Investigations into Resistance and Susceptibility to Johne’s Disease in Red Deer

By Blake Gibson

Thesis component of MSc

28/2/2012
Abstract

Johne’s disease (JD), caused by the pathogen *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic inflammatory bowel disease of ruminants that is characterised in its clinical stage by progressive weight loss/wasting and profuse diarrhea. Immune responses to MAP infection are initially characterised by a Th1 T cell response that subsequently decreases over disease progression. Detection of MAP occurs via pathogen recognition receptors (PRR) including NOD2 and the toll like receptors (TLR). Mutations in NOD2 have been identified as a susceptibility factor to JD. To cause infection MAP has a documented role in manipulating its environment including gene expression profiles. The aims of this project was to map the NOD2 gene for mutations associated with resistance or susceptibility to JD in red deer (*Cervus elaphus*), and to investigate the gene expression profiles of deer of demonstrated resistance and susceptibility for informative factors in disease progression.

Foundational work, carried out at AgResearch Invermay investigating the heritability of resistance/susceptibility to JD in red deer, included artificially infecting 18 4 month old fawns with MAP. These deer then had mesenteric lymph node biopsies at weeks 4, 12, and 49 of the infection trial. Messenger RNA (mRNA) was extracted from the biopsied tissue from 6 animals chosen to represent the polarised extremes of resistance and susceptibility, purified, and reverse transcribed to produce cDNA that was used for qPCR of immune related genes. Blood collected via vascular venepuncture at the week 49 time point was used for mRNA extraction for reverse transcribing to investigate the CARD15 sequence.

The primary finding from the CARD15 sequence evaluation identified one non-synonymous mutation named 1816 and five synonymous mutations. Using histopathology data from the 14 deer (the CARD15 sequence was obtained from) as a measure of disease severity indicated that this mutation does not affect resistance or susceptibility. Profiling of multiple immune related genes indicated likely disease severity phenotypes, including heightened IFNβ related genes at week 4 being associated with resistance as well as heightened NLRP3 expression. Expression of immune genes throughout this infection trial has led to some insightful factors, including IFNs and NLRP3, influencing susceptibility and resistance to JD in red deer.
Acknowledgements

This MSc has been a huge learning curve for me personally and I would like to thank those who have helped me along the way. Firstly I have to thank the DRL, especially my supervisors Rory O’Brien and Frank Griffin for their continued patience with my stupid questions and never ending source of revisions from material I burdened them with. Secondly I would like to thank Collin Mackintosh for the blood samples and tissue biopsies from his cervine infection experiment, without which there would be no experimental ground for my project. Thirdly I thank all of my class mates and lecturers for helping me out over the course of this MSc, with a specific thanks to Ros for her help and encouragement. Also a big thanks must be said to Simon Liggett who helped me with the automation of my experimental procedures.

My Family has always been a great source of advice and encouragement, even if at the time I didn’t want to hear it (just joking Dad!!!)! Mum and Dad, you guys are great and have helped me become who I am today! Thank you for your love and support as well as your patience.

Tabi you have been fantastic since I met you! Thank you for helping me keep my sanity and for putting up with me in those odd occasions when I lost it!

Finally thanks to the University of Otago!
# Table of Contents

Investigations into Resistance and Susceptibility to Johne’s Disease in Red Deer....... i

Abstract ........................................................................................................................................ ii

Acknowledgements .................................................................................................................... iii

Abbreviations ................................................................................................................................ 8

Chapter 1: Introduction.................................................................................................................. 9

1.1 Purpose of Research .............................................................................................................. 9

1.2 Johne’s Disease ................................................................................................................... 9

1.3 MAP Host Range, Models, and Detection of Disease ....................................................... 10

1.3.1 Host range, models and resistance/susceptibility to disease ...................................... 10

1.3.2 Detection of JD .................................................................................................................. 11

1.4 *Mycobacterium avium* subspecies *paratuberculosis* ..................................................... 11

1.4.1 Mycobacterial Pathogens ............................................................................................ 11

1.4.2 MAP Infection and Disease Progression ..................................................................... 12

1.4.3 MAP in Crohn’s Disease .............................................................................................. 14

1.5 Innate Immunity .................................................................................................................. 14

1.5.1 Toll like receptors ............................................................................................................. 17

1.5.1.1 Cellular Membrane TLRs ..................................................................................... 18

1.5.1.2 Intracellular TLRs ............................................................................................... 20

1.5.2 NOD like receptors ......................................................................................................... 21

1.5.2.1 NOD Like Receptor Pyrin 3 (NLRP3) .................................................................. 21

1.6 CARD15/NOD2 Genetic Polymorphisms .......................................................................... 22

1.7 Nuclear factor-κB (NF-κB) ............................................................................................... 23

1.8 Cytokines, Chemokines and Interferon ............................................................................. 26
1.8.1 Interleukin-1β and Interleukin-18 .......................................................... 26
1.8.2 Interleukin-6 and Interleukin-2 ............................................................... 27
1.8.3 Type one Interferon .................................................................................... 27
1.8.4 Type two Interferon and RANTES ........................................................... 27
1.8.5 Interleukin-4 ............................................................................................... 28
1.8.6 GM-CSF ................................................................................................. 29
1.8.7 RANKL ..................................................................................................... 29
1.8.8 Interleukin-10 .......................................................................................... 30
1.9 Signal transducer and activator of transcription ........................................ 30
1.10 Mitogen activated Protein Kinases (MAPK) ............................................... 36
1.11 Adaptive Immunity ..................................................................................... 37
1.12 Host Immune Response against MAP ....................................................... 39
  1.12.1 MAP Manipulation of the Host Immune Response ................................. 39
  1.12.2 MAP Manipulation of TLR2 ................................................................. 40
  1.12.3 Macrophage Phagosomal Killing Inhibition ........................................... 40
  1.12.4 Inhibition of Macrophage Apoptosis ..................................................... 41
  1.12.5 Global Manipulation of the Host Immune Response ............................. 42
1.13 Quantitative Polymerase Chain Reaction (qPCR) ........................................ 42
1.14 Automation of Laboratory techniques ....................................................... 45
1.15 Significance of research ............................................................................. 46
1.16 Hypothesis and Aims ................................................................................ 47

Chapter 2: Methods ......................................................................................... 48
  2.1 Experimental infection model (Previous Work) ........................................... 48
  2.2 TRizol RNA extraction .............................................................................. 48
  2.3 Qiagen RNeasy RNA cleanup .................................................................... 49
  2.4 Bioanalyser ............................................................................................... 50
2.5 RNA reverse transcription ................................................................. 50
2.6 CARD15 genotyping – Primers and PCR protocol .............................. 50
2.7 Gel purification and Sequence interpretation ...................................... 51
2.8 qPCR Primers, Protocol and data interpretation ................................. 52
2.9 384 well qPCR ................................................................................. 53
2.10 qPCR Automated plate loading trials ................................................. 53
2.11 qPCR Automated 96 well to 384 well Automated blotting ................. 54
2.12 Data analysis .................................................................................. 56

Chapter 3: Results .................................................................................. 57
3.1 Previous Results ................................................................................ 57
3.1.1 JD Infection States ......................................................................... 57
3.2 RNA Integrity Verification ................................................................. 60
3.3 Aim 1: CARD15 Polymorphisms ...................................................... 60
3.4 Aim 2: production of a working model ............................................ 65
3.4.1 Normalising gene selection ............................................................... 65
3.4.2 qPCR Primer Specificity ................................................................. 65
3.4.3 qPCR gene expression ratios .......................................................... 67
3.4.4 Interpretation of Results ................................................................. 68
3.5 Aim 3: qPCR analysis of immune genes ............................................ 70
3.5.1 PRR pathway ................................................................................ 70
3.5.2 Type one interferon ....................................................................... 73
3.5.3 Type two interferon ....................................................................... 76
3.5.4 IL-6 signalling .............................................................................. 79
3.5.5 IL-12 signalling pathway ............................................................... 82
3.5.6 GM-CSF ....................................................................................... 85
3.5.7 IL-4 signalling pathway ............................................................... 88
3.5.8 NLR pathways ................................................................. 91
3.5.9 NF-κB pathway ............................................................ 94
3.5.10 MAPK pathway ............................................................ 97
3.6 Automation of qPCR ........................................................ 100
Chapter 4: Discussion .......................................................... 101
  4.1 CARD15 polymorphisms ............................................... 101
  4.2 Establishment of the working model .................................... 102
    4.2.1 B2M normalising gene ............................................... 103
    4.2.2 Automation of Laboratory techniques ............................... 103
    4.2.3 JD infection states ...................................................... 104
  4.3 Expression Levels of Candidate Immunological Factors .......... 104
    4.3.1 Innate Immunity ......................................................... 104
    4.3.2 Acquired Immunity ..................................................... 110
Chapter 5: Conclusion .......................................................... 114
  5.1 CARD15 polymorphisms and genetic profiles ......................... 114
  5.2 Future experiments ........................................................ 115
  5.3 Final model of Resistance and Susceptibility to JD ......... 116
Appendix ........................................................................ 117
References ........................................................................ 126
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johne’s Disease</td>
<td><em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em></td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td></td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay</td>
<td></td>
</tr>
<tr>
<td>T helper 1</td>
<td></td>
</tr>
<tr>
<td>T helper 2</td>
<td></td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>Major histocompatibility complex 2</td>
<td></td>
</tr>
<tr>
<td>Granulocyte macrophage colony stimulating factor</td>
<td></td>
</tr>
<tr>
<td>Regulated upon activation, normal T-cell expressed and secreted</td>
<td></td>
</tr>
<tr>
<td>Toll like receptor</td>
<td></td>
</tr>
<tr>
<td>Interferon gamma</td>
<td></td>
</tr>
<tr>
<td>Interferon beta</td>
<td></td>
</tr>
<tr>
<td>Mitogen activated protein kinase</td>
<td></td>
</tr>
<tr>
<td>Antigen presenting cell</td>
<td></td>
</tr>
<tr>
<td>Mannose-capped lipoarabinomannan</td>
<td></td>
</tr>
<tr>
<td>Early endosomal antigen 1</td>
<td></td>
</tr>
<tr>
<td>Protein kinase G</td>
<td></td>
</tr>
<tr>
<td>Phenolic glycolipid</td>
<td></td>
</tr>
<tr>
<td>Pathogen associated molecular patterns</td>
<td></td>
</tr>
<tr>
<td>Pattern recognition receptors</td>
<td></td>
</tr>
<tr>
<td>NOD like receptors</td>
<td></td>
</tr>
<tr>
<td>Damage associated molecular patterns</td>
<td></td>
</tr>
<tr>
<td>Lipopolysacharide</td>
<td></td>
</tr>
<tr>
<td>Myeloid differentiation primary response gene 88</td>
<td></td>
</tr>
<tr>
<td>Nuclear factor κ B</td>
<td></td>
</tr>
<tr>
<td>Inhibitor of NF-κB</td>
<td></td>
</tr>
<tr>
<td>Interferon regulatory factor 3</td>
<td></td>
</tr>
<tr>
<td>NOD like receptor pyrin 3</td>
<td></td>
</tr>
<tr>
<td>g-D-glutamyl-meso-diaminopimelic acid</td>
<td></td>
</tr>
<tr>
<td>Muramyl dipeptide</td>
<td></td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Purpose of Research

Foundational work for this thesis, carried out at AgResearch Invermay under Dr. Colin Mackintosh, explored the heritability of resistance and susceptibility to Johne’s disease (JD) in Red Deer. An artificial infection model was utilised whereby the deer were infected with MAP and subsequently investigated at 4, 12, and 49 weeks after infection using sequential intestinal lymph node biopsies. From this experiment the ability to investigate the topics covered in this thesis was obtained; firstly the pathogen recognition receptor NOD2 was investigated in deer of demonstrated phenotype and secondly genetic profiling of candidate immune genes of known function were investigated. The value of gaining a greater understanding of these two points includes not only the ability to select deer biased upon their ability to produce healthy offspring, but also to gain a greater level of insight into the immune activities of red deer, within the mesenteric lymph nodes, during JD progression.

1.2 Johne’s Disease

JD, caused by the bacterial pathogen Mycobacterium avium subspecies paratuberculosis (MAP), also known as paratuberculosis, is a chronic inflammatory bowel disease of ruminants that is characterized in its clinical stage by progressive weight loss/wasting, profuse diarrhea, granulomatous enteritis and typically enlarged mesenteric lymph nodes (Mackintosh et al., 2007). First described in 1895 as an intestinal disease affecting cattle, JD is an economically significant disease that has been estimated to cost the United States agricultural industry up to $1.5 billion a year (Eda S., 2005; Whittington et al., 2011). Within New Zealand this disease has significantly negatively impacted farmed deer; one conservative report published in 1998 estimated that JD costs this industry $205,000 to $341,000 per year (Brett E., 1998). The New Zealand dairy industry may suffer estimated losses of $3.8 to $18.9 million a year (Brett E., 1998). Recent estimates suggest that between 10-68% of cattle herds in developed countries are affected by JD (Eda S., 2005; Whittington et al., 2011). Within New Zealand bovine JD has an estimated dairy herd prevalence of 60 % (Eda S., 2005; Pant et al., 2007). Disease prevalence in young (8-15 month old) red deer (Cervus elaphus) can account for losses of up to 15 % (Griffin et al., 2005). Spread of JD within a deer herd can occur horizontally as infected individuals spread disease amongst their herd mates and vertically during pregnancy (Mackintosh et al., 2005). Infection may be exacerbated by risk factors including the age of the animal, size of infectious dose,
organism/strain involved, genetic susceptibility/resistance of the animal and lastly environmental factors and other stressors (Chacon et al., 2004; Mackintosh et al., 2007).

1.3 MAP Host Range, Models, and Detection of Disease

1.3.1 Host range, models and resistance/susceptibility to disease

A recent survey undertaken in the South Island of New Zealand found MAP has not only a broad ruminant host range it also has the ability to survive within the gastrointestinal tract of a variety of animals and birds as diverse as possums, hares, hedgehogs, cats, rabbits and the paradise shelducks (Nugent et al., 2011). Within these species MAP can actively infect possums, cats, ferrets, and hares (Nugent et al., 2011). In a similar study in Scotland the range of animals that can be infected by MAP also included foxes, stoats, weasels, rats, mice and crows (Beard et al., 2001). In some of these species this infection results in pathologically detectable disease and bacterial shedding, whereby these hosts act as a reservoir for MAP providing the potential for infection in previously uninfected livestock as these infected animals shed bacteria contaminating livestock feed or water (Beard et al., 2001). Chronic wasting and profuse diarrhoea associated with disease are not usually observed until 3 to 10 years after infection in cattle and 3 to 4 years in sheep (Beard et al., 2001; Mackintosh et al., 2007; Secott et al., 2004; Whittington et al., 2011). The delay in disease manifestation makes most ruminant species unsuitable as experimental infection models, however a cervine infection model has recently been developed which allows for the development of clinical JD within one year (Mackintosh et al., 2007). While mouse models for disease are available that can take as little as 8 months to observe severe gastrointestinal pathology (Hamilton et al., 1991); the current study was undertaken in a natural host and the animal of interest the red deer, the most commonly farmed deer worldwide, as this disease represents a clear challenge to farmers productivity.

Innate resistance or susceptibility to JD in red deer is influenced by the genetic makeup (genotype) of the animal; parental genotypes may have a significant impact on the phenotype of their progeny (Mackintosh et al., 2011). Heritable resistance to JD has been demonstrated in cattle, sheep, goats, and more recently in red deer (Gonda et al., 2006; Korou et al., 2010; Mackintosh et al., 2011; Reddacliff et al., 2005). Furthermore, heritability of resistance has been previously reported for bovine tuberculosis infection in red deer; offspring of resistant sires exhibited similar responses to pathogenic challenge giving an estimated heritability of disease resistance of 0.48 (± 0.096 P<0.01) (Mackintosh et al., 2000).
1.3.2 Detection of JD

Early detection of infected individuals is paramount to farmers’ profit as infected animals not only rapidly lose condition and therefore value due to decreased carcase weight, but also these infected animals can go on to infect other members of the herd. In severe cases of JD in cattle infected adults can lose over 100 kg (Hamilton et al., 1991). The “gold standard” for the detection of mycobacterial infection remains the bacteriologic culturing method whereby samples are taken from an animal either from the faeces or from tissue samples collected usually after necropsy and cultured for mycobacterial growth (Bhide et al., 2006). A major disadvantage to this method is that it may take 5-16 weeks for detectable growth to become evident (Eda et al., 2005). Indirect methods for the detection of MAP infection in animals include immunodiagnostic testing for circulating antibodies against MAP in peripheral blood using an enzyme-linked immunosorbent assay (ELISA). The adaptive immune system is characterised by two branches of immune function and will be discussed in greater detail in the adaptive immunity section (Section 1.11). It includes the cell mediated immune response (Th1) and the antibody immune response (Th2). As MAP is an intracellular pathogen the Th1 immune response is considered to be of great importance for the clearance of this pathogen. Serum antibody levels are inversely related with cellular effector mediated immunity and are found later in disease progression; high antibody levels are suggestive of the failure of the Th1 immune response to clear the pathogen (Burrells et al., 1991). Along with the ELISA and culturing method for MAP detection there is also a polymerase chain reaction (PCR) based technique usually conducted using faecal or tissue samples that provides a fast and highly specific detection method using either the IS900 MAP specific insertion sequence or other species specific genes (Bhide et al., 2006).

1.4 Mycobacterium avium subspecies paratuberculosis

1.4.1 Mycobacterial Pathogens

The mycobacterial genus includes several major species of pathogens including M. avium, MAP, M. leprae, M. bovis and M. tuberculosis all of which are obligate intracellular pathogens (Chacon et al., 2004). While these diverse pathogenic members of the mycobacterium genus affect different hosts and have a predilection for different tissues they share patterns of disease as such each mycobacterial pathogen produces pathology and patterns of pathology that is generic for other mycobacterial species (Whittington et al., 2011). Within the mycobacterial pathologies one common hallmark includes the formation of
granulomas defined as “a compact (organized) collection of mature mononuclear phagocytes which may or may not be accompanied by accessory features such as necrosis” (Adams, 1976).

*Mycobacterium avium subspecies paratuberculosis* (MAP), the causative agent of JD, is itself a slow growing obligate intracellular parasite with an acid fast cell wall and a bacilli (rod shaped) morphology that causes infection via the faecal oral route (Chacon et al., 2004; Mackintosh et al., 2007; Secott et al., 2004). Two different strains of MAP have been identified which exhibit genotypic differences, these being the ‘bovine’ strain and the ‘ovine’ strain named as they were first identified in and predominantly infect cattle and sheep respectively (Begg and Whittington, 2008; Collins et al., 2002). Both of these strains of MAP may cause infection in other ruminant species such as deer and goats with varying levels of infectivity and severity (Collins et al., 2002). While the ‘ovine’ MAP strain may establish infection and disease in deer this species is typically more susceptible to the ‘bovine’ strain of MAP (Mackintosh et al., 2011).

### 1.4.2 MAP Infection and Disease Progression

Disease progression, as outlined in Table 1, begins with MAP ingestion after which the bacteria are taken up by inducing M cells found upon Peyer’s patches (intestinal lymph nodes) in the intestine, mediated by a fibronectin attachment protein homologue (Secott et al., 2004). Following M cell infiltration MAP bacilli are phagocytosed by macrophages where they reside within the phagosome, actively inhibiting phago-lysosomal fusion as well as suppressing immune activation of the infected cell, metabolizing and dividing (Chacon et al., 2004). Post ingestion MAP survives the acidity of the stomach and then the low-oxygen, hyperosmotic conditions found in the small intestine and subsequently the cationic antimicrobial environment found within macrophages due to the complex nature of its cell wall and cellular envelope (Alonso-Hearn et al., 2010). Responding to these changes in environmental conditions MAP utilises quorum sensing molecules (a system of communication, based around production/detection of small proteins, utilised by bacteria affecting gene expression altering this expression to suit different environmental factors) specifically a LuxR homologue detected through a LuxI quorum sensing system. These quorum sensing mechanisms are upregulated during an infection affecting the gene expression of the mycobacterium cell wall and envelope composition as well as affecting other metabolic pathways that facilitate pathogen survival (Alonso-Hearn et al., 2010).
Following infection with MAP the infected animal goes through a subclinical phase during which there are few or no detectable consequences of the infection although the animal may be actively excreting MAP organisms capable of infecting other susceptible animals (Begg et al., 2010). Shedding of MAP may continue during the subclinical phase of disease, typically increasing in severity, throughout disease progression to the death of the host (Whittington et al., 2004). MAP can persist in the environment from weeks up to over one year before the cell loses integrity (becoming nonviable) (Whittington et al., 2004). The shedding of MAP from the intestinal lumen occurs when environmental conditions within the infected macrophage become unsuitable for continued mycobacterial replication triggering the mycobacteria to cause the host cell to undergo a necrotic cell lysis to aid in the further dissemination of the pathogen (Whittington et al., 2011).

**Table 1**

<table>
<thead>
<tr>
<th>Stages of disease progression</th>
<th>Time after infection</th>
<th>Pathological phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP is ingested and attaches to M cells overlying Peyer’s patches</td>
<td>Hours</td>
<td>Silent infection</td>
</tr>
<tr>
<td>Phagocytosed by macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Replication within macrophages and subsequent dissemination to other macrophages within the Peyer’s patches, intestinal lamina propria and draining lymph nodes</td>
<td>Days</td>
<td>Disease regression or progression</td>
</tr>
<tr>
<td>Macrophages and lymphocytes recruited and development of early granulomas</td>
<td>Weeks</td>
<td></td>
</tr>
<tr>
<td>MAP survives the acquired immune response and is actively shed</td>
<td>Months</td>
<td>Subclinical, heavy shedding</td>
</tr>
<tr>
<td>Th1 response progresses to become a non-protective Th2 response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected animal develops clinical disease, losing body condition and developing extensive gastric lesions.</td>
<td>Years</td>
<td>Clinical disease</td>
</tr>
</tbody>
</table>

*Table 1. Disease Progression/Regression Post Infection with MAP*

Table 1 has been adapted from Whittington et al. 2011. The infection cycle of MAP, shown above, in red deer JD can result in clinical disease in susceptible animals in less than one year.
1.4.3 MAP in Crohn’s Disease

Currently MAP is considered a potential zoonosis and has been suggested to have an etiological role in Crohn’s disease (CD) due to similarities observed between JD and CD pathology (Beard et al., 2001; Collins et al., 2002; Pinedo et al., 2009). An association between CD and JD is seen by the recovery of MAP from some CD patients (Hamilton et al., 1991). In addition to the common pathology seen between CD and JD, MAP has been observed in CD tissues and a pathology called creeping fat (unique to CD in humans) seen in CD is identical to lymphatic engorgement observed in JD (Pierce, 2009). Evidence of an association of MAP and CD can be deduced from a recent American Academy of Microbiology colloquium in which it was concluded that people with CD had a seven fold greater risk of being infected with MAP than healthy individuals (Whittington et al., 2011). While the role of MAP in CD is unclear, it could act as either a causal or exacerbating pathogenic agent (Whittington et al., 2011). While CD is not the focus of this thesis and will not be explored in detail, the pathogenesis of CD does resemble JD and has led to some authors using CD associated susceptibility genes to look into JD. Association between MAP and CDs importance is further emphasised as there is great potential damage to export markets and food safety considerations to account for if MAP was found to be a contributing factor.

1.5 Innate Immunity

The vertebrate immune system is comprised of multiple cell types sharing a common origin that differentiate from hematopoietic stem cells, providing two broad branches of immune protection; the innate and the adaptive immune system (Geissmann et al., 2010; Williams et al., 2010). The innate arm of the immune system represents the first response cells of the immune system and is not specific to individual pathogens while the adaptive arm provides a very specific targeted response (Williams et al., 2010). Innate immunity is an evolutionarily conserved system of protection mediated by phagocytes such as macrophages and neutrophils (Kawia and Akira, 2009). While the innate arm of the immune system is controlled by phagocytes the adaptive immune system relies on antigen specific T and B lymphocytes (Kawia and Akira, 2009). Pathogenic organisms have been shown to exhibit conserved antigens termed pathogen associated molecular patterns (PAMP), that can be sensed by germline encoded pattern recognition receptors (PRR) in innate immune cells (Williams et al., 2010). One key mechanism by which PRR function involves a Leucine rich repeat (LRR) region that is found in some PRRs consisting of the conserved ‘xLxxLxLxx’
amino acid motif, where by ‘x’ represents a non-conserved amino acid and ‘L’ is Leucine, responsible for recognition of specific cognate ligands (Akira, 2003; Kawia and Akira, 2009). There are multiple classes of PRR including Toll Like Receptors (TLR) and NOD like receptors (NLR) that collectively recognise a variety of targets (PAMPs) as well as others such as RIG-I like receptors that are responsible for the detection of viral RNA (Williams et al., 2010). Cells expressing these PRR can differentiate self from non-self and therefore cause immune activation in response to conserved PAMP antigens specifically, however they can also in some instances detect self-antigens in specific contexts (Kawia and Akira, 2009). Detection of self-antigens by PRR is tightly linked to autoimmune and inflammatory diseases although in some instances this activation is appropriate; for example TLR and NLR can detect necrotic cellular debris including damage associated molecular patterns (DAMP) (Kawia and Akira, 2009). An overview of the innate immune system involved in JD is represented diagrammatically below in Figure 1.
Figure 1

Figure 1 represents a simplified overview of the innate immune response against PAMP and DAMP molecules are shown. This cartoon has been simplified to represent specific pathways aspects of the innate immune response investigated in this thesis.
1.5.1 Toll like receptors

Members of the TLR family are an example of PRRs involved in activating the innate immune system by sensing conserved microbial PAMPs (Barton and Medzhitov, 2002; Bhide et al., 2009). TLRs consist of a family of transmembrane receptors that have an intracellular Toll/interleukin-1 domain and an extracellular-vesicle LRR region responsible for pathogen recognition in either the extracellular environment or within the lumen of intracellular vesicles (Akira, 2003; Bell et al., 2003; Gay and Gangloff, 2007; Kawia and Akira, 2009; Medzhitov, 2001; Xu et al., 2000). Currently over 12 members of the TLR family have been identified in mammals (11 of which are active in humans), illustrated in Figure 2, that can confer protection from a wide range of PAMP including lipids, lipoproteins, proteins, glycans and nucleic acids (Kawia and Akira, 2009). Due to localisation differences TLRs can be divided into two subpopulations based on their cellular location. The first TLR subpopulation consists of those localised on the cell surface which include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 and are responsible for the recognition of pathogenic microbial membrane components (Kawia and Akira, 2009). While the first subpopulation is found on the cellular surface the second population are found in intracellular vesicles such as endosomes, lysosomes or the endoplasmic reticulum and includes TLR3, TLR7, TLR8 and TLR9 (Kawia and Akira, 2009). This second population of TLRs is predominantly responsible for sensing microbial/viral nucleic acid in the host cell (Kawia and Akira, 2009). In order to activate the appropriate response to the antigenic challenge TLRs have specific adaptor proteins associated with their cytosolic region (Kawia and Akira, 2009). MyD88, which is utilized by all of the TLRs, with the exception of TLR3, drives the activation of NF-κB and the MAPK pathways (Kawia and Akira, 2009).

Aberrant activation of TLRs has been associated with various diseases including inflammatory diseases, autoimmune diseases and tumour progression (Kawia and Akira, 2009). While aberrant activation of TLRs is associated with inflammatory disease, mutations in these pathogen recognition and binding mechanisms have been associated with increases in susceptibility to diseases such as tuberculosis and leprosy as well as being associated with disease conditions such as acute rheumatic fever and CD (Bhide et al., 2006; Franchimont et al., 2004 A; Henckaerts et al., 2007; Xu et al., 2000). Mutations in TLR1, TLR2, TLR4 genes along with CARD15 have been identified as factors increasing susceptibility to MAP infections in sheep and cattle (Minozzi et al., 2010).
Figure 2

Figure 2. Human TLRs

A simplified representation of the 11 TLRs found in humans with their corresponding ligands and the subsequent activation of inflammatory mediators following activation of the TLRs is shown in Figure 2. Depicted above these TLRs are spanning the cytoplasmic membrane, however these TLRs are not all found on this membrane (refer to section 1.5.1). This is a modified figure from Medzhitov (Nature Reviews Immunology 2001).

1.5.1.1 Cellular Membrane TLRs

TLR4 is responsible for detecting lipopolysacharide (LPS), one of the major components of the gram negative bacterial cell wall and functions by forming a multimer with the non-TLR molecules CD14 and MD-2 (Akira, 2003; Kawia and Akira, 2009). Conversely TLR2 is responsible for the recognition of a range of PAMPs derived from various pathogens ranging from bacteria, fungi, parasites and viruses, sensing in particular triacyl and diacyl lipopeptides, and lipoteichoic acid (Kawia and Akira, 2009). In relation to MAP TLR2 can sense not only lipoteichoic acid but also peptidoglycan and glycolipid mannose-capped
lipoarabinomannan (LAM) (Kawia and Akira, 2009). Functionally TLR2 operates in association with TLR1 or TLR6 forming heterodimers or alternatively like TLR4 may form a multimer with non-TLR molecules such as CD14, CD36 and or dectin-1 allowing TLR2 to discriminate and respond to a broad range of PAMPs (Bhide et al., 2009; Kawia and Akira, 2009; Taylor et al., 2008). In the instance of TLR2 forming a heterodimer with TLR6, with the help of CD36, the resulting PRR complex can recognise mycobacterial diacylated lipopeptide, while TLR2/TLR1 heterodimers recognise bacterial triacylated lipopeptides (Kawia and Akira, 2009). When TLR2 associates with the CD14 protein the resulting multimer is responsible for the detection of LAM (Kawia and Akira, 2009). Once activated TLR2 can cause the production of proinflammatory or anti-inflammatory cytokines while TLR4 activation is associated with proinflammatory cytokine production (Kawia and Akira, 2009; Taylor et al., 2008). Some PRR can detect endogenous molecules, these include TLR2 and TLR4 that can sense heat shock proteins along with other markers of inflammation related tissue damage and necrotic cells (Kawia and Akira, 2009). Other TLRs such as TLR5 and TLR11 recognise flagellin and uropathogenic bacteria respectively and, like TLR4 and TLR2 (along with TLR2 associated TLR1 and TLR6), are also found on the surface of the cell (Kawia and Akira, 2009).

TLR2 and TLR4 have both been implicated in MAP infection (Ferwerda et al., 2007). Pathogen detection properties for TLR2 is achieved by the TLR2 molecule forming heterodimers with either TLR1, TLR6, or non-TLR proteins. The TLR2 – TLR6 heterodimer is the main complex responsible for recognition for mycobacterial antigens (Whittington et al., 2011). Activation of TLR2 and TLR4 involves the adapter protein TIRAP that binds myeloid differentiation primary response gene 88 (MyD88), which signals through the TRAF6 protein intermediary to activate the MAPK pathways and NF-κB (Ferwerda et al., 2007; Kawia and Akira, 2009). TLR4 also has the adapter protein TRAM that links TLR4 to TRIF that is responsible for the activation of interferon regulatory factor 3 (IRF3), which post activation forms a homo dimer and translocates to the nucleus to initiate a type one IFN and inflammatory cytokine response following challenge (Kawia and Akira, 2009). Upon antigenic challenge of TLR4 by lipopolysaccharide or the self-antigen heat shock proteins activation results in activating the two signalling pathways: the MyD88 and TRIF pathways (Ferwerda et al., 2007; Kawia and Akira, 2009). Activation of IRF3 occurs after the initial activation of TLR4, and post the MyD88 pathway, as first TLR4 and its corresponding
A natural infection surveillance study in outbred sheep, carried out by Taylor et al. in 2007, demonstrated that both TLR2 and TLR4 are upregulated in the intestine and mesenteric lymph nodes of animals infected with MAP (Taylor et al., 2008). TLR2 and its heterodimeric partner proteins TLR1 or TLR6 have recently also been shown to be unregulated in ovine MAP infection (Plain et al., 2010). Excess of TLR2 activation causes expression of conventional type 2 immune cytokines as well as IL-10 and as such if TLR2 is blocked this has been demonstrated to enhance bovine macrophage clearance of MAP (Taylor et al., 2008). While excess TLR2 activation is involved in production of anti-inflammatory cytokines TLR4 produces Th1 promoting IFN as well as the proinflammatory cytokines TNF-α and IL-12 as well as activating costimulatory molecules such as CD80/B7-1 and CD86/B7-2 via activation of NF-κB (Dabbagh et al., 2002).

1.5.1.2 Intracellular TLRs

Intracellular TLRs are not involved directly in the detection of MAP during an infection and are discussed only briefly here. The detection of PAMP by TLR3, TLR7, TLR8 and TLR9 occurs within intracellular compartments primarily on the endoplasmic reticulum and act as sensors of foreign nucleic acids; once activated these PRRs are involved in triggering antiviral innate immune responses by producing type one interferon and other inflammatory cytokines (Kawia and Akira, 2009). As with TLR4, TLR3 is linked to the adapter protein TRIF, leading to the activation of IRF3 and subsequently type one interferon production (Kawia and Akira, 2009). TLR3 is responsible for the detection of double stranded RNA, a virus specific motif, along with small interfering RNA and is highly expressed in dendritic cells (Kawia and Akira, 2009). To carry out its sensing role TLR7 recognises guanosine rich, uridine rich RNA along with some small interfering RNA and once activated TLR7 causes production of type one interferons (Kawia and Akira, 2009). TLR8 is physiologically similar to TRL7. Pathogenic bacterial and viral DNA is detected by TLR9 distinguishing self and non self DNA via recognition of unmethylated ‘CpG motifs’ found in bacteria/viruses, and as the other intracellular TLRs upon activation causes the production of type one interferon (Kawia and Akira, 2009).
1.5.2 NOD like receptors

NOD like receptor proteins (NLRs) also known as Nucleotide-binding domain Leucine-rich repeat proteins, are a class of pattern recognition molecules that specifically interact with soluble intracellular PAMPs or DAMPs within the cytoplasm of a cell (Chaplin et al., 2010; Turvey and Broide, 2010). Presently there are over 20 NLR proteins identified in the human genome with conserved C-terminal associated Leucine rich repeats (Chaplin et al., 2010). NLRs consist of three domains including a protein binding N-terminal domain containing CARD motifs which post activation lead to trans homophilic CARD-CARD protein bonds, a central nucleotide binding domain that has potential ATPase activity and is responsible for self oligomerisation, and a LRR region responsible for either PAMP or DAMP sensing either directly or indirectly (Inohara et al., 2003; Kawia and Akira, 2009). Post activation the CARD domains trans homophilic interaction with other CARD containing proteins, RICK and CARD9 in the case of NOD1 and NOD2, results in the activation of NF-κB and subsequently an inflammatory immune response (Inohara et al., 2003; Kawia and Akira, 2009; Pant et al., 2007). NOD1, NOD2, and NOD like receptor pyrin 3 (NLRP3) are the three NLR discussed in this thesis due to their role in shaping the immune response to MAP infection.

NOD1 and NOD2 are well studied members of the NLR family and are responsible for the detection of bacterial products, in the cytoplasm, such as g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) respectively (Kawia and Akira, 2009). While the NOD1 ligand is mainly associated with gram negative bacterial cell walls some gram positive bacteria have been reported to contain iE-DAP in their cell wall (Kawia and Akira, 2009). NOD2 senses the minimal bioactive unit MDP of the cell wall of both gram positive and negative bacteria (Pant et al., 2007). Activated NOD1 and NOD2 lead to the activation of the inflammatory mediator NF-κB (Kawia and Akira, 2009).

1.5.2.1 NOD Like Receptor Pyrin 3 (NLRP3)

NLRP3 is a component of a complex of proteins collectively termed the inflammasome and is responsible for the detection of metabolic stress related ligands (Chaplin et al., 2010; Turvey and Broide, 2010). Different NLRs have different functions and are responsible for the detection of different ligands; for example NOD2 is responsible for sensing intracellular MDP (Pant et al., 2007). Sensing microbial structures, as is the case for
NOD2, is not the only function of NLR proteins; they can also recognise endogenous signals of cellular damage such as uric acid crystals, extracellular ATP and potassium efflux all of which NLRP3 is responsible for detecting/responding to (Chaplin et al., 2010). Once activated NLRs can proceed to activate NF-κB, as described for NOD1 and NOD2, exhibit a specific enzymatic function, as NLRP3 has, or lead to cellular apoptosis via recruiting and activating cysteine proteinases (Chaplin et al., 2010; Turvey and Broide, 2010). For the production of the active NLRP3 inflammasome to occur two danger signals are required, the activation of NF-κB by a PRR or STAT signal which causes the production of the NLRP3 unit of the inflammasome and the DAMP signal which results in NLRP3 activation (Williams et al., 2010). NLRP3 binds to the adaptor protein ‘apoptosis-associated speck-like protein containing a caspase recruitment domain’ (ASC), which as the name suggests binds to and activates Caspase 1 (Chaplin et al., 2010). This three protein complex, the inflammasome, functions to activate inflammatory mediators such as IL-1β and IL-18 by cleaving the biologically inactive form of pro-IL-1β and pro-IL-18 into diffusible bioactive cytokines (Chaplin et al., 2010; Turvey and Broide, 2010; Zaki et al., 2010).

Gain of function mutations in NLRP3 have been linked to several auto-inflammatory disorders typically characterised by skin rashes and prolonged episodes of fever with no apparent infection, while loss of function mutations have been linked to CD (Zaki et al., 2010).

1.6 CARD15/NOD2 Genetic Polymorphisms

MAP is an intracellular pathogen and as such is sensed by the intracellular cytoplasmic pathogen recognition protein NOD2 (nucleotide-binding oligomerisation domain 2) encoded by the CARD15 gene (Pinedo et al., 2009). The NOD2 PRR consists of three distinct regions and senses MDP, the minimal bioactive component of the bacterial cell wall of both gram positive and gram negative bacteria (Pant et al., 2007). Primarily NOD2 is expressed in cells involved in the innate immune system, including monocytes, macrophages, DC, epithelial cells and Paneth cells (Stronati et al., 2008). Genetic susceptibility studies in CD patients have revealed that CARD15 mutations are associated with an increase in risk for development of CD (Ferwerda et al., 2007). In the western hemisphere 30% - 50% of CD patients carry NOD2 mutations on at least one allele (Turvey and Broide, 2010). Three main susceptibility mutations have been identified in CARD15 in humans with CD, which are located in or near the LRR domain or in the near vicinity suggesting an alteration of this area results in susceptibility to CD potentially through impaired bacterial sensing (Gaya et al.,
These polymorphisms are designated R702W, G908R, and 1007fs due to their position and effect (Taylor et al., 2006). Two of these mutations are missense mutations while the third is a frame shift resulting in a truncation mutation. These amino acids are highly conserved (indicative of their importance) between humans, cows and mice with the exception of R702 which is only conserved between humans and cows (Taylor et al., 2006). Heterozygotes with one of these mutations have been associated with 1.5-4 fold increased risk, while homozygotes/compound heterozygotes (compound heterozygotes: a genotype in which both alleles are mutated but in different locations) have a 15-40 fold increased risk (Buning et al., 2005; Chacon et al., 2004). An association study between CARD15 gene polymorphisms and JD in cattle, performed by Pinedo et al. 2009 identified a single nucleotide polymorphism (SNP) within the LRR domain that resulted in an amino acid substitution, designated C733R, that increases JD risk by 3.35 times in heterozygotes (Pinedo et al., 2009). In CD patients it has been shown that increased levels of CARD15 expression is associated with increased infiltrates of mononuclear cells within the inflamed tissues supporting NOD2 having a pivotal role during and/or following the formation of a granuloma (Berrebi D, 2003). Once again in CD, it was shown by Berrebi et al. (2003), that the expression ratios of CARD15 mRNA were more than five times higher in CD compared to healthy controls indicating a potential role of NOD2 ratios in inflammation (Berrebi D, 2003). In a similar line of study Stronati and colleagues found elevated NOD2 expression levels in children suffering from CD and interestingly were able to link the active NF-κB binding to the promoter of the CARD15 sequence (Stronati et al., 2008).

1.7 Nuclear factor-κB (NF-κB)

Originally identified as a factor that regulated the expression of antibody in murine B lymphocytes in 1986 by Sen and colleagues, NF-κB is an integral transcriptional activator of immune activity responsible for the activation of early response genes that exist in virtually all cell types (Epstein et al., 1997; Ghosh et al., 1998; Sen and Baltimore, 1986). NF-κB belongs to a family of transcription factors known as the Rel family, a family sharing a 300 amino acid homologous domain responsible for protein dimerisation, nuclear localisation, and binding to a target element in genes promoters called the κB element (Barnes, 1997). Structurally NF-κB is a heterodimer consisting of two subunits, while there are 6 distinct potential subunits (p50, p65/Rel A, c-Rel, Rel B, v-Rel, and p-52) the most common composition is comprised of the p50 subunit bound to a p65 subunit that together comprise the transcription factor (Barnes, 1997; Epstein et al., 1997). While the p50 p65 heterodimer
NF-κB is the most common the different forms may allow for some variation in the target gene activation profiles (Barnes, 1997). The activation of NF-κB is achieved by some NLRs as well as by TLR and other sensory mechanisms (Kawia and Akira, 2009). These PRR activate NF-κB via the MyD88 and MAPKs pathways, as well as activating other transcription factors such as AP-1, ATF and CREB (Barnes, 1997; Dabbagh et al., 2002; Kawia and Akira, 2009). Inactive NF-κB is found in the cytoplasm bound to one of the four inhibitor of NF-κB activation (IκB) proteins (IκB-α, IκB-β, IκB-γ, and Bcl-3) that in the case of IκB-α and IκB-β causes inhibition of NF-κB by blocking the nuclear localization signal (Barnes, 1997). After an activation signal IκB is phosphorylated by a protein kinase and then ubiquitinated for proteasomal degradation causing IκB to release NF-κB allowing NF-κB to subsequently translocate to the nucleus (Barnes, 1997; Barnes and Karin, 1997; Ghosh et al., 1998). Within the nucleus NF-κB causes transcription of various target genes via the previously mentioned κB binding target characterised by the consensus sequence 5’ – GGGPuNNPyPyCC - 3’ (Barnes, 1997; Ghosh et al., 1998). Some NF-κB targets transcribed are shown in Table 2 below, however it is important to note that in different cell types different expression profiles due to NF-κB will be observed.

**Table 2**

<table>
<thead>
<tr>
<th>Targets</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
<td>TNF-α, GM-CSF, M-CSF, G-CSF, IL-1β, IL-2, IL-6, IL-11, IL-17, and IL-18</td>
</tr>
<tr>
<td>Chemokines</td>
<td>IL-8, RANTES, Macrophage inflammatory protein 1α, MCP-2, Macrophage chemotactic protein 1, and Eotaxin</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>ICAM-1, VCAM-1, and E-selectin</td>
</tr>
<tr>
<td>Enzymes</td>
<td>INOS and inducible cyclooxygenase</td>
</tr>
<tr>
<td>Receptors</td>
<td>IL-2 receptor, NOD2 and T cell receptor (β chain)</td>
</tr>
</tbody>
</table>

**Table 2. Genes Transcribed by Active NF-κB**

Above can be seen a representative sample of different effector molecules for immune function expressed by NF-κB classed according to their specific function. This table has been adapted from Barnes and Karin 1997.
Infection and inflammation can result in tissue damage causing cellular necrosis which activates TLR to signal NF-κB producing further inflammation and activating tissue repair mechanisms (Li et al., 2001). The downstream products of activated NF-κB, such as the proinflammatory cytokines IL-1β and TNF-α, cause further expression of NF-κB causing a positive regulatory loop, shown in Figure 3, that can perpetuate a local inflammatory response (Barnes, 1997; de Prati et al., 2005). Combating uncontrolled NF-κB activation one of the IκB genes IκB-α has a κB target site that allows for NF-κB binding and subsequent transcription of this gene that can once translated into protein can enter the nucleus and bind to NF-κB and cause its export out of the nucleus terminating the NF-κB induced gene expression (Barnes, 1997; Barnes and Karin, 1997).

**Figure 3**

Figure 3. Positive Feedback Loop of NF-κB Activation

There are many activators of NF-κB that post activation leads to target gene expression. In the case of the proinflammatory cytokines TNF-α and IL-1β, transcribed as a result of NF-κB activation, can activate NF-κB causing a positive feedback loop. Figure 3 has been taken from Barnes and Karin 1997.
1.8 Cytokines, Chemokines and Interferon

Immune cell signalling is achieved through the use of small secreted proteins called cytokines, chemokines and or interferons. These signalling molecules have effects on the immune response to a stimulus through effecting cell growth, differentiation, and activation functions. Different cell types produce different cytokines in a response to various antigenic stimuli. Some antigen presenting cells (APC) such as dendritic cells and macrophages are extremely effective in signalling both the innate and adaptive elements of the immune system (Commins et al., 2010). APC produce cytokines in response to activation of their PRR such as the TLR and NLR classes of receptors, or alternatively they can cause the production of cytokines as a result of activating other immune cells such as T helper lymphocytes (Commins et al., 2010). Signalling molecules produced by APC differ depending on the immune activation pathway and which kind of APC is stimulated. For dendritic cells and macrophages some common cytokines include TNF-α, IL-1β, IL-6, IL-12, IL-18, and GM-CSF and the chemokine RANTES (Commins et al., 2010; Medzhitiov, 2001).

1.8.1 Interleukin-1β and Interleukin-18

Interleukin-1β (IL-1β) is a key participant in the generation of a response to infection and cellular injury, produced in response to NF-κB activation (Barnes and Karin, 1997; Zaki et al., 2010). This cytokine carries out this role by generating fever (pyrexia), activating lymphocytes and causing leukocyte infiltration at the sight of infection or injury (Zaki et al., 2010). IL-18 is a cytokine related to IL-1β and has an effect on several secondary inflammatory cytokines, chemokines, cell adhesion molecules and nitric oxide synthesis (Zaki et al., 2010). During natural MAP infection of red deer it has been documented by Galindo and colleagues that IL-1β was upregulated (Galindo et al., 2010). While IL-1β has been linked with pyrexia and generation/activation of cellular mediators IL-18 has been linked with not only a role in promoting secondary inflammatory signals, but also tissue repair (Zaki et al., 2010). Zaki and colleagues found in a mouse model of experimental colitis representative of inflammatory bowel syndrome in humans (CD and ulcerative colitis) that IL-18, activated via NLRP3, is a crucial mediator of epithelial repair (Zaki et al., 2010). TLR4 was also identified by Zaki and colleagues as important in intestinal immunity, via its MyD88 NF-κB signalling pathway, in maintaining epithelial cell homeostasis and conferring protection against colitis (Zaki et al., 2010). This effect could be due to the ability of TLR4 activated NF-κB to produce the cytokine IL-18 (Zaki et al., 2010).
1.8.2 Interleukin-6 and Interleukin-2

Primarily IL-6 is produced by APCs, with some secondary production by T and B cells, and is involved in activating B cells to differentiate into mature plasma cells, T cell activation particularly into Th17 cells, as well as sharing some activities with IL-1β (Commins et al., 2010). In a manner similar to IL-1β, IL-6 can induce pyrexia and activation of leukocyte function (Commins et al., 2010). Unlike IL-6, the primary source of IL-2 is from activated T cells and is essential for T cell proliferation, activation of NK cells, B cells, CD8+ cells, and macrophages (Commins et al., 2010). Production of IL-2 from activated T cells requires previous exposure to IL-1 and IL-6 followed by stimulation of the T cell by the T cell specific antigen and costimulation via CD80 or CD86 (Commins et al., 2010).

1.8.3 Type one Interferon

Interferons are a class of cytokine that have been named after their ability to interfere with viral growth. Within the IFN class of cytokines there are three subtypes; type one, type two, and type three. Produced primarily by APC, B lymphocytes and NK cells, in response to various PAMPS type one IFN consisting of the three main types IFNα/β/ω each encompassing a range of different subtypes (Commins et al., 2010). These type one IFNs have immunologic effect by increasing the ability of cellular effectors to combat infection, primarily by stimulating CD8+ lymphocytes and NK cells, as well as by increasing the expression of MHCI (Commins et al., 2010). While type two IFN will be discussed below type three IFN shares some commonality with type one IFN having antiviral and antitumor activates (Commins et al., 2010).

1.8.4 Type two Interferon and RANTES

IFNγ “The most important cytokine responsible for cell-mediated immunity” (Commins et al., 2010) is the sole member of the type two IFN family commonly referred to as the signature cytokine of Th1 cellular immunity and is produced by Th1 T cells, cytotoxic T cells and NK cells (Commins et al., 2010). Following signalling by IFNγ there is an increase in both MHCI and MHCII expression, antigen presentation, and cytokine production from APC; specifically for macrophages it induces adherence, phagocytosis, cellular product transportation mechanisms, and production of INOS and other respiratory burst associated mechanisms accumulatively resulting in macrophage infiltration and activation (Commins et al., 2010). Macrophages, like T lymphocytes, have different subsets including the proinflammatory antimicrobial M1 phenotype and the Th2 promoting M2 macrophage
phenotype (Geissmann et al., 2010). Detection of PAMP and exposure to IFN\(\gamma\) results in macrophages being directed along a M1 phenotype associated with potent antimicrobial properties and enhanced IL-12 production that selectively upregulates a Th1 response (Geissmann et al., 2010). In addition to macrophage activation IFN\(\gamma\) also activates NK cells and neutrophils. Neutrophil infiltration into the site of cellular immune response is due to IFN\(\gamma\), in association with IL-8 which has a similar effect upon neutrophils, by upregulates the cell adhesion molecule ICAM-1 upon the granulocytes (Commins et al., 2010). Specifically for JD, IFN\(\gamma\) has a reported heightened expression ratio in animals suffering from JD particularly during early stages of infection that decreases during disease progression (Khalifeh and Stabel, 2004). This observed decrease in IFN\(\gamma\) results in defective bacterial handling, as exogenous IFN\(\gamma\) supplementation can partially restore bacterial killing (Khalifeh and Stabel, 2004). Modulation of IFN\(\gamma\) production could be a direct result of the observed upregulation of IL-10 and TGF\(\beta\) that occurs during JD progression (Khalifeh and Stabel, 2004). Activated T cells, specifically Th1 T cells, produce cytokines such as IFN\(\gamma\) along with many others that activate macrophages and other T cells (Khalifeh and Stabel, 2004). The chemokine RANTES shares functionality with both IFN\(\gamma\) and IL-12 as it acts in a proinflammatory manner. Chemokines are small proteins that induce cellular migration to the site of inflammatory process. Responsible for the recruitment and activation of leukocytes RANTES can actively promote the development of an IFN\(\gamma\) Th1 T cell response either directly or by causing the upregulation of IL-12 from APC (Commins et al., 2010). In some conditions, such as hypersensitivity reactions (found in granulomas) resulting in exposure to TNF\(\alpha\) and IFN\(\gamma\), RANTES is also produced by epithelial cells causing accumulation of memory T cells and monocytes (Marfaing et al., 1995).

### 1.8.5 Interleukin-4

While IFN\(\gamma\) has potent Th1 stimulating immune properties, IL-4 has Th2 stimulating properties (Commins et al., 2010). Signalling via STAT6, IL-4 promotes the activation of the transcription factor GATA3; IL-4 is primarily produced from active Th2 T cells, basophils, NK T cells, and mast cells, although initial differentiation into Th2 T cells can be due to Th0 T cells producing IL-4 (Commins et al., 2010). In the context of macrophage subpopulations, M1 is enhanced by IFN\(\gamma\) and supports a Th1 immune response, while the M2 phenotype is enhanced by IL-4 (Geissmann et al., 2010). M2 macrophages support Th2 immune responses
and play an important role in not only this function but also during the resolution of inflammatory processes (Geissmann et al., 2010).

1.8.6 GM-CSF

Granulocyte macrophage colony stimulating factor (GM-CSF) is a cytokine that helps the differentiation of hematopoietic stem cell progenitor cells into dendritic cells, macrophages, and neutrophils, as well as aiding the maturation of these immune cells (Commins et al., 2010; Morrissey et al., 1989). Specifically GM-CSF is associated with, in combination with IL-4, the differentiation of monocytes into dendritic cells, as well as the previously stated enhanced activation and maturation of pre-differentiated monocytes (Geissmann et al., 2010). While dendritic cells are produced from monocytes via GM-CSF macrophages are produced in response to macrophage colony stimulating factor (M-CSF) (Geissmann et al., 2010). Morphologically similar to IL-3 and IL-5 GM-CSF signals through the STAT5 pathway (Commins et al., 2010).

1.8.7 RANKL

Receptor activator of NF-κB ligand (RANKL) is a member of the TNF family of cytokines that has the function of stimulating T cells, macrophages, and dendritic cells, as well as being involved in lymphocyte differentiation, signalling through RANKL receptor RANK (Franchimont et al., 2004 B; Lam et al., 2001). Different TNF cytokines have different functions ranging from modulation of inflammatory processes, organogenesis, autoimmunity, and apoptosis (Lam et al., 2001). Predominantly TNF cytokines have the pleiotropic capacity to coordinate the development and function of different tissue groups; RANKL has important roles in the immune system, osteogenesis, and mammary epithelium modulations (Lam et al., 2001). In the immune system RANKL is involved in T cell and dendritic cell processes as RANKL mediates the differentiation of T and B lymphocytes and dendritic cell survival and cytokine production (Franchimont et al., 2004 B). CD4+ T cell precursors require autocrine RANKL signalling for survival and differentiation during migration from the bone marrow to lymphoid tissues (Lam et al., 2001). RANKL detection by the receptor RANK causes the downstream activation of the MAPK and NF-κB pathways (Lam et al., 2001). RANKL-RANK interactions occur post CD40-CD40L interactions implicating this cytokine to have a role in later stages of T cell function. Supporting this role RANKL mRNA is constitutively expressed in memory T cells (Franchimont et al., 2004 B). Mature dendritic cells and macrophages in JD tissues and colonic tissues from CD patients’
express high levels of RANK protein on their cell surface as well as having increased mRNA expression of both RANK and RANKL within colon tissues (Franchimont et al., 2004 B).

1.8.8 Interluekin-10

Some key cytokines have been discussed due to their ability to promote inflammatory processes or direct T cell responses have been discussed. Without mediators of inflammation excessive levels of these factors may result in hypercytokinemia, also known as a ‘cytokine storm’, with the potential to cause the death of the host. The key anti-inflammatory mediator is the cytokine IL-10, which is primarily produced from T cells, macrophages, and B cells (Commins et al., 2010). The immunosuppressive function of IL-10 is achieved by forming a homodimer that signals via STAT1 STAT3 heterodimers to cause the inhibition of IFNγ production by Th1 T cells, down regulation of costimulatory ligand on T cell CD28, as well as down regulating the expression of IL-4 and IL-5 by Th2 T cells (Commins et al., 2010). Monocytes exposure to IL-10 causes down regulation of IL-1β, IL-6, IL-8, IL-12, and TNFα production, as well as the inhibition of expression of MHCII, ICAM-1, CD80 and CD86 expression (Commins et al., 2010). Finally IL-10 causes down regulation of IFNγ and TNFα from NK cells (Commins et al., 2010). In a healthy individual the expression of IL-10 is associated with tolerance to allergens while in the JD infection model increased expression of IL-10 during MAP infection has been associated with a worsening disease state (Coussens, 2004).

1.9 Signal transducer and activator of transcription

The inflammatory response to MAP is achieved through signal transducer and activator of transcription (STAT) pathways via cytokine signalling, shown in Figure 4. Phosphorylation of the STAT proteins, carried out by the Janus kinase (JAK) family of tyrosine kinases in response to cytokine binding, is required for dimerisation of the STAT proteins after which the STAT molecule translocates to the nucleus where they have their role as transcription factors activating appropriate genes to the given stimuli (Kaplan et al., 1996). In Figure 5 each STAT is shown with one of its corresponding ligands and the downstream effect is shown in response to the activation of the STAT molecule. Each STAT has a different subset of ligands allowing for specific responses to different stimuli; STAT-1 responds to IFNγ, STAT-2 in association with STAT1 responds to type 1 interferons, STAT-3 is activated via IL-6, STAT-4 by IL-12, STAT-5 by GM-CSF, and STAT6 by IL-4 (Greenhalgh et al., 2005; Kaplan et al., 1996; Mudter et al., 2005). STAT1 signal cascade
also have cross talking functions as in response to IFNγ signalling not only STAT1 is activated, but also the MAPK pathway (de Prati et al., 2005). This is due to the requirement of STAT1 to be further phosphorylated post dimerisation to enhance its transcriptional potential resulting in MAPK activation (de Prati et al., 2005). Cytokine signalling causes the production of numerous downstream effector molecules including nitric oxide a signature molecule for inflammation (de Prati et al., 2005). The production of the enzyme inducible NO synthase (iNOS) is upregulated in response to pro-inflammatory cytokines including IFNγ and IL-1β as well as in response to TLR signalling (de Prati et al., 2005).

Aberrant activation of the immune system is responsible for negative consequences such as autoimmune diseases and inflammatory diseases so to combat over expression of immune activating molecules different mechanisms exist to regulate immune activation. A constitutively expressed mechanism employed by the cell to prevent excesses of cytokine signalling are the protein inhibitors of activated STATs (PIAS) family that act to repress activated STAT activity (Shuai and Liu, 2005; Wormald and Hilton, 2004). Of these PIAS molecules PIAS1, PIAS2 and PIAS4 will be looked at in further detail due to their role in limiting the extent of inflammatory processes. PIAS target various STAT members specifically; PIAS1 targets STAT1 as does PIAS2 (also known as PIASx), while PIAS4 (also known as PIASγ) targets STAT4 (Wormald and Hilton, 2004). Mode of function for the PIAS inhibition of STAT signalling occurs through different mechanisms, PIAS1 inhibits STAT1 by binding to the active form and preventing DNA interactions, while both PIAS2 and PIAS4 appear to prevent the downstream activation of STAT activated genes by acting as the E3 subunit of a 3 component ubiquitin ligase system that results in the modification of the STAT via a process called sumoylation (Shuai and Liu, 2005; Wormald and Hilton, 2004). This sumoylation occurs near the site of phosphorylation (Lys-703 being sumoylated while position Tyr-701 is phosphorylated) of the STAT protein, however the mechanism for the inhibitory function of the PIAS via sumoylation is not fully understood (Wormald and Hilton, 2004). One potential mechanism proposed for the function of the sumoylation involves the targeting of the STAT to an alternative cellular location, preventing the function of the activated STAT, such as nuclear bodies (Wormald and Hilton, 2004).

A class of cytokine signalling suppressors, activated in response to prolonged/excessive activation of a signal pathway, are known as suppressors of cytokine signalling (SOCS) molecules that are investigated in this context due to their ability to cause anti-inflammatory mechanisms specifically targeting the JAK/STAT pathway (de Prati et al.,
This results in a down regulation of inflammatory immune activation. There are currently 8 known members of the SOCS family several of which share high levels of promiscuity in targets however within specific cells SOCS have a clear physiological role (Greenhalgh et al., 2005). SOCS are composed of a variable N-terminal domain, a central SH2 domain and a 40-amino acid long C-terminal domain known as the SOCS box that acts as a binding site for components of the ubiquitin ligase system (Wormald and Hilton, 2004). Suppression by SOCS is achieved either by the SH2 domain binding the phosphotyrosine residue in the activated STAT protein (as is done for SOCS2 and SOCS3) or alternatively by suppressing the activity of the JAK molecule (Wormald and Hilton, 2004). Inhibition of the JAK protein function is achieved by out competing STAT molecules for the phosphorylation binding site or alternatively by ubiquitination of the JAK molecule for proteosomal degradation (Wormald and Hilton, 2004). Examples of physiological function of SOCS inhibition include IFNγ signalling inhibition by SOCS1, growth hormone signalling by SOCS2, and IL-6 and GM-CSF signalling by SOCS3 (Greenhalgh et al., 2005; Wormald and Hilton, 2004). In the context of a mycobacterial infection up regulation of SOCS-1 and SOCS-3 has been previously identified (Whittington et al., 2011).
Figure 4

Illustrated in Figure 4 is a simplified cartoon of the signalling cascades activated in response to cytokine signalling. This figure depicts pathways involved with the cytokine signalling cascade.
Figure 5

A)
**Figure 5 Continued**

B)

![Diagram of cytokine signalling and downstream effectors](image)

**Figure 5 Cytokine Signalling and Downstream Effectors**

Above is a representation of the signalling through the JAK/STAT pathway in response to cytokine/growth factor stimulation (A and B). Shown in green boxes are the effector transcription factors formed post activation and dimerisation of the STAT molecules and accessory factors where required. Conversely the purple boxes representing the constitutively expressed PIAS and the induced SOCS are shown repressing their specific ligands. This figure is a modified figure originally designed from Aaronson and Hovarth, 2002, obtained from Invitrogen life technologies (www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Signaling-Pathways/Jak-STAT.html).
1.10 Mitogen activated Protein Kinases (MAPK)

MAPK are a highly conserved family of serine-threonine kinases containing the highly conserved ‘Thr-Xxx-Tyr’ phosphorylation motif, composed of the extracellular signal-regulated kinases (ERK), p38, and c-Jun N-terminal kinases (JNK) pathways activated in response to PAMPs/cytokine signalling and or DAMPs (Schorey and Cooper, 2003). This pathway of intra cellular signalling is responsible for a range of cellular functions including proliferation, apoptosis, cytokine production and cytoskeletal reorganization (Schorey and Cooper, 2003). Typically the ERK pathway of the MAPK family is activated in response to detection of growth factors, while both the p38 and JNK pathways are activated in response to environmental stress and pathogen detection (Schorey and Cooper, 2003). A diagrammatic representation of the MAPK pathway can be seen in Figure 6. Phosphorylation of the MAPK pathway leads to the activation of various transcription factors (shown in Figure 6), leading to the subsequent expression of pro-inflammatory mediators including TNF-α, IL-1β, IL-6 and iNOS (Schorey and Cooper, 2003). Mycobacteria have been shown to activate the MAPK pathway, however the more virulent strains have the ability to limit the extent of this activation accounting for some of the previously described reductions in inflammatory potential of the infected macrophages (Schorey and Cooper, 2003).
Figure 6

**Figure 6. MAPK Pathways of Cell Signalling**

Representation of the MAPK pathway post activation by TLR4 signalling in response to LPS. As can be seen the three main families of MAPK signal transduction are present; JNK, ERK and p38. Activation of these pathways results in the activation of different transcription factors including AP-1, SRE, CRE and NF-κB, via activation of their individual components shown below each transcription factor. Figure 6 has been based on Schorey and Cooper, 2003.

1.11 Adaptive Immunity

Once CD4\(^+\) T helper lymphocytes have been activated by their specific antigen via APC interaction coupled with costimulatory activation they can differentiate into various effector cell lines with variable function and cytokine profiles (Bonilla and Oettgen, 2010; Szabo et al., 2000). Active T helper cells can differentiate into different effectors including Th1, Th2 and Th17 cells produced in response to pathogenic challenge and subsequently play a pivotal role in responding to set challenge (Jenner et al., 2009). Th1 cells are critical for protection against intracellular pathogens such as viruses and intracellular bacteria, while Th2 and Th17 cells confer protection against extracellular parasites and bacteria (Jenner et al., 2009). As previously described even macrophages differentiate into multiple different subsets
based upon the nature of the immune reaction to a given stimuli. The M1 and M2 phenotype actively promote the Th1 and Th2 immune response respectively; however the differentiation of these macrophages may have a plasticity in vivo (M1 macrophages under the right conditions can class switch to M2 and vice versa), sharing similarity to T cell plasticity, changing phenotype in response to changing immune cues (Geissmann et al., 2010).

For the optimal generation of Th1 cells to occur IL-12 and IFNγ signalling via STAT4 and STAT1 respectively is required (Jenner et al., 2009; Szabo et al., 2000). STAT1 and STAT4 signalling in response to IFNγ and IL-12 induces the expression of the transcription factor T-box expressed in T cells (Tbet) and Eomesoderm that combined have activity through transcription of Th1 specific pathways while simultaneously suppressing Th2 and Th17 pathways (Jenner et al., 2009; Szabo et al., 2000; Yang et al., 2008). While Th1 differentiation requires IFNγ and IL-12, and Th17 requires IL-6 signalling via STAT3, Th2 cell production requires IL-4 that signals through the STAT6 pathway, which promotes the transcription of the transcription factor GATA-3 (Jenner et al., 2009). After differentiation into either Th1, Th2, or Th17 cells the T-bet, GATA-3, or RORC expression actively represses the expression of the other T cell profiles inducing these cells signature cytokine (Jenner et al., 2009). T-bet induces expression of IFNγ, RANTES, CCR5 (the ligand for RANTES), transcription factors including CREB, and the repressor of GATA-3 RUNX1 (Jenner et al., 2009; Szabo et al., 2000). GATA-3 conversely activates expression of IL-4 along with STAT6 (Jenner et al., 2009). Although T-bet and GATA-3 are hall marks of Th1 and Th2 cell differentiation respectively, it has been previously found that Th1 cells express not only T-bet, but also GATA-3 and the range of genes that these transcription factors target overlap with few T-bet specific targets (Jenner et al., 2009). The extent of the targeting overlap in Th1 cells due to GATA-3 compared to Th2 cells is as high as 78% (Jenner et al., 2009). Within Th2 cells the Th1 lineage specific genes that can be activated by GATA-3 in Th2 cells are targeted in Th1 cells via GATA-3 in 92% of instances (Jenner et al., 2009). Taken together this indicates a significant overlap of these two transcription factors function in targeting similar genes that are required in Th1 and Th2 lymphocyte differentiation. Although the target sequences of these two transcription factors overlap differential expression of genes required to produce the Th1 and Th2 phenotype is obtained via the ability of T-bet to act as a repressor of transcription of some Th2 genes including IL-4 (Jenner et al., 2009; Szabo et al., 2000). Interestingly Susanne Szabo and colleagues have shown that retroviral gene transduction of T-bet into polarized Th2 cells was sufficient to re-direct these
cells into a Th1 phenotype (Szabo et al., 2000). As with CD4+ T cells, CD8+ cells also differentiate into type 1 and type 2 phenotypes (Tc1 and Tc2 respectively) and as has been seen for redirecting Th2 CD4+ cells into a Th1 phenotype using retroviral T-bet this also holds true for polarised CD8+ Tc2 cells as it will redirect them into a Tc1 phenotype (Szabo et al., 2000).

1.12 Host Immune Response against MAP

MAP infection causes the formation of chronic inflammation of the Peyer’s patches and draining lymph nodes often resulting in hyperplasia of T cells, B cells and infiltrating macrophages (Coussens, 2004). Active MAP infection causes intestinal granulomas that can present as a paucibacillary form that is mediated by a type 1 cellular immune response and associated with small scattered granulomas containing few organisms and a multibacillary form that is mediated by a type 2 humoral immune response and is associated with ‘sheet-like’, macrophage-dense granulomas (Burrells et al., 1998; Gillan et al., 2010; Nalubamba et al., 2008; Stabel, 2006). Granulomas observed in JD share pathological similarities with those found in human leprosy and CD patients (Chamberlin and Naser, 2006; Whittington et al., 2011).

In both susceptible and resistant animals the immune response to MAP infections is characterized by an initial Th1 T-cell immune activation. In susceptible animals the initial immune response fails to contain or eliminate the infection and intestinal damage occurs causing a malabsorption syndrome responsible for the wasting observed during disease progression (Chacon et al., 2004; Stabel 2006). While the Th1 immune response in the resistant animal is responsible for the clearance of infection, in the susceptible animal this Th1 cellular immune reaction decreases to weak or undetectable levels gradually shifting to a Th2 immune response that is ineffective against intracellular pathogens, indicative of the animal succumbing to disease (Burrells et al., 1998; Coussens, 2004; Pant et al., 2010). Suppression of the Th1 immune response by T regulatory cells may play a major role in disease progression as in susceptible animals the proliferation of suppressor T cells and their subsequent production of IL-10 play an important role in the down regulation of immune pathways capable of clearing the pathogenic challenge (Coussens, 2004).

1.12.1 MAP Manipulation of the Host Immune Response

MAP may have a role in modifying gene expression profiles in infected ruminants causing macrophage dysfunction (Galindo et al., 2010). Pathogenic mycobacteria manipulate
the activation of the Mitogen activated Protein Kinases (MAPK) pathway, diminishing the level of inflammatory mediators produced by that cell (Schorey and Cooper, 2003; Hestvik et al., 2005). Along with MAPK manipulation mycobacterial inhibition of phagolysosomal fusion is considered a hallmark of mycobacterial infection, representing one of the definitive key mechanisms that the mycobacterial pathogens employ to avoid host killing mechanisms and survive within macrophages (Hestvik et al., 2005).

1.12.2 MAP Manipulation of TLR2

Pathogenic mycobacteria have evolved intra-cellular survival mechanisms within macrophages that can down regulate major histocompatibility complex 2 (MHCII) expression along with pro-inflammatory cytokines like GM-CSF, chemokines such as RANTES, and reactive oxygen and nitrogen species (i.e. nitric oxide) as well as upregulation of the anti-inflammatory and anti-apoptotic cytokine; IL-10 (Buza et al., 2003; Coussens, 2004; Chacon et al., 2004; Chamberlin W. M., 2006; Hestvik et al., 2005; Koul et al., 2004; Rao et al., 2000; Russell et al., 1997). MAP, M. avium and M. tuberculosis hyper-activate toll-like receptor 2 (TLR2) to produce an anti-inflammatory response hindering the immune activation of the cell and subsequent clearance of infection (Netea et al., 2004; Noss et al., 2001; Weiss et al., 2008). Hyper activation of TLR2, in the macrophage, is achieved by the use of a mycobacterial phenolic glycolipid (PGL) produced by the mycobacterium resulting in production of IL-10- preventing infected macrophage apoptosis as well as actively interfering with interferon γ (IFNγ) production, which is essential for complete macrophage activation and MAP clearance (Khalifeh and Stabel, 2004, Hestvik et al., 2005). These MAP survival mechanisms also help it block the Th1 T cell dependent immune response that is required for disease clearance (Burrells et al., 1998). In addition to infected macrophage abnormalities including reduced responsiveness to IFNγ, and reduced production of cytokines and reactive oxygen and nitrogen species, is the macrophages’ reduced efficiency in antigen processing (Hestvik et al., 2005).

1.12.3 Macrophage Phagosomal Killing Inhibition

Following antigen/bacterial uptake to a phagosome a series of events designed to result in microbial killing ensue including: acidification of the phagosome due to proton-ATPase pumps located in the phagosomal membrane, phagosome lysosomal fusion resulting in bactericidal proteolytic enzymes in the phagosome, induction of reactive oxygen and nitrogen species and finally antigen processing (Hestvik et al., 2005). Mycobacteria contain
the cell wall glycolipid mannose-capped lipoarabinomannan (LAM) that causes the exclusion of early endosomal antigen 1 (EEA1) from the phagosomal membrane. This is a necessary intermediary protein for phagosomal maturation, that the exclusion of causes inhibition of phagosomal maturation and as a result the inhibition of the accumulation of ATPase proton pumps on the phagosomal membrane (Hestvik et al., 2005). The inhibition of the EEA1 protein also results in decreased calcium signalling which in turn abrogates the activity of the PI3-kinase pathway blocking the trans-Golgi network from the phagosome (Hestvik et al., 2005; Schorey and Cooper, 2003). LAM also has a role in mycobacterial survival in that it causes fusion events of phagosome and early components of the endocytic pathway resulting in the transfer of nutrients to the phagosomal compartment (Hestvik et al., 2005). So far the mechanisms behind the inhibition of phagosomal maturation have been discussed as being based upon the cell wall glycolipid LAM, however the mechanism behind the prevention of phagolysosomal fusion is based around a mycobacterial serine/threonine kinase known as protein kinase G (PknG) (Walburger et al., 2004). PknG is actively secreted by mycobacteria (following phagocytosis) and can be detected in the phagosome as well as in the cytosol of the infected macrophage where PknG exhibits its effect of blocking lysosomal delivery (Walburger et al., 2004).

1.12.4 Inhibition of Macrophage Apoptosis

The apoptosis of infected macrophage acts as a host defence mechanism to counter mycobacterial pathogens. This is seen from studies using attenuated M. bovis vaccine strain BCG where macrophage infection results in significantly more infected macrophages undergoing apoptosis than macrophages infected with a wild-type M. bovis strain (Hestvik et al., 2005). Once an infected cell undergoes apoptosis that cell is subsequently taken up by the process of efferocytosis, whereby the apoptotic cell is phagocytosed by another primary phagocyte (i.e. a macrophage or neutrophil), further enhancing MAP killing due to mechanisms related to the degradation of the apoptosed cell within the phagocyte (Whittington et al., 2011). Efferocytosis not only enhances mycobacterial killing it also promotes Th1 immune response by processing of the mycobacterial antigens on antigen presenting cells (APC) that phagocytosed the apoptotic cell (Whittington et al., 2011). One mechanism employed by mycobacteria to circumvent host cell apoptosis is the up regulation of anti-inflammatory cytokines such as IL-10 (Hestvik et al., 2005). To infect other macrophages and continue to proliferate MAP initiates a necrotic cell death of the infected
macrophage, which due to the chemo-attractant nature of cellular lysates further enhances macrophage infiltration and the potential for infection of these cells (Whittington et al., 2011).

1.12.5 Global Manipulation of the Host Immune Response

Mycobacterial manipulation of the host immune response does not stop at the cellular level as seen by the increased expression of anti-inflammatory mediators such as IL-10, but also by decreasing the expression of MHCII on the surface upon the infected macrophage (Hestvik et al., 2005). This decrease in MHCII disrupts the activation of the adaptive arm of the immune system as macrophages play a key role in the activation of T cell populations, preventing the amplification of the inflammatory response (Hestvik et al., 2005). One mechanism by which mycobacteria can manipulate the production of pro-inflammatory mediators in macrophages is by the production of the mycobacterial PGL that decreases the release of pro-inflammatory mediators via the sustained activation of TLR2 causing the subsequent increase in IL-10 (Khalifeh and Stabel, 2004; Hestvik et al., 2005). Current understanding as to the mechanism by which mycobacteria suppress the expression of MHCII are based upon the interference that mycobacteria exert on the INF-γ pathway. This may be due to a negative feedback loop as a consequence of sustained TLR2 signalling, or due to the anti-inflammatory effects that occur as a result of a successful mycobacterial infection such as the inhibition of the MAPK pathway (Fulton et al., 2004; Hestvik et al., 2005). While the production of IFNγ and its subsequent binding to a cytokine receptor, to STAT activation is unaffected by the mycobacterial pathogen, there is evidence to indicate suppression of the activated STAT at the transcriptional level via attenuation of gene transcription by the transcription factor inhibiter p19 effectively suppressing the transcription of not only pro-inflammatory mediators including the MHCII genes (Hestvik et al., 2005). Another proposed mechanism for the down regulation of MHCII is by blocking the transport and processing of the MHCII by the pre-described mycobacterial manipulation of the phagosomal lysosomal systems (Hestvik et al., 2005).

1.13 Quantitative Polymerase Chain Reaction (qPCR)

Analysing the genetic makeup of an organism has had an important role in advancing current knowledge in the field of biology. In 1996 several authors developed different methods for improving the efficiency of qPCR, for example Heid and colleagues created a method for real time quantification of cDNA by measuring the accumulation of PCR products using a fluorogenic probe activated during amplification of the target gene (Heid et al., 1996).
An alternative, cheaper and less complicated method involves the inclusion of dyes such as SYBR Green that binds to double stranded DNA (Wittwer et al., 2001). These new methods became available in 1997 as part of the LightCycler system, providing a new and less labour intensive technique for measuring the amount of the target product without the need for post PCR handling preventing post PCR contamination and allowing for increased efficiency of the assay (Klein, 2002). Using these systems has enabled researchers to measure the expression of genes of interest by taking mRNA preparations and reverse transcribing them into cDNA (Bustin et al., 2005). While the process of converting RNA into cDNA (reverse transcription) template is a fundamental step in the qPCR process this step has been reported to add an element of variability and therefore adversely affect the reproducibility of the experiments (Bustin et al., 2005). Factors influencing quality and reproducibility of qPCR specifically during reverse transcription include the dynamic state of cells RNA pools, the quality of the RNA, and the method of the RNA to cDNA conversion and the efficiency of this conversion (Bustin et al., 2005). During the PCR itself several parameters also influence the efficiency of the amplification such as magnesium and salt concentrations, reaction conditions including time and temperatures, PCR target size and composition, primer efficiency and specificity, and sample purity (Heid et al., 1996). To test the specificity of primers one of the best methods utilises the melt curve method achieved using melting curve analysis at the end of a qPCR assay (Bustin et al., 2005).

Quantification of the cDNA is based upon the cycle threshold (Ct) of detection value, an “arbitrary number” that changes based on the variability of the base-line data shown in Figure 7, intersecting the log phase of the qPCR reaction (Heid et al., 1996). Using this Ct detection method samples with fewer starting copies of cDNA will have a higher Ct value and targets with a high starting number will conversely have a very low Ct value (Heid et al., 1996).
Figure 7

A

\[ \Delta RQ \] (Fluorescence units)

0 10 20 30 40

Cycle

0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8

Baseline

Threshold

Plateau

C_t

Figure 7. Representation of a typical qPCR amplification plot

C_t value intersects the log phase (represented by the blue line) of increase in fluorescence. In this instance the C_t value is at cycle 23. This figure has been taken from Heid et al. 2006.

To take into account differences in initial cDNA concentration a normalising gene is included for every sample which allows for comparison of different samples as a good normalisation gene is considered to be expressed at a constant rate (Heid et al., 1996). These genes are preferably constitutively expressed at a constant rate for instance a “housekeeping” gene (Klein, 2002). Some commonly utilised normalising genes include GAPDH, β-actin and the MHC I component β2-microglobulin (Livak and Schmittgen, 2001). Using these normalising genes allows for the comparative quantification method known as the delta C_t method (ΔC_t) (Livak and Schmittgen, 2001). This method allows for the determination of the fold change in gene expression across the samples using the equation shown in Figure 8. Initially delta C_t (ΔC_t) is found via gene of interest (GOI) minus the normalising gene. Using the ΔC_t is informative for looking at expression ratios of a target against the normalising
gene. Fold change can then be determined by either assuming the primer efficiency is 100 % or by using the primer specific efficiency (Livak and Schmittgen, 2001).

**Figure 8**

A) $\Delta Ct = \text{GOI} - \text{normalising gene}$

B) Fold change $= 2^{-\Delta Ct}$

**Figure 8. Delta Ct method of qPCR data interpretation**

An equation commonly used to obtain the fold change for target genes after which fold change differences can then be compared and conclusions drawn. A) Equations to obtain the $\Delta Ct$, B) the fold change. Fold change in B) is assumed to be 100 % as each cycle the target doubles, however if the primer efficiency is known it would replace the absolute maximum efficiency of 2. This equation has been adapted from (Livak and Schmittgen, 2001).

1.14 Automation of Laboratory techniques

Using robotics to automate repetitive tasks as a time and labour saving procedure is a practise that has been utilised in and for many laboratory tasks (Greenspoon et al., 2006). In laboratories automation of techniques such as PCR allows for enhanced throughput without sacrificing quality of the results gained (Greenspoon et al., 2006). Automation can also help to reduce some of the common sources of contamination of PCR samples including sample mix-up and aerosolised contagions (Greenspoon et al., 2006; Wilke et al., 1995). During the course of Wilke and colleagues trial comparing the practicality of automation, specifically the effect on PCR setup time and contamination, it was determined that of the manual PCR reactions run over a 21 month period 7% of the total 3404 reactions were invalidated due to carryover contamination (Wilke et al., 1995). This 7% contamination for the manual PCR procedure was compared to a robotic automated procedure that not only greatly decreased the time required for PCR setup but also decreased the contamination rate to a contamination rate of <0.1% of 7002 reactions (Wilke et al., 1995). Wilkes concluding remarks “we recommend that automation be seriously considered when establishing a PCR diagnostic laboratory” further emphasises the potential of this technology (Wilke et al., 1995).
1.15 Significance of research

Informed alteration of the genetic makeup of domestic livestock is one potential application of the knowledge of whether an animal is resistant or susceptible to JD (Pant et al., 2007). Using this knowledge could reduce the incidence of JD via selective breeding and or culling of susceptible animals and result in a greater profit margin for farmers (Pant et al., 2007; Beard et al., 2001). As the control strategies of JD is hampered not only by difficulties in disease diagnosis, especially during the subclinical stage of the disease, but also the complex epidemiology of JD, selective breeding of resistance associated traits may be a viable option to combat this disease (Beard et al., 2001). While vaccination programs have been shown to reduce the severity JD symptoms and spread of infection they do not prevent infection (Brett E., 1998). Current vaccination strategies use heat killed or attenuated MAP that interferes with diagnostic tests for bovine tuberculosis and as such vaccination regimes are not usually appropriate (Mackintosh et al., 2005; Muskens et al., 2002). Treating infected animals is not feasible due to the high cost per animal and poor clinical response (Whittington et al., 2011). While new generation vaccines and treatments for JD are still in development selective breeding is a age old proven method of increasing a desired phenotype in livestock (Pant et al., 2007). Natural resistance against pathogens produced by selective breeding can cause an increase in generic resistance to multiple other pathogens due to enhanced immunologic factors.
1.16 Hypothesis and Aims

Mutations in the CARD15 gene will be contributing to susceptibility in deer, linked to the LRR region of the CARD15 gene, to Johne’s disease caused by MAP infection. The second hypothesis investigated questioned the immune response against MAP; susceptibility to JD maybe a result of abnormal expression of immune response genes in animals of this phenotype. Finally, expression patterns of inflammatory mediators in resistant animals will be elevated earlier in the infection trial representing resistance mechanisms that will show different patterns of expression to susceptible animals.

To test these hypothesis the following aims were established:

1. Scan/map the cervine CARD15 LRR region for potential polymorphisms associated with JD severity in red deer of demonstrated phenotype.
2. Establish a model by which the expression ratios of immune related genes can be compared between the resistant and susceptible animals.
3. Compare gene expression ratios of established JD susceptibility genes along with immune genes of established function in this cervine infection model to identify any resistant/susceptible phenotypes.
Chapter 2: Methods

2.1 Experimental infection model (Previous Work)

A previous study of heritability, immunological, and pathological responses of red deer (Cervus elaphus) to an experimental challenge of MAP, was conducted at AgResearch Invermay, Otago, New Zealand ending in 2010 (Mackintosh et al., 2011). To carry out the experimental infection model 24 mixed aged hinds were artificially inseminated using frozen semen from either a resistant or susceptible stag. The resistance or susceptibility of the stags was tested in a previous experiment based on intensive observation of their offsprings incidences of natural JD infection. Of the 24 hinds inseminated 18 became pregnant with a final pool of 9 resistant and 9 susceptible fawns birthed 8 months later. These 18 fawns were left for four months and then challenged with high doses of a virulent bovine strain of MAP isolated directly from the lymph node of a clinical JD animal resulting in an inoculation of approximately $10^9$ colony forming units, each day for 4 days using this virulent bovine strain of MAP. There currently is no standard dose for MAP infection models, but typically doses of $10^9$-$10^{12}$ are used (Begg and Whittington, 2008). These deer were studied for 49 weeks after experimental infection, with the animals that developed clinical JD being electively slaughtered at time of disease manifestation. Tissue biopsies of the jejunal lymph node were recovered at weeks 4, 12 and at the slaughter time point (week 49) as described in Mackintosh et al., 2011. Samples of these biopsies were used for histopathology and culturing of MAP independently of this study using the BACTEC 12 B liquid culture medium, and the subsequently described qPCR research (Section 2.3 onwards).

2.2 TRIzol RNA extraction

One week prior to time of necropsy 10 ml of peripheral blood was acquired by jugular venepuncture and temporarily preserved using heparin anticoagulant. The TRIzol (Invitrogen Ltd.) extraction method was carried out utilising consumables supplied with the kit. This is a method that utilises the phenol-chloroform extraction technique. Briefly; white blood cells (WBC) were separated from whole blood samples via centrifugation at 13,000 rpm for 10 minutes. Using a pasteur pipette the WBC were removed from the sample and placed into a 50 ml falcon tube containing 30 ml of chilled erythrocyte lysis buffer to lyse any red blood cells carried over. These samples were incubated at room temperature for 10 minutes after which the WBC were pelleted via centrifugation at 13,000 rpm for 10 minutes. After centrifugation the supernatant was removed and 1 ml of TRIzol was added to the pelleted
WBC. These samples were incubated for 5 minutes at room temperature and 200 µL of chloroform was added, to separate the organic phase and aqueous phase, and the samples mixed. After mixing the samples were then centrifuged at 10,000 rpm for 15 minutes at 4 °C to further help the separation of the aqueous phase and the organic phase. The aqueous phase contained the RNA that was transferred to a clean eppendorf tube and 500 µL of isopropyl alcohol was added to precipitate out the RNA, after which the samples were incubated for 15 minutes at room temperature then the tubes were centrifuged at 13,000 rpm for 10 minutes at 4 °C. Following centrifugation the supernatant was removed and the RNA pellet was washed with 1 ml of 75 % ethanol via vortexing. Post vortexing the samples were then centrifuged for 5 minutes at 7,500 rpm at 4 °C. The ethanol was aspirated off and the sample was allowed to air dry after which 50 µL of water was then added to each sample. At this stage RNA recovery was quantified by UV spectroscopy, resulting in a range of 370-3144 ng/µl over all RNA preparations undertaken during this study. RNA samples were stored at -80 °C. Crude RNA was supplied to the researcher from the biopsied samples; RNA extracted via the tissue TRIzol method (based upon the manufacturers methods, independently at the time of sampling) and then had been stored at -80 °C.

2.3 Qiagen RNeasy RNA cleanup

Crude extracted RNA (produced via the above Invitrogen TRIzol method) was further purified using Qiagen RNeasy Mini Kit RNA cleanup protocol according to the manufacturers specifications (consumables supplied with kit). Samples were diluted using RNase free water to a concentration of 1ng/µL in a final volume of 100 µL (Qiagen). Following dilution, 350 µL RLT lysis buffer was added to the RNA and mixed, after which 250 µL of 95 % ethanol was added. The resulting solution was placed into an RNeasy Mini spin column (Qiagen) within a collection tube; one spin column and collection tube per sample. Following centrifugation at 13,000 rpm for 15 seconds the RNA was bound to the spin column and the flow through discarded. Each spin column was then washed with RPE buffer twice via centrifugation initially using 500 µL for 13,000 rpm for 15 seconds, and then using 500 µL for 2 minutes to allow the column to dry, removing the flow through each time. The column was then centrifuged for a further minute at 13,000 rpm to ensure all ethanol had been removed. The ethanol free column was then transferred to a fresh 1.5 ml collection tube that had 50 µL of water added to it then was centrifuged for 1 minute at 13,000 rpm to elute the RNA.
2.4 Bioanalyser

RNA integrity was assessed using an Agilent BioAnalyser instrument as per manufacturer’s protocol. An RNA Nano microfluidics chip was used to measure the relative ratio of 28S and 18S ribosomal RNA to produce an RNA integrity score (RIN). RIN values greater than 8 are considered to represent high quality RNA preparations (Fleige and Pfaffl, 2006). As a test of RNA integrity 12 samples were chosen from animals 77, 79, 80, 81, 82, 84, 85, 86, 88, 89, 90 and 91, tested using the Bioanalyser to produce RNA integrity number scores.

2.5 RNA reverse transcription

The RNA samples were reverse transcribed using Invitrogen SuperScript VILO cDNA Synthesis Kit as per the manufacturers’ recommended method (Invitrogen). For each sample 4 μl of 5 × VILO Reaction Mix, 2 μl of 10 × superscript Enzyme Mix, 1 μg of RNA, and DEPC-treated water to make a final volume of 20 μl was prepared. The contents of this tube were mixed gently and incubated at room temperature for 10 minutes, then at 42 °C for 60 minutes and lastly to terminate the reaction at 85 °C for 5 minutes. The resulting cDNA was diluted by a factor of 100 and stored at -20 °C.

2.6 CARD15 genotyping – Primers and PCR protocol

In total 12 primer pairs (Sigma) were tested to span as much of the Leucine Rich Repeat (LRR) region of CARD15 as possible, those used are shown below in Table 3; all were tested via PCR reactions that were checked via gel electrophoresis using 1 % agarose. The primers were based upon bovine sequence, for primer pair B 3 reverse primer to obtain the extreme 3’ end of the CARD15 LRR sequence. The cervine sequence available, independently produced previously, was utilised to obtain the forward B 3 primer and the forward B 12 primer. Primer pair 3 was used for getting initial LLR 3’ sequence upon which to make new more specific primers such as the reverse primer for B 12. Each primer pair was optimised using temperature gradients, from 55 to 65 °C, and MgCl₂ curves ranging from 0.5 mM to 5 mM raised incrementally by 0.5 mM to find the optimum concentration.
Table 3

<table>
<thead>
<tr>
<th>Primer</th>
<th>Purpose and sequence length</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 3 Forward</td>
<td>PCR for extreme 3’ end of CARD15; forward primer was</td>
<td>GGGGCTCAGAACTAACAACCTCCTT</td>
</tr>
<tr>
<td></td>
<td>based on cervine sequence while the reverse primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>based on bovine sequence NM_001002889. These primers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>produced a 695bp product.</td>
<td></td>
</tr>
<tr>
<td>B 3 Reverse</td>
<td></td>
<td>GGCTGCCCCTCTTCAACATCC</td>
</tr>
<tr>
<td>B 12 Forward</td>
<td>820bp CARD15 LRR for sequencing.</td>
<td>TCCGGAGCTGTACGAGATGC</td>
</tr>
<tr>
<td>B 12 Reverse</td>
<td></td>
<td>ACTTCCGAATGCTGTCA</td>
</tr>
</tbody>
</table>

Table 3. Primers for sequencing CARD15

The primers listed above were used for attaining the CARD15 sequence and are shown along with the purpose of each primer pair.

For each PCR reaction the final concentration of reagents used consisted of: 1 × Bioline reaction buffer, 1.5 mM MgCl₂, 0.2 μM primers, 0.2 μM dNTPs, 1.5 units of Taq polymerase/reaction, 5 μl of the diluted cDNA, produced from the reverse transcriptions, per reaction, 5 % DMSO and water to make 300 μl (for 10 PCR reactions as PCR were done in 30 μl volumes).

PCR regime for the sequencing of the LRR region consisted of: 94 °C for 1.30 minutes, 94 °C for 30 seconds, annealing temperature (64 °C for B 3 and 55 °C for B 12) for 30 seconds, 72 °C for 1.30 minutes, followed by cycling from stage 2 to 4 30 times, and finally 72 °C for 5 minutes.

The PCR products were gel purified over 0.7 % agarose and were stained using ethidium bromide and visualised under UV light.

2.7 Gel purification and Sequence interpretation

Purification of the amplified CARD15 LRR region from the agarose gel was done using the Promega Wizard SV Gel and PCR Clean-Up System following the manufacturers’ instructions (consumables supplied with kit). Briefly; the DNA band was visualised and excised from the gel under UV illumination. Following band excision membrane binding
solution was added to each tube at a ratio of 1 ml of binding solution to 1 gram of agarose, these tubes were then mixed via vortexing and subsequently incubated in a water bath at 60 °C for 10 minutes to melt the agarose. DNA purification was performed on the molten agarose by placing each sample into a SV minicolumn inside a 2 ml collection tube. The sample was then passed through the spin column, after a 1 minute incubation, at 13,000 rpm for 1 minute. The spin column, now with the DNA bound to the column, was then washed using 700 μl of membrane solution via centrifuging as previously at 13,000 rpm for 1 minute. Flow through was discarded and the wash was repeated using 500 μl of wash solution and centrifuged as before. Post washing the spin column was centrifuged for 1 minute to allow all remaining ethanol to pass through after which the spin column was then placed into a new collection tube and the DNA was recovered in 50 μL of water via centrifugation at 13,000 rpm for 1 minute. The gel purified PCR products were then submitted to the Allan Wilson Centre, Massey University, for sequencing. Sequence data has been interpreted using DNASTAR Lasergene 8 SeqMan software. Previous CARD15 sequencing work in the DRL lab provided a template cervine sequence to act as a reference against which to align the obtained sequences against.

2.8 qPCR Primers, Protocol and data interpretation

qPCR assays were performed using ABgene’s Absolute qPCR SYBR Green Low ROX Mix (ABgene, UK) for the genes TLR1, TLR2, TLR4, TLR6, MyD88, TRAF6, MAPK8, MAPK11, MAPK14, P53, ATF2, CREB, cFOS, cJUN, GM-CSF, NOD1, NOD2, NF-κB, IL-1β, IL-18, NLRP3, IRF3, INOS, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6, Tbet, IFNγ, IL-19, GATA3, RORC, RANK, PIAS1, PIAS2, PIAS4, SOCS1, SOCS2, SOCS3, IFNβ, HLA-DMB and B2M as a control. These primers were designed using Primer Express (Applied Biosystems) software. The concentration of primers used for qPCR was 100 mM for all qPCR reactions. The primers designed for MAPK14, P53, NOD1, IL-19, and SOCS1 failed to produce specific targets and will not be discussed further. Primers for the forward and reverse reaction for each gene can be seen in Appendix 1.

The qPCR temperature and time settings for both the 384 and 96 well format plates includes an initial temperature of 95 °C for 15 minutes to denature the cDNA. Following this step the machine cycles between 95 °C for 15 seconds followed by 60 °C for 1 minute forty times. The reaction is then terminated at 95 °C for 15 seconds and a subsequent melt curve run to ensure product purity concludes the qPCR run.
2.9 384 well qPCR

Six animals that exhibited polarised disease states following necropsy were selected to represent the resistant and susceptible phenotypes. These included the three most resistant animals R92, R91 and S89, while the three most susceptible animals were represented by R86, S90 and S96. The qPCR reactions were carried out on a Roche LightCycler 480 machine using 384 well plates. Data obtained was then analysed using LightCycler 480 software version 1.5.0.39.

Each well for the 384 well plate contained a final volume of 10 μl, with the cDNA used per reaction at 2 μl. As with the automated protocol the qPCR was done using master mix 2 × Absolute qPCR SYBR Green low ROX mix (Thermo Scientific), primers at a final concentration of 100 nM, and water to make a final volume of 10 μl per reaction. Use of 2 × Absolute qPCR SYBR low ROX mix was optimum as this mix, with the exception of primers and template, contains all the required components for the qPCR reaction.

Fluorescence data was obtained from the 384 well plates and then converted and standardised in Microsoft Excel via the Delta Ct (DCT) method in which the Ct values are converted to relative expression of B2M (B2M expression representing the standard 1) by standardising the Ct values against the control gene B2M. For this to be viable the Ct values of the replicates had to be within 1 Ct value of each other to provide meaningful results. The resulting values were then determined using the previously determined primer efficiency score. Primer efficiency scores are important for defining the efficiency of the PCR reaction and are obtained using dilution series of DNA, making a standard curve and utilising the equation ‘Efficiency = 10^(-1/slope)’ to obtain the efficiency score. At this stage of data manipulation the results are referred to as the E-Mean DCT (E-DCT). E-DCT values were then used in GraphPad Prism 5 (GraphPad Software, USA) software to produce line graphs.

Plates with 384 wells were selected for the qPCR format as a large selection of genes had been selected for study due to their nature in the immune systems reaction to MAP infection.

2.10 qPCR Automated plate loading trials

To test whether the potential of loading 384 well plates using a Biomek 2000 automated liquid handling system was viable to produce informative consistent data a 96 well plate format was tested due to the reduced demand on materials. This 96 well plate format was set up for eight genes done in triplicate, with cDNA from animals 92 and 96, as a proof
of concept study for this system. This initial proof of concept QPCR was done in a Micro Amp Fast Optical 96 well plate (Applied Biosystems) using optical adhesive covers to seal the plates. Each well was set up for a final volume of 20 µl. The QPCR were done using master mix 1 × Absolute QPCR SYBR Green low ROX mix (Thermo Scientific), primers at 100 nM, and 5 µl of cDNA per reaction. Primers for the genes B2M, HLA-DMB, RORC, STAT1, STAT6, TLR2, PIAS1 and RANK were used. qPCR protocol followed the pre-described method in the 384 well QPCR section.

Following promising results utilising the automated plate loading system for the 96 well plate a 384 well plate format was tested in this system. Each well was set up as previously described for the 96 well plate. As with the 96 well QPCR protocol the 384 well automated plate was run in the manner described in the 384 well QPCR section of the methods.

2.11 qPCR Automated 96 well to 384 well Automated blotting

To test the application of a 96 channel ‘Biomek NXP multichannel’ pipette machine 96 well plates were set up as shown in Figure 9 below. For each well an initial master mix was made following previously described QPCR protocol with the exception that each well has a final volume of 45 µL. After loading the 96 well plate 40 µL of the mix was pipetted out using the Biomek NXP multichannel pipette machine into the format seen in Figure 10, effectively quadrupling the 96 well plate allowing for four replicates of each sample.
**Figure 9. 96 Well Plate Format**

Above is shown the layout of the 96 well plate. Each well is set up with the corresponding cDNA with each colour representative of a different gene target allowing for the testing of 12 genes and one normalising gene.
Figure 10

Figure 10. 384 Well Plate Format.

The corresponding 384 well plate format after blotting from the 96 well plate format. The colour coding indicates the origin of the target in the 96 well plate. This protocol has allowed for quadruplicates to be done of the 96 well plate.

2.12 Data analysis

Analysis of the sequence data for the CARD15 gene was carried out on Seqman (DNASTAR) as described above. Interpretation of QPCR Ct values was carried out using a manual technique on Microsoft Excel (Microsoft) using the Delta Ct method described in the introduction (section 1.13); this data was checked for significance via Graph Prism 5 (GraphPad Software, USA).
Chapter 3: Results

3.1 Previous Results

Work undertaken during the challenge/biopsy trial (by Dr. Mackintosh and colleagues) determined for each of the sampling points the histopathology of the 14 deer that survived to the end of the trial measured via histopathology scoring (Clark et al., 2010; Mackintosh et al., 2011). Table 4 outlines the histopathology of the deer at the time of necropsy as well as the infection status of each animal. At the necropsy time point based upon their histopathology grade the three most polarised disease state deer representing resistant and susceptible phenotypes were selected for subsequent gene expression investigation and for most of these animals the CARD15 LRR sequence was determined. Initial work on the LRR region of the CARD15 gene was performed using blood taken at week 49 of the infection model. Genotype of these animals is depicted in Table 4 as either an R (resistant) or S (susceptible) beside the animal identification number. Based upon their ability to combat the disease these animals have been classed phenotypically resistant or susceptible with the animals R 92, R91 and S 89 representing the resistant cohort of animals in the QPCR experiments and the animals R 86, S 90, and S 96 represent the susceptible cohort.

3.1.1 JD Infection States

While susceptibility is a comparatively straightforward phenotype to identify as susceptible animals will display clinical symptoms within the time frame, resistance on the other hand is more problematic as a seemingly resistant animal may succumb to the disease given more time. Histopathology scores are based upon Clark et al., 2010 criteria: in short a histopathology score of 0 represents a healthy gut, 1 and 2 as very mild non-specific enteric infection, 3 as very mild JD, 4-7 as mild JD, 8-10 as moderate JD, and 11-13 as severe JD as suggested through histopathological examination of biopsied material (Mackintosh et al., 2011). As can be seen in Table 4, at week 4 there was no disease detected with the exception of animals S 77 and S 80 that exhibited histopathology of 5 and 2 respectively. The 14 week biopsies all of the animals show histopathological scores of 6 or higher, of which animals R 92, R 91 R 88, and S 90 had the histopathological score of 6 while the remainder of the animals had severe JD with histopathological scores of 11 or higher. At week 14 the majority of animals had paucibacillary disease with the exception of animals S 80 and S 81 which had multibacillary disease. Finally when considering the necropsy samples, animals that developed clinical cases of JD during this study were euthanised, of the 14 animals that
survived until the end of the trial 8 of these animals had histopathology scores of 5 or under. These animals therefore had either mild JD/non-specific disease or no disease. While these animals had more severe pathology at the week 12 time point indicative of at least partial recovery but the question remained would the animals now with mild JD progress to clinical disease or carry on with their current trend and successfully overcome the infection had the infection model time frame been extended? Unfortunately this will remain an unanswered question. Of the total pool of animals that survived until the end of the trial only animal R 92 showed no sign of MAP infection at this final time point having a histopathology of 0 and no detectible MAP via Ziehl-Neelsen staining of intestinal biopsies and mesenteric lymph nodes (Mackintosh et al., 2011). The next most resistant animal R 91 had a histopathology of 2 as well as having no detectible organisms in the biopsied tissues in a similar manner to animal S 89 with the exception that S 89 had a histopathology of 3. These three animals, R 92, R 91 and S 89 being the three most resistant animals based upon disease severity represent the resistant group of animals in the QPCR trials. Following the three most resistant animals in order of histopathological score are five animals, R 79, R 84, R 88, R 94, and R 98 that all have a histopathology of 5 as well as having paucibacillary infections. Next three animals have a histopathology of 11 including: S 77 that has a multibacillary disease state, S 80 and S 81 that both have paucibacillary disease. While the three most resistant animals are represented by R 92, R 91 and S 89, the three most susceptible animals include R 86, S 90, and S 96 all of which have a histopathology of 13 and multibacillary disease (Mackintosh et al., 2011).
Table 4

<table>
<thead>
<tr>
<th>Animal</th>
<th>1816</th>
<th>Sex</th>
<th>Disease state</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>12 weeks</td>
<td>Necropsy</td>
</tr>
<tr>
<td>R92</td>
<td>Yes</td>
<td>F</td>
<td>PB</td>
<td>None</td>
</tr>
<tr>
<td>R91</td>
<td>Yes</td>
<td>M</td>
<td>PB</td>
<td>None</td>
</tr>
<tr>
<td>S89</td>
<td>No</td>
<td>M</td>
<td>PB</td>
<td>None</td>
</tr>
<tr>
<td>R79</td>
<td>Yes</td>
<td>M</td>
<td>PB</td>
<td>PB</td>
</tr>
<tr>
<td>R84</td>
<td>Yes</td>
<td>M</td>
<td>PB</td>
<td>PB</td>
</tr>
<tr>
<td>R88</td>
<td>Yes</td>
<td>M</td>
<td>PB</td>
<td>PB</td>
</tr>
<tr>
<td>R94</td>
<td>No</td>
<td>F</td>
<td>PB</td>
<td>PB</td>
</tr>
<tr>
<td>R98</td>
<td>No</td>
<td>M</td>
<td>PB</td>
<td>PB</td>
</tr>
<tr>
<td>S77</td>
<td>No</td>
<td>M</td>
<td>PB</td>
<td>MB</td>
</tr>
<tr>
<td>S80</td>
<td>No</td>
<td>M</td>
<td>MB</td>
<td>PB</td>
</tr>
<tr>
<td>S81</td>
<td>Yes</td>
<td>M</td>
<td>MB</td>
<td>PB</td>
</tr>
<tr>
<td>R86</td>
<td>Yes</td>
<td>F</td>
<td>PB</td>
<td>MB</td>
</tr>
<tr>
<td>S90</td>
<td>No</td>
<td>M</td>
<td>PB</td>
<td>MB</td>
</tr>
<tr>
<td>S96</td>
<td>No</td>
<td>M</td>
<td>PB</td>
<td>MB</td>
</tr>
<tr>
<td>S85</td>
<td>No</td>
<td>F</td>
<td>MB</td>
<td>MB</td>
</tr>
<tr>
<td>S93</td>
<td>No</td>
<td>M</td>
<td>MB</td>
<td>MB</td>
</tr>
<tr>
<td>R82</td>
<td>No</td>
<td>F</td>
<td>PB</td>
<td>MB</td>
</tr>
</tbody>
</table>

Table 4. Animal Histopathology and Infection States over the course of the Trial

Each animal was graded for its respective JD histopathology and disease state (paucibacillary/PB or multibacillary/MB). All of the animals displayed above had CARD15 sequences produced from them. The animals in blue (and the green animal S 89) represent the resistant cohort for the subsequent qPCR expression assays. Those in red (and the green animal R 86) represent the susceptible cohort based upon the severity of disease at the end time point of the trial. The green animals are still included in but are separate from the resistant and susceptible cohort as these animals are genotypically opposed from the other animals in their respective cohorts and act as confounding factors in the qPCR assays. Animals shown in yellow did not survive to the week 49 time point and as such were not investigated in the qPCR trials. This table has been adapted from (Mackintosh et al., 2011).
3.2 RNA Integrity Verification

To determine the quality of the RNA extracted from the tissue biopsies taken from the animals a representative sample was tested using a Agilent 2100 Bioanalyser. Bioanalysers are a useful tool for checking the feasibility of an experiment via ensuring the quality of the sample tested. Over the course of this thesis the Bioanalyser was utilised to ensure the quality of extracted mRNA preparations. The intensity and clarity of the 18S and 28S ribosomal RNA subunits are measured via fluorescence and the clarity of this is used to find the RNA concentration, while the RNA integrity score is a measure of the entire electrophoretic trace of the RNA sample sensitive to RNA degradation. An example of the results from the Bioanalyser is shown in Appendix 2. This assay indicated the RNA integrity score for the representative sample were all within acceptable limits for subsequent work. RNA integrity numbers range from 0 to 10, with anything above 8 considered high quality; all of the representative sample were above 8.

3.3 Aim 1: CARD15 Polymorphisms

Following verification of the quality of the RNA via the Bioanalyser, the cDNA produced from these samples was used as a substrate for primer pair B 3. Using primer pair B 3 the extreme 3’ end of the LRR region was mapped and B 12 was developed that spanned the LRR sequence. Sequence data was generated using primers B 12 by first using gel electrophoresis and then by extracting the LRR band from the gel and purifying the DNA for sequencing. Figure 11 shows a gel photo prior to CARD15 band excision. Using the primer pair B 12 the LRR region has been spanned for the animals 77, 79, 80, 81, 86, 89, 90, 91, and 98 with incomplete sequence data for 84, 88, 92, and 94. The sequence data, aligned against the bovine CARD15 sequence NM_001002889 and the available cervine sequence, highlighted several areas of interest identified by comparison of the produced sequence.

Five areas of synonymous heterogeneity have been detected and one non-synonymous nucleotide substitution that caused an amino acid change, at nucleotide position 1816 where a cytosine (C) is substituted with a guanine (G) causing a glycine to arginine polymorphism, this nucleotide substitution is illustrated in Figure 12. The polarities of the amino acid glycine compared to arginine are different and this shift changes this neutral hydrophilic glycine to the positive hydrophobic arginine potentially altering the ability of this protein to function. When comparing the effect of this polymorphism against the disease state of the animals with
this genotype there was no significant difference between the histopathology between the two genotypes, shown in Figure 13. The substitution at position 1816 was found in 79, 81, 84, 86, 88, 91 and 92 all of which are heterozygotes and have both cytosine and guanine at position 1816. Interestingly of the 7 animals with 1816 6 are R genotype and only animal, 81, is S genotype and 5 of the 7 of this group of animals histopathological scores are 5 or less. As the R animals are fathered from a single resistant sire the 1816 genotype may have been passed down paternally potentially causing an association between this polymorphism and resistance. Although the majority of these animals have low histopathological scores the exceptions to this trend prevented the potential significance of this data set.

**Figure 11**

An example of a agarose purification gel. Left to right the lanes are 1 Kb Molecular weight ladder (Sigma) followed by the PCR product for the representative animals A, B, C, D and E using primer pair B 12. Numbers on the left edge are indicative of the band size of the ladder and are measured in kilo bases. The CARD15 LRR products size is 820 base pairs in length and can be seen as expected.
**Figure 12. Polymorphism 1816**

Polymorphism 1816 has been highlighted (red box), shown in samples 92, 91, 88 and 82 to represent the group of animals showing this genotype. Animals 90, 89 and 77 are displayed to represent the animals without this polymorphism.
**Figure 13. Histopathology of deer wild-type versus 1816**

Above the wild-type versus animals containing the 1816 polymorphism shows no significant difference in the distribution of disease severities between these two genotypes.

Areas of synonymous heterogeneity were found at positions 1728, 1740, 1935 and 2232 shown in Figure 14. In these four areas of heterogeneity the animals found within, with two exceptions, were heterozygotes for both alleles. The polymorphism 1728 has a common base G that in six samples has both G and adenine (A). Polymorphism 1740 is an area where the common base C is co-expressed with a thiamine (T) in three samples, however in samples 84 and 93 only the T residue is present at this locus. Like 1728 1935 is only seen in heterozygotes and it also represents a G common base with a less common A, however 1935 unlike 1728 has only been seen in three samples. The most common polymorphism/area of heterogeneity was 2232, which like 1935 and 1728 had a G common base co-expressed with an A, that was heterozygotic in seven samples and in sample 86 only the allele with A at this locus was present. A diagrammatic depiction of the CARD15 gene including nucleotide domains, the area sequenced using primer pairs B 3 and B 12, and the sites of the
polymorphisms detected are shown in Figure 14. While analysing the cervine CARD15 LRR domain 9 putative LRR motifs have been identified, which differs from the 10 reported for the bovine CARD15 LRR region (Taylor et al., 2006).

**Figure 14**

A) Nucleotide domains. The blue arrows represent the primer pair B3, while the red arrows represent B12. B) Bovine sequence with the available cervine sequence. C) Area that has been amplified using B12. The black arrows represent areas of synonymous heterogeneity while the red arrow represents polymorphism 1816.

**Figure 14. The CARD15 gene sequence**

A) Nucleotide domains. The blue arrows represent the primer pair B3, while the red arrows represent B12. B) Bovine sequence with the available cervine sequence. C) Area that has been amplified using B12. The black arrows represent areas of synonymous heterogeneity while the red arrow represents polymorphism 1816.
3.4 Aim 2: production of a working model

3.4.1 Normalising gene selection

The inclusion of a normalising gene is extremely important to provide the ability to compare different samples. A commonly used normalising gene was selected (B2M) based on its previously identified ability to be used in this role as a gene expressed at a standard rate across different animals. Validation of this normalising gene was carried out using qPCR with 3 well known normalising genes available for cervine work (independently made), including B2M, PPIB and TMBIM4. As seen in Figure 15, the spread of the Ct value fold change for the genes validates the selection of B2M as the normalising gene used as when it was tested it resulted in the most consistent spread of data points with the least extreme fold changes in between samples. However there was a slight decreasing trend in B2M expression in the resistant animals and an increasing trend in the susceptible animals over the course of this trial. During the course of the normalising gene assay an intermediary disease state animal was selected as the calibrator; animal R79 that had a mid-range histopathology score.

3.4.2 qPCR Primer Specificity

Specificity of the primers produced (Appendix 1) using the relevant cervine sequence (obtained via correspondence) was tested utilising a melt curve function at the end of each qPCR run. Using this method to test the primers was the most practical due to time constraints, as sequencing each product was not feasible, and as this allowed checking of qPCR contamination of other primers. Specific primers were obtained for the genes seen in Table 5, while the primers for MAPK14, P53, NOD1, IL-19, and SOCS1 failed to produce specific products. An example of both a specific and non-specific primer pair are shown in Appendix 3.
Figure 15. B2M verification via testing with other normalising genes

Above can be seen the normalising genes tested against each other in an animal specific setting using the animal 79 as a calibrator. Top graphs, B2M as the gene being tested using PPIB and then TMBIM4 as the calibrator. Middle graphs, testing PPIB first using B2M and subsequently by TMBIM4. Bottom graphs, using B2M and PPIB to test TMBIM4. The differences in fold change between samples supports B2M selection.
3.4.3 qPCR gene expression ratios

Using B2M as the gene against which the target genes would be normalised, the qPCR expression of genes related to various immune functions were measured. For clarity the genes tested have been divided into specific pathways representative of the genes primary function, illustrated in Table 5.

Table 5

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Representative genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRR</td>
<td>TLR1, TLR2, TLR6, IRF3, MyD88, TRAF6, NOD2, and NF-κB</td>
</tr>
<tr>
<td>Type one interferon</td>
<td>IFNβ, STAT1, STAT2, and PIAS2</td>
</tr>
<tr>
<td>Type two interferon</td>
<td>IFNγ, STAT1, PIAS1, and SOCS3</td>
</tr>
<tr>
<td>IL-6</td>
<td>STAT3, RORC, and SOCS3</td>
</tr>
<tr>
<td>IL-12</td>
<td>STAT4, Tbet, IFNγ, SOCS3, and PIAS4</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>GM-CSF, STAT5a, STAT5b, and SOCS3</td>
</tr>
<tr>
<td>IL-4</td>
<td>STAT6 and GATA3</td>
</tr>
<tr>
<td>NLR</td>
<td>NLRP3, IL-1β, and IL-18</td>
</tr>
<tr>
<td>NF-κB</td>
<td>NF-κB, GM-CSF, HLA-DMB, RANK, INOS, RANTES, and NLRP3</td>
</tr>
<tr>
<td>MAPK</td>
<td>MAPK8, MAPK11, CREB, cFOS, cJUN, and ATF2</td>
</tr>
</tbody>
</table>

Table 5. Representative immune genes

The immune pathways investigated have been shown with the corresponding representative genes tested. The term pathways have been used over the course of this thesis for simplicity sake even though this analogy may not be entirely correct.
3.4.4 Interpretation of Results

For easy interpretation of the results of the expression assays the mean expression of each pathway (obtained by averaging out the results of the total sample) represented relative to expression of B2M are shown on a graph with the animal specific information displayed in the corresponding Table. Animals with an increased expression relative to the mean are depicted by a ‘↑’ while animals with a relative decrease in expression are depicted by a ‘↓’; animals showing little difference from the mean are represented by a ‘0’. Figure 16 below illustrates how the interpretation of the results have been achieved. The table, Figure 16 B), show the layout of the subsequent results. These data are assigned the ↑/0/↓ value based on a number chosen to illustrate robust differences in expression profiles, found in the figure legends.

An example of how this data was interpreted: taking the Ct values of the target gene and normalising them against B2M (target minus B2M) provides the Delta Ct (ΔCt). As B2M has been expressed at such high levels in this trial compare to the target genes selected this ΔCt is a positive value. Due to the inverse relationship between the Ct value and expression a low Ct depicts high expression. The efficiency of the primer pairs are then taken into account and this value is then squared by the minus ΔCt. The resulting value is target gene expression relative to B2M. These values have been used to produce the tables and graphs displayed throughout the results. The values the assigned symbol used in the tables was chosen to represent clear differences between animals and is representative of the expression levels of this gene relative to B2M.
Figure 16

A)  

![Graph showing expression over time](image)

B)  

<table>
<thead>
<tr>
<th></th>
<th>Week 4</th>
<th>Week 12</th>
<th>Week 49</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
<td>Resistant</td>
</tr>
<tr>
<td>Animal</td>
<td>Animal 89</td>
<td>Animal 86</td>
<td>Animal 89</td>
</tr>
<tr>
<td></td>
<td>↑ ↑ 0 0</td>
<td>↑ ↑ 0 0</td>
<td>↑ ↑ 0 0</td>
</tr>
</tbody>
</table>

Figure 16. Data interpretation model

Above is illustrated the method for the interpretation of the results, the black line within the blue zone (the ‘neutral zone’ indicating little difference from the mean expression is depicted as a 0 in the subsequent tables) represents the target and its expression over the trial, while the red line represents B2M expression A). Table B) shows an example of the layout of the subsequent results. The cohorts, row not subsequently included, are separated by dark lines, however the animals that show genotypical and phenotypical reversal (animals 89 and 86) are shown in green as the qPCR of these animals frequently behaves differently from the phenotype and genotype ‘pure’ resistant/susceptible animals.
3.5 Aim 3: qPCR analysis of immune genes

3.5.1 PRR pathway

Gene expression qPCR assays of the PRR pathway focused on the genes TLR1, TLR2, TLR6, IRF3, MyD88, TRAF6, NOD2, and NF-κB as displayed in Figure 17. The results for the expression of these genes, using B2M as the calibrator (results are displayed as a fraction of B2M) are shown in Figure 18 A. Overall expression of the TLR pathway shows two trends in expression, one in which the sample increases in expression by the end of the trial, while the second trend shows genes decreasing in expression. Compared to TLR1 and TLR6 TLR2 has a greatly decreased expression over the entire trial and unlike these two TLRs follows the declining trend. NOD2 was the other gene showing this declining trend, while the remainder of the genes increased in expression by the end of the trial. When considering animal specific expression (Figure 18, B.) at the week 4 time point each animal shows variation in the expression of these genes compared to the mean with few consistent trends. Animal 92 showed in general increased or neutral expression of these genes, along with animal 91, and 89 with the exception of the TLR genes. This mixed expression carries on into the 12 week time point excluding the animals 90 and 96 that show a general decreases across this gene set. While both the first two time points for this set of genes have a lot of variation within each animal the 49 week time point shows more consistent expression of these gene profiles. The resistant cohort has with few exceptions a decreased expression of these PRR genes while the susceptible cohort have an increased expression the exception being animal 96 that has a mixed expression of these genes.
Figure 17. Pathogen recognition receptor immune activation pathway

This Figure depicts the signal cascades associated with PAMP/DAMP detection. In this instance the relevant members of the PRR pathway are represented diagrammatically in a simplified format.
Figure 18

A) Mean Expression of PRR Pathway

B) Animal specific trends of expression over the trial is illustrated by B). Expression of these genes is +/- 0.02 from mean for TLR1, 0.0001 TLR2, 0.02TLR6, 0.001IRF3, 0.001MyD88, 0.002TRAF6, 0.001NOD2, and 0.001NF-kB. Missing data in the table is represented by *.
3.5.2 Type one interferon

Type one interferons are represented by IFNβ, STAT1 and STAT2 the receptors responsible for detection of type one interferon and PIAS2 the constiutively expressed suppressor of STAT2 activation shown diagramatically in Figure 19. Figure 20 below shows the expression of these genes (measured as a fraction of B2M) over the time course of the infection trial along with the animals specific expression. The mean expression the IFNβ pathway shows increases in the expression of these genes over the course of the trial, the greatest relative increase displayed by PIAS2 that increased by over a factor of 10, with the exception of STAT1 that to a small degree decreased in expression (Figure 20 A.). Animals specific values for the expresion of this gene set shows variation of expression at the week 4 time point with the genotypically resistant deer (92, 91 and 86) have a more positive expression of these genes compared to the genotypically susceptible deer. At the 12 week time point the expression of these genes is varied with differnt animals showing limited trends; animals 92 and 91 have neutral expression of the IFNβ gene and negative expression of the rest of this pathway, while animal 96 has a negative IFNβ expression but a positive expression of the remaining genes. The ‘green’ animals (89 and 86) at the 12 week time point show very similar mainly positive expression profiles. The trends set at the week 49 time point show an increase in expression within 92 and 91, with the exception of the STAT1 gene, and a decrease in expression for the remainder of the samples, excluding animal 90 that has an increase in IFNβ and STAT1 at this time point.
Signalling by IFNβ affects the cell via STAT signalling causing a change in the transcriptional profile of the cell. Above is a simplified version of this signal cascade.
The time line graph shows the mean expression of the IFNβ representative genes A). Expression of the animal specific expression of this pathway +/- 0.002 for IFNβ, 0.02 for STAT1, 0.02 for STAT2, and 0.002 for PIAS2 illustrated in B).
3.5.3 Type two interferon

The IFNγ pathway of cell signalling is represented by the genes IFNγ, STAT1, PIAS1, SOCS3, and Tbet as depicted in Figure 21 in a simplified manner. Below in Figure 22 the line graph of mean gene expression is displayed along with the animals specific expression table. Considering the expression of IFNγ and Tbet the expression does not change to a large extent over the course of the trial although there is a decrease by the end of the trial in IFNγ while Tbet has a marginaly heightened expression. While STAT1 has been discussed previously PIAS1 shows a similar expression to STAT1 with the exception that it has a slightly increased expression at the week 49 time point. Conversely to the other genes representative of this group SOCS3 shows a consistent increase in expression over the time course with over a 10 times higher expression than the begining of the trial. The animal specific expression of these genes at the 4 week time point shows little correlation between animals or groups of animals, with the exception of the expression of Tbet that is primarily expressed neutrally between all animals. Only animals 92 and 91 have an increase in expression of IFNγ at the 12 week time point that carries on to the week 49 time point while the remainder of the genes are varied within the cohorts.
Figure 21

Figure 21. IFNγ signalling

Above the cellular response to IFNγ signalling in a simplified manner is shown. The effect of this signalling occurs in response to homo-dimerisation of two STAT1 molecules entering the nucleus and altering the genetic profile of the cell as well as resulting in the production of the transcription factor Tbet.
**Figure 22**

**A)**

**Mean Expression of IFNγ Pathway**

<table>
<thead>
<tr>
<th>Time point of trial (Weeks)</th>
<th>Expression (Relative to B2M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0001</td>
</tr>
<tr>
<td>10</td>
<td>0.001</td>
</tr>
<tr>
<td>20</td>
<td>0.01</td>
</tr>
<tr>
<td>30</td>
<td>0.1</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
</tr>
</tbody>
</table>

**B)**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Week 4</th>
<th>Week 12</th>
<th>Week 49</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>↓ 0 ↑ 0</td>
<td>↓ 0 ↑ 0</td>
<td>↓ 0 ↑ 0</td>
</tr>
<tr>
<td>STAT1</td>
<td>↓ ↑ ↓ 0</td>
<td>↓ ↓ 0 ↑</td>
<td>↓ ↓ ↓ 0</td>
</tr>
<tr>
<td>PIAS1</td>
<td>↑ ↓ ↓ 0</td>
<td>0 0 ↑ 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>SOCS3</td>
<td>↑ ↓ ↓ 0</td>
<td>0 0 ↑ 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>Tbet</td>
<td>0 0 0 0</td>
<td>0 0 ↑ 0</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

**Figure 22. IFNγ representative data**

The mean expression of the pooled data for the expression of the IFNγ pathway representative genes is shown in A). The expression of these genes within the animals is displayed showing +/- value of 0.0002 for IFNγ, 0.02 for STAT1, 0.02 for PIAS1, 0.002 for SOCS3, and 0.001 for Tbet B).
3.5.4 IL-6 signalling

IL-6 signal pathway is represented by the genes STAT3, RORC and SOCS3 and is shown in Figure 23. As shown in Figure 24 the mean expression of the combined data for STAT3 shows an initial consistent expression between the first 4 weeks to the 12 week time point that by the week 49 time point has increased by almost a factor of 10. While SOCS3 has previously been described, RORC has a heightened initial expression that decreased by the 12 week time point and subsequently returns at the week 49 time point to almost the same level of expression as the start of the trial. For each animal and cohort there is mixed expressions at the week 4 time point with animals 92 and 96 showing neutral to increased expression while the remainder of the samples are varied. This varied expression remains the case to the 12 week time point with the exception of animals 92, 89 and 86 that have neutral or increased expression at this time. The green group at this time point do show similarities in expression profiles however 89 does have heightened expression. By the week 49 time point animals 92 (excluding STAT3) and 91 have heightened expression of these genes while the remaining 4 animals, the phenotype genotype conflicting animals (89 and 86), cluster with the susceptible animals having negative expression of these genes.
In a manner similar to IFNβ and IFNγ signalling, the detection and response to IL-6 is achieved via STAT molecules becoming active, entering the nucleus and having transcriptional properties. In this instance the transcription factor RORC is produced that can then further affect the genetic profile of the animal.
Figure 24

A)

**Mean Expression of IL-6 Pathway**

Line graph shows the mean expression of the pooled data of the sample group combined for the IL-6 pathway genes in A). Expression, compared to B2M, of each gene for each animal is +/- 0.02 for STAT3, 0.001 for RORC, and 0.002 for SOCS3 B).
3.5.5 IL-12 signalling pathway

IL-12 signalling has been represented in this study by STAT4, Tbet, IFNγ, SOCS3, and PIAS4 shown in the representative diagram Figure 25. The expression of the mean trend of the combined data for the expression of the IL-12 pathway is shown in Figure 26 A. It is apparent that both STAT4 and the inhibitor of STAT4 PIAS4 are expressed at similar levels at each of the time points and both of these genes increase by nearly a factor of 10 over the 49 week trial. The genes IFNγ, Tbet and SOCS3 have already been described. Expression of these genes in an animal specific manner shows that at the 4 week time point there is little difference between the animals or cohorts (Figure 26 B). Moving to the 12 week time point there is still a spread of values across the animals, with the exception of animal 92 that has a neutral to increased expression of these genes and animals 90 and 96 that have a neutral to decreased expression of these genes. Interestingly the next most resistant animal 91 has a positive value for and IFNγ and a neutral value for Tbet while the expression of the rest of these genes are negatively expressed at this time point. At this 12 week time point the green animals (89 and 86) have similarities with the two genotype phenotype resistant animals however by the week 49 time point they have the appearance of the susceptible animals. At the 49 week time point only the animals 92 and 91 have positive expressions of these genes with the exception of Tbet whereby 92 is neutral and 91 is negative. Animal 90 showed the only exception to this trend for the genes Tbet that is positive and IFNγ for which it is neutral.
As has been previously described the detection of IL-12 is achieved by STAT molecules altering the genetic profile of the cell, along with production of other transcription factors including Tbet.
Figure 26

A)

Mean Expression of IL-12 Pathway

![Graph showing mean expression of IL-12 pathway genes over time.]

B)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Week 4</th>
<th>Week 12</th>
<th>Week 49</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>92</td>
<td>91</td>
<td>89</td>
</tr>
<tr>
<td>STAT4</td>
<td>0</td>
<td>0</td>
<td>↓</td>
</tr>
<tr>
<td>Tbet</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IFNγ</td>
<td>↓</td>
<td>0</td>
<td>↑</td>
</tr>
<tr>
<td>SOCS3</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>PIAS4</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

**Figure 26. IL-12 representative pathway**

The mean pooled data for the entire sample group depicted in a line graph of the IL-12 pathway genes over the 49 week trial A). Expression for each animal is shown compared to B2M, +/- 0.02 for STAT4, 0.001 for Tbet, 0.0002 for IFNγ, 0.002 for SOCS3, and 0.002 for PIAS4 B).
3.5.6 GM-CSF

Cell signalling via GM-CSF, depicted in Figure 27, is detected by the STAT5a STAT5b receptor motif and this signalling is supressed by SOCS3. Below in Figure 28 is the expression ratios of GM-CSF, STAT5a, STAT5b and SOCS3. When observing GM-CSF expression profile there is an initial rise in expression of greater than 5 times higher from the week 4 to 12 time point that decreases to between the week 12 and week 4 values at the week 49 mark. The expression of STAT5a and STAT5b are closely expressed and both increase by around 10 times their starting values between the week 4 and week 49 time points with a semi linear increase in expression. Specifically for each animal at the week 4 time point the animals 92 and 96 have neutral or positive expression of these genes while the remainder of the animals have varied expression, with the exception of animal 90 that has negative expression of these genes. This varied expression continues into week 12, with the exception of animal 91 that has negative expression of these genes and 90 the only animal with an increase in the expression of GM-CSF versus the mean and neutral expression of the remaining genes. The expression of the GM-CSF pathway by the week 49 time point resembles the IL-12 pathway whereby the phenotypically and genotypically resistant animals 92 and 91 have increased expression while the remaining animals, both the phenotypically and genotypically susceptible animals and the two conflicting animals, have a reduced expression profile, with the exception of animal 86 that has positive expression of GM-CSF.
**Figure 27. Cell signalling via GM-CSF**

Cellular reactivity to GM-CSF is achieved via detection and subsequent genetic manipulation by STAT molecules. This signal cascade is inhibited by SOCS3.
Above is the expression of the pooled data from all the animals for the genes representing this pathway A). Animal specific expression, using B2M as the calibrator, is based upon +/- 0.00002 for GM-CSF, 0.02 for STAT5a, 0.02 for STAT5b, and 0.002 for SOCS3, B).
3.5.7 **IL-4 signalling pathway**

Representing the signalling pathway of IL-4 are the genes STAT6 and GATA3 shown interacting in the cell in Figure 29. The results of these two genes mean expression, calculated using the combined data of all the animals, is shown below in Figure 30 A. While the expression of STAT6 does not change to any large extent between week 4 and 12 by week 49 it has increased to around 10 times the starting expression. Conversely GATA3 does change between the week 4 and 12 time points where it decreases by around 5 times its starting value after which it returns to close to its starting value by the week 49 time point. The expression of these two genes is quite closely conserved, at the 4 week time point, between individual animals and both cohorts that remains the case to the 12 week time point. By the week 49 time point the phenotypically and genotypically resistant animals 92 and 91 show positive expression of these two genes while the remaining animals have reduced expression of these genes.
The affect upon a cell of detection of IL-4 is achieved via STAT activation and translocation into the nucleus affecting the transcriptional properties of that cell and in some instances causing the production of the transcription factor GATA3.

Figure 29. IL-4 signalling
Above the mean expression, of the combined data from the entire animal set, changes over the course of the trial for the genes STAT6 and GATA3 A). For each animal the expression as a fraction of B2M is expressed whereby the specific values are +/- 0.01 for STAT6 and 0.001 for GATA3 B).
3.5.8 NLR pathways

This pathway has been represented by the genes NLRP3, IL-1β and IL-18 shown interacting below in Figure 31. Expression profiles of these genes shown in Figure 32 include the mean expression of the pooled data for the genes NLRP3 and IL-1β that have an initial rise in expression from the 4 week time point to the 12 week time point after which, excluding minor changes, remain at a similar expression level. Conversely IL-18 has a minor decrease between the week 4 and 12 samples that subsequently increases to finish with an increase in expression on the 4 week sample. Similarly to the IL-4 and IL-12 pathways, the initial expression of these genes for the two cohorts show that the resistant cohort has either a neutral or increased expression of these genes at the 4 week time point while the susceptible cohort has neutral or reduced expression. The week 12 time point shows similar trends with the resistant cohort, with the addition of the phenotypically/genotypically conflicting animal 86 appering as a resistant animal, being mainly neutral with some increased and reduced expression, however the susceptible cohort is more varied. The other members of the susceptible cohort, which are otherwise negative (the exception being 90 with an increase in IL-1β). By the week 49 time point the resistant cohort including the genotypically susceptible animal 89 has a decreased expression of these genes while the susceptible cohort including the genotypically resistant animal 86 have an increased expression of these genes.
Figure 31

Once the inflammasome has been produced in response to inflammatory mediators the detection of DAMP causes the activation of the pre-cytokines IL-1β and IL-18 into their biologically active form.
**Figure 32.**

A) The inflammasome

Above the mean expression of the pooled data set for the genes NLRP3, IL-1β and IL-18. Expression for individual animals as a fraction of B2M has been shown as the value of (+/-) 0.0001 for NLRP3, 0.001 for IL-1β, and 0.002 for IL-18 in B).
3.5.9 NF-κB pathway

The NF-κB pathway, shown below in Figure 33, includes NF-κB, GM-CSF, HLA-DMB, RANK, INOS, NLRP3, and RANTES. Expression profiles of these genes are shown below in Figure 34. While the mean expression of the genes NF-κB, GM-CSF and NLRP3 have been previously addressed, the expression of HLA-DMB has an initial decrease in expression between the 4 week and 12 week time points that finishes at a elevated level by the week 49 time point. Mirroring this RANTES has a similar expression through out the trial, albeit at a slightly reduced level. The expression of INOS has a large initial decrease in expression at the 12 week time point dropping around 10 times from the initial starting 4 week value. It subsequently finishes between the week 4 and week 12 values. Investigation of the specific expression levels at the 4 week time point indicates there is a range of different values of these genes with a great deal of variability within animals as well as between them, the exception to this generality is animal 96 that has at this time point an increase in expression of most of these values with the remainder being expressed neutrally. In a similar manner to the 4 week time point the expression of these genes at the 12 week time point is very varied with only weak trends. The week 49 time point shows interesting clustering of animals and animal groups, the resistant animals (genotypically and phenotypically) have very similar expression profiles of these genes with only minor disimilarities, while the conflicting animals (89 and 86) share most similarity with the susceptible cohort. All 4 of this group of animals (89 and the susceptible cohort) have increased expression of NF-κB, with the exception of animal 90 that has a missing data point for this gene.
Figure 33

Figure 33. Transcription of NF-κB targets

Post activation NF-κB translocates to the nucleus where it has its effect transcribing its target genes. The activation of NF-κB can be caused by pathogen recognition or via cytokine signalling; however in this simplified representation it is the pathogen recognition. Activated NF-κB has many target genes producing proteins of varied functions, some of which are shown above.
Figure 34

A.

Mean Expression of NF-κB Pathway

B.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Week 4</th>
<th>Week 12</th>
<th>Week 49</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>92</td>
<td>91</td>
<td>89</td>
</tr>
<tr>
<td>NF-κB</td>
<td>↓</td>
<td>0</td>
<td>↓</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0</td>
<td>↑</td>
<td>0</td>
</tr>
<tr>
<td>HLA-DMB</td>
<td>0</td>
<td>↓</td>
<td>0</td>
</tr>
<tr>
<td>RANK</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>INOS</td>
<td>↑</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NLRP3</td>
<td>↑</td>
<td>↑</td>
<td>0</td>
</tr>
<tr>
<td>RANTES</td>
<td>0</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>

Figure 34. NF-κB representative targets

Mean expression of the combined data sets for the NF-κB representative gene subset is shown in A). The expression as a fraction of B2M +/- 0.001 for NF-κB, 0.00002 for GM-CSF, 0.01 for HLA-DMB, 0.01 for RANK, 0.0001 for INOS, 0.0001 for NLRP3, and 0.002 for RANTES in B).
3.5.10 MAPK pathway

The MAPK pathway will be represented by the genes for MAPK8, MAPK11, CREB, FOS, JUN, and ATF2 shown in cellular pathways in Figure 35. Gene expression represented as a fraction of expression of B2M acting as the constant marker within these genes are shown below in Figure 36. The expression profiles of the genes CREB and JUN are closely linked and strongly resemble each others expression. Initially these genes start at a similar level at the 4 week time point and both increase by week 12 in expression by over a factor of 5 before decreasing marginally at the week 49 time point. Similarly to CREB and JUN MAPK8 and MAPK11 also share a similar profile pattern, however MAPK11 has over a factor of 10 times lower expression than MAPK8 throughout the entire trial. By the week 12 mark these two genes have reduced in expression by around a factor of 5 and then by week 49 increase in expression to just over the starting amount. There is a slight increase in the expression of FOS over the trial; however the data for FOS is missing the week 12 time point. Finally ATF2 has an increasing expression trend throughout the entire trial that increases in an almost linear fashion to end at around a factor of 5 times higher than where it started. The expression of the individual animals and cohorts show varied results for these genes. Interestingly for the 4 week time point the expression of the transcription factors activated by this pathway (excluding NF-κB as it was not included) was increased in the resistant cohort. The animals 92 and 96 have neutral or increased expression of this gene set, while 89 has a consistent neutral expression while the remaining samples have varied expression. Varied expression, with few consistent trends of these genes remains the case for the 12 week time point as well as the week 49 time point.
Figure 35

While the MAPK pathway is also activated in a response to cytokine signalling for simplicities sake this pathway is shown above being activated in response to pathogen detection. Upon activation this pathway results in the activation of various transcription factors that subsequently affect the genetic profile of the cell and that cells ability to combat pathogenic challenge.

Figure 35. The MAPK pathway
Figure 36

A)

Mean Expression of MAPK Pathway

Expression (Relative to B2M)

Time point of trial (Weeks)

B)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Week 4</th>
<th>Week 12</th>
<th>Week 49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>92</td>
<td>91</td>
<td>89</td>
</tr>
<tr>
<td>MAPK8</td>
<td>↑↓</td>
<td>0</td>
<td>↑0</td>
</tr>
<tr>
<td>MAPK11</td>
<td>↑↓</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CREB</td>
<td>0</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>FOS</td>
<td>↑</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>JUN</td>
<td>↑↑</td>
<td>0</td>
<td>↓</td>
</tr>
<tr>
<td>ATF2</td>
<td>0</td>
<td>↑↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

Figure 36. MAPK pathway

Above in Figure 36 the expression of the mean of the total sample and the trend of these genes over the time course are shown in A). Expression of these genes, as calibrated to B2M, are +/- 0.001 for MAPK8, 0.0001 for MAPK11, 0.001 for CREB, 0.001 for FOS, 0.001 for JUN, and 0.001 for ATF2 B).
3.6 Automation of qPCR

The automation of the 384 well qPCR by the Biomek 2000 automated liquid handeling system initially presented some technical issues, despite working well for a 96 well trial plate. Due to the complexity of the initial setting up coupled with the high demand on materials it did not initially prove to be a viable option. An alternative was then identified whereby the qPCR sample master mixes were prepared in a 96 well plate and then using the ‘Biomek NXP multichannel’ pipette machine blotted the 96 well plate into the 384 well plate format (as displayed in the methods section 2.11). The application of this technique proved to be not only a time saving technique, but also it provided more consistent data with tighter Ct values between replicates. Results obtained using this technique includes part of those shown above. Observed differences between replicates for the automated qPCR was generally less than 0.4 Ct, while for the manual qPCR there was up to 1 Ct difference between replicates.
Chapter 4: Discussion

4.1 CARD15 polymorphisms

Five areas of synonymous heterogeneity were identified, in the CARD15 gene of the NOD2 PRR, during the course of this research project which do not affect the amino acid sequence. There is no indication that they are associated with the resistant or susceptible host phenotype. A non-synonymous polymorphism 1816, which was only found in heterozygotes, results in a glycine to arginine amino acid substitution. Amino acid substitutions have been previously identified as a factor that can result in higher prevalence of CARD15 related diseases such as JD and CD. Pinedo and colleagues found an amino acid substitution, where a cysteine was substituted with an arginine at amino acid position 733, that in heterozygotes resulted in increased risk of JD in cattle (Pinedo et al., 2009). This particular SNP has not been detected in this study. Unlike the polymorphism identified by Pinedo and colleagues when comparing the histopathology scores against the animals with polymorphism 1816 the effect of this polymorphism at nucleotide position 1816 may or may not be involved as a factor influencing severity of JD as although 1816 is in the LRR domain it is found in between the individual LRR motifs. When considering the 1816 amino acid substitution the amino acid polarities as well as the side chain dimensions are very different so this substitution may warrant further study to see if the protein/function is affected. Animals with this allelic state have a range of histopathology scores from 0 to 13. This trait was mainly associated with the resistant cohort (Table 4), having passed down paternally as 6 of the 9 resistant animals have this polymorphism. Of the resistant animals with this polymorphism 5 of the 6 have histopathological scores of 5 or less. Only one susceptible animal had this polymorphism, animal 81 which had a histopathological score of 11 at the time of necropsy. The high prevalence of this polymorphism in resistant deer indicates that this polymorphism maybe a contributing factor in JD resistance, acting in a protective capacity. Although in this study this trend was not significant to animal health, the sample size in this study is too small to state this with any certainty.

The LRR region was selected for investigation due to the previously reported susceptibility associated polymorphisms that have been identified within it for both the pathologically similar disease CD and in other studies of animals with JD. As previously described the three primary human susceptibility polymorphisms to CD are found within or in
the region of the LRR and the bovine CARD15 mutation that was found in the LRR domain. The LRR region is responsible for the interaction of the NOD2 PRR and bacterial cell wall peptidoglycan (Gaya et al., 2006; Pinedo et al., 2009). The frequency of CARD15 mutations in homozygotes with CD is around 1 in 25 indicative of there being other genetic and or environmental factors that account for total disease penetrance (Gaya et al., 2006). Supporting the complexity of incomplete disease penetrance due to a single causal factor, CARD15 polymorphisms in Taylor and colleagues study found no evidence of a simple causal relationship between CARD15 mutations and disease severity in 11 JD positive animals (Taylor et al., 2006). In this study there was no evidence for a causal link between CARD15 polymorphisms and disease severity.

4.2 Establishment of the working model

Selecting deer genotypes from resistant and susceptible sires was carried out by testing their respective offspring, via ELISA for circulating anti MAP antibodies, over the course of 8 years to identify the incidence and severity of naturally occurring JD (Mackintosh et al., 2011). Using these observations the resistant and susceptible sires were selected. From these sires frozen semen was used for artificial insemination of crossbred red deer hinds resulting in progeny being born within 7 days of each other. Subsequently these animals were run in a single mob and as such were exposed to similar environmental factors that might affect disease resistance (Mackintosh et al., 2011). Gender represented the only variable not accounted for in the trial, however gender bias was not detected (Mackintosh et al., 2011). Of the deer artificially infected all of the animals became infected and 17 % of those these animals declined to clinical disease similar to the previously reported 15 % potential JD prevalence in young animals (Mackintosh et al., 2011).

The research in this thesis has been carried out as a pilot study and as such the qPCR data presented in this thesis is preliminary and many of these results have yet to be replicated in separate experiments. The deer used in this study were the bred from the two sires however the genotypes of the dams (as they were from an out-bred population) were unaccounted for effecting the individual animal variation. Also it is important to consider that this experiment has been performed under conventional farming conditions and although these deer were run in a single mob to help control outside influences such as disease and stressors that had the potential to affect the deer to differing levels. Environmental factors like this (that are unaccountable) could mask/exaggerate immunological responses. Despite these limitations
studying out-bred populations are appropriate to identify robust differences in gene expression ratios (Taylor et al., 2008).

A recent study carried out by Galindo and colleagues found that the expression profiles among red deer and cattle in response to mycobacterial infection resulted in similar expression patterns for IL-1β and p53 genes (Galindo et al., 2010). Other similarities observed between red deer and cattle included modulation in calcium processing, metabolic processes, cytokine signalling and cellular processes including proliferation and motility (Galindo et al., 2010). Immune responses to MAP as well as disease progression and genetic resistance have been well documented in bovine and ovine models (Burrells et al., 1998; Coussens, 2004; Mackintosh et al., 2011), however there is much less information concerning disease progression and heritable resistance in red deer (Mackintosh et al., 2011). The initial innate and then subsequently the adaptive immune response greatly influence the severity of disease (Mackintosh et al., 2011).

4.2.1 B2M normalising gene

Utilising B2M as a normalising gene is common in qPCR experiments (Livak and Schmittgen, 2001). This gene was selected as the normalising gene due to previous use of this gene for this role, however during the course of writing this thesis it became evident that this gene’s expression is affected by interferons and due to the nature of this experiment this potentially acted as a confounding factor (Commins et al., 2010). Multiple normalisation genes were not used due to the high number of gene targets already being investigated and the demand for space required for extra genes.

4.2.2 Automation of Laboratory techniques

Similarly to Wilke and colleagues investigations into automating laboratory techniques using the ‘BioMek NXP mutichannel’ pipette machine to load the 384 well qPCR plates, compared to loading the same plates manually, resulted in more consistent results with less sample mix up as well as less contamination due to the automated nature of this method and therefore less human error (Wilke et al., 1995). The quality of the results increased as the replicates showed an increase in consistency with fewer outliers attributable to the increase in pipetting accuracy achievable using an automated system. Manual qPCR assays have a spread between replicates of less than 1 Ct while the automated system usually had less than a 0.4 Ct spread. Automation of the qPCR also resulted in a significant reduction in the time to
complete the assay. This automation trial was not scientifically measured due to its secondary nature; this system was a definite advantage and worthy of recognition.

4.2.3 JD infection states

In an ovine JD infection model there is typically an asymptomatic phase that is superseded by a paucibacillary phase that culminates in the multibacillary form of paratuberculosis (Gillan et al., 2010). When considering the histopathology data it is of interest to note that the three worst effected deer/those with the worst histopathology have the multibacillary end state form of MAP infection as is seen in the bovine model (Gillan et al., 2010). As suggested previously, multibacillary disease is associated with a humoral immune response that is inappropriate for clearance of intracellular pathogens (Nalubamba et al., 2008). The paucibacillary form of JD was seen in two grade 11 deer and whether this is the end state of disease for these animals is unknown, as they may have progressed to multibacillary disease. Paucibacillary disease, is associated with a self-limiting disease in both tuberculosis and leprosy, is in ovine JD one end state of MAP infection (Nalubamba et al., 2008). The paucibacillary form of JD represents a state where the growth of MAP is still under regulation by IFNγ and TNFα and therefore there is still some activation of macrophages and as such it is unknown if these animals would succumb to JD or clear the infection (Nalubamba et al., 2008). There are various factors influencing whether or not an animal will become sick, develop paucibacillary disease as an end state, progress to multibacillary disease or develop an asymptomatic paucibacillary diseased state (Mackintosh et al., 2007). In this model outside influence was accounted for as the age of the deer was constant, each reaching four months of age prior to being infected with approximately the same infectious dose of bovine MAP and they were exposed to similar environmental factors/stressors as they were kept in the same paddock to minimise complicating factors.

4.3 Expression Levels of Candidate Immunological Factors

4.3.1 Innate Immunity

The expression of the TLRs did not show the level of consistency that was anticipated, with each gene having dissimilar expression ratios over the course of the trial. Expression of TLR1 decreased in between the 4 and 12 week time point to rise to similar to starting level; TLR2 increased at the 12 week time point before returning to starting level; TLR6 increased in a near linear fashion throughout the trial. As the functionality of TLR2 is achieved by forming heterodimers with TLR1 or TLR6 (or non TLR molecules such as CD14) the
expression ratios were expected to be similar for these molecules to interact functionally, subsequently providing the pathogen recognition ability (Bhide et al., 2009). The TLR dimer TLR2 – TLR6 is the main constitutive responsible for the detection of MAP, however the expression of TLR2 was much lower than TLR6 indicative of these genes not being tightly linked (Whittington et al., 2011). The other PRR investigated NOD2 showed an expression profile similar to that of TLR1. Changes in the expression ratios of these genes could be due to several different reasons including that the expression of these genes could be upregulated/down regulated in individual cells or potentially the nature of the cells in the tissue has changed to those with a heightened expression of these genes potentially caused by innate/adaptive immune cell influx.

Comparison of the gene expression ratios for the representative PRR genes between the resistant and susceptible animals at the 4 week mark is uninformative due to the lack of uniform consistent expression within these groups. The 12 week time point however does show a slight trend favouring the expression in the resistant animals with the exception of the (genotypically resistant) animal 86 (part of the susceptible cohort). By week 49 the week 12 trend is reversed as at this time point the susceptible animals show increased expression in these PRR genes. In an ovine challenge experiment the response to MAP infection caused an upregulation of TLR2 at the site of infection (Taylor et al., 2008). These results could be suggestive of an increase in innate immune cells (versus cells with no expression of these genes) or an increase in cellular effectors or expression of these genes within the cell responding against MAP in the resistant animals at the 12 week time point (Taylor et al., 2008). By the week 49 time point the resistant animals have cleared most of (if not all in the case of animal 92) the pathogenic challenge. The need for heightened innate cell activation/presence in the mesenteric lymph nodes, at week 49 is not required, while the susceptible animals are still actively fighting the challenge and as such still have a heightened expression of these genes. An explanation for the observed ratios at the 12 week time point could be in the way that MAP can hyper-activate TLR2 and subsequently cause an anti-inflammatory response producing IL-10 and reducing INF-γ production in the susceptible animals and subsequent immune activation causing reduced immune activity at the site of infection (Khalifeh and Stabel, 2004). Bridging the gap between PRR and transcription factor are the intermediary messenger molecules MyD88 and TRAF6; the trends laid down by the resistant and susceptible animals PRR are very similar for these genes.
Downstream of TLR activation due to a pathogen/self-antigen is the activation of response regulators including IRF3, MyD88, and TRAF6. When comparing the mean expression of these PRR against their downstream signalling pathways shows limited similarity in the expressions of these two groups of genes. Within the expression of MyD88 and IRF3 these two genes are expressed in the mesenteric lymph node tissue during this trial at similar level and share a similar expression profile over the course of the trial, while TRAF6 expression initially mirrors TLR2s expression, except is expressed at a higher level, after the 12 week time point it runs parallel to the MyD88 IRF3 genes (except at a higher expression level). Induction of the PRR pathway eventually leads to transcription factors such as IRF3 and NF-κB; the expression profile of NF-κB (as the profile of IRF3 has previously been described as very similar to MyD88) is similar to NOD2 and TLR1. The similarity in expression could be explained by the regulator function of NF-κB as NF-κB (if active) will cause an increase in NOD2 expression in cells of the innate immune system (Stronati et al., 2008). The expression of IRF3 shows strong correlation to IFNβ as active IRF3 causes the production of IFNβ (Kawia and Akira, 2009). Decreases in NF-κB at the 12 week time point could be a consequence of an increase in B2M expression as it is well known that B2M increases in expression due to exposure to IFN signalling potentially compromising its role as the normalising gene (Commins et al., 2010).

Interpretation of the CARD15 expression ratios indicates that there are no consistent differences between the resistant animals and the susceptible animals at the 4 week time point. By the 12 week time point the CARD15 expression profile is similar to the trends in the NF-κB profile, with the resistant animals showing neutral expression while the susceptible animals have individual consistency but as a cohort are completely mixed (86 has neutral expression of NF-κB and CARD15, 90 has negative expression of these genes, and 96 has positive expression of these genes). At the week 49 time point the expression levels appear lower in the resistant animals. As has been documented in CD patients there is an increase in expression of CARD15 mRNA in the inflamed tissues of the gut due to inflammatory processes (Berrebi et al., 2003); increases of CARD15 in the susceptible animals could be indicative of the fact that there is still an active MAP infection and as such there is active inflammation.

It is apparent that the transcription factors IRF3 and NF-κB genes are expressed at equivalent levels in the resistant and susceptible groups, with the exception of the 4 week time point. The resistant animals have heightened expression of the IRF3 gene and IRF3
downstream target IFNβ and reduced expression of NF-κB while the susceptible group of animals has the opposite expression of these genes. This result could represent a mechanism of resistance to MAP infection as IRF3 is a potent activator of type one IFN, helping to activate/enhance a Th1 acquired immune response to clear the intracellular pathogen (Kawia and Akira, 2009). In a manner similar to the reduced level of PRR in the resistant animals at the week 49 time point the heightened level of NF-κB expression in the susceptible animals could relate to the active infection in these animals, activating NF-κB and producing the positive feedback loop causing this heightened expression of this gene (Barnes, 1997). One animal (92) showed very high expression of the IFNβ genes, while this may represent an outlier it may be associated with naturally heightened expression of these genes. The resistant genotype animal in the susceptible cohort, animal 86 had elevated levels of STAT1 at this time point and neutral expression of IFNβ giving it the appearance of a resistant animal at this stage. While the resistant group had the heightened expression of this pathway at the 4 week time point the susceptible animals have the increased expression at the 12 week time point, which may represent an initial delay in the expression profile of these genes. At week 49 it is however the resistant animals that have the increased expression of these genes (almost exclusively animals 92 and 91) reflecting a decrease in expression of this pathway in the susceptible animals due to MAP immune modulation. Alternatively it could relate to a relative decrease in MHC I expression in the resistant animals and therefore a decrease in B2M expression due to the decreased immune activation as the pathogenic challenge had been cleared (Netea et al., 2004; Commins et al., 2010).

Expression of the IFNβ group of genes shows that some of these genes are similarly expressed throughout the trial including IRF3 and IFNβ both presenting low increase throughout the trial; as anticipated due to the fact that IRF3 is responsible for the transcription of IFNβ in APC (Kawia and Akira, 2009). The effect achieved in response to IFNβ binding actives STAT1 and STAT2 heterodimers that subsequently have transcriptional activity. While both STAT1 and STAT2 are required for the signalling of IFNβ these two genes are expressed differently throughout the trial, STAT2 increasing at both time points while STAT1 remains the same between the 4 and 12 week time points and decreases at the week 49 time point. The actual expression levels of these genes show that at the 4 week time point STAT2 is expressed less than STAT1 by around a factor of 3, however at the 12 week time point the expression of these two genes is almost identical after which at the 49 week time point STAT2 finishes around 3 times higher than STAT1; considering the nature of activation of
these transcription factors the increase in STAT2 to similar levels as STAT1 could be attributable to the requirement of IFNβ signalling having both STAT1 and STAT2 at similar levels (Commins et al., 2010). Due to the levels of IFNβ versus IFNγ the requirement for similar levels of these STAT molecules becomes apparent as IFNβ is more highly expressed than IFNγ. The expression of the inhibitor of STAT2 activation PIAS2 is initially expressed in a parallel manner to IRF3 and IFNβ, but at a slightly higher level than these two genes. As PIAS2 is constitutively expressed this increase in expression could be attributed to increases in STAT2 expression levels or cells containing STAT2, however the expression of these two genes is not parallel potentially indicating an initial increase in STAT2 expression or inhibition of PIAS2 transcription.

The inflammasome component NLRP3 showed a large increase in expression, over a factor of 5, between the week 4 and 12 time points, attributable to the heightened inflammatory response in the biopsied tissue (Kawai and Akira, 2009). The expression of the cytokines that require NLRP3 activation to be expressed in their biologically active form, IL-1β and IL-18, have different expression profiles and both are expressed at a higher rate than NLRP3. The expression of IL-1β initially resembles the expression of NLRP3, although the initial change in expression was not as large, as a result of the pro-inflammatory nature of the mesenteric lymph node and the increased requirement of the pro-inflammatory IL-1β (Galindo et al., 2010). The expression of the secondary inflammatory cytokine IL-18 did not change significantly over the course of the trial. Considering that one of the primary functions of IL-18 is the repair of damaged tissue via inflammatory mechanisms the expression of this cytokine was anticipated to have increased over the course of the trial (Zaki et al., 2010). The expression of these genes in a cohort specific manner indicates a clear and interesting role of these genes over the course of the MAP infection trial. Resistant animals had a heightened expression of these genes over the 4 and 12 week time points and reduced at the 49 week time point as the pathogenic challenge was cleared. The Inflammasome potentially acts as a mechanism of resistance as a higher initial expression correlated with the resistant animals ability to clear the disease while maintaining superior enteric health and as the susceptible animals only gained relative increased expression of these genes at the week 49 time point this is attributable to decreases in resistant profiles of these genes (Mackintosh et al., 2011; Zaki et al., 2010).

Expression of the NF-κB pathway was not as clear as was anticipated, as the expression of NF-κB and many of its transcriptional products were not expressed in a parallel
manner as expected. The expression of INOS and NF-κB was the main exception to this rule, as the expression of these two genes indicates linked expression, although INOS is expressed at a lower level. Initially these two genes decrease in expression by over a factor of 5 at the 12 week time point before increasing to near or above the starting level. This expression trend could be representative of the repressive nature of MAP infected tissue and therefore un-activated NF-κB causing less accumulation of NF-κB products or alternatively this pathway was less expressed in the lymph node level due to T and B cell influx, however along with T and B cell influx macrophages, one of the most prominent sources of INOS, are also recruited and as such these results were unexpected (Coussens, 2004). Along with INOS the MHCII component HLA-DMB did show some similarity in the level of expression compared to NF-κB though not to the same extent as INOS. The lower level of expression of this gene was not anticipated and may be due to MAPs ability to down regulate the expression of MHCII in macrophages (Hestvik et al., 2005). Expression of MHCII is essential for the full activation of the acquired immune system, and is controlled by the transcription factor NF-κB (Hestvik et al., 2005). Showing similar expression patterns to MHCII the cytokine RANTES was expressed in a parallel fashion to HLA-DMB in a reduced manner, both of these genes are expressed in APC as a result of the activation of NF-κB and as such the similarity in expression is not surprising (Commins et al., 2010). While RANTES is primarily produced by T cells in the granulomas found in JD the relative high concentrations of TNFα and IFNγ were expected to cause an increase in RANTES expression from epithelial cells, however this was not the outcome as RANTES did not positively change expression to a large extent (potentially explainable by a relative increase in B2M expression) (Marfaing et al., 1995). The expression of RANK did not change over the course of the trial. While RANTES is a chemo-attractant of memory T cells, RANK is constitutively expressed in memory T cells, and as such the expression of these genes was expected to rise reflective of an increase of these cells (as memory T cells were produced) (Franchimont et al., 2004 B).

The gene expression profiles of the NF-κB pathway genes show that there was little uniformity between the animals in each cohort. Some animals with decreases in NF-κB expression had positive expression of the majority of the remainder of the gene set; it is interesting to note that in this trial the expression of NF-κB and the transcription of its downstream products showed little similarity; the transcriptional properties of NF-κB are acquired post activation of NF-κB and its subsequent translocation into the nucleus of the cell and as such the expression levels of NF-κB and its products are not tightly linked (Barnes,
Toward the end of the trial the susceptible animals shared a trend towards increased expressions of these genes, which may relate to persistent infection in these animals.

Expression levels of the MAPK genes MAPK8 and MAPK11 were very similar, with the exception that MAPK8 was expressed one log higher, both having a dip in expression then finishing at around starting levels. This expression profile is very similar to NF-κB; this was expected due to these molecules being involved in the same signal transduction pathways (Shorey and Cooper, 2003). While MAPK8 and MAPK11 showed parallel expression at different levels JUN and CREB showed near identical expression over the course of the trial, while these two proteins do not cooperate in producing a transcription factor the equivalent expression levels could be due to their expression being linked to common transcription factor. The transcription factor ATF2, which works in association with JUN, was the only gene in this pathway that showed near linear increased in expression over the course of the trial. In an animal specific sense the expression of these pathways was quite consistent, some animals have increases in expression of these genes while others show reduced expression; in a cohort specific manner there is little over all trends in this gene set. It is important to note that the expression of these genes, similarly to NF-κB, does not represent the signal transduction of the activated proteins and transcription of specific targets and as such the information gained here represents maximum signal transduction potential via this pathway and as well as being informative as an indication into profiling the expression of these genes.

### 4.3.2 Acquired Immunity

Expression of IFNγ parallels STAT1, as STAT1 is responsible for the detection of IFNγ. Although STAT1 was expressed by a factor of 100 times higher than IFNγ and the expression of IFNγ decreases toward the end of the infection trial potentially due to up regulated IL-10 and TGFβ (Khalifeh and Stabel, 2004). Previous observations identified the expression of IFNγ decreases over the course of MAP infection. This decrease in expression coincided with patterns seen in bovine JD, where the expression of IFNγ is reduced in clinically affected cows compared to non-affected animals (Coussens, 2004; Khalifeh and Stabel, 2004; Stabel 2006). One area that could add value to these results would be tissue biopsies from the deer pre-MAP infection. This would add insight into the expression of IFNγ at time zero to see if there was an initial increase leading to the 4 week time point. However independent IFNγ ELISAs using peripheral blood were carried out that show similar expression trends (Mackintosh et al., 2011). Surprisingly the expression of the transcription factor responsible for the production of IFNγ, Tbet, did not show any significant change in

110
expression over the course of infection. The intrinsic inhibitor of this signal cascade PIAS1 shows an increase in expression over the trial indicative of the high expression of these genes and the requirement of regulation in this system. While the increase in the intrinsic regulator PIAS1 is mild the increase in expression of the inducible regulator SOCS3 is much greater (×10) over the course of the trial. Expression of SOCS3 is induced via STAT transcription after prolonged exposure to cytokine signalling (de Prati et al., 2005). The SOCS1 inducible suppressor of cytokine function is the main inhibitor of IFNγ signalling, however some over expression studies of SOCS3 has implicated it to have the ability to inhibit STAT4 and therefore inhibit subsequent IFNγ production as well as Th1 T cell production (Seki et al., 2003). While there were no overt differences in expression at the 4 week time point between the resistant and susceptible cohorts by the 12 and 49 week time point only the resistant animals show increased expression of IFNγ. This represents an increased ability of these animals to fight the bacterial infection (Geissmann et al., 2010). The results obtained by Mackintosh and colleagues (2011) showed the resistant animals had heightened levels of IFNγ in the genotypically resistant animals 92 and 91 (Mackintosh et al., 2011). Unlike the intrinsic inhibitor of IFNγ signalling PIAS1 (that showed neutral expression), the expression of the inducible inhibitor of IFNγ signalling SOCS3 was also upregulated exclusively in the animals 92 and 91 exclusively, potentially due to the heightened expression of IFNγ in these animals.

When considering the expression profile of the IL-6 pathway it is apparent that the expressions of STAT3 and RORC showed similarity in expression although STAT3 is expressed by over a factor of 10 more at each time point. One regulator of this pathway SOCS3 shows increase in expression over the trial, but due to the potentially promiscuous nature of SOCS3 range of inhibition this increase in expression could be a result of increases in the cytokine signalling by multiple different cytokines including IFNγ, IL-12, and GM-CSF (Greenhalgh et al., 2005; Seki et al., 2003; Wormald and Hilton, 2004). It has also been proposed that instead of having a promiscuous nature the role of SOCS3 is more constrained, and that its primarily affects STAT3 phosphorylation (Chen et al., 2006). Induction of STAT3 signalling via IL-23 dependent phosphorylation is a major target of SOCS3 inhibition, indicating that SOCS3 plays a significant role in inhibiting the production of STAT3 downstream target IL-17 that subsequently causes production of Th-17 T cells (Chen et al., 2006). The RORC transcription factor is, in association with STAT3 signalling, responsible for the production of Th-17 T cells (Ouyang et al., 2008). The relative expression of RORC
did not increase over the course of the trial relative to B2M; as Th-17 cells are primarily involved in the clearance of extracellular infection this lack of increase in expression is not surprising (Ouyang et al., 2008). Expression of this set of genes shows no consistent trends throughout the 4 and 12 week time point but by the week 49 time point animals 92 and 91 share the only increased levels of expression of these genes. This could be due to the fact that these animals have at this time point cleared the infection and have reduced expression of B2M compared to the remainder of the animals.

Expression of STAT4 and its inhibitor PIAS4 are tightly linked due to the consititively expressed nature of PIAS4; PIAS4 is required to inhibit aberrant activation of STAT4 (Wormald and Hilton, 2004). The expression of SOCS3 is potentially also involved in the regulation of IL-12 signalling, showing similar expression profiles to STAT4 and PIAS4 except that it is expressed at a lower level. This decrease in expression, around a factor of 2, may be due to the inducible nature of SOCS3 as it is activated in response to sustained cytokine signalling. The upregulation of SOCS3 in JD has been previously observed and correlates with these findings (Seki et al., 2003; Whittington et al., 2011). However a study by Chen and colleagues (2006) found that SOCS3 had only minimal affects upon the production of Th1 cells and no ability to effect the IL-12 signalling pathway (Chen et al., 2006). In T cells the expression of Tbet and subsequently IFNγ is a downstream consequence of IL-12 signalling through STAT4; although STAT4 increased in expression over the trial the relative expression of both Tbet and IFNγ did not change to any large extent. Animal specific profiles of these genes had potentially informative trends at the week 12 and 49 time points whereby the only the phenotypically and genotypically resistant group had the heightened expression of these genes. This heightened expression could contribute to the increased ability of these animals to clear this pathogenic challenge (via the IFNγ activated Th1 T cells and M1 macrophage mechanisms), reduced relative level of B2M in these animals or alternatively the down regulation of this pathway in the susceptible animals. Weakening of the Th1 immune response that over the course of an infection was observed in the susceptible animals and in other trials has been associated with the inappropriate Th2 response (Burrells et al., 1998).

The mean expression levels of STAT5a and STAT5b are almost identical over the course of the trial. This was anticipated as to have function STAT5a and STAT5b form heterodimers to have transcriptional properties (Aaronson and Hovarth, 2002). One signal responsible for the activation of STAT5a STAT5b heterodimers is GM-CSF that over the
course of this trial showed an initial increase in expression of over a factor of 5 before returning to starting levels. The initial rise in expression of GM-CSF is mirrored in the rises in SOCS3 and both of the STAT5 molecules. SOCS3 is involved in the regulation of this system due to its ability to inhibit STAT5 phosphorylation (Chen et al., 2006). The initial spike in GM-CSF could be explained by the increased demand for monocytes in the mesenteric lymph nodes that decreased toward the end of the infection due to the infection being contained by the resistant animals and the MAP affected anti-inflammatory mechanisms affecting this tissue in the susceptible animals (Hestvik et al., 2005). The expression of these genes at the end of the trial was elevated in the resistant animals 92 and 91, potentially due to the ratio of B2M to this pathway or the down regulation of these genes in the susceptible animals.

Signalling via IL-4 activates STAT6 that has its effect by activating the transcription factor GATA3 in T cells (Commins et al., 2010). The initial expression of GATA3 dropped after the 4 week time point by a factor of 5. This could be due to an increase in mononuclear cells in the mesenteric lymph nodes or an increase in Th1 T cell activation/recruitment (Jenner et al., 2009). Both GATA3 and Tbet are co-expressed in Th1 T cells, but only GATA3 is expressed in Th2 T cells; however the activation of GATA3 is inhibited in Th1 cells (Szabo et al., 2000). Examination of the expression ratios obtained in this infection model indicated that at the week 12 time point there is a greater expression of Tbet compared to GATA3 suggesting a predominantly Th1 profile of the immune reaction at this time point (Burrells et al., 1998; Szabo et al., 2000). The decrease of Tbet and GATA3 expression seen at the 12 week time point could potentially be attributed to an influx of mononuclear cells into the mesenteric lymph node associated with an inflammatory response (Whittington et al., 2011). Examination of the week 49 results indicates in a manner similar to most of the other cytokine pathways observed in this thesis that the animals 92 and 91 have the highest expression of these genes at the end of the trial. This is most probably due to these animals having contained the infection as the nature of the immune profile in these animals mesenteric lymph nodes contain less mononuclear cells compared to T cells increasing the relative expression of these genes. In support of this theory is the observation that the susceptible animals have the highest expression of the PRRs at the week 49 time point indicating a higher incidence of monocytes in these tissues.


Chapter 5: Conclusion

5.1 CARD15 polymorphisms and genetic profiles

Investigations into the sequence polymorphisms in the CARD15 gene, encoding the NOD2 PRR, yielded five synonymous nucleotide polymorphisms and one non-synonymous polymorphism named 1816 that resulted in a glycine to arginine amino acid change. This polymorphism was only observed in heterozygotes. Interestingly 6 of the 7 animals with this polymorphism in this trial were sired by the resistant stag but in this context 1816 could not be associated with resistance or susceptibility to JD; further investigation is justified. While in the context of this project 1816 could not be proven to be a factor in resistance to JD, however in a larger trial containing homozygotes for this polymorphism this could be clarified. With a larger trial further insight would be gained into any advantage/handicap this polymorphism causes in the context of JD.

The scope of the qPCR trials into the genetic profiles of the immune pathways focused upon in this thesis has highlighted the complexity of this disease as well as difficulties working in outbred populations in non-controlled environments, many of the pathways were not as consistent as was anticipated. Of those that were informative to potential differences in expression profiling between resistant and susceptible animals IFNβ versus NF-κB, the inflammasome component NLRP3 and the cytokines IL-1β and IL-18 did show interesting differences. The heightened expression of IFNβ versus NF-κB along with the heightened expression of NLRP3, and the cytokines it is involved in activating (IL-1β and IL-18), in the resistant animals compared to the susceptible animals provide potential clues to the manner in which these animals immune systems are activated in response to MAP challenge. While the results obtained for other pathways also add insight into the nature of the mesenteric lymph node tissue response at the times of biopsy, the inconsistent findings limited the value of the observations. Due to the nature of this experimental procedure, and the small numbers of animals tested, the use of mesenteric tissues rather than separated primary cell lines, added extra layers of complexity to the interpretation and value of the results. Different tissue samples inevitably contain different population of cells which confounded the analysis of distinct sets of genes.

While the use of B2M as a normalising gene is common place in qPCR assays it was identified to be expressed differently between the resistant and susceptible cohort of animals, mainly at the week 49 time point. This is due to the heightened inflammatory nature of the
susceptible animals mesenteric lymph nodes as these animals are still actively combating the
disease compromising this gene as a normalising gene. While B2M did show differences in
expression between the animals the other normalising genes tested showed even greater
differences.

5.2 Future experiments

Given a greater amount of time several areas of the research undertaken in this thesis
could have been reinforced to provide a greater depth. Repetition of experiments to ensure
data integrity and reproducibility would be justified due to the unstable nature of mRNA and
the potential of human error at any stage of the experimental process.

One major challenge for finding meaningful differences between these groups was the
limited number of animals tested, due to high cost per animal, and the need to select a number
of polarised phenotypes for resistance and susceptibility. It would also have been beneficial to
obtain pre-challenge biopsy samples to establish base levels of gene expression of the qPCR
gene targets before the animals were infected. It would also be valuable to include an
uninfected group of animals to act as controls. This unfortunately was not possible for this
trial due to the cost required for major surgery from multiple deer and the cost of maintaining
each deer under experimental conditions for long periods.

Increasing the validity of the qPCR data obtained could have been achieved using
multiple normalisation genes which would have had the potential to account for any variation
in expression of B2M. This may be important as the expression of this commonly used
normalisation gene is affected by exposure to interferon.

With more time another approach to better understand the complexities of the immune
response to MAP infection would involve the use of flow cytometry to investigate actual
amounts of proteins rather than mRNA transcripts in the cell. This method of
experimentation, based on having fluorescently labelled protein specific antibodies, allows for
the detection of activated proteins, for example showing phosphorylated STAT dimers or
activated NF-κB. Unfortunately for this method of investigation the nature of the samples
available for experimentation were unsuitable as each sample was frozen in Trizol in
preparation of mRNA extraction. Furthermore the antibodies required for this cytometry are
very limited due to the uniqueness the deer model of JD.
5.3 Final model of Resistance and Susceptibility to JD

Figure 37 below illustrates a proposed model developed from the findings in this thesis, it illustrates the importance of having a strong IFN response over the course of the infection, but most importantly at the very beginning of the infection (for IFNβ), to produce a resistant phenotype as well as the importance of upregulation in the NLRP3 inflammasome. In contrast the susceptible deer showed an initial increase in expression of NF-κB suggestive of a less specific immune activation.

**Figure 37**

Above the proposed model of immune reactivity shows the difference observed during the course of this study. Resistant animals favoured the IRF3 pathway at the beginning of the trial and IFN signalling over the course of the trial while at the first time point the susceptible animals had an increase in NF-κB.
## Appendix

### Appendix 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Pathway</th>
<th>Gene</th>
<th>Primer-sequence</th>
<th>Amplicon length (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervine</td>
<td>Normalising genes</td>
<td>B2M</td>
<td>Forward: GAGGATGGAAAG CCAAATTACCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>B2M</td>
<td>Reverse: CATTCTTCAGCAA TTCGATTTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>PPIB</td>
<td>Forward: TGGCTACAAGAC AGCAAATTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>PPIB</td>
<td>Reverse: CCAGGCCCAATAAT GTTTAAGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TMB1M4</td>
<td>Forward: TACAGCATCCTTT CTCTGCAAGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TMB1M4</td>
<td>Reverse: CTAAAAATCAAAACC CAAGGATCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>PRR</td>
<td>TLR1</td>
<td>Forward: ATGCCGAGAGCCT TCAAGAC</td>
<td>151</td>
<td>NM_001046504</td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TLR1</td>
<td>Reverse: CACCCATTATCAT CAAGCACACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TLR2</td>
<td>Forward: CAGTCATGATCTC AGTCCATTATTGA</td>
<td>151</td>
<td>NM_174197.2</td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TLR2</td>
<td>Reverse: CAAAGAACACCC CTCCAGACAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TLR4</td>
<td>Forward: ACCCGCAAGAAGC GACAAC</td>
<td>151</td>
<td>NM_174198</td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TLR4</td>
<td>Reverse: GGCTCGCGTCACCA CTGAATTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TLR6</td>
<td>Forward: TTCCTCTTTGAAA GCGAATTTGTG</td>
<td>151</td>
<td>NM_001001159</td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>TLR6</td>
<td>Reverse: GCCCTGAGAGAAA GCTGACATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td></td>
<td>IRF3</td>
<td>Forward:</td>
<td>151</td>
<td>NM_001029845</td>
</tr>
<tr>
<td>Cervine</td>
<td>IRF3</td>
<td>Reverse: TTGCCCGCGCTATG TCTACAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
<td>---------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>MyD88</td>
<td>Forward: CTGGTAGGATAGT CCCACCACAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>MyD88</td>
<td>Reverse: TTGCTCTGCGCTT CACTGAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>TRAF6</td>
<td>Forward: AACTGTCCCTTTGG CAAATGTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>TRAF6</td>
<td>Reverse: CCAAGTGATTCTT CTGCATCTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>CARD15</td>
<td>Forward: TGGGTGCGCAAGC CTTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>CARD15</td>
<td>Reverse: ACAGCTTCAGGAC TTTCAAACCTTGA TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>NF-κB</td>
<td>Forward: GCTGGCGGCTCTT CTTCAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>NF-κB</td>
<td>Reverse: CCTTCATCCTCTCC ATCCTCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>Type one Interferon</td>
<td>IFNβ</td>
<td>Forward: GGTGCCTCCTCCA GATGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>IFNβ</td>
<td>Reverse: TCGAGGCAATGT GAGAAGTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT1</td>
<td>Forward: TCGTGGTGATCTC CAATGTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT1</td>
<td>Reverse: TGCCAACCTCAGCA CCTCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT2</td>
<td>Forward: CCCTGGTGCAAGG AGCAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT2</td>
<td>Reverse: CTTACCTCGTGAC CCCTTGGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>PIAS2</td>
<td>Forward: 156 XM_002697814</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Gene Symbol</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Length</td>
<td>Accession</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>----------------</td>
<td>----------------</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>Cervine PIAS2</td>
<td>Reverse: CAGCATCAAAAACA CTGTAGGTGTGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine Type two Interferon IFNγ</td>
<td>Forward: CTCTGAGAAACTG GAGGACTTCAAA</td>
<td>Reverse: GGCTTTGCGCTGG ATCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine IFNγ</td>
<td>Reverse: CAGCATCAAAAACA CTGTAGGTGTGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine STAT1</td>
<td>Forward: TCGTGGTGATCTC AAATGTCA</td>
<td>Reverse: TGCCAAACTCAGCA CCTCTGA</td>
<td>151</td>
<td>NM_001077900</td>
<td></td>
</tr>
<tr>
<td>Cervine STAT1</td>
<td>Reverse: TGCCAAACTCAGCA CCTCTGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine PIAS1</td>
<td>Forward: GTGAAAGTGAATA CAAAACCTTGCA</td>
<td>Reverse: GTTTTACAAGATA CACTGCCATGGA</td>
<td>198</td>
<td>NM_001075396</td>
<td></td>
</tr>
<tr>
<td>Cervine PIAS1</td>
<td>Reverse: GTTTTACAAGATA CACTGCCATGGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine SOCS3</td>
<td>Forward: TCGGACCAGCGCC ACTT</td>
<td>Reverse: CACTGGATGCAGCA GGTTCT</td>
<td>71</td>
<td>NM_174466</td>
<td></td>
</tr>
<tr>
<td>Cervine SOCS3</td>
<td>Reverse: CACTGGATGCAGCA GGTTCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine IL-6 STAT3</td>
<td>Forward: GCCTCTCAGACCC AGAAGCA</td>
<td>Reverse: ACCTGTCACCCC CTTGTCCTTG</td>
<td>151</td>
<td>NM_001012671</td>
<td></td>
</tr>
<tr>
<td>Cervine STAT3</td>
<td>Reverse: ACCTGTCACCCC CTTGTCCTTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine RORC</td>
<td>Forward: GAGCCCTGTGTAGC CAGCATGT</td>
<td>Reverse: TCCCTCCTCCAGG TCACTTG</td>
<td>152</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine RORC</td>
<td>Reverse: TCCCTCCTCCAGG TCACTTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine IL-12 STAT4</td>
<td>Forward: GTTGCTCGTGGCC TGAACCTC</td>
<td>Reverse: CTTCAAGCCAGGT</td>
<td>154</td>
<td>XM_002685461</td>
<td></td>
</tr>
<tr>
<td>Cervine STAT4</td>
<td>Reverse: CTTCAAGCCAGGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>Tbet</td>
<td>Forward: CCGGAGAAACTTTG AGTCCCATGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>----------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>Tbet</td>
<td>Reverse: TCCCCCAAGCAGT TGACAGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>IFNγ</td>
<td>Forward: CTCTGAGAAACTG GAGGACTTCAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>IFNγ</td>
<td>Reverse: GGCTTTTGCCTGG ATCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>SOCS3</td>
<td>Forward: TCGGACCAGCGCC ACTT 71 NM_174466</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>SOCS3</td>
<td>Reverse: CACTGGATGCAGCA GGTCTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>PIAS4</td>
<td>Forward: GGAACGTGGTGAG GCCAAA 181 NM_001083482</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>PIAS4</td>
<td>Reverse: CTCGTAAGAGCTCC TTAATTTTCTTTGA A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>GM-CSF</td>
<td>GM-CSF</td>
<td>Forward: CCCTGGCAGCATG TGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>GM-CSF</td>
<td>Reverse: GCGAGTCTGCAGGGCATGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT5a</td>
<td>Forward: TTGATGCCATGTC CCAGAAAC 136 NM_001012673</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT5a</td>
<td>Reverse: CTCTCCTGATACT GGATGATGAAATA CTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT5b</td>
<td>Forward: TGTMTTGGGAATCT GATGCCCTTTT 110 NM_174617</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>STAT5b</td>
<td>Reverse: TTTGGGGCCTGCA GGAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>SOCS3</td>
<td>Forward: TCGGACCAGCGCC ACTT 71 NM_174466</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>SOCS3</td>
<td>Reverse:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward</td>
<td></td>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>----------------------------------</td>
<td>-------</td>
<td>----------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Cervine</td>
<td>IL-4</td>
<td>STAT6</td>
<td></td>
<td>CACTGGATGCGCA GGTCTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>XM_002687570</td>
</tr>
<tr>
<td></td>
<td>STAT6</td>
<td>Forward: GCGGCTCTATGTC GACTTTCC</td>
<td></td>
<td>Reverse: TGTCTGGAGGGCTC TGGACAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATA3</td>
<td>Forward: CCACAAGATGAAC GGACAGAAC</td>
<td></td>
<td>Reverse: GACAGTTTGCAACA GGACGTACCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NLR</td>
<td>NLRP3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward: AACCGAGATGTGA AGGTCTCTT</td>
<td></td>
<td></td>
<td>165</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CAGCAGCTCCAGC CTGATTT</td>
<td></td>
<td></td>
<td>NM_001102219</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>Forward: AACTCCAGGACAG AGAGCAAAAA</td>
<td></td>
<td></td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GCCACAGGAATCT TGTTGTCTCCTT</td>
<td></td>
<td></td>
<td>AB246787</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>Forward: AACTCCAGGACAG AGAGCAAAAA</td>
<td></td>
<td></td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GCCACAGGAATCT TGTTGTCTCCTT</td>
<td></td>
<td></td>
<td>NM_174091</td>
</tr>
<tr>
<td></td>
<td>IL-18</td>
<td>Forward: AGAAGCTATTGGAG CACAGGCCATAA</td>
<td></td>
<td>Reverse: TCAAGTTTGCCAA AGTGATCTGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NF-κB</td>
<td>NF-κB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward: GCTGGCCGCTCTT CTCAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NF-κB</td>
<td>Forward: CACTCACTCCTCCTCA CATCCTCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>Forward: CCCCTGGCAGCATG TGGAT</td>
<td></td>
<td>Reverse: GCGAGTCTGCAGG CATGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>Reverse: GCGAGTCTGCAGG CATGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLA-DMB</td>
<td>Forward: CTCAACAGTAACG ATGCCCTGAT</td>
<td></td>
<td></td>
<td>NM_001040481.2</td>
</tr>
<tr>
<td>Gene</td>
<td>Primer Set</td>
<td>Sequence</td>
<td>Length</td>
<td>Accession</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>-------------------------------</td>
<td>--------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>HLA-DMB</td>
<td>Reverse: CAGACTCCCTCGT GTAAAAGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>RANKL</td>
<td>Forward: CAACATCTGCTTC CGACATCA</td>
<td>151</td>
<td>AF019047</td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>RANKL</td>
<td>Reverse: AGAATTCGCCGAC CAGTACTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>INOS</td>
<td>Forward: GAAGAGGCTGAG AAGCAGAGGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>INOS</td>
<td>Reverse: TCCAGCACCCTCA GGAATGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>NLRP3</td>
<td>Forward: AACCGAGATGTGA AGGTCCCTTCT</td>
<td>165</td>
<td>NM_001102219</td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>NLRP3</td>
<td>Reverse: CAGCAGCTCCAGC CTGATTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>RANTES</td>
<td>Forward: ACACCACACCCTG CTGCTTT</td>
<td></td>
<td>AJ007043</td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>RANTES</td>
<td>Reverse: GCCGGTTCTTTCT GGTGATAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>MAPK8</td>
<td>Forward: ACACTAATTTCCT ATGCTTTCCCAAGT</td>
<td>151</td>
<td>NM_001192974</td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>MAPK8</td>
<td>Reverse: TCTTCTTTACTGAT GCAAGGTATATAT TTTACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>MAPK11</td>
<td>Forward: CAAGTGCTGGTGTA CTGTACTGTTG</td>
<td>86</td>
<td>NM_001080335</td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>MAPK11</td>
<td>Reverse: CCCAGCAGGCTCT TAGCAAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>P53</td>
<td>Forward: CAGTGCTGGTGTA CCCTATG</td>
<td>161</td>
<td>NM_174201</td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>P53</td>
<td>Reverse: TCCGTCCCAGCAG GTTACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine</td>
<td>CREB</td>
<td>Forward: GTGCGAAGGATTG</td>
<td>151</td>
<td>AF006042</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Primer</td>
<td>Forward, Reverse Accession Numbers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine CREB</td>
<td>TACCATTGTAGC CAGTTGTATTGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine cFOS</td>
<td>GCAACGAGCCTCC CTCTGACT</td>
<td>AY322482</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine cFOS</td>
<td>GATAAGGTCCTCC CTAGGTCTATGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine cJUN</td>
<td>GCAGAGAGGAAG CGCATGAG</td>
<td>NM_001077827</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine cJUN</td>
<td>CCTGTTCCCTGAG CATATTGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine ATF2</td>
<td>TGCCTAGCGTTCC AGGAATT</td>
<td>XM_002685316</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervine ATF2</td>
<td>GAGTCCTAACCAA ACCACTACTGTGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Appendix 1. Primer sequence, accession numbers and product size**

Above, where applicable, the primer sequences of the primers used, product size and accession numbers are recorded. Gaps in this table are attributed to these primers having been previously made and validated in other research projects. The primers have been divided into their specific previously described pathways. The accession numbers above were sent to AgResearch Invermay where they were blasted against a not yet published cervine genome. Relevant sequence was sent back and from these primers were designed.
Appendix 2

A representative sample of RNA was tested for integrity; results for the RNA samples 77, 79, 80, 81, 82, 84, 85, 86, 88, 89, 90 and 91 are shown above. The graphs indicate RNA integrity, the first main peak representing the control marker, and the next two representing the ribosomal RNA subunits 18S and 28S. Beneath the graphs is the RNA integrity number (RIN) highlighted in a blue box, in this case 9.2 indicative of a good mRNA sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA Integrity Number (RIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>5.2 (8.32-0.7)</td>
</tr>
<tr>
<td>Sample 2</td>
<td></td>
</tr>
<tr>
<td>Sample 3</td>
<td></td>
</tr>
<tr>
<td>Sample 4</td>
<td></td>
</tr>
<tr>
<td>Sample 5</td>
<td></td>
</tr>
<tr>
<td>Sample 6</td>
<td></td>
</tr>
<tr>
<td>Sample 7</td>
<td></td>
</tr>
<tr>
<td>Sample 8</td>
<td></td>
</tr>
<tr>
<td>Sample 9</td>
<td></td>
</tr>
<tr>
<td>Sample 10</td>
<td></td>
</tr>
<tr>
<td>Sample 11</td>
<td></td>
</tr>
<tr>
<td>Sample 12</td>
<td></td>
</tr>
</tbody>
</table>

RNA Area: 234.3
RNA Concentration: 168 ng/μl
rRNA Ratio (28S / 18S): 1.5
Result Flagging Color: 
Result Flagging Label: RIN: 9.20
Appendix 3

A)

Appendix 3. Examples of Primer Specificity Assays

To ensure specificity each primer pair was tested using a melt curve. A single peak represents a specific product as the DNA melted simultaneously representative of the products being the same size A). As can be seen in B), this primer pair is not specific as different product sizes have been amplified. The above melt curves are examples produced using Applied Biosystems ViiA 7 software A) and Roche Light Cycler 480Software release 1.5.0 B).
References


70. Morrissey, P.J., Grabstein, K.H., Reed, S.G., Conlon, P.J., 1989, Granulocyte/Macrophage Colony Stimulating Factor. International Archives of Allergy and Immunology 88, 40-45.


86. Schorey, J.S., Cooper, A.M., 2003, Macrophage signalling upon mycobacterial infection: the MAP kinases lead the way. Cellular Microbiology 5, 133-142.


99. Whittington, R.J., Begg, D.J., de Silva, K., Plain, K.M., Purdie, A.C., 2011, Comparative immunological and microbiological aspects of paratuberculosis as a model mycobacterial infection. Veterinary Immunology and Immunopathology.