 hours. The siRNA and RNAiMAX complex was generated by adding 6 μ l of the 20 μ M siRNA stock (50nM final transfection concentration) and 6 μ l of Lipofectamine RNAiMAX (Life Technologies) to 400 μ l Opti-Mem I serum free medium (Life Technologies) followed by a 20 minute incubation at RT. The cells were transfected with the siRNA by replacing the medium of the well with 2mls of fresh medium and adding the siRNA/RNAiMAX complex solution. The controls included non-transfected cells (NTC), transfection reagent only (TC) and Mission siRNA universal negative control (NC siRNA) (Sigma-Aldrich), which shares no known homology to any mammalian gene.

5.2.3 SiRNA duplexes

The ovine and bovine NOD1/NLRP3/GAPDH protein sequences were aligned using BLAST and found to be 98% (NLRP3), 96% (NOD1) and 99% (GAPDH) identical. The accession numbers were sent to Sigma-Aldrich for the custom design of siRNA duplexes, which were cross-reactive to both cattle and sheep (Table 5.1). SiRNA stocks of 20 μ M were prepared and sub-aliquoted for storage at -20°C.

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Table 5.1: Duplex siRNA targeting ovine and bovine NLRP3, NOD1 and GAPDH.

	Accession numbers (bovine/ovine)	SiRNA sequence (5' – 3')	
		Sense	Antisense
Ruminant NLRP3 version 1	NM_001102219/ XM_004008740	GUGUAUAUCUUCUUCCUCUdTdT	AGAGGAAGAAGAUAUACACdTdT
Ruminant NLRP3 version 2	NM_001102219/ XM_004008740	CAUUCCUCCUUCAGCAUUAdTdT	UAAUGCUGAAGGAGGAAUGdTdT
Ruminant NLRP3 version 3	NM_001102219/ XM_004008740	GAAACUUUACCAGGAUAGAdTdT	UCUAUCCUGGUAAGUUUCdTdT
Ruminant NOD1 version1	NM_001256563/ XM_004007930	GUGUGAACCCAGAUACUGAdTdT	UCAGUGAUCUGGUUCACACdTdT
Ruminant NOD1 version 2	NM_001256563/ XM_004007930	GUGAGAUCAUCUUCGUGUdTdT	AACACGAAGAUGAUCUCACdTdT
Ruminant NOD1 version 3	NM_001256563/ XM_004007930	CAGAAUUCUGGACCUGGUAdTdT	UACCAGGUCCAGAAUUCUGdTdT
Ruminant GAPDH version 1	NM_001034034/NM_001190390	CAGGUUGUCUCCUGCGACUdTdT	AGUCGCAGGAGACAACCUGdTdT
Ruminant GAPDH version 2	NM_001034034/NM_001190390	GCAACAGGGUGGUGGACCUdTdT	AGGUCCACCACCCUGUUGCdTdT
Ruminant GAPDH version 3	NM_001034034/NM_001190390	CCAUCACUGCCACCCAGAAdTdT	UUCUGGGUGGCAGUGAUGGdTdT

5.2.4 Measuring siRNA uptake

The MDMs were incubated for 24 hours following the transfection with AllStars negative control siRNA labelled with Alexa Fluor 488 (Qiagen) and harvested using TrypLE Express. The cells were washed in 1xPBS and then stained with LIVE/DEAD® Fixable violet stain for 30 minutes before analysis on the MACSQuant. The cells were gated on size, live cells and single cells as described in section 4.2.6. The expression of FITC was measured and the percentage positive cells calculated by comparing between the non-transfected cells and cells transfected with fluorescently labelled siRNA.

5.2.5 Measuring siRNA knockdown

RT-PCR was used to assess the knockdown of siRNA targets using the protocol and primer/probes described in section 2.9.

5.2.6 Infection of siRNA transfected MDMs

The MDMs were incubated for 24 hours at 37°C following transfection. The medium was then replaced with fresh medium and MDMs incubated for a further 24 hours at 37°C. The MDMs were then infected with an MOI 4 *C. abortus* or medium alone for 24 hours before the supernatants and lysates were harvested.

5.2.7 Measuring innate immune cytokine/chemokine production

The production of IL-1 β , CXCL8 and TNF- α from siRNA transfected MDMs was measured using ELISAs detailed in section 2.6. IL-1 β mRNA was measured by RT-PCR as described in section 2.9.

5.2.8 FAM-FLICA™ caspase-1 activation

Caspase-1 activation was measured using the FAM FLICA™ caspase-1 kit (Bio-Rad AbD Serotec) according to the manufacturer's instructions for adherent cells. Briefly, the MDMs were dissociated using TrypLE and centrifuged at 200g for 5 minutes. The cells were resuspended in MDM culture medium before the addition of 1:30

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30xFLICA. The cells were incubated at 37°C for 1 hour, mixing every 10 minutes, before the addition of 1xapoptosis wash buffer for centrifugation at 200g for 5 minutes. The pellet was resuspended in 1xapoptosis wash buffer and incubated for 10 minutes at 37°C. The cells were centrifuged again at 200g for 5 minutes and resuspended in 1xapoptosis wash buffer with 1:10 fixative. The cells were analysed on the MACSQuant and gated on size, single cells and fluorescence.

5.2.9 IL-1 β SDS-PAGE and Western blot

The IL-1 β western blot was optimised using IL-1 β CHO transfected cells and CHO untransfected cells, generated as described previously (Wattegedera et al., 2010). The MDMs were washed with 1xPBS before replacing the medium with Opti-MEM I reduced serum medium (Life Technologies). The MDMs were infected with *C. abortus* or cultured with medium alone for 24 hours. The cell pellets were collected and solubilised in 0.8% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in PBS for 30 minutes at RT. The pellets were spun down at 10000g for 10 minutes. Serumless supernatant was concentrated using Amicon ultra 0.5ml 10K centrifugal filters (Merck Millipore, Watford, UK) at 14000g for 10 minutes. The soluble portion of the cell pellet and the concentrated cell supernatant was denatured using reduced sample buffer (250mM Tris (pH 6.8), 8% sodium dodecyl sulphate (SDS), 20% β -mercaptoethanol, 15% glycerol and 0.1% bromophenol blue) and incubated at 100°C for 5 minutes. Samples were loaded onto NuPAGE 4-12% Bis-Tris gels (Life Technologies) with SeeBlue Plus 2 Prestained standards (Life Technologies) and run in NuPAGE MES SDS Running buffer (Life Technologies) for protein separation using X-cell surelock mini-cell electrophoresis system (Life Technologies). The protein bands were transferred onto a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) in transfer buffer (48mM Tris base, 0.0375% SDS, 39mM Glycine, 20% methanol, dH₂O) using a semi-dry blotter (Sigma-Aldrich).

The blots were washed in wash buffer (1xPBS, 0.5M NaCl and 0.5% Tween 80) and blocked in 4% Marvel (diluted in 1xPBS with 0.05% Tween 20) for 1 hour with shaking at RT. The blots were washed in wash buffer before the proteins were

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probed with 4 µg/ml rabbit anti-sheep IL-1β polyclonal antibody (Bio-Rad AbD Serotec) for 1 hour at RT with shaking. Following three wash steps the blots were incubated with 1:1000 goat anti-rabbit HRP (Dako) for 1 hour at RT with shaking. The blots were vigorously washed three times before incubating with SuperSignal West Pico Chemiluminescent Substrate (Thermoscientific) for 5 minutes. Chemiluminescence was visualized using the ImageQuant LAS4000 imaging system (GE Healthcare).

5.2.10 Statistical analysis

The siRNA target knockdown was assessed using 2-sample T-tests comparing the siRNA to the scrambled siRNA. The cytokine data was not normally distributed and therefore was logarithmically or SQRT+1 transformed. A linear mixed model was applied where treatment was fitted as a fixed effect and animal as a random effect. If there was a significant F-statistic ($p < 0.05$) from the model, two-sided probabilities of comparisons of means were obtained. The probabilities were adjusted using an FDR approach (Benjamini and Hochberg, 1995).

5.3 Results

5.3.1 Efficacy of siRNA transfection in ruminant MDMs

The uptake of fluorescently labeled siRNA was measured in ruminant MDMs (Figure 5.1). The uptake experiment was repeated three times with MDMs from three different cattle and sheep. The ovine MDMs consistently demonstrated over 80% uptake (95.71%, 97.44% and 86.65%) whereas the bovine MDMs demonstrated more variable uptake (87.39%, 63.36% and 66.17%).

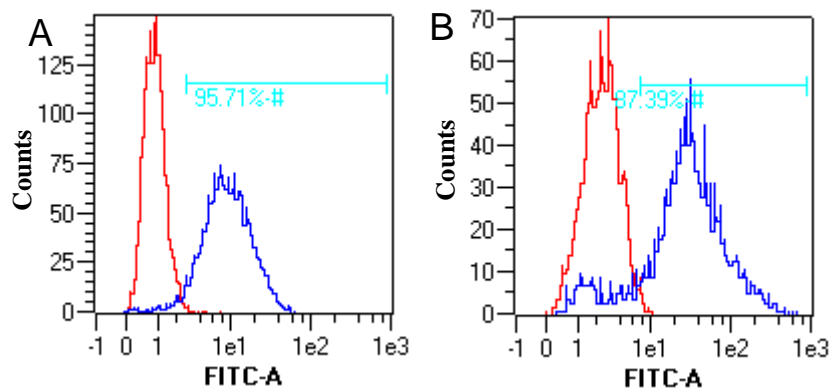


Figure 5.1: The uptake of fluorescently labelled siRNA by ovine (A) and bovine (B) MDMs. The red histogram represents the non-transfected control MDMs and the blue histogram represents the MDMs transfected with FITC labelled siRNA. The light blue bar and percentage indicates the FITC positive cells. The histograms are from one animal but are representative of the profiles observed in three animals.

5.3.2 Knockdown of GAPDH, NOD1 and NLRP3 in ruminant MDMs

Detection of fluorescently-labelled siRNA effectively demonstrates transfection efficiency. However, it is also essential to confirm that the transfection method can facilitate gene knockdown. Two siRNAs were used in each experiment, due to limited numbers of cells, and were selected as NLRP3#3 was identified to be toxic to cells (through visual inspection) and lower levels of knockdown were observed with NOD1#3 and GAPDH#3. GAPDH is a ubiquitously expressed gene which is commonly used as a positive control to confirm gene knockdown. A preliminary

study identified that GAPDH#1 and GAPDH#2 siRNA knocked down mRNA expression by over 70% in both ovine and bovine MDMs. The NLRP3 siRNAs did not induce GAPDH knock-down indicating the effect was specific to GAPDH siRNA and not just an off-target effect of siRNA transfection (Figure 5.2).

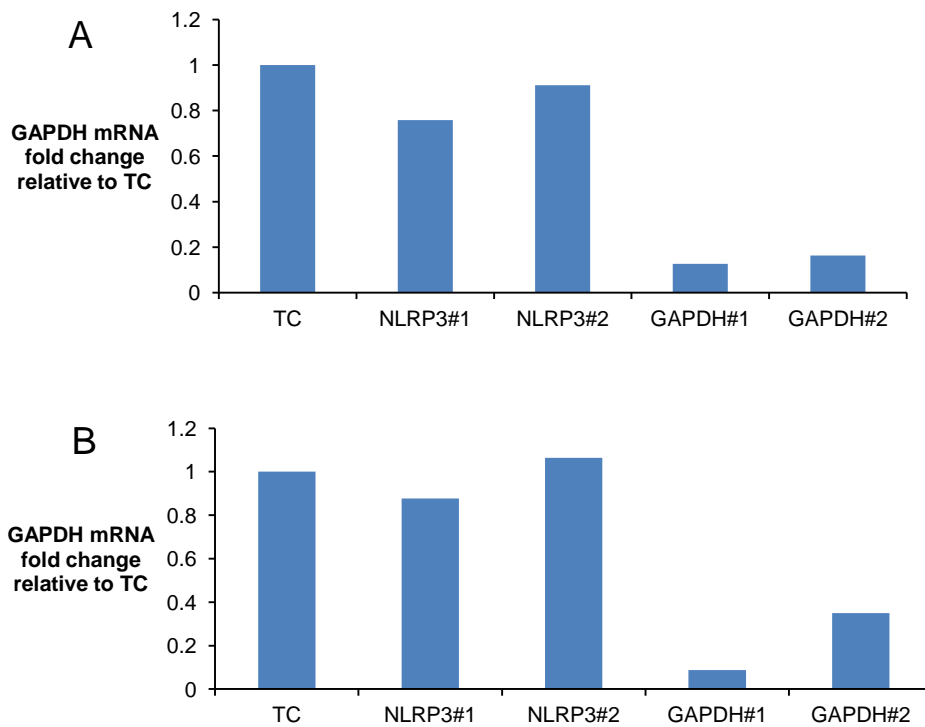


Figure 5.2: Knockdown of GAPDH mRNA in ovine (A) and bovine (B) MDMs. Ruminant MDMs were transfected with NLRP3 and GAPDH siRNA for 24 hours. The GAPDH mRNA fold changes relative to the transfection reagent only control (TC) are presented.

A preliminary study investigating siRNA gene knockdown over time identified that NLRP3 knockdown was stable at 24, 48 and 72 hours following transfection in both ovine and bovine MDMs (Figure 5.3).

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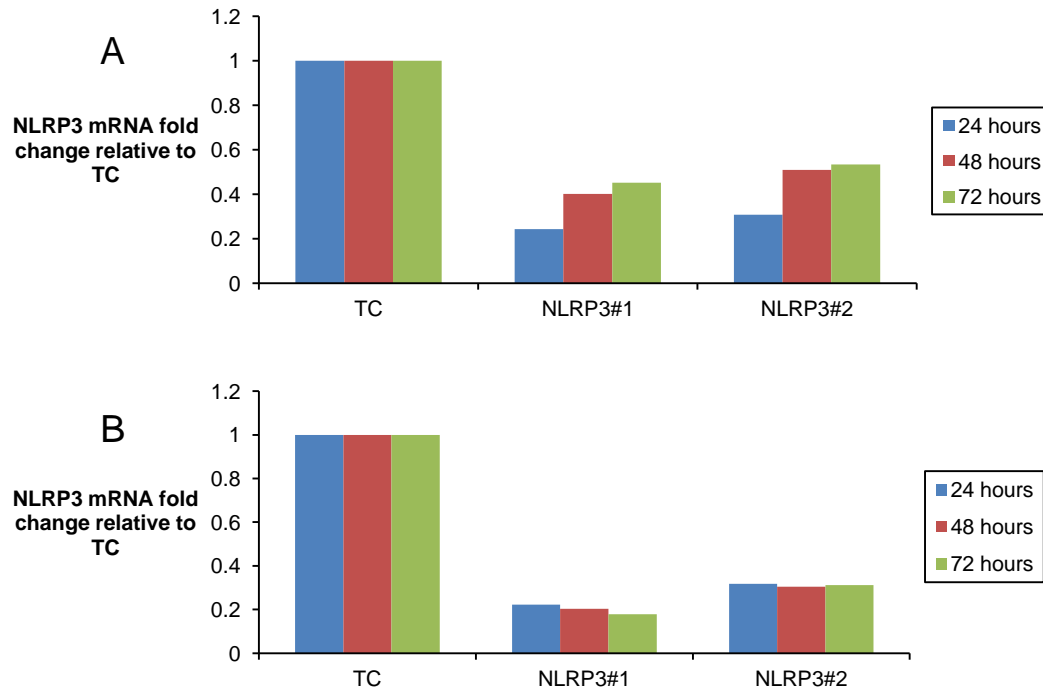


Figure 5.3: Knockdown of NLRP3 across 24, 48 and 72 hours in ovine (A) and bovine (B) MDMs. Ruminant MDMs were transfected with NLRP3#1 and NLRP3#2 and fold change was calculated by comparing the NLRP3 siRNA treated cells to the transfection reagent only control (TC).

Ruminant MDMs were transfected with NOD1 and NLRP3 siRNA. Controls included NC siRNA, TC and NTC. The expression of NLRP3 and NOD1 was measured at 72 hours post transfection and fold change calculated relative to the NTC. NOD1 expression was significantly reduced with both NOD1#1 and NOD1#2 compared to the NC siRNA control in both ovine and bovine MDMs ($p < 0.05$). NOD1#1 reduced NOD1 expression by over 65% in bovine and 50% in ovine MDMs and NOD1#2 reduced expression by over 80% in bovine and 64% in ovine MDMs. NLRP3 expression was significantly reduced compared to the NC siRNA ($p < 0.05$) by around 45% with NLRP3#2 in bovine MDMs. NLRP3#1 and NLRP3#2 reduced NLRP3 expression by over 40% in ovine MDMs, although this was not significant compared to the NC siRNA control ($p > 0.05$). Similarly, NLRP3#1 reduced NLRP3 expression in bovine MDMs, but this was not significant compared to the NC siRNA control ($p > 0.05$) (Figure 5.4).

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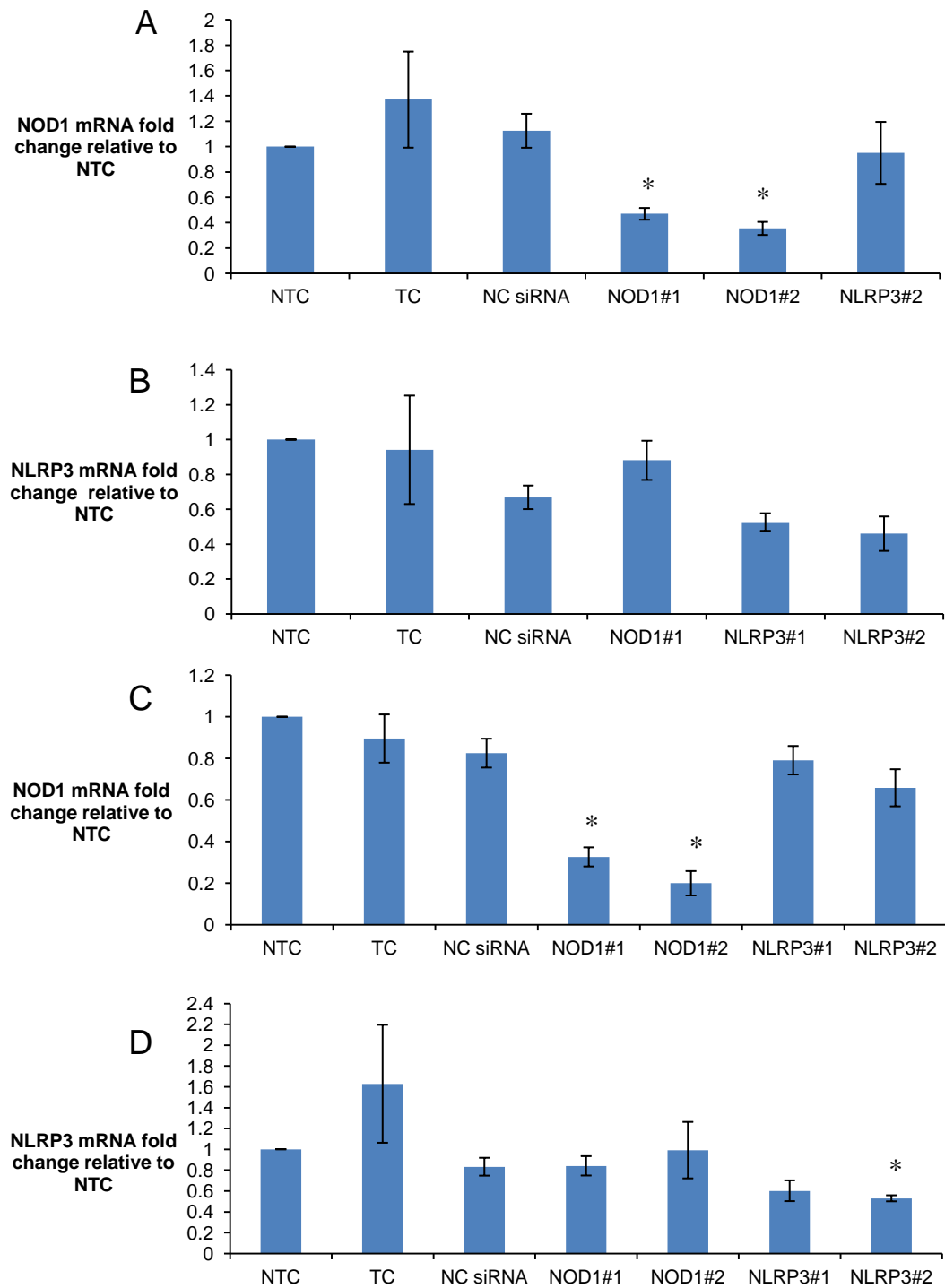


Figure 5.4: Knockdown of NOD1 (A, C) and NLRP3 (B, D) in ovine (A, B) and bovine (C, D) MDMs. The fold change of NLRP3 and NOD1 was calculated by comparing expression to the non-treated control cells (NTC). The data are the means of results using MDMs from at

least 3 different animals and the error bars represent standard error between the replicates. Significant reductions in NOD1 and NLRP3 relative to the negative control (NC) siRNA ($p < 0.05$) are denoted with asterisks.

5.3.3 Impact of NOD1 and NLRP3 knockdown on IL-1 β production

The impact of NOD1 and NLRP3 knockdown on the IL-1 β produced by ruminant MDMs infected with *C. abortus* was assessed (Figure 5.5). The knockdown of NOD1 but not NLRP3 resulted in significantly reduced IL-1 β production by over 50% compared to the NC siRNA in ovine MDMs ($p < 0.05$). In contrast, the knockdown of both NOD1 and NLRP3 results in significantly reduced IL-1 β production by over 55% compared to the NC siRNA in bovine MDMs ($p < 0.05$). There was no significant difference between the different control groups ($p > 0.05$).

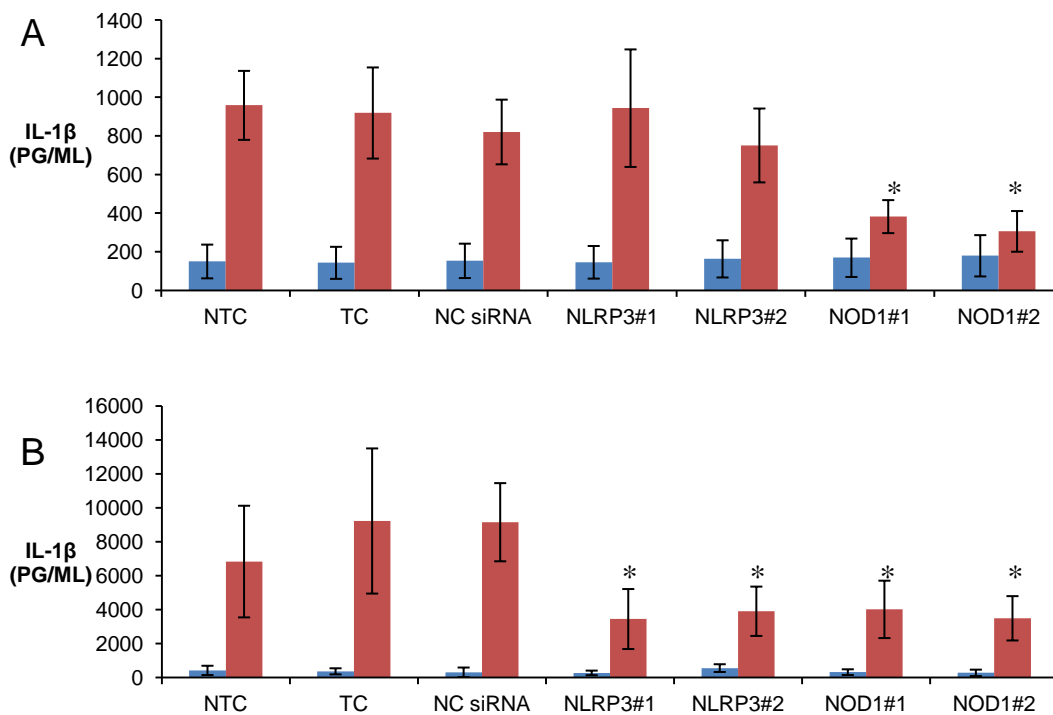


Figure 5.5: IL-1 β protein production from siRNA treated ovine (A) and bovine (B) MDMs that were infected with *C. abortus* (red bars) and non-infected control cells (blue bars). The presented data are means of results from 4 animals and the error bars represent standard error between the replicates. Significant reductions in IL-1 β relative to the scrambled siRNA ($p < 0.05$) are denoted with asterisks.

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Preliminary investigation into the impact of NOD1 and NLRP3 knockdown on IL-1 β mRNA revealed a similar pattern of IL-1 β mRNA expression in ovine MDMs as observed with protein production (Figure 5.6). A decrease in IL-1 β mRNA was observed when NOD1 but not NLRP3 was knocked down.

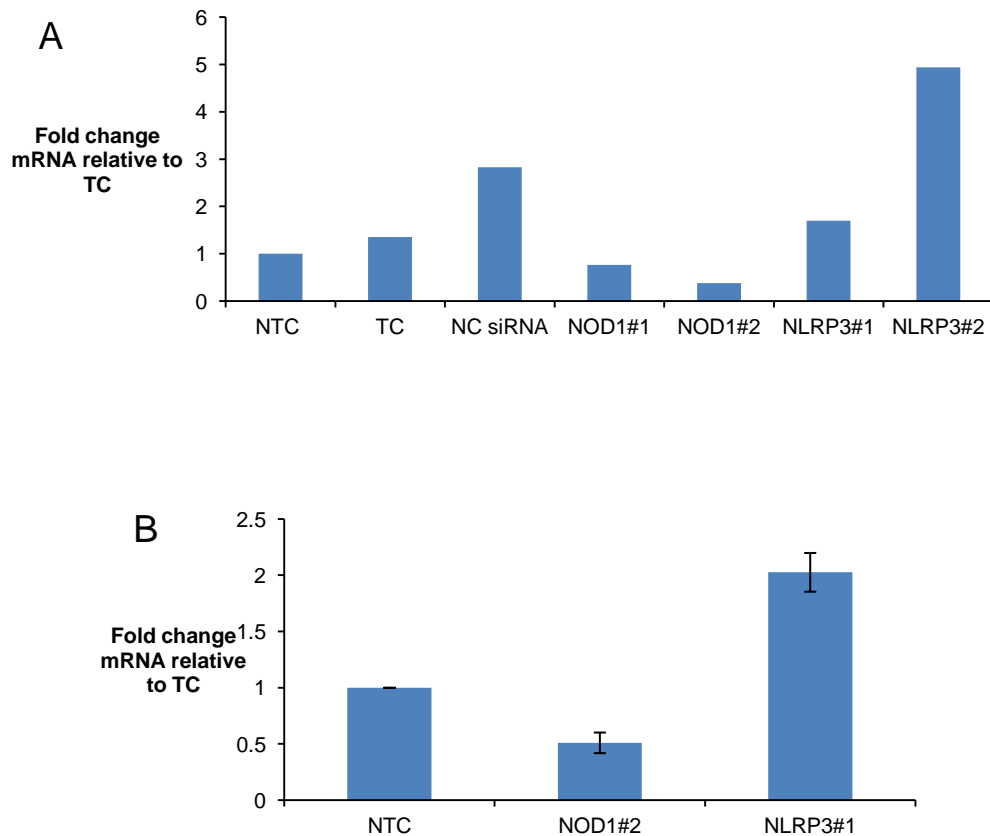


Figure 5.6: IL-1 β mRNA expression from siRNA treated ovine MDMs following *C. abortus* infection. The fold change of NLRP3 and NOD1 was calculated by comparing expression to the non-treated control cells (NTC). The presented data in A is representative of results from MDMs from 2 different animals and the data in B are means of results from 3 different animals with error bars which represent standard error between the replicates.

The RT-PCR primers and probes do not distinguish between pro and mature IL-1 β and this information is critical for assessing IL-1 β signalling pathways activated by NOD1 and NLRP3.

5.3.4 Assessing the production of pro and mature IL-1 β by Western blot

The production of IL-1 β requires two processing steps: the transcription of a pro-form of the cytokine and cleavage of the pro-form into mature IL-1 β (as discussed in Chapter 1). There are no published protocols for the differentiation of ruminant pro and mature IL-1 β , however previous studies in model organisms have successfully used Western blot to distinguish between the two (Kavathas et al., 2013). Western blotting separates proteins based on molecular weight and therefore can be used to distinguish the pro and mature form of IL-1 β . The pro-form of IL-1 β weighs 31 kilodalton (kDa) and the mature form weighs 17kDa (Chen et al., 2013b; Soller et al., 2007). Therefore a Western blot using an anti-ovine IL-1 β antibody (which cross-reacts with bovine) was optimised to identify the different components of ruminant IL-1 β . Transfected CHO cells stably expressing recombinant ovine IL-1 β secreted mature cytokine into the supernatant and a single clear band was detected at 17kDa. The soluble lysate expressed a band at around 17kDa, but also a number of larger bands around 31kDa that may represent pro-IL-1 β (Figure 5.7).

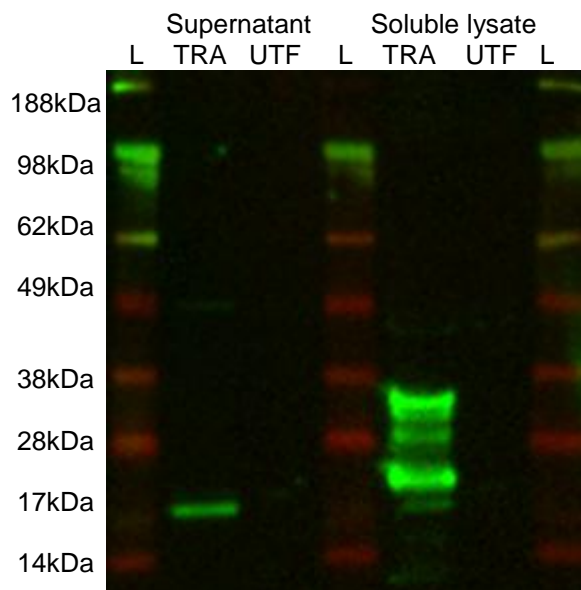


Figure 5.7: The expression of pro- and mature IL-1 β by CHO cells transfected to express ovine IL-1 β . Supernatant and soluble lysate from CHO cells transfected with ovine IL-1 β (TRA) and untransfected CHO cells (UTF) were analysed using Western blot and band sizes attained compared to the molecular weight marker ladder (L).

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The same optimised Western blot was used to assess supernatants and cell lysates from ovine MDMs that were infected with *C. abortus* and compared to non-infected control MDMs. A single protein band of around 17kDa was present in both the supernatants and cell lysates of the infected MDMs. Larger bands around 31kDa were present in the supernatants but not the soluble lysates of infected MDMs. The Western blot detected an extra band in animal 2 which was larger than 31kDa. There were no bands detected in the non-infected control MDMs (Figure 5.8).

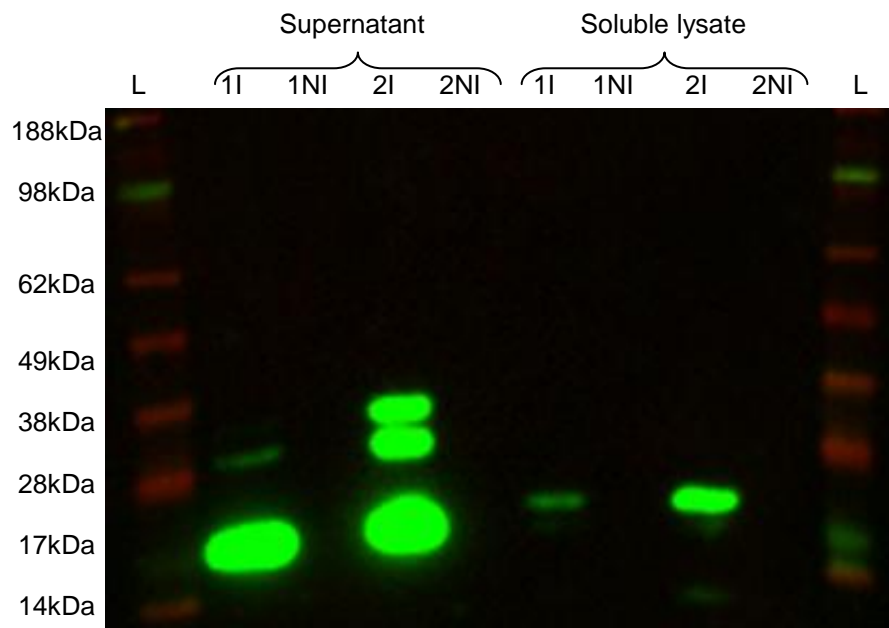


Figure 5.8: Pro- and mature IL-1 β expression by ovine MDMs from two sheep (1, 2).

The supernatant and soluble lysate from non-infected (NI) and *C. abortus*-infected (I) MDMs were analysed using Western blot and band sizes attained compared to the molecular weight marker ladder (L).

5.3.5 Caspase-1 activation by ruminant MDMs in response to *C. abortus*

The cleavage of pro-IL-1 β into mature IL-1 β is mediated by caspase-1 (Franchi et al., 2012). Therefore, caspase-1 activation was measured using the FAM-FLICA[™] caspase-1 kit. The kit contains fluorescently labelled inhibitors of caspase-1 that bind to active caspase-1. Cells that have activated caspase-1 retain the fluorescent signal

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following the wash steps, unlike the cells that have not activated caspase-1. The kit is cross-reactive with cattle and has previously been used to measure caspase-1 activation in bovine monocytes (Hussen et al., 2012). Caspase-1 activation was measured in ruminant MDMs at 6 and 24 hours post *C. abortus* infection. There was no caspase-1 activation detected at 6 hours post-infection, but a shift in fluorescence was detected at 24 hours post-infection, which was higher in bovine than ovine MDMs (Figure 5.9). However, the fluorescence measured in ruminant MDM infected with *C. abortus* was variable and the observed shift in fluorescence often not distinct. The aim was to use this method of measuring caspase-1 activation to assess the impact of NOD1 and NLRP3 knockdown. However, preliminary data from siRNA treated ruminant MDMs was difficult to interpret and this caspase-1 detection method may not be sensitive enough to detect small but critical differences within MDMs following infection with *C. abortus*.

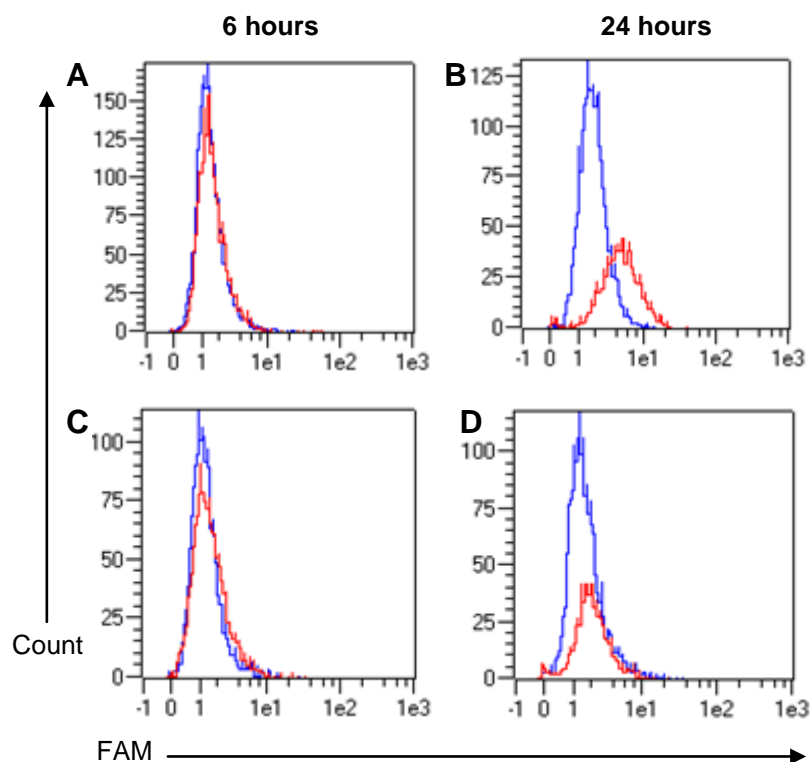


Figure 5.9: Caspase-1 activation by bovine (A, B) and ovine (C, D) MDMs in response to *C. abortus*. The histogram of the non-infected cells (blue) is shown compared to the *C.*

abortus-infected cells (red). The histograms are from one animal but are representative of the profiles observed in three animals.

5.3.6 Impact of NOD1 and NLRP3 knockdown on CXCL8 and TNF- α production

The role of NOD1 and NLRP3 in the production of CXCL8 in response to *C. abortus* was also assessed. NOD1 knockdown significantly reduced CXCL8 production from ovine MDMs in response to *C. abortus* compared to the NC siRNA treated MDMs ($p < 0.05$). NLRP3 knockdown had no effect on CXCL8 production from the ovine MDMs infected with *C. abortus* ($p > 0.05$). The bovine MDMs did not produce significantly different levels of CXCL8 in response to *C. abortus* with NLRP3 or NOD1 knockdown compared to the NC siRNA ($p > 0.05$) (Figure 5.10).

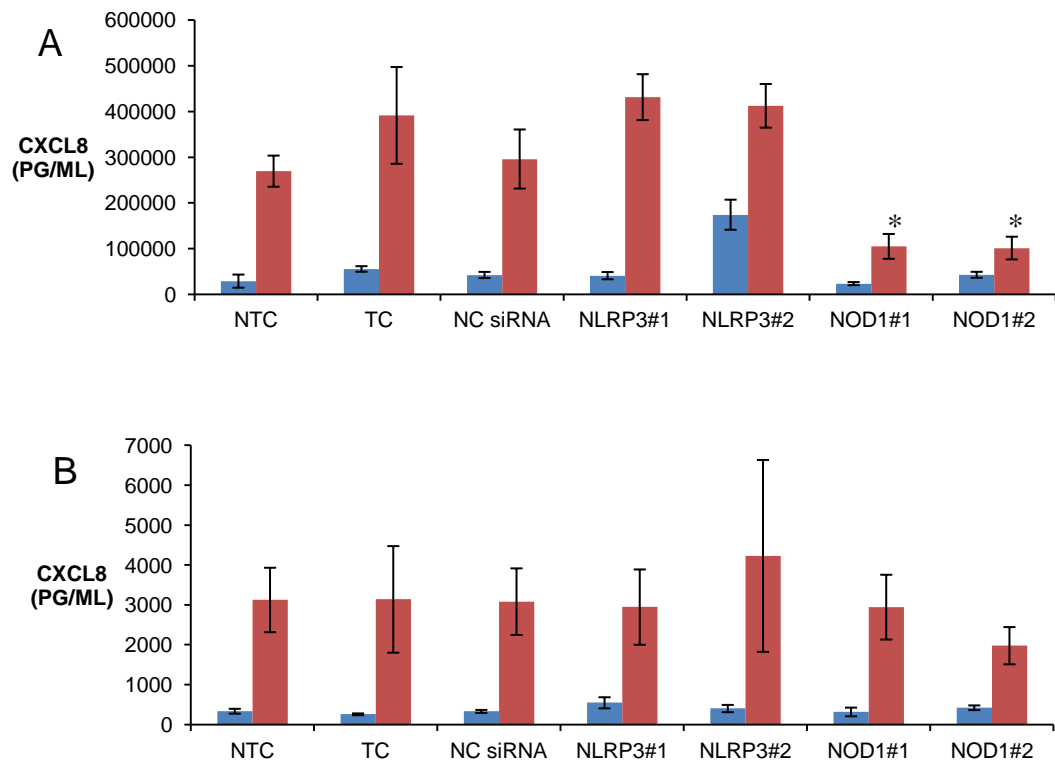


Figure 5.10: CXCL8 protein production from siRNA treated ovine (A) and bovine (B) MDMs which were challenged with *C. abortus* (red bars) and non-infected control cells (blue bars). The presented data are means of results from 4 animals and the error bars represent standard error between the replicates. Significant reductions in CXCL8 relative to the NC siRNA ($p < 0.05$) are denoted with asterisks.

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The role of NOD1 and NLRP3 in the production of TNF- α protein was also assessed. The background production of TNF- α was high in the ovine MDMs and there was no difference in production between the cells infected with *C. abortus* and non-infected cells. NLRP3 and NOD1 knockdown had no significant effect on TNF- α production in ovine MDMs infected with *C. abortus* ($p > 0.05$). The bovine MDMs had a lower level of TNF- α background production and significantly reduced ($p < 0.05$) TNF- α production with NOD1 knockdown (although only with NOD1#2 siRNA) (Figure 5.11).

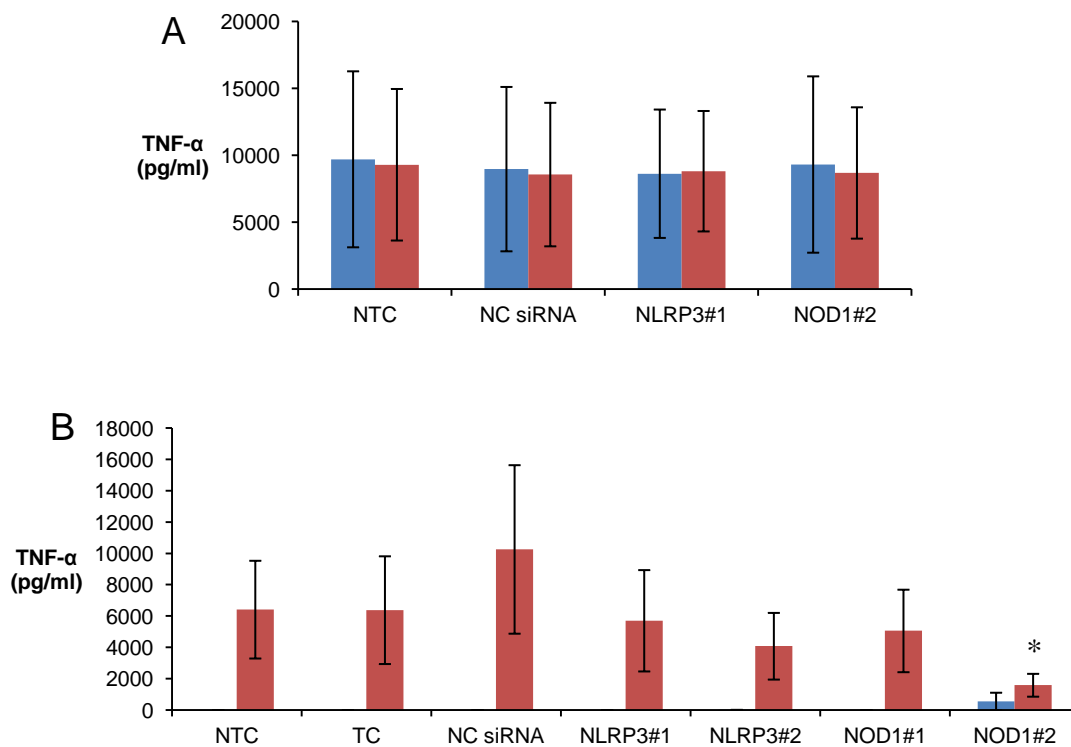


Figure 5.11: TNF- α protein production from siRNA treated ovine (A) and bovine (B) MDMs which were challenged with *C. abortus* (red bars) and non-infected control cells (blue bars). The presented data are means of results from at least 3 different animals and the error bars represent standard error between the replicates. Significant reductions in TNF- α relative to the scrambled siRNA ($p < 0.05$) are denoted with asterisks.

5.4 Discussion

The use of siRNA for gene knockdown is an effective method for studying gene function in various cell types. Recently, a siRNA protocol was optimised for the effective transfection of siRNA into bovine MDMs. The protocol has been successfully used to knockdown a number of different genes in bovine MDMs, including MEFV (Jensen et al., 2014) and TLR5 (Tahoun et al., 2015). Similarly, we found that the protocol demonstrated high transfection efficiency and knockdown of the expression of NOD1, NLRP3 and GAPDH in both cattle and sheep MDMs. The knockdown of NLRP3 mRNA was lower than that of NOD1 and not found to be statistically significant in ovine MDMs. Ideally, the level of knockdown should be measured at both the mRNA and protein level (Mocellin and Provenzano, 2004), however commercial ruminant antibodies for the detection of NLRP3 or NOD1 protein are not available. Recently, however, human NLRP3 antibodies have been demonstrated to recognise bovine NLRP3 using western blot (Jensen et al, unpublished) and may provide tools for further analysis.

NOD1 knockdown significantly decreased IL-1 β production in both ovine and bovine MDMs in response to *C. abortus* infection. The same result was obtained using two siRNA that target different parts of the gene, thereby increasing confidence in the result (Mocellin and Provenzano, 2004). Previous studies have identified that NOD1 is involved in the recognition of *Chlamydia* and production of pro-inflammatory cytokines, but most did not investigate the impact of NOD1 on IL-1 β production (Buchholz and Stephens, 2008; Opitz et al., 2005; Welter-Stahl et al., 2006). A previous study demonstrated a role for NOD1 in *C. trachomatis* mediated IL-1 β production from a human trophoblast cell line. The study identified that IL-1 β protein produced in response to *C. trachomatis* was independent of TLR2, TLR4, NLRP3 and ASC, but NOD1 knockdown decreased production compared to the non-target shRNA control (Kavathas et al., 2013). A role for NOD1 in IL-1 β production has also been identified in response to other intracellular bacteria. Following lung infection with *Legionella pneumophila*, NOD1 KO mice had reduced IL-1 β detected in lung homogenates at 4 hours post infection and also displayed delayed neutrophil

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recruitment and impaired bacterial clearance compared to the wild-type mice (Berrington et al., 2010). Similarly, NOD1 KO mice intratracheally infected with *C. pneumoniae* displayed impaired bacterial clearance compared to wild-type mice, although the level of IL-1 β production was not measured (Shimada et al., 2009).

NOD1 recognises peptidoglycan expressed by Gram-negative bacteria (Opitz et al., 2005), including *Chlamydia* in which expression has recently been identified. The development of a fluorescently labelled D-amino acid dipeptide probe, which is incorporated into peptidoglycan during synthesis, allowed the investigation of the expression of peptidoglycan by *C. trachomatis*. Peptidoglycan was detected from 8 hours post-infection, which supports the current theory that peptidoglycan is synthesised during the conversion of EBs into RBs (Liechti et al., 2014). Therefore, the potential role for NOD1 in the detection of *C. abortus* and consequential activation of pro-inflammatory signalling pathways, may explain why the production of IL-1 β protein in ruminant MDMs infected with *C. abortus* is observed within 24 hours post infection (see Chapter 4).

Upon recognition of peptidoglycan, NOD1 interacts with the adaptor molecule RIP2. RIP2 mediates a signalling cascade, which involves the activation of NF- κ B and MAPK signalling pathways (Kaparakis-Liaskos, 2015). The production of pro-IL-1 β is mediated by the activation of NF- κ B (Latz et al., 2013) and therefore NOD1 may mediate pro-IL-1 β production (Kersse et al., 2011). Preliminary investigation identified that the production of IL-1 β mRNA mirrored the protein profile of ovine MDMs, which suggests that NOD1 may mediate the production of pro-IL-1 β . NOD1 also contains a CARD domain and therefore may activate caspase-1 for cleavage and production of mature IL-1 β . A role for NOD1 and RIP2 in caspase-1 activation and induction of IL-1 β secretion in response to LPS was identified using transfection assays in human cell lines (Yoo et al., 2002). The knockdown of NOD1 specifically decreased the quantity of mature IL-1 β but not pro-IL-1 β released into the supernatant by human trophoblasts following infection with *C. trachomatis*. This indicates that NOD1 is involved in the cleavage of pro-IL-1 β ; however, a limitation

of this study was that the level of intracellular pro-IL-1 β was not measured (Kavathas et al., 2013).

A role for NLRP3 in stimulating IL-1 β production was also identified in bovine MDMs. Therefore, the relatively low level of knockdown achieved with these siRNA was sufficient to have a functional effect. Previous studies into the role of NLRP3 have also confirmed the receptors involvement in stimulating IL-1 β production with chlamydial infection. The knockdown of NLRP3 using shRNA in a human monocytic cell line decreased IL-1 β production in response to both *C. muridarum* and *C. trachomatis* compared to cells transfected with the non-targeting shRNA (Abdul-Sater et al., 2010). The knockdown of NLRP3 using siRNA in a human monocytic cell line reduced the production of IL-1 β with *C. pneumoniae* infection compared to cells treated with scrambled siRNA (Matsuo et al., 2015). BMDM from NLRP3 KO mice produced reduced IL-1 β in response to *C. pneumoniae* compared to BMDM from wild-type mice (He et al., 2010; Itoh et al., 2014; Shimada et al., 2011). The knockdown of NLRP3 in human PBMC reduced IL-1 β produced in response to *C. pneumoniae* compared to PBMC treated with non-silencing control siRNA (Eitel et al., 2012)

In contrast, there was no impact of NLRP3 knockdown on IL-1 β production from ovine MDMs infected with *C. abortus*. The lack of a role for NLRP3 may be reflective of the low levels of knockdown achieved with NLRP3 siRNA or indicative of species-specific difference in *C. abortus* recognition. Studies have previously identified a redundancy for NLRP3 in the induction of IL-1 β secretion in response to chlamydial infection. NLRP3 knockdown did not reduce *C. trachomatis* induced IL-1 β production in a human trophoblast cell line (Kavathas et al., 2013). The production of IL-1 β was not reduced in peritoneal macrophages, which were pre-stimulated with *E. coli* LPS, derived from NLRP3 KO mice but was reduced in peritoneal macrophages derived from ASC KO mice following infection with *C. muridarum* (Nagarajan et al., 2012). Therefore, other inflammasome receptors may be involved in the detection of chlamydial infection and may be worth further investigation.

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In order to fully understand the IL-1 β production pathway, a Western blot that could detect the ruminant pro and mature form of IL-1 β was optimised. The detection of a clean 17kDa band represents mature IL-1 β and a number of bands were detected around 31kDa that may represent the pro-form of IL-1 β . The multiple bands detected around 31kDa added complexity to the interpretation of the Western blot. The multiple bands may be indicative of the action of various proteases, which can cleave IL-1 β at different cleavage sites (Afonina et al., 2015), or may be due to glycosylation (Livi et al., 1991; Opdenakker et al., 1995). Mature IL-1 β was detected in the supernatant and lysate of *C. abortus*-infected ovine MDMs by Western blot, which confirms previous ELISA and RT-PCR results (see Chapter 4). Bands around the size of pro- IL-1 β were also detected in the supernatant of *C. abortus*-infected ruminant MDMs. The secretion of pro- IL-1 β may be indicative of necrotic cell death (Lopez-Castejon and Brough, 2011), but pro-IL-1 β is also produced by mouse macrophages independent of cell death. Released pro- IL-1 β can be cleaved by extracellular proteases, which can be secreted by neutrophils at the site of infection, into the more potent mature form (Pelegrin et al., 2008). Further analysis including N-terminal protein sequencing of the bands could be used to identify what the bands represent. Thereafter this technique could be used to assess the effect of NOD1/NLRP3 knockdown on pro-IL-1 β production, which would inform on the type of signalling pathways activated by these receptors.

Caspase-1 activation was detected in bovine MDMs at 24 hours post *C. abortus* infection using the FAM-FLICA™ assay. The early activation of caspase-1 in response to chlamydial infection is consistent with previous studies. Caspase-1 activation was detected using an active caspase-1 antibody and fluorescently labelled secondary antibody for flow cytometric analysis of primary mouse mast cells at 24 hours following infection with *C. pneumoniae* (Rodriguez et al., 2015). The activation of caspase-1 was detected in HeLa cells at 24 hours following *C. trachomatis* infection using flow cytometry. The shRNA mediated knockdown of NLRP3 decreased caspase-1 activation by around 50%, compared to cells treated with the non-target shRNA control, following *C. trachomatis* infection of HeLa cells

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(Abdul-Sater et al., 2009). The use of a cell line rather than more variable primary cells may explain the clear up-regulation of caspase-1 and ability to measure differences with gene knockdown unlike in ruminant MDMs.

Caspase-1 activation has been identified as an important component in IL-1 β secretion in response to chlamydial infection. Peritoneal macrophages harvested from caspase-1 knockout mice expressed significantly less IL-1 β in response to *C. muridarum* compared to macrophages from wild-type mice. Caspase-1 knockout macrophages could still respond through TNF- α and IL-6 production at a similar level to WT macrophages indicating the specificity of its action (Cheng et al., 2008). Caspase-1 is also involved in the production of IL-18 as demonstrated in HeLa cells, which expressed decreased IL-18 in response to *C. trachomatis* in the presence of a caspase-1 inhibitor. Peritoneal macrophages from caspase-1 knockout mice also expressed reduced IL-18 in response to *C. trachomatis* or *C. muridarum* compared to macrophages from wild-type mice (Lu et al., 2000). A role for caspase-1 in *C. pneumoniae* mediated IL-1 β and IL-18 production was identified in a human monocyte/macrophage cell line using a caspase-1 inhibitor (Matsuo et al., 2015). Similarly, the addition of a caspase-1 inhibitor to murine BMDMs reduced the production of IL-1 β production with *C. pneumoniae* infection (Itoh et al., 2014).

Caspase-1 activation was not detected in ovine MDMs infected with *C. abortus*. Caspase-1 is not the only inflammatory caspase that has been implicated in IL-1 β processing. Murine caspase-11 (orthologous to human caspase-4 and caspase-5) can activate the non-canonical inflammasome, which potentiates caspase-1 activation but can also function independently of caspase-1 (Kayagaki et al., 2011). The combined expression of caspase-1 and caspase-5 was required for optimal pro-IL-1 β cleavage in a human monocyte cell line (Martinon et al., 2002). A combined caspase-1 and caspase-5 inhibitor had an increased effect on reducing the infectivity of *C. trachomatis* in HeLa cells compared to the caspase-1 inhibitor (Abdul-Sater et al., 2009). Therefore, further analysis of the expression of other inflammatory caspases may more fully inform on the role of caspases in *C. abortus* mediated IL-1 β production. The production of IL-1 β may also be caspase-1 independent. Previous

studies in *C. muridarum* and *Mycobacterium tuberculosis* have identified that IL-1 β production may be produced *in vivo* in a caspase-1 independent manner (Mayer-Barber et al., 2010; Nagarajan et al., 2012). A potential caspase-1 independent mechanism of pro-IL-1 β cleavage is mediated by serine proteases, which can be expressed by macrophages and neutrophils (Netea et al., 2010). A role for serine proteases in IL-1 β production in response to chlamydial infection has previously been demonstrated. The production of IL-1 β from LPS-primed murine peritoneal macrophages infected with *C. muridarum* was inhibited by treatment with a serine protease inhibitor (Prantner et al., 2009).

NOD1 activates the NF- κ B and MAPK signalling pathways that are involved in the production of other pro-inflammatory cytokines, including CXCL8 and TNF- α (Kaparakis-Liaskos, 2015; Sabio and Davis, 2014). The knockdown of NOD1 decreased CXCL8 production from ovine MDMs following *C. abortus* infection, which is consistent with previous studies. The knockdown of NOD1 in HeLa cells using siRNA resulted in reduced CXCL8 expression induced by *C. muridarum* infection compared to the cells treated with non-target siRNA (Prantner et al., 2010). The knockdown of NOD1 decreased CXCL8 production by human trophoblast cell lines in response to *C. trachomatis* compared to the cells treated with non-target shRNA (Kavathas et al., 2013). The knockdown of NOD1 in human endothelial cells decreased CXCL8 produced in response to *C. pneumoniae* compared to cells treated with the non-silencing control siRNA. This study also identified that the over-expression of NOD1 in human embryonic kidney cells increased NF- κ B activation mediated by *C. pneumoniae* (Opitz et al., 2005). NOD1 also contributed to the activation of the NF- κ B pathway in HeLa cells infected with *C. muridarum* (Welter-Stahl et al., 2006). The knockdown of NOD1 in ovine MDMs had no effect on TNF- α expression indicating differences in the activation of specific cytokine signalling pathways. Similarly, NOD1 knockdown in human endothelial cells resulted in decreased CXCL8 compared to the non-silencing control siRNA, but had no effect on TNF- α production in response to *C. pneumoniae* (Opitz et al., 2005). The knockdown of NOD1 decreased TNF- α but not CXCL8 expression in bovine MDMs,

indicating species-specific differences in the signalling pathways activated in ruminant MDMs in response to *C. abortus* infection.

NLRP3 has primarily been associated with the assembly of the inflammasome complex and regulation of IL18 and IL-1 β production (Kersse et al., 2011). The NF- κ B and MAPK signalling pathways can be activated independently of NLRP3 as shown by knockout experiments (Kanneganti et al., 2006). The knockdown of NLRP3 had no effect on CXCL8 or TNF- α production in either ovine or bovine ruminant MDMs. Similarly, BMDMs from NLRP3 KO mice produced similar levels of TNF- α in response to *C. pneumoniae* compared to BMDMs from the wild-type (Shimada et al., 2011). Therefore, NLRP3 does not appear to be involved in activating the signalling pathways necessary for CXCL8 or TNF- α production by ruminant MDMs in response to *C. abortus* infection.

The role for NOD1, NLRP3 and caspase-1 in determining the outcome of chlamydial infection *in vivo* is controversial. RIP2 KO mice displayed inhibited *C. pneumoniae* clearance from the lung and increased inflammation, which resulted in increased mortality compared to the wild-type controls (Shimada et al., 2009). However, the production of inflammatory cytokines and chemokines, including IL-6, IL-1 β and MIP-2, in the genital tract was unaffected in NOD1 deficient mice challenged with *C. muridarum*. There was also no difference in the clearance of infection in NOD1 deficient mice compared to wild-type mice (Welter-Stahl et al., 2006). NLRP3 KO mice had reduced IL-1 β in genital secretions at 2 days post *C. muridarum* infection, but levels were comparable to wild-type mice levels from 3 days post-infection onwards. In the same study, NLRP3 KO mice did not have a significantly different rate of chlamydial clearance or incidence of hydrosalpinx pathology compared to the wild type mice (Nagarajan et al., 2012). *In vivo* caspase-1 KO mice infected with *C. muridarum* presented with significantly reduced signs of inflammatory pathology, but had a similar course of infection to the wild-type mice (Cheng et al., 2008). Caspase-1 KO mice exhibited delayed bacterial clearance and increased mortality in response to *C. pneumoniae* infection compared to wild type mice. The early addition of recombinant IL-1 β rescued caspase-1 KO mice from increased mortality rates

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(Shimada et al., 2011). Although *in vitro* the role for different PRRs can be clearly demonstrated, often *in vivo* there is no clear effect of knockdown of a single receptor. This highlights the redundancy of single PRRs and the potential of the immune system to compensate for missing PRRs (Darville et al., 2003; Welter-Stahl et al., 2006). In addition, these studies have all been conducted in mouse models and care needs to be taken when extrapolating the data to other hosts and different chlamydial infections.

The host cell response to an infection is likely to be controlled by multiple PRRs, which is why knockdown or KO studies rarely show complete ablation of cytokine production. Studies have identified that TLRs and NLRs often collaborate in order to produce cytokine/chemokines in response to stimuli (Oviedo-Boyso et al., 2014). A number of studies have identified that a single pathogen can be recognised by multiple PRRs (Kersse et al., 2011; Oviedo-Boyso et al., 2014). Therefore, future studies investigating the combinatory knockdown of NOD1, NLRP3, TLR2, TLR4 and caspase-1 or other inflammasome components in ruminant MDMs may further illuminate the signalling pathways activated by *C. abortus*.

In order to summarize the five chapter objectives were revisited. The first objective was to establish the siRNA procedure in ovine MDMs, which were successfully transfected with fluorescently labelled siRNA and target genes knocked down. Secondly, siRNA-mediated knockdown of NOD1 and NLRP3 in ruminant MDMs was investigated and were found to induce knockdown of both receptors. The knockdown of NLRP3 was low, but future experiments with pooled siRNA may increase knockdown level. The third objective was to investigate the role of NOD1 and NLRP3 in *C. abortus* driven IL-1 β production. In both ovine and bovine MDMs, NOD1 was found to be important for the production of IL-1 β and this may be mediated through a number of different pathways. NLRP3 was important for IL-1 β production in bovine but not ovine MDMs, which indicates potentially important species-specific differences in *C. abortus* recognition (Table 5.2). The impact of *C. abortus* infection on the different components of the IL-1 β processing pathway was then assessed, and further optimisation of the techniques used to detect the

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components of the ruminant IL-1 β pathway will aid future studies. The final aim was to investigate the role of NOD1 and NLRP3 in the production of other pro-inflammatory cytokines/chemokines. Species-specific differences were identified in the role of NOD1 in CXCL8 and TNF- α production, but NLRP3 knockdown did not affect CXCL8 or TNF- α production in either species (Table 5.2).

Table 5.2: Summary table of the impact of NOD1/NLRP3 knockdown on cytokine production in ruminant MDMs following *C. abortus* infection.

	IL-1 β	CXCL8	TNF- α
Ovine NOD1	↓	↓	-*
Ovine NLRP3	-	-	-*
Bovine NOD1	↓	-	↓**
Bovine NLRP3	↓	-	-

↓ Detectable down-regulation in production; -no change in production; *only tested one siRNA; **down-regulation identified with one siRNA and not the other.

Therefore, NOD1 and NLRP3 are involved in recognising *C. abortus* and activating the likely complex pro-inflammatory signalling pathways in ruminant MDMs.

Chapter 6: RNA-seq analysis of ruminant MPS cells and MDMs infected with *C. abortus*

6.1 Introduction

The term ‘transcriptome’ describes the expression profile of mRNA, non-coding RNA and small RNA in an organism (Wang et al., 2009). Transcriptomic analysis can be used to investigate the unique gene expression profiles that characterize different cell types (Vu Manh et al., 2015b) and define the cellular response to different pathogenic stimuli (Hossain et al., 2006). The availability of annotated reference genomes for both sheep and cattle facilitates the study of the ruminant transcriptome. Major advances in transcriptomic analysis, including the development of microarrays, and more recently RNA-seq, have facilitated deeper understanding of gene expression and potential function in these species (Bai et al., 2012).

Microarrays involve the hybridisation of probes to target sequences (Miller and Tang, 2009) and can be used to assess a large number of genes at a relatively low cost (Roy et al., 2011). Microarray technology continues to improve and several microarray platforms that use different methods of hybridization are commercially available (Kim et al., 2010). Microarray technology has been used to study cell differentiation and host cell responses to pathogens, including *Chlamydia*. The Immunological Genome Project (ImmGen) was established to assess the gene expression profile of all immune cell subsets in the mouse (Heng and Painter, 2008). Already, the data from ImmGen has led to the improved understanding of the genes contributing to cell function and the differential expression of genes between cell subsets (Chow et al., 2011; Kim and Lanier, 2013). The FANTOM consortium has developed novel technology to interrogate genomic data, which has contributed to the understanding of gene function and regulation in mice and humans (Hoon et al 2015). Information from the FANTOM consortium has been used to set up macrophages.com, which is an online resource combining macrophage multi-omic data and bioinformatics tools to facilitate further analysis (Robert et al, 2011). Microarray data have also been used for the cross-species comparison (including

sheep and pigs) of the gene expression profiles of MPS cells (Vu Manh et al., 2015b). The host gene expression profile of human cells in response to chlamydial infection has also been studied using microarrays (Coombes and Mahony, 2001; Hess et al., 2003; Natividad et al., 2010; Ren et al., 2003; Schrader et al., 2007).

However, microarray technology has a number of limitations. The technology uses pre-designed probes to detect transcripts and is therefore dependant on prior knowledge of the genome sequence. The probes can also cross-hybridize to transcripts that have a similar sequence to the intended target, generating false results (Shendure, 2008; Wang et al., 2009). The quantified level of gene expression is dependent on the binding affinity of the probe, which is determined by technical factors such as probe length and design (Kim et al., 2010). These technical issues affect the dynamic range of detection and hinder the quantification of low or highly expressed transcripts (Roy et al., 2011; Wang et al., 2009). The comparison of results across different microarray studies is also often difficult due to the variability in results from diverse microarray platforms (Kim et al., 2010).

Transcriptomic analysis can also be conducted using sequencing-based approaches. The first studies using high-throughput RNA-seq technology were published in 2008 (Roy et al., 2011). In brief, this technology involves fragmentation of RNA transcripts and the preparation of a cDNA library from the fragmented RNA, which is then amplified using PCR. The library is sequenced and aligned to the relevant reference genome. Alternatively, *de novo* assembly can be used if no reference genome is available (McGettigan, 2013; Roy et al., 2011). The expression is quantified based on the number of sequences aligned to the gene (Wang et al., 2009). Although there are also limitations with RNA-seq (difficulties associated with analysing/storing large amounts of data and potentially subject to PCR artefacts) (Wang et al., 2009) the technology has a number of advantages over microarray, including that less starting material is required (McGettigan, 2013), knowledge of the genome sequence is not necessary and cross-hybridization is not an issue because RNA-seq does not use probes (Wang et al., 2009). RNA-seq technology can also lead to the discovery of novel non-coding RNAs, including miRNAs and siRNAs,

which can be associated with important cellular processes, whereas microarrays are restricted to the detection of known non-coding RNAs (Morozova et al., 2009). The technique is also more sensitive than microarrays as measures the transcript abundance directly instead of being limited by the hybridization features of the probe (Morozova et al., 2009).

RNA-seq has also been used to differentiate between cell types and define host-pathogen interactions but is still a relatively new technology, hence there are fewer published studies compared to the vast number of microarray studies. A recent study used RNA-seq to characterize gene expression profiles of monocytes as they differentiated into macrophages. The data correlated well with previously published microarray data on the monocyte-to-macrophage differentiation process, with RNA-seq detecting more lower-expressed genes, suggesting higher sensitivity (Dong et al., 2013). Microarray and RNA-seq have also been compared for the assessment of the gene expression profiles of polarized human macrophages. An increased number of significantly differentially expressed (DE) genes and two novel gene networks, of potential biological importance, were identified using RNA-seq compared to microarray analysis (Beyer et al., 2012). The gene expression profile of bovine MDMs infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was assessed using both RNA-seq and microarray technology. RNA-seq identified an increased number of DE genes compared to microarray analysis, which potentially have important roles in MAP disease pathogenesis (Casey et al., 2015). There is currently only one published RNA-seq study that has characterized the cellular host response to chlamydial infection. This study used a novel RNA-seq method that simultaneously profiled host cell and chlamydial gene expression at early time-points following infection. The identified DE genes included genes that had not previously been associated with chlamydial infection and may be involved in the pathogenesis of scarring (Humphrys et al., 2013).

A major challenge in analysing the transcriptomic data from both microarrays and RNA-seq is in the interpretation of the large data set results into biologically relevant information (Shendure, 2008). Typically, the functional analysis of large-scale data

of this type involves the identification of significantly DE genes, which are associated with a particular sample condition (e.g. infection). The biological relevance of the DE genes can be implied through the identification of functional categories that are enriched with DE genes. The identification of enriched cellular signalling pathways can also be useful for the biological interpretation of the data. Computer programmes are available for this type of analysis, including Ingenuity Pathway Analysis (IPA) which uses knowledge collated from published scientific sources to interpret large data sets (Roy et al., 2011).

Therefore, RNA-seq is an improved method for the in-depth analysis of the transcriptome. RNA-seq analysis has been used to differentiate cell types, but has not yet been used to differentiate cells of the ruminant MPS. RNA-seq can also be used to reveal the full gene expression profile of cells in response to a particular stimulus, such as *C. abortus*. The full profile of receptors and signalling pathways that stimulate the production of innate immune cytokines in ruminant MDMs in response to *C. abortus* infection (as described in Chapter 4) can be investigated using unbiased RNA-seq technology. Transcriptomic analysis can also be used to further explore and analyse the pathways that may underlie the observed differential *C. abortus* pathogenesis between cattle and sheep. The aims of this chapter were to:

- Investigate the transcriptome of ruminant MPS cells (as described in Chapter 2) for identification of differentiation-specific 'signature' profiles.
- Investigate the transcriptomic profile of *C. abortus*-infected ruminant MDMs.
- Infer the biological function of DE genes and enriched cellular pathways in *C. abortus*-infected MDMs compared to non-infected MDMs.

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- Compare the DE genes of cattle and sheep *C. abortus*-infected MDMs to identify species-specific pathways.

6.2 Materials and methods

6.2.1 Ruminant monocytes, MDCCs, MDMs and bovine CD16⁺⁺ cells

Ruminant CD14⁺ monocytes, MDCCs and MDMs were generated as described in section 2.2. The CD16⁺⁺ cells were included as part of a different study and were isolated as described in Corripio-Miyar et al., 2015. Four biological replicates from each cell type were generated.

6.2.2 Infection of ruminant MDMs for RNA-seq analysis

Ruminant MDMs were infected with an MOI of 1 *C. abortus* (S26/3) for 24 hours before harvesting lysates with TRIzol. Four biological replicates from ovine and bovine *C. abortus*-infected MDMs were generated.

6.2.3 TRIzol total RNA extraction

TRIzol (Life Technologies) was added in 1 ml volumes to cells and the cells lysed with repeat pipetting before storage at -80°C. The RNA was extracted according to the manufacturer's instructions. Briefly, the samples were defrosted at RT for 5 minutes before 200µl chloroform was added. The tube was vigorously shaken for 15 seconds and incubated for 2-3 minutes at RT. The sample was centrifuged at 12000g for 15 minutes at 4°C and the clear aqueous phase removed into a new tube. 500µl of 100% isopropanol containing 45µg/ml glycoblue (Ambion, Life Technologies) was added to the aqueous phase and incubated at RT for 10 minutes. The sample was centrifuged at 12000g for 10 minutes at 4°C. The supernatant was removed before the RNA pellet was resuspended in 1ml of 75% ethanol. The sample was vortexed and then centrifuged at 7500g for 5 minutes at 4°C. The supernatant was removed and the pellet air-dried for 10 minutes at RT. The pellet was resuspended in 25µl RNase-free water and quantified using the nanodrop. The quality was assessed using the Agilent RNA 6000 nano kit on the Agilent 2100 Bioanalyzer (Agilent technologies, Cheshire, UK). RNA integrity numbers (RINs) >8.4 were obtained for all RNA samples before submission to ARK genomics.

6.2.4 RNA-seq library preparation and sequencing

The NEBNext ultra directional RNA library prep kit for Illumina (New England Biolabs, Hitchin, UK) was used by ARK genomics to generate 36 sequencing libraries (4 of bovine CD16⁺⁺ cells and 4 each of ovine and bovine CD14⁺ monocytes, MDDCs, MDMs and *C. abortus*-infected MDMs). Briefly, NEBNext Oligo d(T)₂₅ beads were used to bind poly A RNA from 1 µg of total RNA. The bound RNA was then fragmented into 180-200 bp fragments and used as a template for cDNA library generation which involved first and second strand synthesis, blunt end repair, adaptor ligation and PCR amplification. The libraries were clustered and sequenced using the Illumina HiSeq 2500 for 125 bases paired end sequencing (see appendix 9.1 for further details provided by Mr Richard Talbot from ARK genomics).

6.2.5 RNA-seq data analysis

The raw reads data files were quality checked using FastQC software (version 0.10.1). Cutadapt v1.3 was used to trim 3'illumina adaptor sequences-m 50 -q 30 -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC. Reads were aligned to the bovine genome (UMD3.1 annotation, Ensembl release 78) and the ovine genome (UMD3.1 annotation, Ensemble release 78) using TopHat v2.0.13 (Kim et al., 2013) with --b2-sensitive. Reads that mapped to multiple sites were discarded and counts of the uniquely mapped genes were then generated using HTSeq-count v 0.6.0. EdgeR was used to conduct differential expression analysis which involved normalisation using the trimmed mean of M values (TMM) method (Robinson and Oshlack, 2010). Multidimensional scaling (MDS) and principal component analysis (PCA) plots were generated, using EdgeR and DEseq2 respectively, to show sample relationships and quality check the data. The distances in the MDS plot are the leading log-fold-changes between pairs of samples. The leading log-fold-change is the root-mean-square average of the largest log₂-fold changes. The PCA plots use the first two components of a PCA of normalized log transformed counts. Samples were analysed as paired and tag wise dispersion was calculated. A negative binomial generalized log-linear model was fitted to the read counts of each gene and the p-values adjusted

using an FDR approach (Benjamini and Hochberg, 1995). The gene names and descriptions of the DE genes were downloaded from BioMart. Dr Timothee Cezard of ARK genomics should be acknowledged for completing the RNA-seq data analysis. The full lists of DE genes are provided on CD as appendix 9.4. Significantly DE genes were identified using a cut-off of a fold change > 2 and FDR adjusted p-value < 0.01 . In the RNA-seq literature, groups use different cut-offs to identify significantly DE genes and the selected cut-off in this study fall within previously published criteria (Dorr et al., 2015; O'Loughlin et al., 2012).

6.2.6 Ingenuity pathway analysis of RNA-seq DE genes

Ingenuity[®] pathway analysis (Qiagen, Summer Release, June 2015) was used to interpret the biological relevance of the DE gene data sets. The software uses information from a Knowledge Base that contains gene relationship information from peer-reviewed journals and public/private databases, and has also been manually reviewed by experts.

The disease and biological functions that were enriched by DE genes of the datasets were assessed. The functional categories were determined using IPA knowledge base information and are defined using IPA terminology (Jimenez-Marin et al., 2009). The DE genes were also overlaid onto known canonical pathways in the IPA Knowledge Base to identify the enriched pathways. The right-tailed Fisher exact test was used to calculate p-values across all IPA analysis. The p-value is calculated using the number of genes in the data set that participate in a function/pathway and the total number of genes known to participate in the function/pathway based on Knowledge Base information.

6.3 Results

6.3.1 Summary statistics and visualizing relationships between samples

The total numbers of sequenced reads in the RNA-seq libraries were calculated before alignment to the bovine and ovine genomes (Table 6.1). The reads that map to multiple locations (outside the genes or in ambiguous areas) have been removed to generate the reads in the count file used for EdgeR analysis (Table 6.1).

Table 6.1: Summary of read ranges from RNA-seq data.

	Range of total number of sequenced reads	Range of number of reads in the count file
Ovine CD14 ⁺ monocytes	73,533,448 - 82,312,462	33,271,142 - 40,844,480
Ovine MDDCs	77,320,150 - 85,132,224	44,193,284 - 49,487,826
Ovine MDMs	71,688,766 - 83,476,284	42,097,928 - 48,541,272
Ovine <i>C. abortus</i> -infected MDMs	72,115,484 – 74,073,730	42,840,714 - 43,589,304
Bovine CD14 ⁺ monocytes	52,155,064 – 61,995,386	28,513,258 - 34,818,598
Bovine CD16 ⁺⁺ monocytes	48,985,228 – 55,876,038	26,237,258 - 29,145,280
Bovine MDDCs	76,871,814 - 84,104,194	49,117,040 - 54,126,388
Bovine MDMs	70,292,452 – 75,422,960	49,148,386 - 54,090,828
Bovine <i>C. abortus</i> -infected MDMs	69,762,836 – 78,350,202	45,982,098 - 54,403,114

The relationship between the replicates and groups samples was assessed using a multidimensional scaling (MDS) plot. The four replicates of each cell type/treatment cluster tightly together. There is clear separation of the different cell types whereas the *C. abortus*-infected and non-infected MDMs cluster together, which suggests that there is less difference in gene expression as a result of infection than the transcriptomic differences between the various cell subtypes (Figure 6.1).

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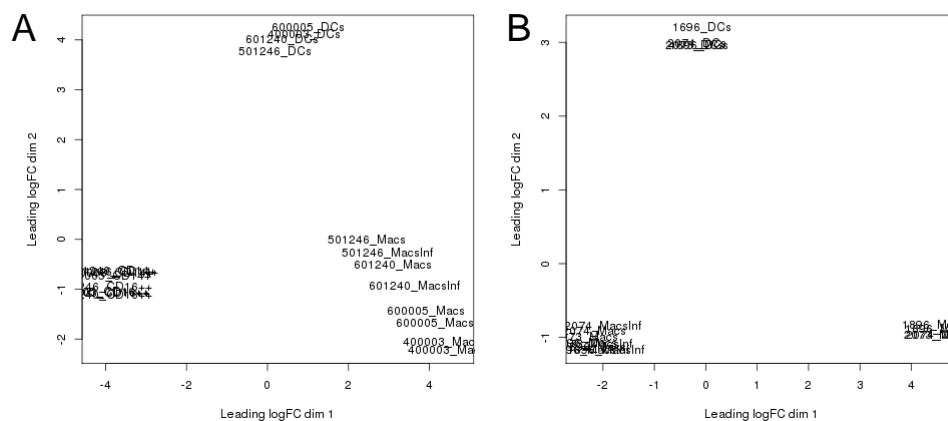


Figure 6.1: Multidimensional scaling plot demonstrating the relationship between bovine (A) and ovine (B) samples. The graphs plot the samples according to the logarithm of fold change (logFC dimension) of genes that most distinguish between samples.

PCA plots were also generated comparing the *C. abortus*-infected MDMs to the non-infected MDMs. The plots demonstrate that there is a higher degree of variance between animals within species than between the non-infected and infected treatment groups (Figure 6.2).

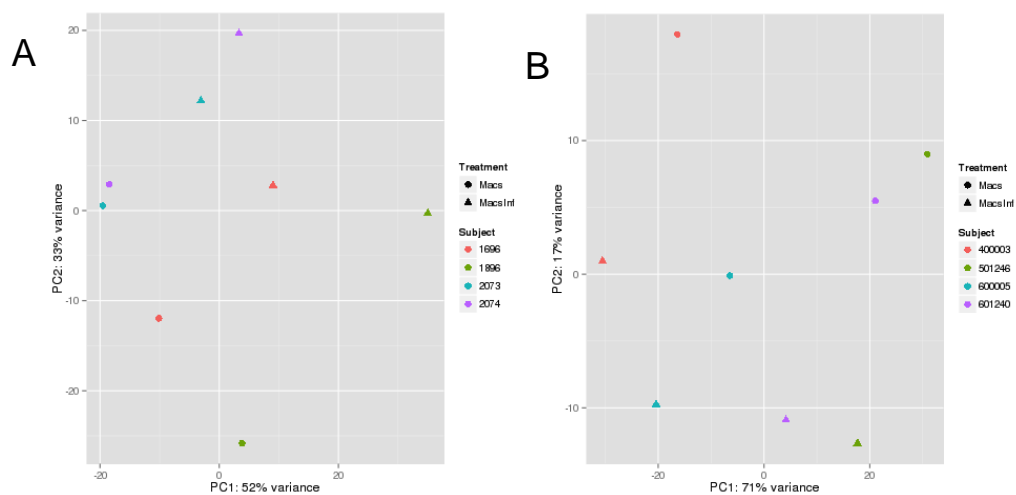


Figure 6.2: PCA plots showing the variance of ovine (A) and bovine (B) *C. abortus*-infected and non-infected MDMs. The graphs plot the samples according to the first two components of a PCA of normalised log transformed counts.

Lists of DE genes were prepared for the different comparisons between cell types and treatment groups. At this point the DE genes in the ruminant *C. abortus*-infected MDMs compared to the non-infected MDMs were further analysed but due to time limitations the ruminant MPS cell subset comparisons were not further analysed. 25196 DE genes were identified in the ovine data set comparing infected MDMs with non-infected MDMs and 24615 DE genes identified in the bovine data set (full list of DE genes available in appendix 9.4) before significantly DE genes were identified. Significant DE genes were identified using a cut-off of a fold change > 2 and FDR adjusted p-value < 0.01 . This cut-off identified 647 ovine genes (540 up-regulated and 107 down-regulated) and 1040 bovine genes (555 up-regulated and 485 down-regulated). Of the significant DE genes 529 ovine genes and 920 bovine genes mapped to the IPA knowledge base.

6.3.2 Comparative analysis of the significant DE gene expression in ovine and bovine MDMs

The DE genes and the direction of expression in both ovine and bovine MDMs infected with *C. abortus* compared to non-infected MDMs were visualised using a Venn diagram. Similar numbers of genes were significantly up-regulated, but not necessarily shared, in both bovine and ovine MDMs with *C. abortus* infection. More genes were down-regulated in bovine versus ovine MDMs with *C. abortus* infection (Figure 6.3).

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Table 6.2: The common DE genes in *C. abortus*-infected ovine and bovine MDMs compared to non-infected MDMs.

	Genes
Ovine up-regulated, bovine up-regulated	ABCG1 ADAMTS4 ADM ANKRD22 APOL6 ARG2 ARNTL2 ARRDC4 ATP10B B3GNT3 BACE2 BATF2 BATF3 BCL2A1 BIRC3 BTG3 CASP7 CBLN3 CCBE1 CCL20 CCL5 CD274 CD40 CD80 CFB CHI3L2 CMPK2 CPNE6 CSF2 CSF3 CXCL8 DMXL2 EBI3 EDN1 FOXS1 FUNDC1 G0S2 GBP5 GLT1D1 GPR84 GRAMD1C HERC6 HES4 HIVEP2 HSH2D IER3 IFIH1 IFIT2 IGSF3 IL15 IL17REL IL1A IL1B IL23R IL27 IL6 IL7R IRF1 IRF7 IRG1 ISG15 ISG20 KIF5C KLF5 MCEMP1 MCOLN2 MEFV MOV10 MST1R MUC1 NEURL3 NFE2L3 NFKB2 NFKBIE NFKBIZ NLRC5 NLRP12 NMI OLR1 PARP14 PDE4B PIM3 PML POU2F2 PSMB10 PSMB9 PSME2 RARRES2 RELB RGS16 RHOH RNF19B RNF213 RSAD2 RTP4 SDC4 SERPINB10 SLAMF1 SLAMF8 SLC28A3 SLC2A6 SOCS3 SOD2 STX11 TAP1 TBC1D9 TBX18 TGM1 TIFA TNF TNFAIP3 TNFSF13B TNIP3 TRANK1 TREML2 TUFT1 UBA7 WARS WNT5A ZC3H12A
Ovine up-regulated, bovine down-regulated	ASGR2 CLEC12A FCGR3A FGD2 LY86 SELE SEMA4A SPIC SYNGR1 TLR10
Ovine down-regulated, bovine up-regulated	THBD
Ovine down-regulated, bovine down-regulated	ALOX5 ATOH8 C19orf35 CPM F13A1 FOS HCRTR1 KCNC1 MAMDC2 MBP NECAB3 PHOSPHO1 PIK3IP1 SH2D6 SLC7A8 SMIM4 STAB1 TPBGL ZNF704

6.3.3 Top 20 up-regulated and down-regulated ruminant MDM DE genes

The top 20 significantly up-regulated ovine DE genes in *C. abortus* infected compared to non-infected MDMs included cytokines/chemokines and enzymes that had immune functions, such as type 1-interferon response genes (Table 6.3).

Table 6.3: The top 20 up-regulated DE genes for ovine *C. abortus*-infected MDMs compared to non-infected MDMs.

Ensembl ID	Gene symbol	Gene name	Log ₂ Fold Change	FDR-adjusted P-value
ENSOARG00000000895	-	Uncharacterized protein	8.40	7.85E-19
ENSOARG00000014648	RSAD2	Radical S-adenosyl methionine domain containing 2	8.16	1.05E-41
ENSOARG00000001815	-	Uncharacterized protein	8.15	4.08E-94
ENSOARG00000020576	CCL20	Chemokine (C-C motif) ligand 20	7.74	1.55E-31
ENSOARG00000007233	ISG15	Interferon stimulated gene 15 (ISG15)	7.67	3.43E-109
ENSOARG00000016213	APOBEC3Z1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide	7.53	1.35E-52
ENSOARG00000015177	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	7.48	3.76E-60
ENSOARG00000011222	CLEC4F	C-type lectin domain family 4, member F	7.37	7.00E-15
ENSOARG00000016668	CXCL11	Chemokine (C-X-C motif) ligand 11	7.19	1.18E-29
ENSOARG00000010283	MX1	Myxovirus (influenza virus) resistance 1	7.00	1.52E-92
ENSOARG00000010964	ISG20	interferon stimulated exonuclease gene 20kDa	6.89	2.79E-73
ENSOARG00000016611	CXCL10	Ovis aries chemokine (C-X-C motif) ligand 10 (CXCL10)	6.74	2.00E-88
ENSOARG00000011372	BATF2	Basic leucine zipper transcription factor	6.67	1.05E-61
ENSOARG00000001757	FOXS1	Forkhead box S1	6.35	3.03E-50
ENSOARG00000015072	-	Uncharacterized protein	6.33	2.06E-66
ENSOARG00000001535	-	Uncharacterized protein	6.21	4.20E-29
ENSOARG00000014800	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	6.10	2.59E-69
ENSOARG00000020215	-	Uncharacterized protein	6.08	3.49E-06
ENSOARG00000009241	CCL8	Chemokine (C-C motif) ligand 8	5.89	4.79E-36
ENSOARG00000016617	TRANK1	Tetratricopeptide repeat and ankyrin repeat containing 1	5.74	1.35E-45

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The top 20 down-regulated genes included a number of enzymes and transporters involved in a range of cellular processes including growth, differentiation, adhesion and apoptosis (Table 6.4).

Table 6.4: The top 20 down-regulated DE genes for ovine *C. abortus*-infected MDMs compared to non-infected MDMs.

Ensembl ID	Gene symbol	Gene name	Log ₂ Fold Change	FDR-adjusted P-value
ENSOARG00000020956	ST6GAL2	ST6 beta-galactosamide alpha-2	-11.93	4.48E-06
ENSOARG00000014010	CX3CR1	Chemokine (C-X3-C motif) receptor 1	-8.13	1.56E-07
ENSOARG00000012933	MAMDC2	MAM domain containing 2	-6.25	0.001319134
ENSOARG00000011251	TPBGL	Trophoblast glycoprotein-like	-5.76	6.74E-06
ENSOARG00000003312	DIRAS2	DIRAS family	-5.43	0.000830803
ENSOARG00000015249	CDH26	Cadherin 26	-4.98	0.002003027
ENSOARG00000014027	C19orf35	Chromosome 19 open reading frame 35	-4.81	1.05E-06
ENSOARG00000011582	CYP26B1	Cytochrome P450	-4.75	3.92E-20
ENSOARG00000012285	VPS37D	Vacuolar protein sorting 37 homolog D (<i>S. cerevisiae</i>)	-4.73	0.003205787
ENSOARG00000007858	RASAL1	RAS protein activator like 1 (GAP1 like)	-4.42	5.48E-05
ENSOARG00000017394	NRG2	Neuregulin 2	-2.13	0.003215491
ENSOARG00000001269	STAR	Ovis aries steroidogenic acute regulatory protein (STAR)	-2.10	0.001532902
ENSOARG00000020255	SLC4A3	Solute carrier family 4 (anion exchanger)	-2.08	0.000130044
ENSOARG00000015923	CHDH	Choline dehydrogenase	-2.06	0.001950291
ENSOARG00000006108	PALD1	Phosphatase domain containing, paladin 1	-2.05	0.00016121
ENSOARG00000019421	IGFBP5	Insulin-like growth factor binding protein 5 (IGFBP5)	-2.0	0.001265343
ENSOARG00000013875	HUNK	Hormonally up-regulated Neu-associated kinase	-1.94	9.23E-06
ENSOARG00000004332	DOC2A	Double C2-like domains	-1.93	0.000120304
ENSOARG00000002088	GRK5	G protein-coupled receptor kinase 5	-1.89	0.000885939
ENSOARG00000018445	F13A1	Coagulation factor XIII	-1.85	1.38E-06

The top 2 up-regulated bovine genes were micro-RNA precursors that were removed from the analysis as were only found to be present and up-regulated in one animal. Further investigation into the micro-RNA precursor genes identified that they

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mapped to an intron and not fully to an exon suggesting that their identification was probably an anomaly. These micro-RNA precursors were also excluded from the Venn diagram (Figure 6.3) for the same reasons. The top 20 up-regulated bovine DE genes included immune related genes, such as pro-inflammatory cytokines/chemokines and enzymes involved in antimicrobial defence (Table 6.5). CCL20 is the only top 20 up-regulated gene shared between ovine and bovine *C. abortus* infected compared to non-infected MDMs.

Table 6.5: The top 20 up-regulated DE genes for bovine *C. abortus*-infected MDMs compared to non-infected MDMs.

Ensembl ID	Gene symbol	Gene name	Log ₂ Fold Change	FDR-adjusted P-value
ENSBTAG00000021462	CSF3	Colony stimulating factor 3 (granulocyte) (CSF3)	5.53	1.00E-05
ENSBTAG00000015214	CA3	Carbonic anhydrase III	5.06	0.00259806
ENSBTAG00000013675	IL37	Interleukin 37	4.86	5.50E-18
ENSBTAG00000007423	TNFRSF11B	Tumour necrosis factor receptor superfamily	4.70	1.17E-14
ENSBTAG00000023431	-	Uncharacterized protein	4.65	0.004124423
ENSBTAG00000016886	MT3	Metallothionein 3 (MT3)	4.64	8.51E-31
ENSBTAG00000004714	MCEMP1	Mast cell-expressed membrane protein 1	4.43	8.15E-13
ENSBTAG00000006894	NOS2	Nitric oxide synthase 2, inducible	4.35	1.49E-10
ENSBTAG00000021326	CCL20	Chemokine (C-C motif) ligand 20 (CCL20)	4.18	9.55E-30
ENSBTAG00000001321	IL1B	Interleukin 1 beta	4.14	1.09E-14
ENSBTAG00000020602	IDO1	Indoleamine 2,3-dioxygenase 1	4.14	1.83E-07
ENSBTAG00000008096	EDN1	Endothelin 1 (EDN1)	4.09	1.78E-34
ENSBTAG000000037800	APOBEC3A	Apolipoprotein B mRNA editing enzyme	3.90	5.18E-11
ENSBTAG00000004710	HNF1B	HNF1 homeobox B	3.82	0.001599711
ENSBTAG000000044676	U6	U6 spliceosomal RNA	3.82	4.15E-06
ENSBTAG00000012529	IFNG	Interferon gamma	3.74	1.25E-09
ENSBTAG00000001870	CPNE6	Copine VI (neuronal)	3.68	3.93E-10
ENSBTAG00000000597	TMPRSS15	Transmembrane protease, serine 15	3.64	4.16E-09
ENSBTAG00000014921	IL6	Interleukin 6	3.64	0.000346062
ENSBTAG000000046944	-	Uncharacterized protein	3.63	2.67E-14

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The top 20 down-regulated DE genes in bovine MDMs included enzymes and G-protein coupled receptors with cellular functions in growth, chemotaxis, recruitment and binding (Table 6.6).

Table 6.6: The top 20 down-regulated DE genes for bovine *C. abortus*-infected MDMs compared to non-infected MDMs.

Ensembl ID	Gene symbol	Gene name	Log ₂ Fold Change	FDR-adjusted P-value
ENSBTAG00000022570	-	Novel gene with description: Prostaglandin F synthetase II-like (LOC782922)	-4.54	7.85E-19
ENSBTAG00000003327	RUFY4	RUN and FYVE domain-containing protein 4	-4.42	1.05E-41
ENSBTAG000000040153	-	Uncharacterized protein	-4.33	4.08E-94
ENSBTAG000000047501	bta-mir-2887-2	bta-mir-2887-1	-4.3	1.25E-12
ENSBTAG000000038981	-	Uncharacterized protein	-4.20	3.43E-109
ENSBTAG000000014313	PYROXD2	Pyridine nucleotide-disulphide oxidoreductase domain 2	-4.18	1.35E-52
ENSBTAG000000047910	-	Uncharacterized protein	-4.0	3.76E-60
ENSBTAG000000020319	ALOX5	Arachidonate 5-lipoxygenase	-3.98	7.00E-15
ENSBTAG000000038680	-	Uncharacterized protein	-3.88	2.50E-13
ENSBTAG000000017043	GPR1	G protein-coupled receptor 1	-3.81	0.003118113
ENSBTAG000000022039	CMKLR1	Chemokine-like receptor 1 (CMKLR1)	-3.80	3.29E-07
ENSBTAG000000021252	TMEM35	Transmembrane protein 35	-3.71	0.003896717
ENSBTAG000000007268	F13A1	Coagulation factor XIII	-3.65	8.90E-44
ENSBTAG000000004950	BRN	Brain ribonuclease	-3.61	2.28E-20
ENSBTAG000000007642	ADORA3	Adenosine A3 receptor	-3.60	7.27E-45
ENSBTAG000000022509	DNAH9	Dynein	-3.55	8.88E-14
ENSBTAG000000014782	STAB1	Stabilin 1	-3.54	1.80E-26
ENSBTAG000000017369	MAMDC2	MAM domain containing 2	-3.54	3.73E-16
ENSBTAG000000016997	-	Uncharacterized protein	-3.53	1.06E-14
ENSBTAG000000026181	UGT1A1	UDP glucuronosyltransferase 1 family	-3.40	6.25E-05

6.3.4 Inferring function of ruminant MDM DE genes

The IPA disease and function terms that were enriched by ovine DE genes included cellular function and maintenance, infectious disease and inflammatory response (Table 6.7).

Table 6.7: The top 15 enriched disease and biological functions with DE genes from ovine *C. abortus*-infected compared to non-infected MDMs. The p-value range describes the p-values of the subcategories within the functional category and the # molecules describes the number of molecules from the ovine DE gene dataset associated with the functions in the category.

Functional category	P-value range	# Molecules
Cellular Function and Maintenance	1.27E-37-1.93E-08	166
Infectious diseases	1.30E-37-1.71E-08	174
Immunological Disease	2.63E-33-1.83E-08	183
Hematological System Development and Function	1.60E-29-1.94E-08	188
Tissue Morphology	1.60E-29-1.94E-08	158
Cellular Movement	8.37E-29-1.27E-08	159
Immune Cell Trafficking	8.37E-29-1.83E-08	134
Connective tissue disorders	1.04E-28-6.85E-10	114
Inflammatory Disease	1.04E-28-1.71E-08	155
Skeletal and muscular disorders	1.04E-28-6.85E-10	126
Cell-to-cell signalling	1.27E-27-1.83E-08	163
Cellular development	1.29E-26-1.46E-08	186
Cellular growth and proliferation	1.29E-26-1.38E-08	232
Cell death and survival	3.53E-26-1.91E-08	230

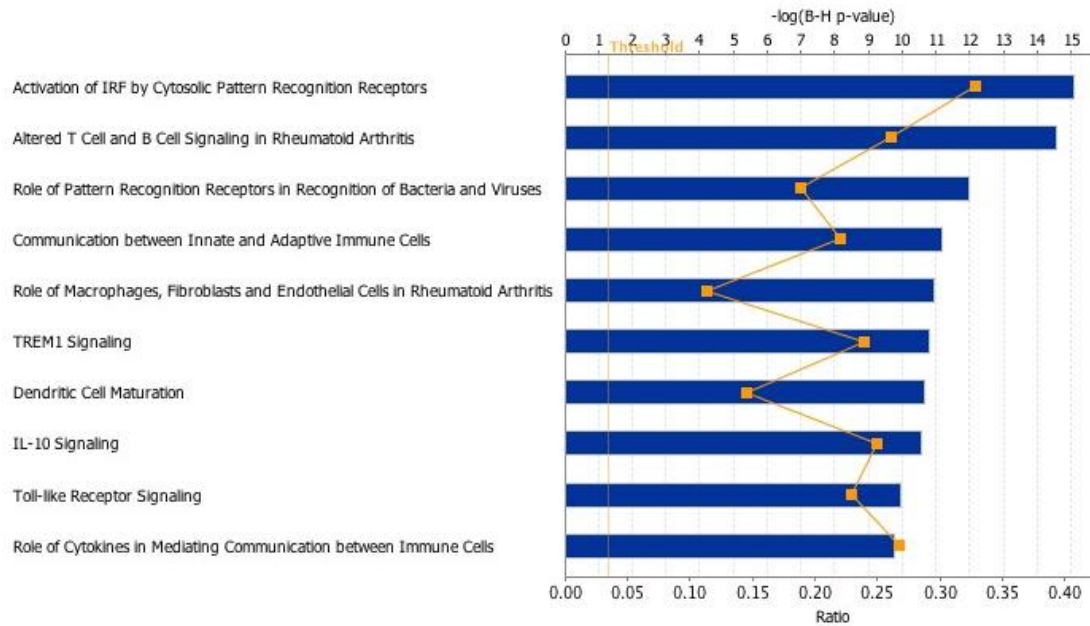
Similarly, functional analysis of the bovine DE genes identified enriched roles in cellular movement, immune cell trafficking and inflammatory response (Table 6.8).

Table 6.8: The top 15 enriched disease and biological functions with the DE genes from bovine *C. abortus*-infected compared to non-infected MDMs. The p-value range describes the p-values of the subcategories within the functional category and the # molecules describes the number of molecules from the bovine DE gene dataset associated with the functions in the category.

Functional Category	p-value range	# Molecules
Cellular movement	7.88E-43-5.51E-09	279
Immune cell trafficking	1.91E-42-5.50E-09	209
Haematological System Development and Function	2.33E-41-5.50E-09	316
Tissue morphology	2.33E-41-2.84E-09	255
Cellular Function and Maintenance	1.77E-37-3.54E-09	260
Cell-To-Cell Signaling and Interaction	2.77E-33-5.50E-09	253
Inflammatory Response	1.01E-32-5.50E-09	294
Cancer	9.23E-28-5.07E-09	788
Organismal injury and abnormalities	9.23E-28-5.46E-09	793
Cellular growth and proliferation	4.29E-27-4.81E-09	377
Infectious diseases	2.13E-25-4.31E-09	207
Cell death and survival	8.17E-24-2.23E-09	347
Cardiovascular system development	2.30E-22-3.51E-09	198
Organismal development	2.30E-22-4.59E-09	276
Metabolic disease	1.32E-21-4.06E-09	365

IPA analysis identified 98 statistically significant (FDR adjusted p-value<0.05) canonical pathways enriched with DE genes from *C. abortus*-infected ovine MDMs compared to non-infected MDMs. All of the top 10 canonical pathways were associated with the immune response (Figure 6.4).

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

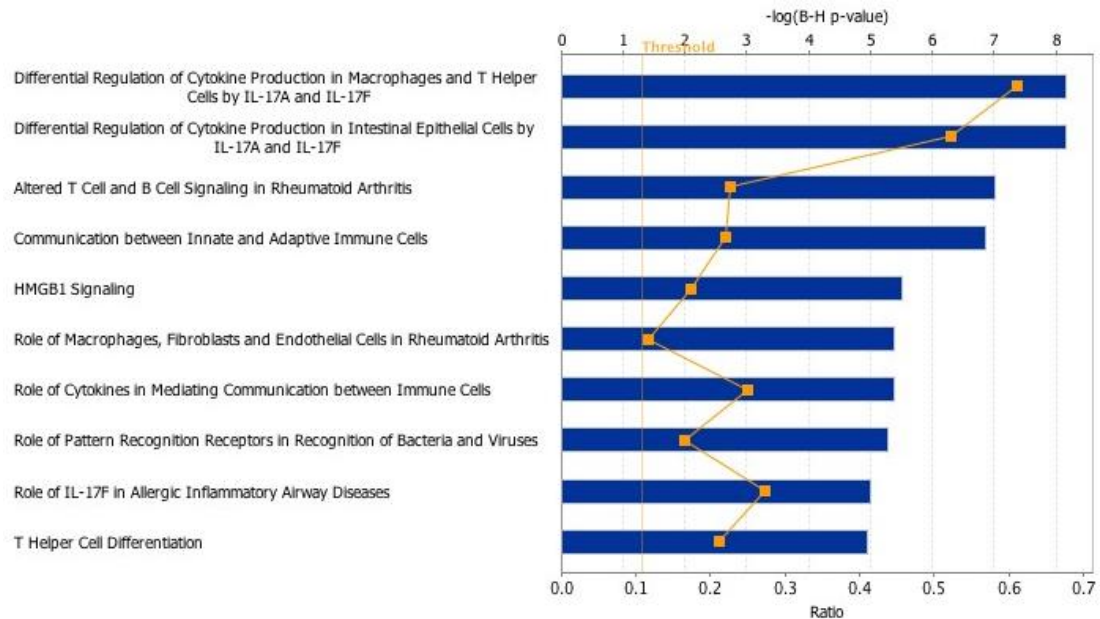


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Figure 6.4: The top 10 canonical pathways enriched with DE genes from ovine *C. abortus*-infected compared to non-infected MDMs. The threshold was set at $p=0.05$. The blue bars represent the FDR adjusted p-value. The yellow line represents the ratio, which was determined using the DE genes in the ovine DE gene dataset compared to the total genes in the pathway according the IPA knowledge base.

IPA analysis identified 73 statistically significant (FDR adjusted p -value <0.05) canonical pathways enriched with DE genes from *C. abortus*-infected bovine MDMs compared to non-infected MDMs. The top 10 canonical pathways in bovine MDMs were all involved in the immune response (Figure 6.5).

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*



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Figure 6.5: The top 10 canonical pathways enriched with DE genes from bovine *C. abortus*-infected compared to non-infected MDMs. The threshold was set at $p=0.05$. The blue bars represent the FDR adjusted P-value. The yellow line represents the ratio, which was determined using the DE genes in the bovine DE gene dataset compared to the total genes in the pathway according the IPA knowledge base.

The top ranked canonical pathways were overlaid with the DE genes from both bovine and ovine datasets. The most significant enriched canonical pathway in ovine MDMs was the ‘Activation of the interferon regulatory factors by cytosolic pattern recognition receptors’. The ovine DE genes that were up-regulated in the pathway included the intracellular receptors, retinoic acid inducible gene-1 (RIG1) and melanoma differentiation gene-5 (MDA5). The intracellular receptors bind to mitochondrial antiviral signalling protein (MAVS) signalsome that mediates the activation of interferon regulatory factor 3 (IRF3) and IRF7, which were also up-regulated in the ovine DE dataset. IRF3 and IRF7 induce the expression of interferon and interferon-stimulated genes (ISGs) of which IFN- β and ISG15 were among the genes up-regulated in the ovine DE dataset (Figure 6.6). The top canonical pathway in cattle was the ‘Differential regulation of cytokine production in macrophages and T-helper cells by IL-17A and IL-17F’. The bovine DE genes which were up-regulated in the pathway included the pro-inflammatory cytokines IL-17A and IL-

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17F, which can mediate the up-regulation of expression of cytokines/chemokines including IL-1 β and TNF- α (also up-regulated in the bovine DE genes) (Figure 6.7).

Inflammasome activation in ruminant cells infected with *Chlamydia abortus*

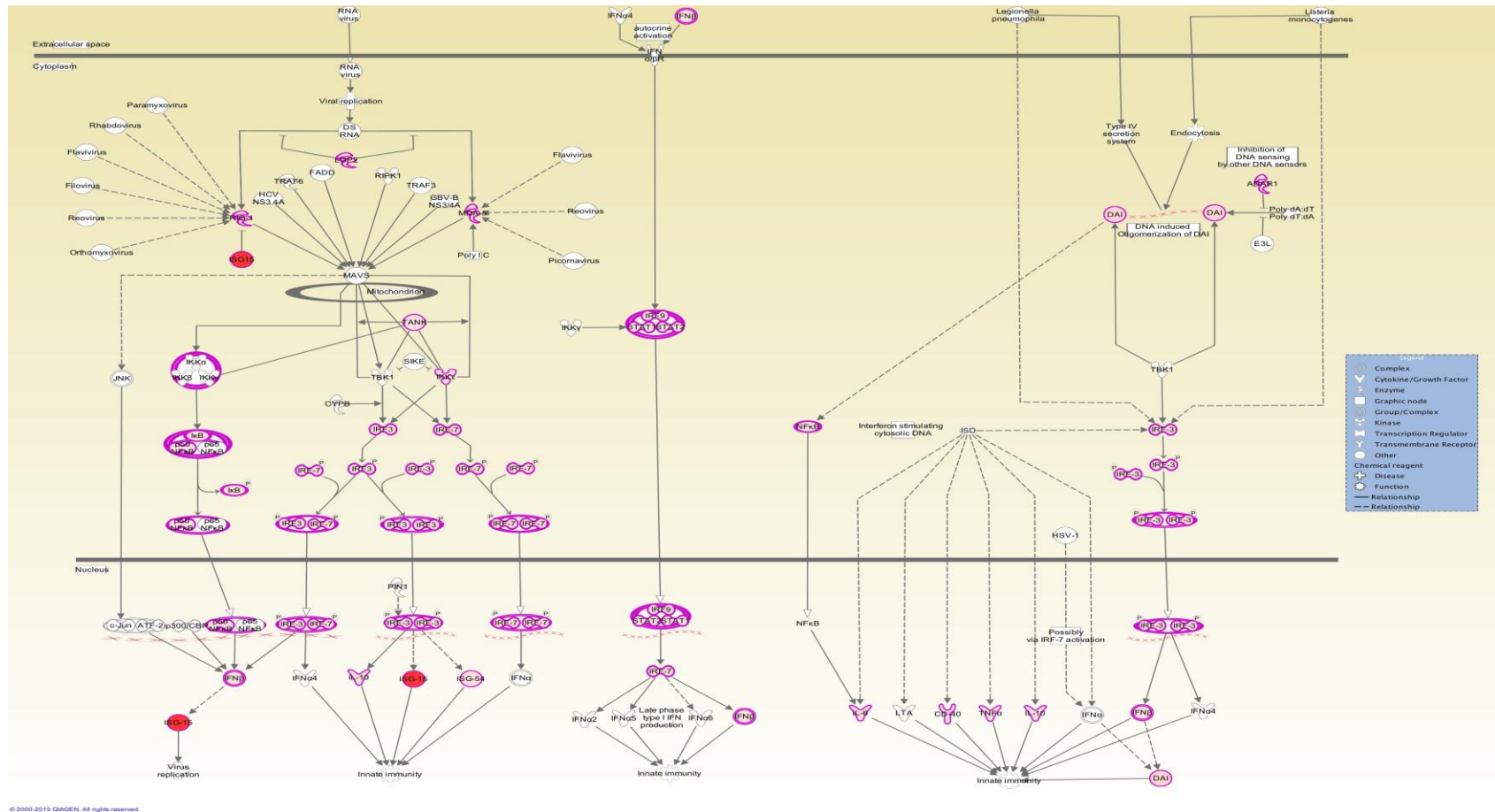


Figure 6.6: The top-ranked canonical pathway enriched with DE genes from ovine *C. abortus*-infected compared to non-infected MDMs: Activation of Interferon regulatory factors (IRFs) by cytosolic pattern recognition receptors. The degree of red shading indicates the level of up-

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regulated expression in *C. abortus*-infected MDMs. Green shading would indicate down-regulated expression and no shading indicates no detected differential expression in dataset (see appendix 9.4 for enlargeable copy of diagram).

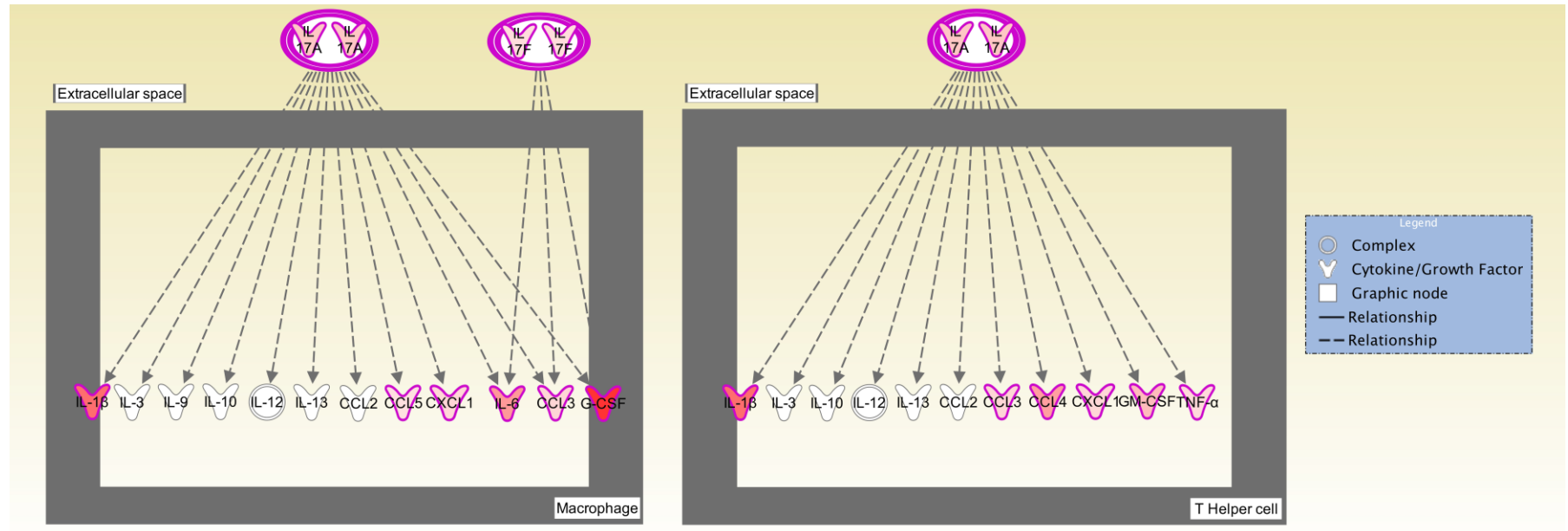


Figure 6.7: The top-ranked canonical pathway enriched with DE genes from bovine *C. abortus*-infected compared to non-infected MDMs: Differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F. The degree of red shading indicates the level of up-regulated expression in *C. abortus*-infected MDMs. Green shading would indicate down-regulated expression and no shading indicates no detected DE in dataset

6.4 Discussion

The experimental samples clustered together on the MDS plot according to cell type, which indicates that the gene expression profiles differed between cell types to a greater degree than between animals. PCA analysis of the ruminant MDMs infected with *C. abortus* and the non-infected MDMs identified that there was more variation between animals of the same species than between treatment groups. This indicates animal specific differences within the MDMs and exemplifies the difficulty of working with an out-bred population. Time limitations meant that the differentiation specific 'signature' profiles of the different MPS cells were not analysed. Future work will involve the analysis of DE genes from the pair-wise comparison of the different cell types. Gene clustering based on co-expression can also be used to simplify the comparison of gene expression profiles across cell types and understand the functional relevance and relationships between genes (Mabbott et al., 2013). The significantly DE genes from the comparison of ovine and bovine *C. abortus*-infected MDMs to non-infected MDMs were selected for further analysis.

Host cells can exhibit a gene expression profile that is commonly induced in response to various pathogenic challenges. Microarray technology has been used extensively to characterize the common gene expression profile (Jenner and Young, 2005). A study compared the responses of human PBMCs to various bacterial stimuli and identified that commonly up-regulated genes included pro-inflammatory cytokines/chemokines and genes involved in NF- κ B signalling. Commonly down-regulated genes included a subset of cytokine/chemokine receptors and also genes involved in antigen processing (Boldrick et al., 2002). Similarly, human MDMs responded to various bacterial stimuli with the up-regulation of pro-inflammatory cytokines/chemokines, cytokine/chemokine receptors, signalling pathway molecules and anti-apoptotic genes. Genes involved in the anti-inflammatory response, transcription and various enzymes were down-regulated in response to infection (Nau et al., 2002). The gene expression profiles from previously published studies using various host cells (including macrophages, T cells and epithelial cells) challenged with different pathogenic stimuli (bacterial, viral and parasitic) were

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compared. Common host responses cluster into functional groups (Jenner and Young, 2005) and a number of genes identified in these groups are up-regulated in both ovine and bovine *C. abortus* infected compared to non-infected MDMs (Table 6.9).

Table 6.9: The ‘common’ host functional groups of genes (Jenner and Young, 2005) and the genes in these groups which were up-regulated in ruminant MDMs following *C. abortus* infection.

Functional groups of common genes	Common genes up-regulated in ruminant MDMs in response to <i>C. abortus</i>
Co-stimulators	CD40, CD80
Cytokine receptors	IL7R
Pro-inflammatory cytokines	IL-1 β , IL-6, TNF, IL1A
Chemotactic cytokines	CCL20, CXCL8, CCL5
Other cytokines	CSF2, CSF3
Transcription factors	NFKBIE, IRF1, IRF7
Interferon stimulated genes	ISG20, PML
Antigen processing	TAP1, PSMB9, PSMB10
Apoptosis	CASP7, BIRC3, BCL2A1

However, gene expression profiles that were specific to intracellular pathogens, cell-types and individual pathogens have also been identified using microarrays. Human MDDCs exhibited up-regulated expression of genes involved in NF- κ B signalling, apoptosis and the TNF- α pathway in response to intracellular *Toxoplasma gondii*, *Mycobacterium tuberculosis*, *Leishmania major*, *Leishmania donovani* but not extracellular *Brugia malayai* (Chaussabel et al., 2003). The up-regulation of particular clusters of genes can also be associated with different cell types and their specific immunological function. A cluster of chemokines including CXCL9, CXCL10, CCL8 and CXCL11, which are involved in T cell recruitment, were more strongly up-regulated in DCs compared to macrophages, T cells, B cells or epithelial cells in response to pathogenic stimuli (Jenner and Young, 2005). Human MDDCs and MDMs displayed unique ISG expression profiles in response to different bacterial pathogens (Chaussabel et al., 2003). Specific gene expression profiles to

individual pathogens have also been identified. The expression of IL-12p40, IL-15 and signal transducer and activator of transcription 2 (STAT2) was shown to be pathogen-specific in human MDMs following infection with *M. tuberculosis*, *E. coli* or *Staphylococcus aureus* (Nau et al., 2002). The comparison of human PBMC gene expression profiles identified a number of genes including the chemokine receptor (CCR1) and monocyte chemoattractant protein 1 (MCP1) that were selectively down-regulated in response to different heat-killed bacteria (Boldrick et al., 2002). A number of ISGs, including IFIT1 and ISG15, were shown to have pathogen-specific profiles when comparing the gene expression of human MDDCs and MDMs in response to different bacterial pathogens (Chaussabel et al., 2003). The transcriptomes of ovine and bovine MDMs in response to *C. abortus* were investigated to identify potentially novel chlamydial-specific gene expression profiles.

A number of DE genes that were significantly up-regulated in response to *C. abortus* infection were shared by bovine and ovine MDMs. However, more genes were not shared and up-regulated in either cattle or sheep. Further, notably more genes were down-regulated in cattle than sheep but the relevance of this requires further analysis. The functional role of the significantly DE genes in ruminant MDMs was implied by identifying the top 20 up and down-regulated genes and using pathway analysis.

Many of the top 20 up-regulated DE genes identified in *C. abortus*-infected ovine MDMs compared to non-infected MDMs were associated with the type 1 interferon response. Type 1 interferons are typical of the innate immune response to a viral infection. The type 1 interferons bind to the IFN α receptor (IFNAR) and induce the expression of interferon-inducible genes including MX1, ISG15, IFIT1, RSAD2 and APOBEC proteins (Sadler and Williams, 2008), which were all identified in the ovine MDM top 20 up-regulated DE genes. The potential role for type 1 IFNs was supported by the finding that the top canonical pathway enriched with ovine MDM DE genes describes the activation of cytosolic PRRs inducing the expression of type 1 interferons and ISGs. The type 1 interferons and ISGs can control viral infection through the inhibition of viral replication or release of virus from infected cells

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(Sadler and Williams, 2008). Although the type 1 interferon response is best characterized in terms of antiviral immune defense, *Chlamydia* has also previously been shown to activate type 1 interferons and ISGs. The increased expression of the ISGs CCL5, CXCL10 and ISG56 was detected in murine fibroblasts infected with *C. pneumoniae* (Chiliveru et al., 2010). Interferon inducible genes (including MX1 and ISG15) were also up-regulated in a human monocytic cell line following infection with *C. trachomatis* (Ren et al., 2003).

The role for type 1 IFNs in bacterial infection is complex and unlike in viral infection not simply a host defense mechanism. Conflicting studies demonstrating both protective and adverse roles for type 1 IFNs with bacterial infection have been published (Decker et al., 2005). The role of type 1 IFNs in chlamydial pathogenesis has been the subject of a number of studies. Type 1 IFNs can activate IDO, restricting chlamydial growth through tryptophan depletion in human MDMs indicating a potentially protective role (Carlin et al., 1989). However, IFNAR KO mice had lower weight loss, bacterial burden and milder lung inflammation compared to wild-type mice following intranasal challenge with *C. muridarum*. The study identified that the increased macrophages in the lung of IFNAR KO mice compared to the wild-type mice following *C. muridarum* infection contributed to chlamydial clearance. The decrease in macrophages in the wild-type was attributed to type 1 IFN mediated apoptosis, which may mediate the pathogenic effect of type 1 IFN *in vivo* with chlamydial infection (Qiu et al., 2008). Similarly, IFNAR KO mice infected vaginally with *C. muridarum* exhibit faster bacterial clearance and reduced incidence of hydrosalpinx compared to wild-type mice (Nagarajan et al., 2008).

A number of pro-inflammatory genes were identified in the top 20 up-regulated DE genes in bovine MDMs, including IL-1 β , G-CSF, IL-37 and IFN- γ . As described in Chapter 1, IFN- γ is thought to mediate defence against chlamydial infection through the induction of IDO. IDO1, the gene that encodes IDO, was also identified in the top 20 up-regulated genes in bovine MDMs indicating that a protective immune response is activated. Inducible nitric oxide synthase (NOS2) is another target gene of IFN- γ , which induces the production of anti-microbial nitric oxide (NO) (Zhang et

al., 2012). NOS2 is also in the top 20 up-regulated DE genes in bovine MDMs. The top two up-regulated canonical pathways in bovine MDMs involve the IL-17A and IL-17F driven production of pro-inflammatory cytokines. Although usually associated with Th17 cells, IL-17A and IL-17F production by macrophages has also been identified in a small number of studies (Reynolds et al., 2010). The addition of recombinant IL-17A to human MDMs induced a distinct inflammatory profile, which up-regulated the expression of pro-inflammatory IL-6, IL-1 α and CCL20 (Erbel et al., 2014). A recent RNA-seq study identified the up-regulation of IL-17A expression in bovine MDMs infected with *M. bovis* (Nalpas et al., 2013). IL-17A has been implicated in the mediation of protective immunity to *C. muridarum* lung infection (Bai et al., 2009). A novel mode of action for IL-17A in the up-regulation of IFN- γ driven iNOS production, which inhibits chlamydial growth in *C. muridarum* infected murine epithelial and macrophage cells was recently identified (Zhang et al., 2012). How this relates to ruminants, and particularly the differential pathogenesis of *C. abortus* in infection in sheep and cattle remains to be investigated.

Chemokine receptor 1 (CX3CR1) is strongly down-regulated in ovine *C. abortus*-infected MDMs compared to non-infected MDMs. CX3CR1 is the receptor that recognises fractalkine, which mediates cellular adhesion and chemotaxis (Imai et al., 1997). The expression of CX3CR1 was also strongly down-regulated on BMDM derived from mice infected with *Listeria monocytogenes* compared to BMDM derived from control mice (Drevets et al., 2010). Cadherin 26 (CDH26) is also highly down-regulated in the ovine DE gene dataset, with similar roles in cellular adhesion and migration and is also down-regulated in bovine MDMs in response to *M. bovis* (Nalpas et al., 2013). Arachidonate 5-lipoxygenase (ALOX5) is strongly down-regulated in the bovine DE gene dataset and is also down-regulated in ovine DE gene dataset. ALOX5 is an enzyme that synthesizes the production of leukotrienes and lipoxins. Leukotrienes can induce chemotaxis and oedema formation, whereas lipoxins can have anti-inflammatory functions including increasing the production of anti-inflammatory cytokines. Studies investigating the role of ALOX5 against different pathogens have conflicting results over whether the enzyme is protective or pathogenic (Fahel et al., 2015). FBJ murine osteosarcoma

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viral oncogene homolog (FOS) is also down-regulated in both bovine and ovine MDM DE gene datasets and is involved in differentiation, cell death and the immune response (Durchdewald et al., 2009). FOS was down-regulated in bovine MDM infected with *M. bovis* (Nalpas et al., 2013) and in epithelial cells at 1 hour post infection with *C. trachomatis* (Humphrys et al., 2013). Further studies are necessary to identify why these genes are down-regulated in response to *C. abortus* infection.

Many of the top 10 canonical pathways enriched with genes from both bovine and ovine MDM DE gene datasets are shared. These include the ‘role of cytokines in mediating communication between immune cells’ and ‘communication between innate and adaptive immune cells’, which describes the expression of pro-inflammatory cytokines/chemokines. Similarly, ‘inflammatory response’ and ‘inflammatory disease’ are functions in the top 15 of the enriched disease and biological functions from bovine and ovine data sets respectively. The induction of a pro-inflammatory response is a characteristic feature of chlamydial infection (Entrican et al., 2004). A number of pro-inflammatory cytokines/chemokines genes including IL-1 β , TNF- α , IL-6 and CXCL8 were up-regulated in both ovine and bovine MDM DE gene datasets, which was supported by results from Chapter 4. One of the first studies to characterize the *Chlamydia* infected host cell transcriptomic profile used a human endothelial cell line infected with *C. pneumoniae*. *C. pneumoniae* infection up-regulated the expression of cytokines/chemokines and growth factors, including CXCL8 and IL-1 β expression (Coombes and Mahony, 2001). The gene expression of human monocytes infected with *C. trachomatis* identified the up-regulation of CXCL8, TNF- α , IL-6 and IL-1 β (Schrader et al., 2007). Microarray analysis of a human epithelial cell line infected with *C. trachomatis* and *C. pneumoniae* identified the up-regulation of pro-inflammatory cytokines, including CXCL8 and IL-6 (Hess et al., 2003). The *C. trachomatis* cryptic plasmid is thought to be an important virulence factor as deficient strains produce attenuated disease. The expression levels of a number of pro-inflammatory genes, including CXCL8 and IL-6, were significantly increased in cells challenged with plasmid-bearing *C. trachomatis* compared to the deficient strain (Porcella et al., 2015).

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A number of pro-inflammatory mediators that have not yet been investigated in response to *C. abortus* were also up-regulated in ruminant MDMs. Mediators included endothelin 1 (EDN1), CCL20 and CCL5. EDN1 has been described to function like a pro-inflammatory cytokine (Teder and Noble, 2000) and has previously been shown to be up-regulated in human MDDC and MDMs in response to intracellular pathogens, including *M. tuberculosis* and *T. gondii* (Chaussabel et al., 2003). EDN1 was also up-regulated in human epithelial cells in response to the plasmid-bearing compared to the plasmid-deficient strains of *C. trachomatis* (Porcella et al., 2015). CCL20 was the highest up-regulated chemokine in both cattle and sheep MDMs in response to *C. abortus*. CCL20 and CCL5 both function in lymphocyte chemotaxis and are up-regulated by *C. trachomatis* in human epithelial cells (Porcella et al., 2015). CCL5 was also up-regulated in conjunctival swabs from individuals with detectable *C. trachomatis* infection and clinical signs of trachoma compared to the control group, which had no clinical signs or detectable *C. trachomatis* infection (Natividad et al., 2010). Therefore, transcriptomic analysis has provided more in-depth and novel information on the pro-inflammatory response profile to chlamydial infection.

The DE gene analysis of *C. abortus*-infected ruminant MDMs identified the up-regulation of other groups of genes, which may be involved in chlamydial pathogenesis. Apoptosis describes a type of programmed cell death that is regulated to prevent the induction of an inflammatory response and is mediated by caspases (Sharma and Rudel, 2009). The ‘cell death and survival’ function that includes apoptosis is highly enriched with genes from the DE ruminant datasets. Genes including Baculoviral IAP Repeat Containing 3 (BIRC3) and BCL2-related protein A1 (BCL2A1) are up-regulated in ruminant MDMs. BIRC3 is an inhibitor of apoptosis protein (IAP) that functions through the inhibition of caspases. BCL2A1 is a member of the BCL-2 family that inhibits the permeabilization of the mitochondrial membrane and therefore inhibits caspase activation. Studies have shown that *Chlamydia* can inhibit apoptosis to facilitate their survival within the host cell (Sharma and Rudel, 2009). Recently, *C. trachomatis* infection was shown to down-regulate p53 expression in human umbilical vein epithelial cells and human

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fallopian primary cells. The down-regulation of p53 may be the main mechanism by which *Chlamydia* inhibits apoptosis and permits intracellular growth (Siegl et al., 2014). *Chlamydia* studies have also previously identified the up-regulation of BIRC3 and BCL2A1 in epithelial cell lines in response to *C. trachomatis* (Rajalingam et al., 2006; Ren et al., 2003). Therefore, these anti-apoptotic genes could contribute to the mediation of *C. abortus* persistence within the host.

Similar results have been identified in other RNA-seq studies investigating the transcriptomic profile of cells infected with intracellular bacteria. The same functional categories that were enriched in ruminant MDMs infected with *C. abortus* were also enriched with DE genes from *M. bovis* infected bovine MDMs including: 'haematological system development and function', 'tissue morphology' and 'cellular movement'. The top 3 canonical pathways activated in bovine MDMs at 24 hours post infection with *M. bovis* (Nalpas et al., 2013) were also the top 3 activated in ovine MDMs infected with *C. abortus*, suggesting a common gene expression profile to the two intracellular pathogens. An RNA-seq study of cervine MDMs identified the up-regulation of type 1 IFN genes (including RSAD2, IFIT2 and ISG15) and chemokines (including CCL8 and CXCL10) with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection. The up-regulation of these genes was higher in MDMs derived from deer susceptible to Johne's disease compared to resistant animals, which the authors suggested may contribute to the inflammatory pathology (Marfell et al., 2013). An RNA-seq study identified that the enriched functions in epithelial cells infected with *C. trachomatis* included the inflammatory and immune response. The study also identified novel host DE genes involved in the remodelling of the extracellular matrix, including mucins, metalloproteinases and collagens (Humphrys et al., 2013). Genes included in these categories are up-regulated in the ruminant MDMs infected with *C. abortus* (including mucin 1 (muc1) and A Disintegrin-Like And Metalloprotease (Reprolysin Type) With Thrombospondin Type 1 Motif, 4 (ADAMTS4)); however, the same extensive range of genes within these categories was not detected which may be due to differences in cell type/*Chlamydia* species.

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Further experiments are necessary to confirm mRNA expression, assess protein expression and identify the biological function of DE genes using knockdown experiments (Chow et al., 2011). Specifically, these studies could investigate the role of ISGs and IL-17 in *C. abortus* mediated pathogenesis. Future analysis will also involve clustering DE genes to identify subset differences in gene expression patterns. The direct DE gene analyses of sheep and cattle *C. abortus*-infected MDMs may also reveal further important species-specific differences. The novel RNA-seq protocol for the simultaneous transcriptomic analysis of the host cell and pathogen could also be used with primary host cells to further elucidate host-*Chlamydia* interactions. An important caveat with RNA-seq data interpretation is that the transcriptomic profile of the sample that is generated is only as good as the genome annotation database (e.g. Ensembl, RefGene) selected. A recent study compared using Ensembl, RefGene and UCSC to analyse the same human RNA-seq data. The study identified that none of the genome annotations were perfect and differed in the definition of some genes, meaning that the annotations of certain genes may not be completely accurate and in some cases may be wrong (Zhao and Zhang, 2015).

In summary, the first aim to assess the transcriptomic profile of ruminant MPS cells was completed using RNA-seq, which will provide the basis for future studies to identify differentiation-specific signature profiles. Secondly, the transcriptome of *C. abortus*-infected ruminant MDMs was investigated using RNA-seq. The RNA-seq data was analysed by identifying the DE genes expressed in *C. abortus*-infected compared to non-infected MDMs. The potential biological function of DE genes and their involvement in signalling pathways was investigated and implied fulfilling the third aim. The fourth aim to compare the DE genes from cattle and sheep *C. abortus*-infected MDMs identified differences in gene expression profiles and enriched signalling pathways, which may have implications for the differential disease pathogenesis of *C. abortus* in sheep and cattle.

Chapter 7: General Discussion

The differential outcome of *C. abortus* infection in sheep and cattle provides an opportunity to identify differences in the ruminant immune system that determine susceptibility to disease. Chlamydial genetics have identified various genes that may play important roles in determining their host specificity and pathogenesis. The genetic analysis of *C. abortus* strains isolated from sheep and cattle suggest that the differences observed with *C. abortus* are due to the host, not the pathogen (Longbottom et al, unpublished). However, as discussed in section 1.1.4.2 small differences in chlamydial isolates can have a large impact on pathology and therefore it is difficult to determine the impact of host or pathogen factors. The focus of this thesis was to investigate the innate immune response to chlamydial infection in sheep and cattle, which, until now, has been primarily characterized in humans and mice. The immune response to pathogens can vary across species (Entrican et al., 2009) and therefore it is important to study the immune response of the natural host. The role of the inflammasome complex and IL-1 β production in response to chlamydial infection has been the topic of many human and mouse studies (Abdul-Sater et al., 2010; Shimada et al., 2012), but prior to this study had not been investigated in ruminants. The improved understanding of the innate immune response to specific pathogens can be used for the design of improved vaccines as innate immune cells infected with *Chlamydia* can polarize the adaptive immune response (Flego et al., 2013). Although commercially available vaccines are available for *C. abortus*, there is clearly room for improvement as it is still the most common infectious cause of ovine abortion in the UK (Animal and Plant Health Agency (APHA), 2014)

The research conducted during this thesis addressed the hypothesis that ruminant hosts differ in their ability to innately sense *C. abortus* infection and activate the inflammasome. A number of aims were formulated in order to address this hypothesis, the outcomes of each and their potential collective impact will be discussed here.

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The first aim was to analyse the expression of selected PRRs by ruminant innate immune cells. TLR2, TLR4, NOD1 and NLRP3 are commonly recognised to be involved in the detection of mouse and human chlamydial species (as described in Chapter 1). Turbinate cells, MDMs and MDDCs from both cattle and sheep were found to express all four receptors (Chapter 3 and Chapter 4). The expression of both intracellular and extracellular PRRs by both ovine and bovine cells indicates that they possess the capability to recognise chlamydial infection at different stages of the developmental cycle. The expression of the four receptors by all of the different cell types supports their role as important innate immune sentinels, which need to be primed to respond to a range of pathogens. The potential role of NOD1 and NLRP3 in *C. abortus* detection was assessed in ruminant MDMs using siRNA, which will be discussed later. Future studies could assess the expression of the TLRs and NLRs that were not investigated in the duration of this thesis. Particularly, the receptors that have only recently been identified to recognise intracellular pathogens may be worth further investigation, including NLRP7 (Broz and Monack, 2013). Candidate gene approaches and genome wide association studies (GWAs) have been used to identify genetic variations in PRRs, which are associated with susceptibility to disease (Pothlichet and Quintana-Murci, 2013). For example, mutations have been identified in TLR1, 2 and 4 that have been associated with increased susceptibility to infection with *Mycobacterium avium* Subspecies *paratuberculosis* in cattle (Mucha et al., 2009). Differences in TLR sequences have been identified between species, which may facilitate the detection of species-specific pathogens (Werling et al., 2009). Mutations do not necessarily affect the function of the protein and therefore it is important to identify whether mutations are present in functional regions and likely to change the function of the receptor (Smith et al., 2014). Therefore, further studies could identify the genetic variation between ovine and bovine PRRs and determine whether variation affects receptor function, impacting on susceptibility to disease caused by *C. abortus*.

Assessing the inflammatory response of innate immune cells to *C. abortus* infection was the second aim. The first step was to assess whether the cells were susceptible to *C. abortus* infection. *C. abortus* formed large inclusions within the ruminant

turbinate cells, unlike in the MDMs and MDDCs where small fragmented inclusions were formed. The inclusion morphology may reflect the differential role of these cells in chlamydial disease pathogenesis. Previous studies have identified that chlamydial species replicate in epithelial cells but replication within mononuclear phagocytes is more limited, to the extent that the growth measured almost never exceeds the starting infectious dose (Beagley et al., 2009a). Studies have shown the *Chlamydiae* can establish a persistent infection within mononuclear phagocytes. Persistence involves the bacteria maintaining viability but not actively progressing through the developmental cycle to produce infectious EBs (Beagley et al., 2009a; Datta et al., 2014). However, the biological and functional relevance for persistence *in vivo* is not clear. *Chlamydia* has been detected *in vivo* in alveolar/peritoneal macrophages and PBMC following intranasal/intraperitoneal infection with *C. pneumoniae*. *C. pneumoniae* was also detected in the lymph nodes and spleen of mice following the adoptive transfer of alveolar/peritoneal macrophages from *C. pneumoniae* infected mice. Theoretically, the macrophages may be facilitating the carriage of the bacteria into the draining lymphatics and lymph nodes, entering the blood stream via the thoracic duct (Moazed et al., 1998). However, this theory of chlamydial dissemination has not yet been experimentally proven. The proposed theory is also complicated by the fact that monocyte-derived cells in the efferent lymph are rare; however, under inflammatory conditions where DC migration from the periphery is induced, increased DCs are detected in the efferent lymph (Randolph et al., 2005). Mononuclear phagocytes could be involved in the dissemination of *C. abortus* from the initial site of infection to the site of persistence and/or to the site of pathology (placenta). However, further studies are required to identify whether *C. abortus* remains viable and infectious, and if so for how long, in the different ruminant cell types.

UV-inactivated bacteria did not form inclusions within ruminant turbinate cells, MDMs or MDDCs, although visible fluorescent specks were present in the immunofluorescent images (Chapter 3 and Chapter 4). This indicates that they were unable to enter the multiplication phase but does not rule out invasion and/or cellular uptake. Confocal microscopy studies are required to determine whether the UV-

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inactivated bacteria are intracellular or attached to the cell surface. Identifying whether the UV-inactivated bacteria were intracellular is important for determining whether cytokine/chemokine production is likely due to intracellular or cell-surface PRR activation.

The production of CXCL8, IL-6, TNF- α and IL-1 β by ruminant turbinate cells, MDDCs and MDMs in response to *C. abortus* infection was then measured. The ruminant turbinate cells responded to infection late in the chlamydial developmental cycle with significantly increased production of CXCL8 only detected at 72 hours post *C. abortus* infection. There was no TNF- α or IL-1 β production detected. In contrast, the ruminant MDMs and MDDCs produced significantly increased CXCL8, TNF- α and IL-1 β by 24 hours post *C. abortus* infection. The cytokine/chemokine production profiles of these cells suggest the activation of signalling pathways that are specific to the different cell types. The unique cytokine/chemokine profiles are likely to be reflective of the specialized roles of different cell types in chlamydial disease pathogenesis. Similarly, a previous study identified that the primary producers of IL-1 β in the murine genital tract following *C. muridarum* infection were macrophages and neutrophils and not epithelial cells (Prantner et al., 2009).

Late CXCL8 production has previously been identified as a feature of the epithelial cell response to chlamydial infection (Buchholz and Stephens, 2008; Rasmussen et al., 1997). Epithelial cells have been shown to respond to other intracellular bacteria, such as *Salmonella Dublin* and *Listeria monocytogenes*, with the rapid production of CXCL8 (Eckmann et al., 1993). Therefore, the delayed CXCL8 production may be a unique feature of chlamydial infection of epithelial cells, which allows the bacterium to enter the cell and begin its developmental cycle before the immune system is alerted. CXCL8 has been implicated in the recruitment of neutrophils to the site of chlamydial infection (Molestina et al., 1999), which were shown to permit chlamydial survival and potentiate replication in macrophages (Rupp et al., 2009). Therefore, late CXCL8 production by ruminant turbinate cells could facilitate the dissemination of *C. abortus* from the oro-nasal mucosa by recruiting neutrophils at a

time when mature EBs are being released from the target cells of the primary infection.

Generally, monocytes can be recruited into infected tissue and under certain conditions can differentiate into MDDC or MDMs (Shi and Pamer, 2011). Mononuclear cell (MHCII⁺) infiltration has been detected in the maternal placental membranes following *C. abortus* infection (Navarro et al., 2004). The contribution of these cells to the *C. abortus* induced inflammatory cascade in the placenta is not fully understood; however, mononuclear cells may contribute to TNF- α production in *C. abortus*-infected placentas (Buxton et al., 2002). Ruminant MDDCs and MDMs respond *in vitro* to *C. abortus* with the production of pro-inflammatory cytokines (Chapter 4) and therefore they may contribute to the inflammatory milieu *in vivo* leading to abortion.

The ruminant turbinate cells did not produce CXCL8 in response to UV-inactivated *C. abortus* (Chapter 3), which contrasts with MDMs and MDDCs. The MDMs and MDDCs were capable of producing both CXCL8 and TNF- α in response to UV-inactivated *C. abortus* (Chapter 4). This may be indicative of the phagocytic ability of monocyte-derived cells and/or the activation of different cell type specific signalling pathways. Viable *C. abortus* was required for IL-1 β protein production in the ruminant MDMs and MDDCs, although mRNA encoding IL-1 β was elevated at 6 hours post infection in MDMs challenged with UV-inactivated *C. abortus* (but not protein). The IL-1 β RT-PCR primers and probe do not distinguish between the pro and mature form of IL-1 β , it is therefore possible that pro-IL-1 β production is induced in response to UV-inactivated *C. abortus* but not subsequently cleaved into the mature form for extracellular secretion. The cleavage of pro-IL-1 β therefore appears to be dependent on a viable chlamydial infection, which fits logically with the theory that cleavage may be mediated by intracellular stress pathways (Tschopp and Schroder, 2010). The production of IL-1 β in murine macrophages was found to be dependent on viable *C. muridarum* infection but not on chlamydial growth, which led the authors to speculate that effector proteins secreted by viable *C. muridarum* were activating the pathway (Prantner et al., 2009). The differences in

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cytokine/chemokine profiles with live and inactivated bacteria should be considered when designing novel vaccines as the live pathogen is uniquely interacting with the host cell to activate host genes that may influence protection/pathology.

The innate immune cytokine profiles were similar in pattern between cattle and sheep cells in response to *C. abortus* infection. Differences in the quantity of production were identified between the two species; however, it was difficult to determine whether ELISA sensitivity issues accounted for these differences and that it wasn't a technical effect rather than a biological effect. From this, it is unlikely that the production of CXCL8, IL-1 β or TNF- α by the ruminant turbinates cells, MDDCs or MDMs contribute to the differential *C. abortus* disease pathogenesis between cattle and sheep. However, it is important to remember that the *in vivo* production has not been assessed and may be different.

The third aim focussed on identifying the role of NOD1 and the NLRP3 inflammasome in IL-1 β and CXCL8 production by ruminant MDMs in response to *C. abortus* infection (Chapter 5). A role for NOD1 was identified in the production of IL-1 β by both ovine and bovine MDMs as well as the production of CXCL8 by ovine MDMs in response to *C. abortus* infection using siRNA technology.

Previously, *C. pneumoniae* and *C. muridarum* studies had identified a role for NOD1 in the production of CXCL8, but most did not investigate IL-1 β production (Opitz et al., 2005; Prantner et al., 2010). The level of target knockdown with NLRP3 siRNA was lower than that observed with NOD1 siRNA. One of the NLRP3 siRNA sequences significantly reduced IL-1 β production in bovine but not ovine MDMs. Therefore, NLRP3 may be involved in the recognition of *C. abortus* and activation of IL-1 β processing pathways in bovine but not ovine MDMs. Further development of the tools to investigate the components of the ruminant inflammasome and IL-1 β production pathway is required for future studies. For example, specific ruminant antibodies that measure ruminant NLRP3, NOD1 and caspase-1 protein (pro-form and cleaved form) may facilitate further studies.

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Targeting NOD1 or NLRP3 may be beneficial for the design of novel *C. abortus* vaccine delivery systems. A subunit vaccine is desirable from the perspectives of safety and for the potential to be used in conjunction with a DIVA (discrimination between vaccinated and infected animals) diagnostic test (Entrican et al., 2012). However, subunit vaccines generally lack immunogenicity and require the addition of an adjuvant to stimulate the immune response. Adjuvants that target PRRs and activate specific signalling pathways that can shape the protective adaptive immune response have been developed. For example, monophosphoryl lipid A extracted from *Salmonella minnesota* LPS is a TLR4 agonist, which is used in licensed human papilloma virus and hepatitis B vaccines (Maisonneuve et al., 2014). Further studies identifying the role of IL-1 β and CXCL8 production *in vivo* with *C. abortus* infection will help identify whether stimulating these pathways will provide protection or cause pathology.

The knockdown of NOD1 or NLRP3 did not completely ablate IL-1 β production, which is a common finding in knockdown studies. The innate immune response to a single pathogen is co-ordinated by various different types of PRRs. This is because pathogens usually contain a number of PAMPs that are recognised by different PRRs and single PAMPs can be recognised by multiple types of PRR. The cross-talk between PRRs has been shown to be necessary for the induction of effective innate immune responses to various pathogens (Kawai and Akira, 2011). PRR cross-talk in the host recognition of chlamydial infection was exemplified in a study which identified that combined TLR2/TLR4 KO mice had increased morbidity and mortality to *C. pneumoniae* infection compared to single KO mice (Rodriguez et al., 2006). Further studies that knockdown combinations of TLRs and NLRs may reveal the full complexity of the signalling pathways activated by *C. abortus*.

The final aim was to use RNA-seq analysis to provide an unprecedented view of the RNA transcripts of ruminant MPS cells and *C. abortus*-infected MDMs (Chapter 6). Future studies will further analyse the RNA-seq data with a view to identifying specific 'signature' profiles for the ruminant MPS cells. This will not only inform on the experiments conducted within this thesis but will be a valuable resource for the

veterinary immunology field. The commonly up-regulated DE genes in *C. abortus*-infected ruminant MDMs included IL-1 β , CXCL8 and TNF- α , which were previously targeted in Chapter 5 as molecules of interest in chlamydial infections. The TLRs and NLRs which were identified to be up-regulated at 6 hours following *C. abortus* infection in ruminant MDMs by RT-PCR (Chapter 4) were not detected to be significantly DE by RNA-seq in both sheep and cattle MDMs at 24 hours following *C. abortus* infection (Chapter 6). The disparity in these results may be due to the different techniques used or the different time points assessed. However, it is important to note that no detectable up-regulated expression does not mean that the receptor is not involved in *C. abortus* recognition. Novel pathways and functional groups of genes involved in inflammatory signalling were enriched with DE genes from both ovine and bovine *C. abortus* infected compared to non-infected MDMs. However, differences in the type of signalling pathways and the top genes that were up-regulated identified potentially important species-specific differences.

The highest up-regulated DE genes in ovine *C. abortus*-infected MDMs included many type 1 IFN related genes. The top enriched ovine canonical pathway described the activation of intracellular signalling PRRs (including RLRs) in the induction of type 1 IFN and ISG expression. The identification of the RLR signalling pathway with *C. abortus* infection highlights a novel PRR pathway worthy of investigation as RLRs are linked to inflammasome activation. For example, RIG-1 has been shown to interact with ASC independent of NLRP3 to mediate IL-1 β production in murine BMDCs in response to the RIG-1 agonist 5'-triphosphate RNA (Poeck et al., 2010). Type 1 IFN signalling has also been shown to contribute to the induction of IL-1 β production in murine BMDMs in response to the intracellular bacteria *Francisella tularensis* (Henry et al., 2007). This reinforces the earlier discussion about the complexity of PRR signalling pathways and the role of cross-talk in coordinating the immune response to a single pathogen. The role of type I IFNs in chlamydial infection is still poorly understood. Previous *in vivo* mouse studies have identified a role for type 1 interferons in *Chlamydia* induced morbidity (Nagarajan et al., 2008; Qiu et al., 2008). Given the RNAseq data with the *C. abortus*-infected ovine MDMs,

further investigation into the role of type 1 interferons in *C. abortus* infection is warranted.

In contrast, the highest up-regulated bovine DE genes were not enriched with type 1 IFN genes and instead contained a number of pro-inflammatory genes, including IFN- γ , IL-1 β and G-CSF. NOS2 is a target gene of IFN- γ , which was also highly up-regulated in bovine MDMs following *C. abortus* infection. NOS2 has been shown to inhibit chlamydial growth *in vitro* in murine cells and therefore may be an important host defence mechanism (Zhang et al., 2012). NOS2 was not significantly up-regulated in ovine MDMs in response to *C. abortus* infection. This echoes previous results in ovine alveolar macrophages, which did not produce detectable NO when stimulated with recombinant ovine IFN- γ in combination with LPS or *C. abortus* (Brown, 1999). Therefore, NO may be involved in IFN- γ driven defence to *C. abortus* in cattle but not sheep, which may be an important species-specific difference that could impact on disease pathogenesis. The top enriched bovine canonical pathway suggested a potentially novel role for IL-17A and IL-17F in driving the production of inflammatory cytokines in bovine MDMs in response to *C. abortus* infection. This is interesting as the analyses were conducted on myeloid cells, not lymphoid cells. A potentially protective role for IL-17A with *C. muridarum* lung infection has been identified (Bai et al., 2009), which may be connected to the recently discovered role for the cytokine in the induction of IFN- γ mediated NOS2 production (Zhang et al., 2012).

A number of challenges associated with working in the veterinary immunology field were encountered in the course of this thesis. Although the veterinary reagents which are commercially available have increased, gaps in availability still exist (Entrican and Lunney, 2012). Ideally, commercially available ELISA kits containing standards for both cattle and sheep would be available for all cytokines/chemokines. However, in the case of CXCL8, IL-6, IL-1 β and TNF- α commercially available reagents are limited to antibody pairs, which were designed for the detection of either the sheep or cattle cytokine. Cattle and sheep are closely related species and therefore reagents are likely to cross-react (Entrican and Lunney, 2012), however reagents can show a

difference in sensitivity (Chapter 3). The commercial IL-6 antibody pairs detected recombinant ovine and bovine IL-6, however the ELISA failed to detect any IL-6 in the supernatants of the cells assessed in this thesis. Previous studies have identified that epithelial cells, monocytes and macrophages produce IL-6 in response to chlamydial infection (Rusconi and Greub, 2011). The up-regulation of IL-6 mRNA was detected at 6 hours post *C. abortus* infection in ruminant MDMs (Chapter 4) and also up-regulated in RNA-seq data from *C. abortus*-infected ruminant MDMs (Chapter 6). Although originally shown to detect native IL-6 in the efferent lymph of sheep (McWaters et al., 2000), results suggest that the current batch of IL-6 antibodies may no longer detect native IL-6. Reagent availability also affects cell phenotyping in veterinary species, as some of the antibodies for the detection of differentiation-specific markers are not yet available. An example is Flt3 (CD135) that has been identified as a specific classical DC marker (Miller et al., 2012) but ruminant Flt3 antibodies are not yet available.

Another challenge in the veterinary immunology field is that protocols that are well established and optimised for human and mouse studies are not established in veterinary species. An example is the generation of ovine MDMs, which has not previously been published. The protocol for bovine MDMs has been published (Jensen et al., 2014), but unfortunately was not transferrable to ovine cells. Various optimisation steps were assessed before the requirement for autologous serum was identified (Chapter 4). Equally, a protocol for the Western blot detection of the pro and mature forms of ruminant IL-1 β had not been published and required development (Chapter 5).

Gene expression technology is helping to bridge the gap in ruminant reagent availability as it uses the annotated ovine and bovine genomes and does not require the development of species-specific reagents. The analysis of gene expression profiles has been used to identify MPS differentiation specific signatures across species (Vu Manh et al., 2015b). Gene expression profiling has also identified novel pathways activated in ruminant cells in response to infection (Nalpas et al., 2013). RNA-seq technology is particularly useful for cross-species comparisons as it

identifies the complete RNA profile of any species without the microarray requirement to design species-specific probes, which requires previously annotated transcripts (Liu et al., 2011). Further validation experiments using other experimental methods (such as RT-PCR) on different biological replicates (different from those used in the RNA-seq experiment) are required to validate RNA-seq results including the results in Chapter 6 (Fang and Cui, 2011).

In some cases *in vivo* studies are necessary to understand disease, however, alternative methods should be used wherever possible (Entrican et al, 2015). *In vitro* studies have provided vast insight into the function of different immune cell types in response to various stimuli. However, studies into human MDDCs and mouse BMDCs have shown that *in vitro* differentiated cells do not display all the functions of their proposed *in vivo* counterparts (Guilliams and Malissen, 2015; Helft et al., 2015; Wimmers et al., 2014). Similarly, *in vitro* studies do not replicate the complex cellular interactions that take place in tissues *in vivo*. *Ex vivo* explant cultures provide an intermediary step between *in vitro* and *in vivo* studies, which allow the investigation of host-pathogen interactions within intact tissue architecture unlike single-cell assays. The recent publication of the protocol for the *ex vivo* culture of bovine endometrial explants (Borges et al., 2012) identified a novel method for the investigation of the maternal immune response to *C. abortus* infection. BKTN PhD top-up funding facilitated the investigation of the potential of *ex vivo* ruminant endometrial explants to investigate maternal immune pathways in response to *C. abortus* infection (see appendix 9. 2). The study identified that the bovine endometrial explants could be infected with *C. abortus*, but the production of cytokines/chemokines to infection was not significant due to high variation between animals. The same protocol also did not generate comparable ovine explants due to anatomical differences in the cattle and sheep uteri. Larger sample sizes and further optimisation of the protocol to facilitate the culture of ovine endometrial explants could form the basis of future work.

Understanding the innate immune response of the natural host to infection will contribute to the design of safer vaccines that can selectively target the protective

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adaptive immune response. The innate immune cytokine profiles of ruminant oronasal turbinate cells and monocyte-derived cells to *C. abortus* infection may reflect the cell-type specific roles in disease pathogenesis. The cytokine profiles were similar between sheep and cattle cells, indicating that more complex interactions may be influencing the differential outcome of infection. NOD1 contributed to IL-1 β production in both sheep and cattle MDMs and further investigation will identify what stage of the signalling pathway the receptor is involved. Finally, novel genes and pathways have been identified in ruminant *C. abortus*-infected MDMs that may impact on disease pathogenesis and will be the topic of future studies.

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Chapter 9: Appendix

9.1 RNA seq library preparation

The total RNA was assessed for quality and quantity using the Agilent RNA ScreenTape and Life Technologies Qubit RNA BR assay kits. RNA with a RNA integrity score (RINe) >6.8 was considered suitable for analysis (range 6.8 – 9.2 when measured by ARK genomics). The Sequencing library was prepared using the NEB Next Ultra directional RNA Library Prep Kit for Illumina according to the manufacturer's protocol. Briefly the poly A RNA was captured from 1 microgram of total RNA using oligo d(T) beads in two rounds of capture to eliminate as much of the non poly adenylated RNA as possible. The poly A RNA was eluted from the oligo d(T) beads using the provided elution and fragmentation solution. This stage was carefully controlled for temperature at 94°C and a time of 8 minutes. The RNA is fragmented into an average insert size of 180-200 bases. The fragmented RNA was used as a template for first strand DNA synthesis with random hexamer oligo primers. The first strand cDNA is used as a template for the second strand synthesis which incorporates dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide. The resulting ds cDNA is then blunt ended before incorporation of a single A base the 3' end of each strand. The addition of an A nucleotide tail prevents the formation of concatamers during the next ligation step. The sequencing adapters to create the library are ligated to the A tailed ds cDNA. Each of the ligated adapters are different for each sample as each contains a 6 base barcode sequence which will allow identification of sequences for each sample in the analysis. The dsDNA with the ligated adapters form the sequencing library with each sample having its own barcoded adapter. This final product is amplified using PCR with only 10 cycle of PCR to amplify the resulting library. The amplified library was assessed for quality and quantity using the Agilent DNA 1000 ScreenTape and Life Technologies Qubit DNA BR assay kits. The Qubit quantification was used to guide the dilution of the library for accurate quantification using qPCR. The nano molar concentration of the library was determined by qPCR using the KAPA Library

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Quantification Kits for Illumina on an ABI 7500 qPCR machine. The quantified libraries were diluted to a concentration of 5nM and stored until sequencing.

The normalised sequencing libraries were pooled at equal concentrations to form two independent pools of libraries which were sequenced over 3 lanes for each pool. The sequencing was performed Illumina HiSeq 2500 for 125 bases paired end sequencing using SBS v4 chemistry.

Briefly the libraries were denatured using NaOH at a final concentration of 0.1N of NaOH and 2nM of library pool in a 20 micro litre reaction volume for a period of 5 minutes at room temperature. After denaturation the libraries were diluted to 14pM by adding 7 micro litres of denatured 2nM library to 986 micro litres of Illumina buffer HT 1. The libraries were loaded and clustered onto the Illumina reaction flow cell using the Illumina cBot instrument using the TruSeq PE Cluster Kit v4-cBot-HS. The clustered flow cell was placed on the Illumina HiSeq 2500 instrument and the HiSeq SBS Kit V4 reagents loaded following the Illumina protocol.

9.2 Investigation of innate immunity and uterine inflammation caused by *C. abortus* infection using endometrial explant cultures

9.2.1 Introduction

Chlamydia abortus is the most common known infectious cause of ovine abortion worldwide (excluding New Zealand and Australia). Interestingly, the bacterium can also infect cattle but is not a causal factor of abortion. *C. abortus* can infect ewes at any time via the oro-nasal route and establish a latent sub-clinical infection. During pregnancy the bacteria colonize the placenta, inducing a powerful innate pro-inflammatory response which can result in pathology (Rocchi et al., 2009). It is not known how this innate pro-inflammatory response is initiated. We hypothesize that sheep and cattle differ in their ability to innately sense and respond to *C. abortus* and that this may explain the differences in pathogenesis observed in ruminants.

The primary focus of the PhD project has been the investigation of *in vitro* responses of various cell types to *C. abortus* infection, including oro-nasal turbinate cells (potentially the primary targets for the bacteria) macrophages and dendritic cells. Interestingly, both macrophages and dendritic cells were found to produce IL-1 β in response to *C. abortus* infection whereas turbinate cells did not. Studies investigating the role of IL-1 β in other chlamydial species suggest that this cytokine has an important role in bacterial clearance but is also involved in pathology. As IL-1 β has been linked with infection associated pre-term birth in animal models it may have a critical role in pathology, hence our interest in investigating the expression of this cytokine in the uterine tissues of sheep and cattle.

9.2.2 Aims

Ex-vivo uterine explants provide an opportunity to study host-pathogen interactions within intact tissue architecture unlike single-cell *in vitro* assays. As a precedent, *ex-vivo* fallopian tube explants up-regulate prokineticin receptor expression in response to *C. trachomatis* infection, which may be linked to ectopic pregnancy (Shaw et al.,

2011). At the start of this PhD project there were no validated *ex-vivo* models that detect cytokine production in the ruminant uterus following infection. However, a bovine endometrial explant model has recently been developed and the protocol published (Borges et al., 2012). The aims of this project were to: (A) learn how to isolate bovine endometrial explants from the group at Swansea University, led by Prof Martin Sheldon, who developed the technology; and (B) transfer the method back to the MRI where the effects of *C. abortus* infection on uterine cytokine production could be investigated and compared between cattle and sheep.

9.2.3 Methodology

A visit to the laboratory in Swansea was arranged for July 2013. Uteri were collected from the local abattoir in Swansea and transported back to the laboratory where the uterine horns were cut open to expose the endometrium. Biopsy punch explants were collected from intercaruncular areas of the endometrium and cultured in 6 well plates for up to 96 hours. This protocol was repeated at the MRI with both cattle and sheep uteri and explants were cultured overnight and then infected with *C. abortus*. Explants were also collected into neutral buffered formalin and snap-frozen for immunohistochemical analysis of infection and inflammation. Culture supernatants were collected at various time points for analysis by cytokine ELISA and cell lysates collected for RNA analysis.

9.2.4 Results

Immediately apparent was the distinct anatomical differences between bovine and ovine uteri. The uteri walls were much thinner in the sheep and the whole of the endometrium was covered in caruncles compared to cattle (Figure 9.1).

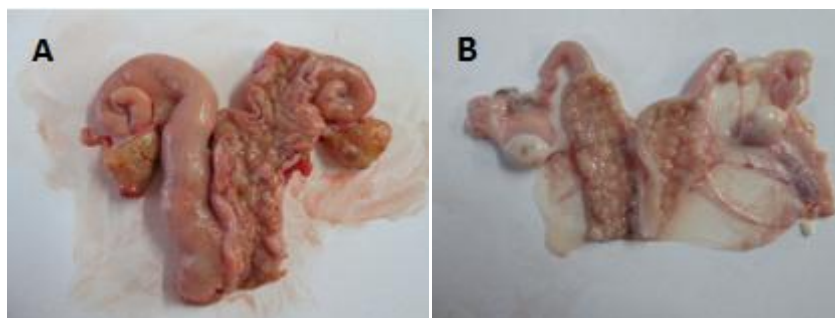


Figure 9.1: Bovine (A) and ovine (B) uteri collected from the abattoir.

Due to these anatomical differences it was not possible to collect the single endometrial layer from the ovine uteri. Therefore biopsy punches through the uterine wall, including both the endometrial layer and stroma were taken and cultured using the bovine explant protocol.

Ovine and bovine explants were found to express mRNA encoding TLR2, TLR4, NOD1 and NLRP3 which are important PRRs for sensing bacterial pathogens. Immunohistochemical analysis identified *C. abortus* within the bovine explants but not the ovine explants. This suggests that the bovine explants were susceptible to *C. abortus* infection but further optimisation is required to establish an active infection in the ovine explants (Figure 9.2).

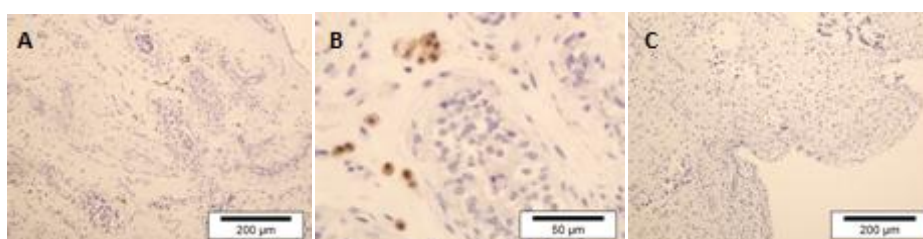


Figure 9.2: Immunohistochemistry with *C. abortus* challenged endometrial explants.

Positive brown staining was present in the bovine endometrial explants (A) which specifically labelled infected cells (B). No positive immunohistochemical staining was observed in ovine explants (C).

The explants produced the innate immune chemokines/cytokines CXCL8, TNF- α , IL-6 and IL-1 β . However, these were highly variable between animals. Moreover, there was no significant effect of *C. abortus* infection on cytokine/chemokine

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production (Figure 9.3), which is different to the effects that have been observed with single-cell ruminant cultures.

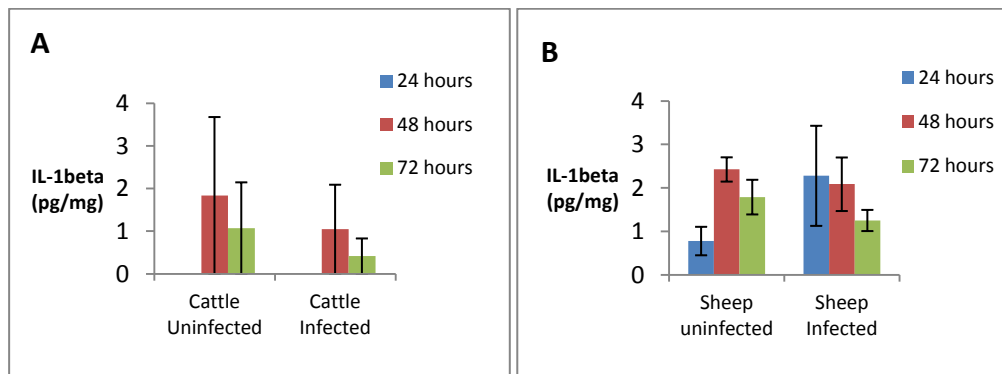


Figure 9.3: IL-1 β production by bovine (A) and ovine (B) endometrial explants in response to *C. abortus* infection.

9.2.5 Conclusions and summary

The development of a protocol for the generation of bovine endometrial explants provided a valuable opportunity to assess uterine immune responses to *C. abortus*. Due to the anatomical differences between cattle and sheep uteri it became clear that direct comparisons could not be drawn between the species as described in the aims. Furthermore, the inherent variation between animals made it difficult to establish any clear effect of *C. abortus* infection on cytokine production. It is possible that further studies on a larger number of uteri would reveal trends. Although the results were overall inconclusive with regards to cytokine production, the project has confirmed the expression of PRRs in both cattle and sheep uteri. The project has added another layer to the PhD ensuring that more than *in vitro* immunology data is considered and has allowed new skill sets to be developed in anatomy and tissue handling. Close collaboration between Swansea University and Moredun Research Institute has allowed the successful transfer of endometrial explant technology which has great potential for future projects in a number of different fields.

9.2.6 References

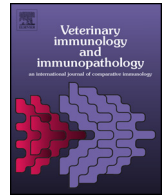
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Short communication

Late production of CXCL8 in ruminant oro-nasal turbinate cells in response to *Chlamydia abortus* infection

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Ruminant

ABSTRACT

Chlamydia abortus is an obligate intracellular bacterium that is an important cause of ovine abortion worldwide. There are reports of abortions in cattle, but these are very rare compared to the reported incidence in sheep. The bacterium is transmitted oro-nasally and can establish a sub-clinical infection until pregnancy, when it can invade the placenta and induce an inflammatory cascade leading to placentitis and abortion. Early host–pathogen interactions could explain differential pathogenesis and subsequent disease outcome in ruminant species. In this study, we assessed the ability of sheep and cattle oro-nasal turbinate cells to sense and respond to *C. abortus* infection. The cells expressed toll like receptor (TLR) 2, TLR4, nucleotide oligomerization domain (NOD) 1 and NOD-like receptor pyrin domain containing 3 (NLRP3) mRNA. In response to *C. abortus* infection, both ovine and bovine turbinate cells produce CXCL8 mRNA and protein late in the bacterial developmental cycle, but do not produce IL-1 β or TNF- α . The UV-inactivated bacteria did not elicit a CXCL8 response, suggesting that intracellular multiplication of the bacteria is important for activating the signalling pathways. The production of innate immune cytokines from cattle and sheep turbinate cells in response to *C. abortus* infection was found to be largely similar.

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1. Introduction

Chlamydia abortus is a major infectious cause of ovine abortion worldwide (excluding Australia and New Zealand). The bacteria can also cause abortion in goats, but interestingly, is rarely linked to abortion in cattle even though cattle are susceptible to infection (Animal and Plant Health Agency (APHA), 2014; Longbottom and Coulter, 2003). *C. abortus* is transmitted oro-nasally and can infect the ewe at any time, persisting sub-clinically (site unknown) until late stage gestation when abortion facilitates the shedding of the bacteria for propagation of the infection cycle. Once a ewe has aborted due to *C. abortus* it does not abort again with re-infection indicative of the development of protective immunity (Rocchi et al., 2009).

During late-stage gestation, *C. abortus* recrudescens from its persistent state and invades the placenta inducing an inflammatory cascade typified by the infiltration of immune cells and production of TNF- α and CXCL8 (Kerr et al., 2005; Wheelhouse et al., 2009). To date, the strongest known single immunological correlate of protection to *C. abortus* is the production of IFN- γ ; however the ovine cellular source of IFN- γ and the innate immune mediators involved have yet to be elucidated (Entrican et al., 2010). Generally, the current understanding of innate and adaptive immune responses in small ruminants is relatively poor due to lack of specific immunological tools (Entrican and Lunney, 2012). The study of *C. abortus* in both small and large ruminants should therefore inform on pathogen–host co-evolution, thereby highlighting potentially important differences in immune recognition which contribute to pathological outcomes.

The genus *Chlamydia* currently comprises 12 species (Sachse et al., 2014; Vorimore et al., 2013) that share a characteristic biphasic developmental cycle, which involves extracellular and intracellular forms. The infectious elementary body (EB) is internalized by the host cell and induces the formation of an intracellular vacuole that allows transformation into the

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metabolically active reticulate bodies (RB), which multiply by binary fission (Abdelrahman and Belland, 2005). Consequently, both intracellular and extracellular pathogen recognition receptors (PRRs) are likely to be important for the detection of the bacteria by the host cell. A number of studies in mice and humans have highlighted the importance of TLR2 and TLR4 found on the host cell surface and intracellular NLRP3 and NOD1 for the activation of signalling pathways and release of pro-inflammatory chemokines and cytokines in response to chlamydial infection (Rusconi and Greub, 2011; Shimada et al., 2012). In particular, CXCL8, TNF- α and IL-1 β have been identified as important innate immune cytokines produced in response to chlamydial infection (Rusconi and Greub, 2011).

Turbinate cells are components of the oral-nasal mucosa, which forms one of the first barriers of immune defence to pathogens. Therefore, the role of cattle and sheep turbinate cells to detect and activate the innate immune response to *C. abortus* infection was investigated. The expression profiles of pathogen recognition receptors (PRRs) by ovine and bovine turbinate cells and their production of innate immune chemokines and cytokines in response to *C. abortus* infection was assessed.

2. Materials and methods

2.1. Propagation of *C. abortus* stocks

C. abortus strain S26/3 was isolated at the Moredun Research Institute (MRI) and grown and titrated in HeLa cells, as previously described (Graham et al., 1995). Harvested bacteria were re-suspended in sucrose-phosphate-glutamate (SPG) (sucrose based medium containing FBS, antibiotics and fungicides) for storage at -80°C .

2.2. Cell culture and infection

Ruminant turbinate cells were generated at post-mortem by harvesting foetal turbinate tissues, which were then trypsinised to make a single cell suspension and cultured as previously reported (McClurkin et al., 1974) in order to create frozen cell banks. The primary cell lines used in this study represent different passages of cattle and sheep turbinate cells that were initially derived from the same fetal bovine or fetal ovine sources, respectively. These cells were screened and shown to be negative for pestivirus. After resuscitation, cells were adjusted to $1 \times 10^5/\text{ml}$ and grown to sub-confluence by adding 1 ml/well in 24 well plates (Corning Costar, High Wycombe, UK) or 500 μl /well in 8-well chamber slides (Nunc, Roskilde, Denmark). The culture medium consisted of Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (PAA, Han-niger, Austria). As for the cell lines, the fetal calf serum was tested for live pestivirus and shown to be negative. The cells were then challenged with live *C. abortus* at a multiplicity of infection (MOI) of 10, with UV inactivated *C. abortus* (equivalent to MOI 10) and medium only. UV treatment was shown to be effective at killing the bacteria as UV-inactivated *C. abortus* failed to form inclusions in HeLa cells (data not shown). Supernatants and lysates were harvested at 24, 48 and 72 h from separate wells. The experiments were repeated on three separate occasions.

2.3. Immunofluorescence

Slides were fixed in cold 90% acetone 10% methanol and stored at -20°C . Slides were rehydrated in PBS prior to the addition of Image-iT[®] FX signal enhancer (Life Technologies) for 30 min in a humidity chamber protected from light. The slides were washed and then incubated with 1:1000 anti-*C. abortus* MOMP mouse

monoclonal antibody (mAb) (4/11) (Santa Cruz Biotechnology, Heidelberg, Germany) for 30 min. After washing the slides three times, the Alexafluor 488 goat anti-mouse IgG (H + L) secondary polyclonal antibody (pAb) (Life Technologies) was added at 1:2000 for 30 min. The slides were washed three more times before mounting with ProLong Gold anti-fade reagent containing DAPI (Life Technologies).

2.4. Total RNA extraction and cDNA preparation

Cell lysates were harvested and stored in RLT lysis buffer at -80°C before total RNA was extracted using the RNeasy plus mini kit (Qiagen, Crawley, UK), following the manufacturer's instructions. The concentration of RNA was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, Rochester, NY, USA) and 1 μg reverse transcribed into cDNA using Taqman[®] Reverse Transcription Reagents (Life Technologies). Reverse transcriptase was omitted from the process for the production of negative controls. Peripheral blood mononuclear cells (PBMC) were stimulated with Concanavalin A (Con A; ICN Biochemical, Cleveland, OH, USA) at 5 $\mu\text{g}/\text{ml}$ for 24 h to generate cDNA to be used as positive controls.

2.5. Standard PCR

Primers were designed using primer 3 (<http://primer3.sourceforge.net/>) and details are shown in Table 1.

PCR cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, the appropriate annealing temperature for 30 s and 72°C for 1 min and then a final extension of 72°C for 5 min. The cycle was finished with 5 min at 72°C . PCR products were run along with a 100 bp ladder (New England Biolabs, Hitchin, UK) on a 1% agarose gel containing Gel Red[™] (Biotum, Hayward, CA, USA) and visualized using UV light.

2.6. Taqman RT-PCR

Taqman RT-PCR was run using custom designed primers and probes for CXCL8. The commercially available eukaryotic 18s rRNA endogenous control (VIC/TAMRA[™], Life Technologies), identified as the most stably expressed compared with GAPDH and β -actin, was used as the reference gene. The bovine CXCL8, TNF- α and IL-1 β primers and FAM/TAMRA probes used were previously designed (Galvao et al., 2012; Leutenegger et al., 2000), as were the ovine IL-1 β and TNF- α primers and FAM/TAMRA probes (Budhia et al., 2006). The ovine CXCL8 forward primer (5'-CACTGCGAAAATTCAGAAATCATTGTTA-3'), reverse primer (5'-CTTCAAAAATGCCTGCACACCTTC-3') and probe (FAM-CCAACGGAAGAGGTGTGCTTAGACCCC-TAMRA) were designed as part of this study using Primer Express (Life Technologies). Samples were analyzed in triplicate on an ABI prism 7000 machine (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions; 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s followed by 60°C for 1 min. Gene expression was quantified using the comparative $2^{-\Delta\Delta\text{CT}}$ method. Cells cultured without any exogenous stimulation in medium alone were harvested at each time point and used as a calibrator for all analyses of the treatment groups at that time point.

2.7. Cytokine ELISAs

Cytokine sandwich ELISAs using commercial pairs of mouse anti-sheep mAbs and rabbit anti-sheep pAbs to CXCL8 and IL-1 β (Bio-Rad AbD Serotec, Kidlington, UK), or rabbit anti-bovine pAb and biotin conjugated pAb to TNF- α (Thermoscientific, Cramlington, UK) were run as previously described (Rothel et al., 1998).

Table 1
Standard PCR primer sequences for the detection of PRRs in ruminant turbinate cells.

Target	Accession number	Forward primer (5'–3')	Reverse primer (5'–3')	Expected product size (base pairs)	Annealing temperature (°C)
Ovine NOD1	XM.004007930.1	CCA TCT TTG AAG TCG GGA TG	CGG GCT TTA TCA AGT TTC CA	378	50
Ovine NLRP3	XM.004008740.1	AAT CCC TTT CCC TGA GGT TG	GGG AAT GGT TGG TGC TTA GA	531	52
Ovine TLR2	NM.001048231.1	GCT CCT GTG ACT TCC TGT CC	AGT GGG AGA AGT CCA GCT CA	555	52
Ovine TLR4	NM.001135930.1	GGA CAA CCA ACC TGA AGC AT	TGA GGT TCC TTG GCA AAT TC	525	50
Bovine NOD1	NM.001256563.1	GCC TTC TTT GCT GCC CTT TTT C	CCC CAC CTT TTC ACT CTG TGT C	395	52
Bovine NLRP3	NM.001102219.1	TCA AGC TGT CCA CCA GAA TG	CAG AAT TCA CCA ACC CCA GT	711	52
Bovine TLR2	NM.174197.2	GGG GAT GAC AGA GGA TGA GA	GGC AGA AAG CTG ATG GAA AG	404	52
Bovine TLR4	NM.174198.6	GAC CCT TGC GTA CAG GTT GT	GGT TGA GTA GGG GCA TTT GA	538	52

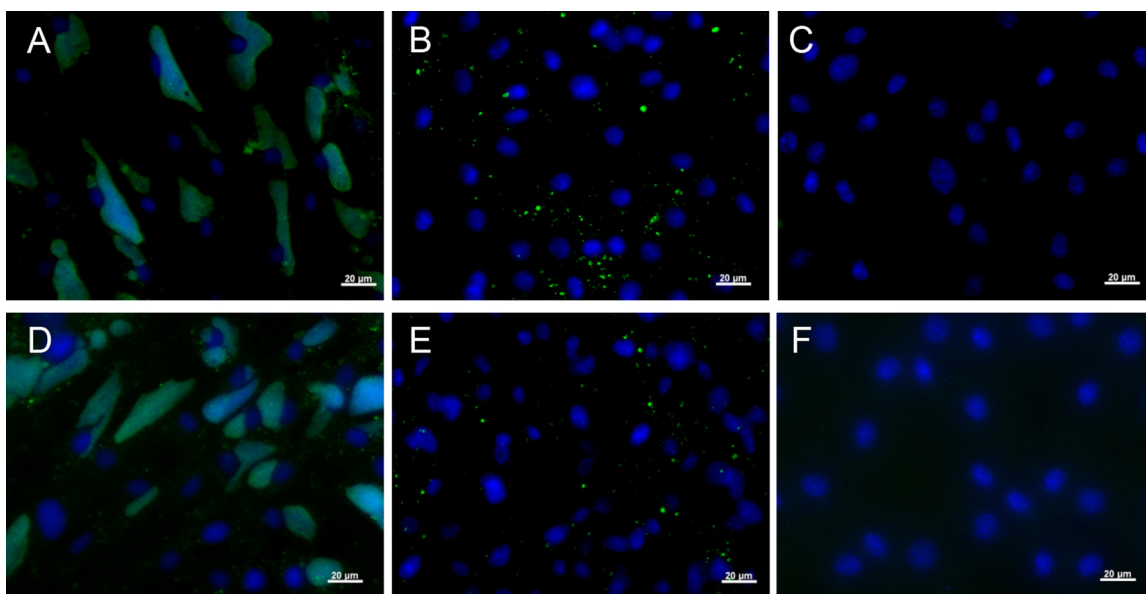


Fig. 1. Immunofluorescent images of bovine (A–C) and ovine (D–F) turbinate cells demonstrating susceptibility to *C. abortus* infection. *C. abortus* was labelled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody and host cell nuclei were stained blue with DAPI. Turbinate cells challenged with MOI 10 live *C. abortus* (A, D), UV inactivated *C. abortus* (MOI 10 equivalent) (B, E) and medium only (C, F) for 72 h. The scale bars correspond to 20 μm.

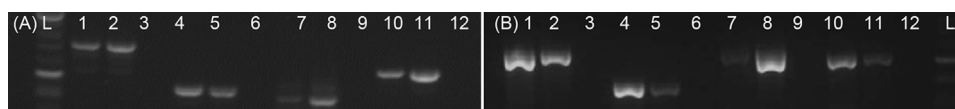


Fig. 2. Expression of NLRP3, NOD1, TLR2 and TLR4 by bovine (A) and ovine (B) turbinate cells. Total RNA was extracted from ruminant turbinate cells and Con A-stimulated PBMC, which was then reverse transcribed and PRRs amplified with specific primers (Table 1). Lanes 1, 4, 7 and 10: turbinate cells; Lanes 2, 5, 8 and 11: PBMC; Lanes 3, 6, 9 and 12: no reverse transcriptase control. Lanes 1–3: NLRP3; Lanes 4–6: NOD1; Lanes 7–9: TLR2; Lanes 10–12: TLR4. L: 100 bp ladder. Products obtained were of the expected molecular weight.

Recombinant ovine CXCL8 expressed in Chinese Hamster Ovary cells by a standard protocol (Wattegedera et al., 2004), recombinant bovine IL-1β (Bio-Rad AbD Serotec) and recombinant bovine TNF-α (Bio-Rad AbD Serotec) were used to generate standard curves. The ELISAs were cross-reactive between cattle and sheep (Caswell et al., 1998; Tahoun et al., 2015; Wheelhouse et al., 2009) and had the following sensitivities: CXCL8 15 pg/ml; TNF-α 1.95 ng/ml and IL-1β 60 pg/ml.

2.8. Statistical analysis

Data included the expression profiles of CXCL8 mRNA and protein, and comprised three different passages of cell lines for each species (bovine and ovine) to which each level of treatment group (3 levels: live *C. abortus*, UV inactivated *C. abortus* and medium only) was applied and measured at three time points (24, 48 and 72 h). The data on mRNA were obtained by comparative $2^{-\Delta\Delta CT}$ method by calibrating the expression for medium control from

each treatment group for each time point. Both mRNA and protein data showed increased variability with increased mean; hence the data on mRNA and protein were transformed by the logarithmic (after adding a constant value of 1) and square root transformation, respectively. The main effect of species, treatment group and time as well as possible two and three-way interaction effects were tested by univariate analysis of variance separately for the expression data of CXCL8 mRNA and protein. The blocking structure included the following categorical variables: cell line and interactions of cell line by treatment group and cell line by time. The overall statistical significance of the interaction effect was assessed using the *F*-statistic. If the overall test was statistically significant ($P < 0.05$), we obtained two-sided probabilities of comparisons of means of treatment groups at each time point. Finally, these probabilities were adjusted using a False Discovery Rate (FDR) approach (Benjamini and Hochberg, 1995) to take into account the multiple comparisons of means so that the expected proportion of false positives among all positives (i.e. rejecting the null hypothesis) was less

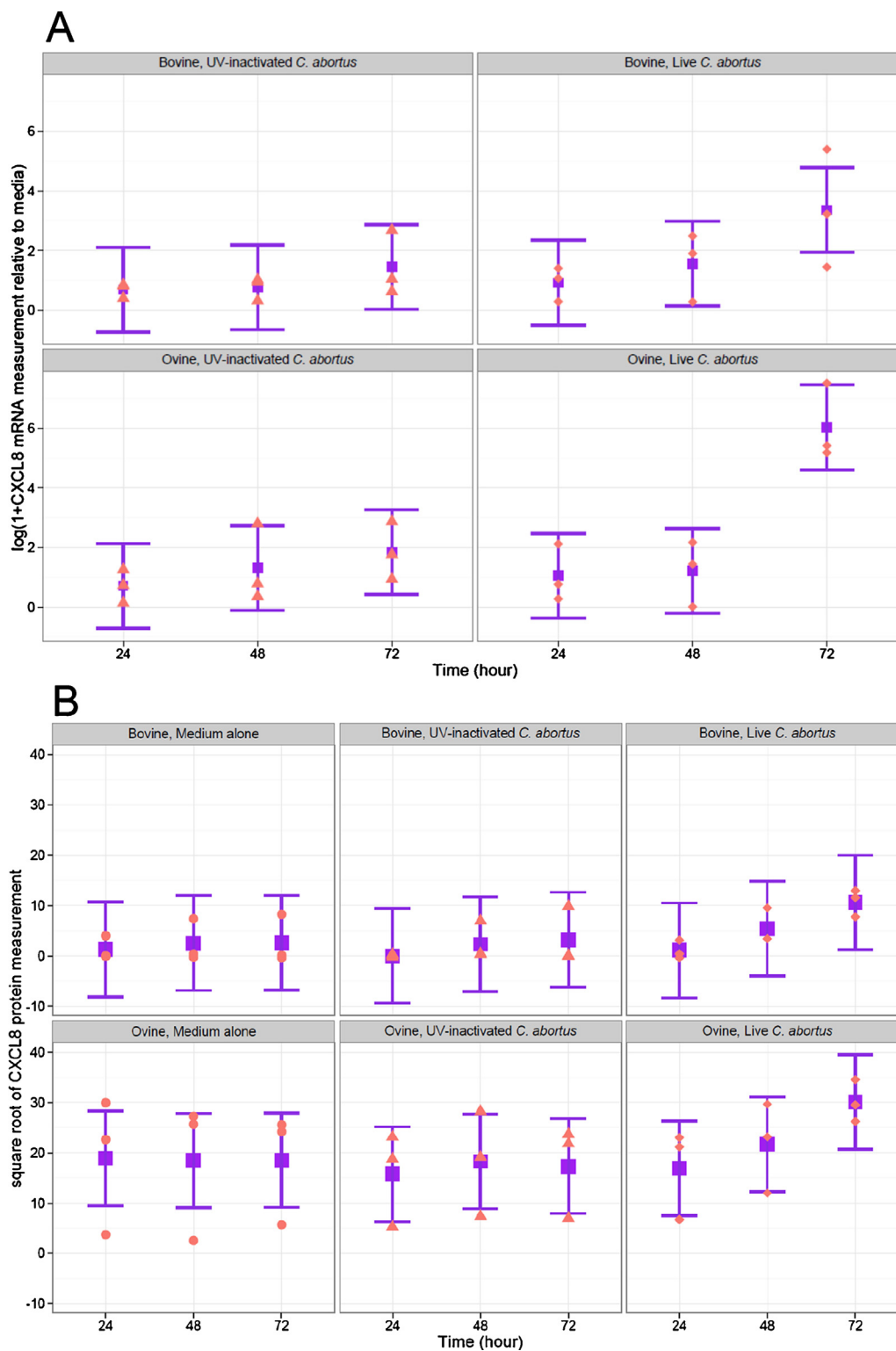


Fig. 3. CXCL8 mRNA and protein production by ruminant turbinates cells in response to *C. abortus*. The expression of (A) CXCL8 mRNA (on the logarithmic scale after adding a constant of one), measured in two treatment groups (response to UV-inactivated *C. abortus* and live *C. abortus*) relative to medium alone and (B) protein (on the square root scale) measured in three treatment groups (response to medium alone, UV-inactivated *C. abortus* and live *C. abortus*) at three different time points (24, 48 and 72 h) in two ruminant (bovine and ovine) turbinates cells. The plots show the mean expression (solid square) of mRNA and protein along with corresponding 95% confidence intervals (error bar). The plots also present the observed mRNA or protein data on each treatment group; with shapes (circle, triangle and diamond) representing each of the three treatment groups. The data on the expression profiles of mRNA and protein were separately analyzed using an analysis of variance approach incorporating main and interaction effects of species, treatment group and time and appropriate blocking structure.

than 5% and we denoted the adjusted probabilities as P_f . Statistical analyses were performed using GenStat (GenStat version 16.2) and R (R Core Team, version 3.1).

3. Results and discussion

Previous *in vivo* studies in sheep have demonstrated that *C. abortus* can induce abortion if inoculated over the tonsillar crypts (Jones and Anderson, 1988) and mucosal nasopharyngeal lymphoid tissues prior to a subsequent pregnancy (Longbottom et al., 2013). This suggests that turbinate cells are an appropriate *in vitro* model for studying initial infection. Initially, the ability of ovine and bovine turbinate cells to sustain an active *C. abortus* infection was assessed. Challenge with live *C. abortus* resulted in the formation of large intracellular inclusions (Fig. 1A, D) at 72 h post-infection. Inclusions were not visible after exposure to UV-inactivated *C. abortus* however, visible fluorescent specks, an indication of dead bacteria, were present (Fig. 1B, E) which were not detected in the non-exposed controls (Fig. 1C, F).

As turbinate cells are a component of the oro-nasal mucosa, they are likely to be important sentinels of the immune system. We observed that ruminant turbinate cells express NLRP3, NOD1, TLR2 and TLR4 (Fig. 2); thus ruminant turbinate cells fit the sentinel role as a varied complement of PRRs would be beneficial in the detection of pathogens. The human nasal mucosa expresses TLR2, TLR4 (Vandermeer et al., 2004), NOD1 and NLRP3 (Bogefors et al., 2010), and this is the first study to demonstrate that ruminant turbinate cells also express these receptors. As these cells express a range of PRRs, they have the potential to respond to both intracellular and extracellular stages of *C. abortus* infection and activate signalling pathways leading to the transcription of cytokines and chemokines.

The expression profiles of CXCL8, TNF- α and IL-1 β mRNA and protein in three treatment groups (live *C. abortus*, UV-inactivated *C. abortus* and medium only) were assessed at three time points (24, 48 and 72 h). There was no IL-1 β mRNA or protein detected at any time points nor any TNF- α protein. Although low mRNA signal was detected in the TNF- α RT-PCR, it was around the threshold limit of detection for the assay. The absence of IL-1 β and TNF- α mRNA expression or protein production from ruminant turbinate cells is perhaps not too surprising as these cytokines are more commonly (but not exclusively) produced by myeloid cells, as has been shown for *C. trachomatis* and *C. pneumoniae* infections (Rusconi and Greub, 2011).

For the expression profile of CXCL8 mRNA, the interaction effect of treatment group and time was statistically significant ($P=0.006$). The mean CXCL8 mRNA expression, relative to the medium only group, in ovine and bovine turbinate cells for the live *C. abortus* group was significantly higher compared with the UV-inactivated *C. abortus* group at 72 h post infection ($P_f < 0.001$) (Fig. 3A). The CXCL8 protein production mirrored the CXCL8 mRNA profiles: the interaction effect of treatment group and time was statistically significant ($P < 0.001$). The live *C. abortus* treatment group produced significantly higher mean CXCL8 protein compared with the UV-inactivated *C. abortus* treatment group at 72 h post infection in both ovine ($P_f < 0.001$) and bovine ($P_f = 0.028$) turbinate cells (Fig. 3B). The live *C. abortus* group also showed higher mean CXCL8 protein production compared with the medium only group at 72 h post infection in both ovine ($P_f < 0.001$) and bovine ($P_f = 0.022$) turbinate cells.

CXCL8 is a pro-inflammatory chemokine which functions as a chemotactic factor driving the recruitment of immune cells including neutrophils. Neutrophils have been identified as potential carriers of *C. pneumoniae*, which may perpetuate the survival and dissemination of the bacteria (Rupp et al., 2009; Zandbergen et al., 2004). Therefore, the production of CXCL8 by ruminant turbinate

cells may aid in the dissemination of *C. abortus* from the site of infection. The increased CXCL8 production, however, occurred only at 72 h, which is late in the chlamydial developmental cycle (Longbottom and Coulter, 2003). Earlier studies identified that the late production of CXCL8 is a feature of *C. trachomatis* infection of epithelial cells (Buchholz and Stephens, 2006; Rasmussen et al., 1997). Potential explanations on CXCL8 production at a later stage include: inhibition of production by *C. abortus*, the pathogen associated molecular pattern (PAMP) threshold is not reached until the bacteria has multiplied or cell lysis is necessary for its production (Buchholz and Stephens, 2008; O'Connell et al., 2006).

We did not find any statistical evidence of interaction effects between species and treatment group, and between species and time for the expression of mRNA ($P > 0.12$) and protein ($P > 0.77$). These observations suggest that the production of CXCL8 from bovine and ovine turbinate cells on average had similar trends across treatment groups and time points. We also did not observe any evidence ($P_f > 0.46$) that the mean CXCL8 protein production differed between the medium only group and UV-inactivated *C. abortus* challenged group at any time point in either species (Fig. 3). This implies that intracellular detection of the active infection is necessary for the stimulation of CXCL8 mRNA expression. A previous study has also shown that CXCL8 production in ovine trophoblasts depends on challenge with live *C. abortus* compared to UV-inactivated *C. abortus* (Wheelhouse et al., 2009). Generally, pathogens are recognized by a range of PRRs which initiate a signal transduction cascade inducing activation of NF- κ B and MAPK kinases and production of pro-inflammatory chemokines and cytokines, including CXCL8 (Arthur and Ley, 2013). Previous studies have identified that the MAPK and NF- κ B signalling pathways regulated the induction of CXCL8 production following chlamydial infection (Buchholz and Stephens, 2006; Buchholz and Stephens, 2008; Rasmussen et al., 1997; Welter-Stahl et al., 2006). TLR2, which co-localises intracellularly with the inclusion, activates CXCL8 production from epithelial cells challenged with *C. trachomatis* (O'Connell et al., 2006). Intracellular NOD 1 mediates production of CXCL8 in an epithelial cell line challenged with *C. trachomatis* (Buchholz and Stephens, 2008). Our results showed that ruminant turbinate cells express TLR2, TLR4 and NOD1 (Fig. 2), which indicates that *C. abortus* may activate similar signalling pathways for the production of CXCL8 within turbinate cells.

Understanding how the early innate immune response activates a protective adaptive immune response is critical for understanding disease pathogenesis and for the delivery of new vaccines. In this study, we observed that *C. abortus* is capable of infecting oro-nasal turbinate cells from cattle and sheep. Both ovine and bovine cells responded similarly to *C. abortus* infection by production of CXCL8 late in the chlamydial developmental cycle with undetectable levels of IL-1 β and TNF- α . This suggests that innate cytokine and chemokine production by turbinate cells is not a discriminating factor for disease pathogenesis between cattle and sheep.

Conflicts of interest

BioRad AbD Serotec distributes and markets ruminant immunological reagents produced by The Moredun Research Institute and by The Roslin Institute at the University of Edinburgh. Both organizations receive royalties from Bio-Rad AbD Serotec from the sale of these ruminant immunological reagents.

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