Genetic Markers to Identify the Resilient and Susceptible Phenotypes to Johne’s Disease in Red Deer (*Cervus elaphus*)

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Abstract

Johne’s disease, a chronic granulomatous enteritis caused by infection of the pathogen *Mycobacterium avium* subspecies *paratuberculosis*, can be a major cause of production losses in farmed ruminants. At present, there are no viable options for treatment or prevention of the disease. With prevalence of Johne’s disease suspected to be on the rise, a method of control is required. Red deer stags with resilient and susceptible phenotypes to Johne’s disease have been identified previously by the DRL, and these phenotypes appear to have a high paternal heritability. The present study is a contribution towards the identification of biomarkers for either phenotype to produce a diagnostic test to proactively identify whether a naïve animal is likely to be resilient or susceptible to the disease. This diagnostic assay would be useful for selection of resilient animals for breeding, producing a genetically resilient herd to reduce Johne’s disease associated production losses.

As susceptibility to Johne’s disease was thought to be due to a dysregulation of the innate immune system, this project investigated differential expression of genetic markers from macrophages of resilient and susceptible animals. Monocyte-derived macrophages were cultured from cervine blood and stimulated with viable *Mycobacterium paratuberculosis*. Quantitative-PCR was used to analyse changes in expression of target genes. Of the investigated gene targets, *ISG15* and *ISG20* showed particular promise, where animals of the resilient phenotype upregulated expression to a greater degree than animals of the susceptible phenotype. Moreover, *IL1A, IL12A, MAPK8, NOS2, PKLR*, and *STAT3* showed sufficient differential expression between the phenotypes to suggest their potential for further investigation. This study also investigated the feasibility of PBMC culture as a platform for a diagnostic assay. This assay presented favorably in terms of volume of blood required and culture duration compared to the MDM assay. However, no consistent differential gene expression was observed from the investigated genes, and more work is required to identify functional genetic markers. Taken together, these results demonstrate the potential for using differential gene expression for phenotypic diagnosis, where further investigations should be carried out to confirm robust and dependable genetic markers of resilience and susceptibility.
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Abbreviations

APC  Antigen presenting cell
B2M  Beta-2-microglobulin
CCL  Chemokine (C-C motif) ligand
cDNA Copy DNA
CR   Complement receptor
Ct   Cycle threshold
CXCL Chemokine (CXC motif)
DC   Dendritic cell
ELISA Enzyme-linked immunosorbent assay
GM-CSF Granulocyte macrophage-colony stimulating factor
GWA  Genome wide association
ICV  Ileocaecal valve
IFN  Interferon
IL   Interleukin
iNOS Inducible nitric oxide synthase
ISG  Interferon stimulated gene
JD   Johne’s disease
JJ   Jejunum
LN   Lymph node
LSS  Lesion severity score
M cell Microfold cell
*M. tb* *Mycobacterium tuberculosis*
MAP  *Mycobacterium avium* subspecies *paratuberculosis*
MDM  Monocyte-derived macrophage
MOI  Multiplicity of infection
mRNA Messenger RNA
MYD88 Myeloid differentiation primary response gene 88
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PKLR</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PPDj</td>
<td>Purified protein derivative-johnin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative-PCR</td>
</tr>
<tr>
<td>R</td>
<td>Resilient</td>
</tr>
<tr>
<td>S</td>
<td>Susceptible</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>T_{REG}</td>
<td>Regulatory T cell</td>
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</table>
1 Introduction

1.1 Johne’s disease

Johne’s disease (JD) is a chronic inflammatory bowel disease of ruminants, caused by the bacterial pathogen *Mycobacterium avium* subspecies *paratuberculosis* (MAP), hence the disease is sometimes known as paratuberculosis. The disease leads to inflammation of the infected intestinal epithelium and associated lymphatic system, in some cases causing severe tissue damage. JD is characterised by weight loss and wasting, diarrhoea, enlarged mesenteric lymph nodes, and granulomatous enteritis. The disease was first described in Germany in 1895 by Heinrich Johne and Langdon Frothingham\(^1\), and has since been reported throughout the world. JD affects ruminant species such as cattle, sheep, goats, and red deer (*Cervus elaphus*) and causes a significant burden on the farming industry; costs associated with detection, control, and loss in production have a considerable economic impact.

1.1.1 Disease impact

JD has the potential to have a serious impact on animal production through decreased milk and meat yields, decreased life expectancy and value of cull animals. Recent estimates suggest JD affects 30% of beef cattle herds, 60% of dairy cattle herds, 79% of sheep flocks, and 52% of deer herds in NZ\(^2\). The disease also poses a significant problem outside of NZ. Estimates suggest 20% of dairy farms in the US are effected by clinical disease\(^3\); however the current situation is likely to be more dire as reported cases of JD have been steadily increasing worldwide\(^4\).

Ongoing intensification of farming processes (such as increasing herd sizes) improves production, yet can have a significant impact on disease. Intensification can lead to a more stressful environment for farmed animals and lead to an increased susceptibility to infection and disease\(^5^-^7\). The recent introduction of techniques such as artificial insemination and embryo transfer may also have led to an increase in the prevalence of JD. These techniques are used to rapidly spread desirable production traits, such as growth rate or milk production; however selection for production may be at the cost of other desirable traits, such as immune competence\(^8\).
As NZ is heavily dependent on the agriculture industry, JD has a significant impact on the NZ economy. Based on estimated prevalence, JD could cost up to $30 million annually to the country’s agricultural industry through surveillance, prevention, and loss of production\(^9\), while some studies suggest it costs the US up to $1.5 billion annually\(^{10,11}\). Along with the well-known reliance of NZ on the dairy and sheep industry, the population of farmed deer is the highest of any country, at approximately 1.3 million, exemplifying the importance of control of the disease in this country.

### 1.1.2 Disease pathology

JD is characterized clinically by non-specific symptoms caused by an inappropriate immune response generated against MAP. Early JD is associated with granuloma formation due to recruitment of macrophages, primarily affecting the ileum of the small intestine\(^{13}\). Formation of granulomas causes thickening and corrugation of the intestinal wall, resulting in a decreased absorptive surface area due to blunting of intestinal villi\(^{14}\). This contributes to a malabsorption of nutrients and leads to the diarrhoea and weight loss seen in clinical disease.

While the pathology of JD is similar amongst ruminant species, subtle differences between species do exist. For example, sheep and goats rarely exhibit scouring, a symptom common in cattle. Other differences can include location, size, and severity of lesions, and timescale of disease progression. Symptoms of clinical disease seen in deer are similar to those seen in cattle and sheep, although thickening of the jejunum and ileum may not be as obvious.

The majority of MAP infections do not result in clinical disease, yet these infected animals can still shed viable organisms into the environment\(^{15}\). This is referred to as ‘subclinical infection’ and represents one of the major barriers to control of the disease, as subclinically infected animals are rarely identified but can transmit the disease throughout a herd. Reductions in milk production\(^{16}\) and slaughter weight\(^{17}\), and increased culling rates\(^{18}\), have been reported in subclinical infection, resulting in potential loses for farmers. Progression from subclinical infection to clinical disease may be accelerated by stressors such as lactation, pregnancy, lack of feed, or change in environment\(^{19}\). Subclinical MAP infection parallels the latent nature of the human pathogen, *Mycobacterium tuberculosis* (*M. tb*); it is estimated that a third of the world’s human population is infected with *M. tb*, while only a tiny proportion of those infected exhibit clinical disease\(^{20}\).
1.1.3 Age-related susceptibility

Both red deer and cattle show an age-related susceptibility to JD, where older animals are more resistant to both clinical and subclinical disease, but not to MAP infection\textsuperscript{21}. Mackintosh \textit{et al.} found that approximately 30\% of three month old red deer challenged with a heavy oral dose of MAP developed clinical disease, whereas no adult deer did when given the same dose\textsuperscript{22}. A similar trend of age-related susceptibility has been seen in cattle; approximately 75\% of calves less than six months old developed disease when exposed to MAP compared to less than 20\% of animals older than 12 months\textsuperscript{23}. This increase in resistance to infection with age may come from repeated exposure to the organism\textsuperscript{24}. Newborn ruminants may also be more susceptible as they have an “open-gut”. An “open-gut” allows molecules such as colostral immunoglobulin to cross the epithelial barrier, but may also allow MAP to easily cross the intestinal wall\textsuperscript{25}. Dobson \textit{et al.} described how young deer have more extensive Peyer’s patches than adult deer\textsuperscript{26}, which could increase the surveillance of the gut contents, allowing for more MAP uptake and contributing to the age related susceptibility.

Red deer are suitable as experimental models for JD as they develop the disease at a young age and disease progression is rapid relative to other ruminants\textsuperscript{22}, improving the efficiency of research. Red deer have been shown to develop disease as young as five months old\textsuperscript{27,28}, compared to two to five years old in sheep and cattle\textsuperscript{11,29}. Infection usually progresses to clinical JD in 4-5 months in red deer or 1-2 years in cattle or sheep. This apparent sensitivity of red deer to JD may be a manifestation of the stress placed on the deer due to their relatively recent domestication compared to the other ruminants\textsuperscript{30}.

1.2 \textit{Mycobacterium avium} subspecies \textit{paratuberculosis}

The genus \textit{Mycobacterium} encompasses many major species of obligate intracellular pathogens, including \textit{M. avium}, \textit{M. tb}, \textit{M. leprae}, \textit{M. bovis}, and MAP. These pathogens share a pattern of disease featuring formation of granulomas containing mononuclear phagocytes, such as macrophages. MAP belongs to the \textit{M. avium} complex, and like other mycobacterial species, it has an acid fast cell wall and rod shaped morphology. Its slow growing nature and reliance on supplementation with the siderophore mycobactin J make the bacteria relatively difficult to culture \textit{in vitro}.
MAP is a robust bacterium that can survive in soil for over a year\textsuperscript{31}. As the primary mechanism of MAP transmission is through the faecal-oral route and ingestion of infected pasture, the ability of this organism to survive in the environment is an important factor for infectivity. Clinically infected animals can shed up to $10^8$ organisms per gram of faeces, leading to heavy environmental contamination\textsuperscript{32}. MAP can also be transmitted to offspring through infected colostrum or milk\textsuperscript{33}, or to the foetus through intra-uterine transmission\textsuperscript{34}. It is important to note that even subclinically infected animals can transmit the disease making control of the infection, not just the disease, of upmost importance.

Two genotypically distinct strains of MAP have been described; the “bovine” and “ovine” strains, named due to their proclivity to infect cattle and sheep respectively. Deer may be infected with both the bovine and ovine strains of MAP making them particularly at risk\textsuperscript{35}; however most naturally occurring cases are due to the bovine strain, which produces more severe pathology\textsuperscript{36}. The sequence of the bovine K-10 strain genome has been published\textsuperscript{37}, showing ~99\% sequence homology with \textit{M. avium}. Despite this genetic similarity, these two bacteria show distinct differences; MAP has a much slower growth rate, infects mammalian instead of avian hosts, and cannot replicate in the environment.

MAP has a broad host range, although is only thought to be pathogenic in ruminant species such as deer, sheep, goats and cattle. All ruminant species are susceptible to infection and reported cases are far more common in domestic ruminants than wild ones, most likely due to the intense use of animal husbandry in farming and high animal density. Non-ruminant species have also been shown to harbour MAP, albeit not showing clinical signs of disease. MAP has been cultured from faeces of infected hedgehogs and rabbits, along with the gut of possums, weasels, stoats, and hares, providing a large environmental reservoir for infection of farmed ruminants\textsuperscript{38}. This is compounded by the ability of the bacteria to persist in the environment for extended periods under appropriate conditions.

Some studies have claimed associations between MAP and Crohn’s disease, a chronic human inflammatory bowel disease of as yet unknown etiology\textsuperscript{39}, which has given rise to theories of MAP having a causal effect. These notions are supported by the striking pathophysiological similarities between JD and Crohn’s disease. While the legitimacy of these claims is controversial, they no doubt pose a threat to the farming sector if products
from MAP infected farms could be considered a risk factor for Crohn’s disease. This further supports the importance of eradication of the disease from the agriculture industry.

1.3 Infection and immunity

The vertebrate immune system has evolved as a dynamic system comprising a multitude of factors aimed at protecting the host from disease. This system comprises a range of structures, cells, proteins, and molecules. The cellular immune response can be broadly characterised into two connected systems: the innate and acquired immune systems.

The innate immune response is not antigen specific yet acts quickly, providing the first line of cellular defence. The main cell types of the innate system include neutrophils, dendritic cells (DC), and macrophages. Neutrophils are the most abundant immune cell in the mammalian immune system. They are recruited to the site of infection through chemotactic molecules, using phagocytosis and degranulation to eliminate the invading organism and limit the spread of infection. DC are specialised antigen presenting cells (APC) which process phagocytosed organisms for presentation to the adaptive immune system. Macrophages are multifaceted cells which are arguably the most important of the innate immune system. They play a role in phagocytosis, tissue repair, antigen presentation, and cell activation depending on their environment.

In contrast, the acquired (or adaptive) immune response is a highly adaptable system which targets specific molecules on invading organisms. Consequently, this system produces a more effective response to infection, yet takes longer to develop and take effect than the innate response. This system is characterised by generation of antigen specific B and T cells and cellular memory, which leads to an improved response against the same pathogen following subsequent exposure. B cells are specialised antibody producing cells, which produce specific antibodies following activation from T cells, while T cells can play a range of roles, such as cell activation and regulation, or direct killing of infected cells.

The immune response towards MAP infection has been investigated extensively in a cattle model of JD; however due to the complex nature of this response, the immune pathways which lead to protection are not well understood. Studies in red deer have been less extensive; however the pathways responsible for protection are expected to draw a parallel.
1.3.1 Model of MAP infection
The most comprehensive model of the immune response against MAP infection to date was proposed by Paul Coussens\textsuperscript{40}. Briefly, gut intraepithelial macrophages and DCs take up the invading bacteria and become activated, triggering these cells to release cytokines to recruit immune cells to the site of infection. This results in chronic inflammation of the Peyer’s patches and draining lymph nodes, and intestinal granuloma formation\textsuperscript{41,42}. The activated macrophages and DC are also able to travel to the lymph nodes where they act as APCs, inducing adaptive immune responses through interactions with T cells. However, MAP is able to down-regulate activation signals and apoptosis in the infected APCs, leading to a reduced ability of the APCs to induce an adaptive immune response\textsuperscript{43–45}. Pro-inflammatory T helper (Th) 1 and Th17 cells are generated, yet only at levels able to control the progression of disease, not completely remove the infection. This is how the subclinical phase of infection develops, which may last for years. As well as controlling infection, these pro-inflammatory T cells also produce host tissue damage through inflammation, which eventually induces the development of regulatory T cells (T\textsubscript{REG})\textsuperscript{46,47}. Regulatory T cells inhibit differentiation of Th1 and Th17, allowing the development of Th2 cells and humoral immunity, which is ineffective at controlling MAP infection. As infection progresses and MAP multiplies, the large numbers of infected macrophages produce excessive amounts of Interleukin (IL)1\alpha, which causes the excessive inflammation and tissue damage characteristic of clinical JD\textsuperscript{48}.

1.3.2 Initial infection
MAP initially infects the Peyer’s patches of the terminal ileum, specifically infecting microfold (M) cells. These M cells act as sentinel cells, sampling the gut contents and transferring antigens to intraepithelial immune cells for surveillance. By this mechanism, M cells take up MAP organisms rather than active invasion by the bacteria. MAP bacilli expresses fibronectin attachment proteins on their surface, which are able to bind host surface intergrins, mediating bacterial uptake\textsuperscript{49}. Bacteria are then phagocytosed by intraepithelial macrophages, from where they establish infection. This phagocytosis is through various receptors, such as complement receptors (CR1, CR3, and CR4), immunoglobulin receptors and mannose receptors\textsuperscript{50–52}. Uptake of mycobacteria by murine or human macrophages can be greatly inhibited by blocking CR3 using anti-CR3
antibodies\textsuperscript{53,54}, suggesting a role of complement opsonisation in the defence against mycobacteria.

Following phagocytosis, macrophages attempt to destroy the bacteria through autophagy. This is a process by which the phagosome containing the endocytosed bacteria fuses with a lysosome, becoming a phagolysosome. The phagolysosome is an acidic environment containing lytic enzymes, which cause destruction of the bacteria. However many mycobacteria, including MAP, are able to inhibit phagolysosome fusion, allowing the bacteria to persist and multiply within the macrophage\textsuperscript{55}.

**1.3.3 Innate immunity**
An interesting consideration of macrophages is that of the M1-M2 paradigm (Figure 1). This model suggests that macrophages can differentiate into different subsets, M1 or M2, dependent on the nature of the inflammatory and cytokine environment\textsuperscript{56}. M1 macrophages are generally pro-inflammatory, while M2 encompasses macrophages that are considered not pro-inflammatory, such as regulatory or those involved in tissue repair. It is important to note in this simplified model that the M1 and M2 subsets are not fixed, but can alternate between or somewhere in the middle if exposed to a different inflammatory environment.

M1 (or classically activated) macrophages develop in the presence of pro-inflammatory cytokines such as interferon-gamma (IFN\(\gamma\)), tumour necrosis factor alpha (TNF\(\alpha\)) and granulocyte macrophage-colony stimulating factor (GM-CSF). The main role of these macrophages is to act as phagocytes during the initial phase of infection, attempting to clear the pathogen. They can also play a pro-inflammatory role by producing cytokines such as IL12 and IL23, which activate and maintain Th1 and Th17 cells, respectively. These cells are important in the initial phase of MAP infection, where they play a role in pathogen clearance.
Naïve macrophages or monocytes can polarize into M1 or M2 macrophages dependent on environment. M1 macrophages can contribute to the pro-inflammatory environment and direct elimination of pathogens, while also stimulating generation of Th1 and Th17 cells. Conversely, M2 macrophages produce anti-inflammatory cytokines and are involved in tissue repair. M2 macrophages also stimulate the differentiation and maintenance of T helper subsets, Th2 and T<sub>REG</sub>.

**Figure 1. Macrophage activation and polarization**

Naïve macrophages or monocytes can polarize into M1 or M2 macrophages dependent on environment. M1 macrophages can contribute to the pro-inflammatory environment and direct elimination of pathogens, while also stimulating generation of Th1 and Th17 cells. Conversely, M2 macrophages produce anti-inflammatory cytokines and are involved in tissue repair. M2 macrophages also stimulate the differentiation and maintenance of T helper subsets, Th2 and T<sub>REG</sub>.
M2 (or alternatively activated) macrophages however, play a more regulatory role in the immune system while clearing damaged host tissue. They develop in the presence of cytokines such as IL4, IL10 and transforming growth factor-beta (TGFβ), and subsequently produce more of these cytokines, thus are sometimes referred to as anti-inflammatory. M2 macrophages are evident late in MAP infection, where tissue damage due to chronic inflammation stimulates the production of IL10 and TGFβ, maintaining the M2 phenotype. At this point the phenotype contributes to the anti-inflammatory environment and repair or clearance of damaged tissues.

Cells of the innate immune system, such as macrophages, can recognise endocytosed mycobacteria through various pathogen recognition receptors such as Toll-like receptors (TLRs) 2, 4, and 9. These receptors recognise conserved antigens on invading microorganisms allowing the phagocytes to discriminate potential pathogens from self-antigens. TLR2 recognises lipoarabinomannan and other constituents of the mycobacterial cell wall, while TLR9 recognises unmethylated CpG sequences. MAP has been shown to be able to block the responsiveness of TLR9, despite over a 10-fold increase in its expression following MAP infection\(^4\). TLR4 recognises lipopolysaccharide (LPS) and is generally considered gram-negative specific, but is also involved in recognition of mycobacteria to a lesser extent. The TLRs signal through the myeloid differentiation primary response gene 88 (MYD88), which drives activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-\(\kappa\)B), a major transcription factor involved in immunity\(^5\). Mutations in TLR1, TLR2 and TLR4 have previously been associated with susceptibility to MAP infection in cattle\(^5\). Nucleotide-binding oligomerization domain-containing protein 2 also plays a role in recognition of mycobacteria by detecting muramyl dipeptide found in bacterial peptidoglycans\(^6\). This receptor also functions by activation of NF-\(\kappa\)B, although through a MYD88 independent manner.

Following pathogen recognition, activation of NF-\(\kappa\)B causes macrophages to upregulate expression of inflammatory immune molecules. These molecules encompass a range of functions from direct killing of an infecting organism to signalling of other immune cells. One such important molecule is inducible nitric oxide synthase (iNOS), which produces reactive nitrogen species, such as nitric oxide (NO). Macrophages can also produce reactive
oxygen species in a “respiratory burst”. These reactive oxygen and nitrogen species act together to oxidize bacterial proteins and molecules, causing damage and leading to bacterial death. Mycobacteria such as \textit{M. tb} and MAP are relatively resistant to reactive oxygen species\textsuperscript{61,62}, likely due to the expression of superoxide dismutase enzymes\textsuperscript{63}; however they are susceptible to reactive nitrogen species \textit{in vitro}\textsuperscript{64}. NO has also been linked to induction of apoptosis, the programmed cell death pathway by which a cell can combat intracellular pathogens\textsuperscript{65}.

Apoptosis is the programmed death of a cell as a defence mechanism in response to infection and tissue damage, or as homeostatic mechanism to maintain cell populations during development and aging. The process is characterised by pyknosis (chromatin condensation), membrane blebbing, DNA fragmentation and compaction of cellular organelles\textsuperscript{66}. Initiation of apoptosis through stress or direct cytokine signalling leads to activation of caspase enzymes, which begins a cascade allowing them to cleave and activate further caspase enzymes. These activated enzymes have wide-ranging proteolytic effects, thus their activation irreversibly leads to the death of the cell. Intracellular pathogens such as mycobacteria have been suggested to directly induce apoptosis in infected macrophages\textsuperscript{67}, an important mechanism of preventing spread of the bacteria by sequestering them within apoptotic bodies, which are then cleared through phagocytosis\textsuperscript{68}.

Along with direct killing of the internalised bacteria, activated macrophages also play an important role in signalling through the release of cytokines. Cytokines are molecules produced by a range of cells (including cells not generally considered part of the immune system, such as epithelial cells) that play roles in many different functions throughout the body. They can be pro- or anti-inflammatory, and play roles in cell communication, activating and recruiting a range of cells. Cytokines bind receptors on their target cells, which induces a signalling cascade within the host cell that generally leads to transcription of effector molecules.

An important macrophage cytokine is TNF\textalpha, which has pro-apoptotic and inflammatory properties. This cytokine is important in the regulation of inflammation, inducing fever, neutrophil recruitment, and phagocytosis. Along with another important pro-inflammatory macrophage cytokine, IL1\alpha, TNF\textalpha is able to stimulate the production of chemotactic
proteins, such as chemokine (C-C motif) ligand (CCL) 2 and C-X-C motif chemokine (CXCL) 5. These chemotactic proteins are critical for the recruitment of immune cells to the site of infection, contributing to both the elimination of mycobacteria and granuloma formation. Granuloma formation aims to limit the spread of infection through “walling off” the infecting organisms; however granuloma formation can lead to significant pathology and allow bacterial growth. In this way, IL1α and TNFα can contribute to both protection and pathology during MAP infection.

Cytokines of the innate immune system can act as an important link between the innate and adaptive immune systems. One such molecule released by macrophages to combat MAP infection is IL12. This cytokine is made up of two discrete subunits: IL12 p35 and IL12 p40. The main role of IL12 is to stimulate the differentiation of T cells into Th1, which is the key cell type in controlling MAP infection\textsuperscript{69}. Activated T cells are able to induce the activation of macrophages via CD40, where CD40L on the T cell interacts with CD40 on the macrophage to further induce expression. The activation of macrophages leads to their enhanced expression of molecules such as IL12, iNOS, and TNFα. However, MAP has the ability to prevent upregulation in expression of IL12 p40 and iNOS, which is thought to be through interference of the CD40-CD40L signalling pathway\textsuperscript{43}. By this mechanism MAP is able to undermine the host immune system and have a direct negative effect on macrophage bactericidal activity.

Type I interferons, including IFNα and β, also play an important role in linking the innate and adaptive immune response\textsuperscript{70}. IFNα/β are produced by virtually any cell type in response to intracellular infection and contribute to a rapidly enhanced rate of DC maturation, greatly improving their ability to present antigens to the adaptive immune system\textsuperscript{71}. IFNα/β can also have a range of effects via secondary molecules, such as interferon stimulated genes (ISG) 15 and 20. ISG15 is an ubiquitin like protein which is thought to play roles in selectively inhibiting the translation of viral mRNA through greatly improving the potency of 4E homologous protein, a protein that binds to the cap of mRNA and prevents translation\textsuperscript{72}. This protein is also important in resistance to infection in humans, where inherited deficiency in ISG15 is associated with a reduced ability to produce IFN-γ, accounting for an increased susceptibility to mycobacterial infection\textsuperscript{73–75}. ISG20 is an exonuclease which acts
specifically on single stranded RNA, which has been shown in vitro to interfere with vesicular somatic virus mRNA transcription without effecting host cellular processes\textsuperscript{76}. These genes have well described roles in anti-viral immunity\textsuperscript{77}; however these roles can likely be extrapolated to mycobacterial immunity due to the intracellular nature of these pathogens and the importance of IFN in their destruction.

An important role of cells of the innate immune system is to act as APCs. DC and macrophages are considered specialised APCs, due to their expression of both major histocompatibility complex (MHC) class I and II. Presentation of antigens via MHC-antigen complexes allows an APC to initiate an adaptive immune response against the particular antigen. MHC-I is expressed on all nucleated cells and presents intracellular antigen, such as viral or cancer derived proteins, to the immune system. Conversely, MHC-II presents antigens of extracellular origin and is limited to expression on professional APCs such as macrophages and DC. MHC-I and MHC-II present antigens to T cells by binding to CD8 or CD4 receptors, respectively. This interaction, in combination with co-stimulatory molecules and the cytokine microenvironment, allows activation of the T cell.

1.3.4 Adaptive immunity

Similar to macrophages, naive T helper cells differentiate into subsets exhibiting proclivity towards different immune responses dependent on the environment in which they developed (Figure 2)\textsuperscript{78}. Such subsets include, but are not limited to, Th1, Th2, Th17 and T_{\text{REG}}. In the current model of the acquired response to MAP infection, Th1 cells are effective at controlling and in some cases eradicating infection, while Th2 responses are ineffective yet associated with clinical disease. Th1 cells primarily mediate the response to intracellular pathogens, such as mycobacteria or viruses, or tumour cells. They are characterized by their expression of pro-inflammatory cytokines IL2, IFN$\gamma$, and GM-CSF, through which they increase the cytotoxicity of other immune cells. Th2 cells mediate the humoral immune response and production of antibody, through cytokines such IL3 and IL4. This makes them important helper cells in allergy and in control of extracellular infections. It is thought that products of Th1 and Th2 cells are able to cross regulate or inhibit responses from the opposite lineage.
Figure 2. Summary of T cell subsets relevant to JD

Graphic representation of the differentiation of naïve CD4+ T cells into effector T helper cells relevant to JD. Shows the major STAT molecule responsible for differentiation, the major transcription factor, and the key cytokines for each T cell subset. Figure adapted from O’Shea and Paul, 2010.
The main cytokine produced by Th1 cells in response to MAP infection is the Type II interferon, IFN\(\gamma\). This cytokine is critical for activating macrophages, improving their antigen presentation and bactericidal activity, which is highly beneficial in MAP infection as macrophages are the primary target of mycobacterial infection. IFN\(\gamma\) stimulation of cells induces activation of receptor-associated Jak tyrosine kinases, which are able to phosphorylate and activate signal transducer and activators of transcription (STAT) molecules capable of upregulating effector molecule expression. Studies have shown that bovine macrophages are more capable of controlling both MAP\(^{62}\) and \(M.\) bovis\(^{79}\) growth when stimulated exogenously with IFN\(\gamma\) \textit{in vitro}. It has been reported that red deer that exhibit a JD resistant phenotype produce higher levels of IFN\(\gamma\) following experimental infection than those that are susceptible\(^{30}\). Expression of IFN\(\gamma\) is also higher in the intestinal tissues of cattle subclinically infected with MAP than those clinically infected\(^{80}\), which is likely a reflection of the strong Th1 response which is able to control MAP infection in subclinical disease, but is dissipated during clinical disease.

The effects of IFN\(\gamma\) on host cells is regulated by the suppressor of cytokine signalling (SOCS) genes, mainly genes 1, 3, and 4. These proteins have been shown to regulate cytokine expression in response to cytokine stimulation, including IFN\(\gamma\), IL10 and IL6\(^{81,82}\). Therefore the SOCS proteins are expected to contribute to the progression of MAP infection, whether in a positive or negative manner. It has been observed that both \(M.\) avium and MAP infected macrophages have an increased expression of SOCS1 and 3, leading to a decrease in IFN receptor expression and thus a decrease in IFN\(\gamma\) responsiveness\(^{44,83}\).

While IFN-\(\gamma\) is the main Th1 cytokine, IL4 is the main Th2 cytokine. It is produced in response to extracellular infection initially by basophils, mast cells, and natural killer T cells, and contributes to the differentiation of naïve T helper cells into Th2. Following differentiation, Th2 cells produce further IL4 to maintain the phenotype. IL4 also plays a role in maintaining the M2 macrophage phenotype, promoting anti-inflammatory responses and tissue repair. This cytokine plays its role late in JD, when Th1 responses are turned down, allowing Th2 cells to develop and contribute to tissue repair\(^{84}\).

T helper 17 cells are another subset of helper T cells which, similar to Th1 cells, produce inflammatory immune responses, primarily through their signature cytokine, IL17. These
cells are maintained by IL23, a cytokine produced by dendritic cells and macrophages, which leads to the phosphorylation of the transcription molecule STAT3. This subset has been proposed to be important in JD due to observations of increased IL17 expression in gut associated lymph nodes in clinically diseased cattle\textsuperscript{85} and red deer\textsuperscript{46} compared to undiseased controls. Th17 cells are also important in the pathogenesis of Crohn’s disease, a disease in humans with similar pathology to JD. As Th17 cells are thought to be important in autoimmunity, and JD is considered to be an autoimmune disease triggered by MAP infection, there is a clear potential role for these cells in the disease state.

Finally, as their name suggests, T\textsubscript{REG} cells are specialised regulatory cells which have anti-inflammatory properties through expression of cytokines IL10 and TGFβ. These cells are thought to play a major role in the progression of subclinical infection into clinical JD, as they downregulate protective Th1 immune responses, meaning the host can no longer effectively control MAP growth. They are likely activated through tissue damage seen in granuloma formation as a mechanism of protection from autoimmunity. IL10 is the primary immunosuppressive cytokine of T\textsubscript{REG} cells, acting through downregulation of IFNγ from Th1 cells, co-stimulatory factor CD28 on all T cells, and IL4 and IL5 from Th2 cells. IL10 also acts on macrophages to downregulate the expression of a range of cytokines and molecules.

1.4 Control of Johne’s disease

1.4.1 Diagnosis

Early diagnosis of JD is crucial as infected individuals must be removed to prevent the disease spreading to the rest of the herd. If the disease develops into clinical JD then the animal will rapidly lose condition and therefore value. Culture of MAP from faeces remains the gold standard \textit{ante-mortem} diagnostic technique as it is highly sensitive, and the strain of MAP may be subsequently characterised; however, it can take up 5-16 weeks for detectable growth to become evident. Faecal PCR to detect MAP has recently been trialled and compared favourably to culture in terms of cost, speed and sensitivity\textsuperscript{86}; however remains more expensive than antibody based diagnosis. Faecal PCR can distinguish between the ovine and bovine strains of MAP and has a higher specificity than other tests. Detecting
MAP in faeces by either method also has the advantage of detecting shedding subclinically infected animals that are difficult to diagnose by other methods.

An alternative method of diagnosis ante-mortem is detection of MAP-specific antibodies in the serum using enzyme-linked immunosorbent assays (ELISA). The Paralisa™ test is such an assay which has been optimized for detection of MAP specific antibodies in NZ red deer\(^87\). However, serological tests can lack sensitivity early in clinical disease and in subclinical infection as antibodies are only produced late in disease.

Diagnosis of JD post-mortem can also be beneficial as an initial indicator that infection is present within the herd. Clark et al. recently described a histopathological grading system, the lesion severity score (LSS), as a method to objectively distinguish disease states and severity in red deer at necropsy\(^88\). This method is useful for challenge studies, especially when determining disease states to group challenged animals into phenotypic R and S groups, as is done the present study and previously by the DRL.

### 1.4.2 Prevention

At present there is no effective treatment for MAP infection or JD. Current options in an infected herd are limited to culling of test-positive stock and depopulation and restocking after two years. These methods are not seen as being cost effective in the eradication of JD, thus prevention of infection in an uninfected herd is of paramount importance. This can be achieved by keeping a closed herd, avoiding buying in animals especially from high risk farms\(^14\). New blood lines can still be introduced through use of artificial insemination.

Several countries have employed vaccination programmes as a means for control of MAP infection. The currently available vaccines are based on oil-adjuvanted whole cell bacterial preparations, containing either heat killed or live, attenuated bacterial strains. Both variations provide moderate protection from incidence and severity of clinical disease; however they do not prevent infection\(^89\)–\(^91\). Furthermore, these vaccines produce strong antibody responses against MAP, which can confound results of routine diagnostic JD and bovine tuberculosis ELISA, and routine skin tests, through cross reactivity\(^92\),\(^93\). Due to these drawbacks of current MAP vaccines, a more effective method of preventing JD may be to use controlled breeding to specifically select for genetically resilient animals.
1.5 Resilience and susceptibility

The ability of an infectious organism to infect a host is, in part, determined by the immune competence of the host. Thus resilience to disease, which is found in many infectious diseases, is controlled by the genetic makeup of an individual. Heritable resilient (R) and susceptible (S) phenotypes have been discovered in purebred red deer by Mackintosh et al., both in *M. bovis* infection\(^94\) and JD\(^36\). R and S phenotypes to JD have also been observed in cattle\(^95\), sheep\(^96\), and goats\(^97\). As resilience to JD most likely arises from an effective immune system, JD resilient animals may also show resilience to other diseases, such as *M. bovis* or parasites, however this idea would require further studies. The polarised phenotypes in red deer exhibit a high paternal heritability of 80% (Frank Griffin, personal communication), indicating that selection of resilient sires for breeding could be a powerful tool for prevention of JD.

Microarray technology has allowed research groups to study the whole transcriptome response of macrophages to mycobacteria, including MAP\(^98\)–\(^101\). These studies are limited by the ability to only detect transcripts of predetermined identity in species from which genomic information is available. Furthermore, these studies have not investigated the differential gene expression in terms of resilience and susceptibility. Previous experimental approaches investigating the R and S phenotype to JD have relied on techniques such as Genome Wide Association (GWA) and have to date been unsuccessful. GWA relies on single genetic markers that show an association with a trait, in this case disease susceptibility, such as single nucleotide polymorphisms (SNP); however these SNPs are often not associated with any known genes or function. These types of study are also ineffective when investigating mechanisms of resilience and susceptibility to infectious diseases as, unlike many inherited metabolic diseases which involve single gene mutations, the mechanisms are complex, involving multiple biochemical and immunological pathways and genes. Rather than attempting to identify single genetic markers, the DRL focuses on multiple genes involved in distinct immune systems in resilience and susceptibility.

A study by Marfell et al. attempted to address some of the shortcomings of previous studies of whole transcriptome responses to mycobacteria by using an RNA-Seq method to investigate differences in innate immune marker expression between genotypically R and S
red deer in response to MAP stimulation. RNA-Seq is a recently developed approach which uses sequencing technologies to give a snapshot of the total messenger RNA (mRNA) in a sample at any one time. The advantages of this technique over hybridization based techniques, such as microarrays, include the ability to measure absolute levels of mRNA expression in a sample as opposed to relative quantification and it does not require prior knowledge of sequence structure. Marfell et al. utilized this technique to identify signature molecules from monocyte-derived macrophages (MDM) associated with either the R or S genotype, and found a more substantial differential gene expression in S animals in response to MAP stimulation. This study identified genes from both the innate and acquired immune system, and also novel genes generally associated with separate systems, such as metabolism. One such novel gene was PKLR, which encodes the enzyme pyruvate kinase. The work by Marfell et al. was expanded by analysis of the expression of selected candidate markers by quantitative-polymerase chain reaction (qPCR). This study found that in response to MAP stimulation, the MDM of S animals showed a significantly higher upregulation of innate inflammatory genes NOS2, IL1A, TNF, and IL23A than the MDM of R animals. Conversely, there was a higher rate of apoptosis in the cells from the R animals post stimulation compared to the S animals.

These initial studies by the DRL suggest that the JD susceptible phenotype of red deer is brought about by an aberrant innate immune response against MAP, whereas a controlled immune system and programmed cell death in R animals leads to a protective response towards the pathogen. The characterisation of R and S animals and the distinguishing markers of either phenotype could allow for marker-assisted exclusion of S animals from breeding programs interested in mitigating the impact of JD.

1.6 Hypothesis and aims

JD is an important disease not only to NZ, but also to global agriculture. The “test-slaughter” eradication method currently used to control the disease is ineffective, as MAP is ubiquitous in the environment in NZ and subclinical disease can allow undetected persistence within a herd. Therefore a new method for control of the disease is important. A more in depth understanding of the immune response to MAP infection could lead to diagnostic tests to determine whether a naïve animal is likely to be R or S. This could allow selection of R sires
and exclusion of S sires for breeding and thus a more genetically resistant herd, decreasing the impact of the disease both on individual farms and the agricultural industry as a whole.

Past research in the DRL has focused on identifying candidate genetic markers with the ability to characterise red deer with extreme R or S phenotypes to JD. This was possible due to identification of purebred red deer lines which showed highly polarised and heritable R or S phenotypes following heavy environmental exposure to MAP. Based on this research, it was hypothesised that there is a dysfunction in the innate immune system of S animals that is contributing to their S phenotype; therefore the host R and S phenotype could be distinguished through differential expression of identified innate immune markers. Initially, this study aimed to validate this hypothesis by addressing the following aim:

- Identify differential expression of innate immune markers providing signatures of the R and S phenotypes

However, the practicality of a diagnostic test for the R or S phenotype would require identifying a combination of both innate and acquired immune markers from a small volume of blood. The feasibility of this was assessed by addressing the following aims:

- Investigate the potential of quantifying differential innate and acquired gene expression from peripheral blood mononuclear cells (PBMCs) cultured from small volumes of blood

- Identify innate and acquired markers of R and S phenotypes in PBMC cultures to be investigated further in future studies

This study is designed as an observational study as an initial exploration into candidate gene expression. Therefore, candidate markers will be investigated for their expression levels and consistency, and for differences between S and R animals, in a small sample of animals. Results from this study will be combined with other studies on both the innate and adaptive immune system of red deer to produce a comprehensive analysis of the immunological mechanisms involved in resilience and susceptibility to JD.
1.7 Rationale

Immunological studies in an exotic animal model, such as red deer, can be difficult due to limited reagents, such as antibodies, compared to more conventional models, such as mice or humans. Techniques utilized in this study included experimental infection and \textit{ex vivo} MDM culture models previously developed and optimized by the DRL. The lack of cervine antibodies means the usefulness of immunoassay techniques such as flow cytometry or ELISA is limited. While some cross-reactivity is seen with certain bovine antibodies, these are too few to create a flow cytometry panel of any meaning.

Consequently, qPCR was employed in the present study to analyse relevant gene expression. This technique is used to amplify and simultaneously quantify target gene transcripts within a sample through the use of fluorescent dyes. The dye preferentially binds to double stranded DNA produced during PCR amplification; the DNA-dye complex then produces a fluorescent signal which can be measured. A higher initial level of DNA results in a stronger signal and thus the sample reaches threshold fluorescence earlier in the PCR cycle. One limitation of using qPCR is that the given gene expression levels cannot be correlated to protein levels, which brings up questions over the biological relevance of a change in expression. While this is of concern for studies aimed at developing a functional understanding of the immune pathways, the primary goal of this study was to discover differences between genetically disparate groups of animals that are R or S to MAP infection for use as biomarkers; therefore it was seen as of little consequence if mRNA levels do not accurately reflect protein levels. Irrespective, mRNA levels could provide markers for each phenotype, which would be relevant as a diagnostic tool assuming mRNA expression levels remain consistent between animals of a given genotype.

The panel of gene targets examined from MDM were determined primarily through previous challenge experiments in the DRL. A preliminary panel of 60 gene targets was identified through a literature search and RNA-Seq data\textsuperscript{102}, and was used in a previous study to gain preliminary data of differential gene expression from R and S animals (Liam Brennan, unpublished data). This set was further condensed to the most informative 14 to be used as a panel in this study, which were selected based on a range of factors, such as consistency of expression, biological relevance, and representation of a range of immunological systems.
It was important that the target genes had grounds for expected differential expression between R and S animals. Therefore, the panel of gene targets included genes which are known to be directly involved in the response to MAP and JD. These included; PRR genes, TLR4 and TLR9; genes involved in chemotaxis, CCL2 and CXCL5; and pro-inflammatory cytokines genes, IL1A, IL12A, and NOS2. IL1A, IL12A, and NOS2 encode for IL1α, IL12 p35, and iNOS, respectively. These genes all contribute to the elimination of pathogens and are thus expected to be upregulated in all animals in response to stimulation. Also included were STAT3 and MAPK8. These genes are involved with intracellular signalling following pathogen recognition; consequently they too were expected to be upregulated following stimulation. ISG15 and ISG20 were included due to the importance of interferons during a protective response to mycobacteria. The regulation of IFNγ is likely also significant, thus the regulatory genes SOCS3 and SOCS4 were included on the panel. Finally, PKLR was identified by Marfell et al. as a novel gene with differential expression between the two groups. While pyruvate kinase is involved in metabolism instead of immunity, these two systems are inexorably linked and this gene may play a role in protection from JD.

A similar method of selection was employed when selecting the target markers to examine from the PBMC assays. However, as this was a new approach taken by the laboratory, the present study did not have the same luxury of transcriptomics data from PBMCs as was available when selecting markers from MDM. Consequently, more weight was added to markers of considerable biological significance. The panel of markers included the macrophage specific genes IL1A, ISG15, and ISG20, markers that were used and showed potential in the MDM assays. Two more macrophage specific markers, IL1B and IL8, were included due to their proposed importance in apoptosis and granuloma formation, respectively. To investigate the lymphocyte response, the remainder of the gene targets were lymphocyte specific. IFNG, IL4, and IL17A were included as the genes encoding the main cytokines produced by Th1, Th2, and Th17 cells, respectively. IL2 was included due to the importance of IL2 in the differentiation of T cells. Finally, IL6 was added due to its role regulating TNFα and IL1α expression.
2 Methods

2.1 Study animals

Ten mixed gender weaner red deer were selected from offspring bred using frozen semen from suspected R and S stags identified from Peel Forest Estate. The animals were housed at AgResearch (Invermay) for the duration of the study.

Over the course of the study, blood samples for monitoring IFNγ and specific antibody production, and faecal samples for monitoring MAP excretion, were taken fortnightly. Results from these tests along with the animal live weights were used to estimate the health and disease status of the animals. Any animal that was seen as severely diseased (loss of 10% of body weight over two weeks) was euthanised. All experimental manipulations performed on the animals were done so with ethical approval from the Invermay AgResearch Animal Ethics Committee (AEC 12928)

2.1.1 Challenge

At approximately six months old, deer were challenged daily with the bovine K10 strain of MAP via oral gavage for 4 days using a dose of $3 \times 10^8$ organisms. The challenge inoculum used in this study was recovered by Geoff de Lisle (AgResearch) from gut associated tissues harvested from two clinically infected animals from a previous study. This particular strain of MAP was used as it is a virulent and well characterized strain.

2.1.2 Phenotype confirmation

At the termination of the study, the surviving deer were euthanised and necropsied. Samples were taken from the anterior, mid, and posterior jejunum (JJ), ileocaecal valve (ICV), and associated lymph nodes and fixed in 10% buffered formalin for histopathological examination. Sections of the posterior jejunal lymph node (JJLN) were also collected into cryotubes and immediately frozen in liquid nitrogen for future gene expression studies. Gross lesions of paratuberculosis in the JJ, ICV, JJLN, and ileocaecal lymph node (ICLN) were described and graded according to blinded lesion severity scores (LSS) by Gary Clark (Wanaka). These scores were then used as a measure of the disease state and thus phenotype
of each animal, where diseased animals were deemed S while undiseased animals were deemed R.

2.2 Blood collection

Due to technical restrictions, four animals were chosen at random from each sire for MDM analysis. These animals were consistent over the study period, with the other two remaining as backups. Blood was collected as required by jugular venepuncture on physically restrained animals at regular intervals up to 37 weeks post challenge. Venepuncture was performed by trained technicians or veterinarians into Citrate-Phosphate-Dextrose containing blood bags (Compoflex®) to a total of 200 ml, and mixed to prevent clotting. As a total PBMC trial was performed at weeks 36 and 37, 10 ml blood was collected into Sodium Heparin-coated vacutainers (BD Biosciences) at this time. Blood was stored at room temperature for no more than four hours until laboratory processing.

2.3 Monocyte-derived macrophage culture

Whole blood was mixed 1:1 with cold citrated-PBS (Appendix 1). The blood mixture was carefully layered over 7.5 ml Histopaque 1083 (Sigma-Aldrich) in 50 ml centrifuge tubes (BD Falcon) and centrifuged for 20 minutes at 600 × g. The PBMCs at the interface were removed using a sterile Pasteur pipette and added to 225 ml centrifuge tubes (BD Falcon), which were filled to 200 ml with cold citrated-PBS and centrifuged for 15 minutes at 500 × g. Cell pellets were resuspended in 10 ml of RBC lysis buffer (Appendix 1) to remove erythrocytes carried over in the isolation process. Cells were washed by addition of 150 ml of cold citrated-PBS and centrifuged for 15 minutes at 500 × g. Cells were resuspended in 60 ml RPMI-1640 (Gibco®, Life Technologies) supplemented with 4 mM L-Glutamine. Ten millilitres of each cell suspension was aliquoted into six 25 cm² vented cap culture flasks (BD Falcon).

Flasks were incubated at 39°C in 5% CO₂ for 2 hours to allow monocytes to adhere to the plastic. Media was removed and cells washed with warm PBS, before addition of warm RPMI-1640 supplemented with 4 mM L-Glutamine, 10% deer serum, 55 µM 2-mercaptoethanol, and 45 µg/ml gentamicin. The flasks were incubated for a further 24 hours and washed with warm PBS, before addition of warm 10% deer serum RPMI-1640. Media
was replaced every two days from this point. The monocytes were allowed to mature into macrophages, as determined by microscope morphology, for 4-7 days then counted on an Olympus IX-71 inverted microscope (Olympus).

Previous studies from our lab using this MDM culture protocol have used α-naphthyl acetate esterase staining and flow cytometry to confirm the cells cultured were from the monocytic lineage. As the same isolation and culture protocol was used, cells isolated in this study were known to be of the same lineage.

2.4 Monocyte-derived macrophage infection

Once cells had taken on typical macrophage morphology, they were infected with the same bovine K10 strain of MAP as used to challenge the animals. Media was removed from the flasks and replaced with gentamicin free 10% deer serum RPMI-1640. Half of the flasks from each animal were infected with MAP at a multiplicity of infection (MOI) of 10:1, whilst the other half remained uninfected controls. Flasks were incubated for 2 hours at 39°C in 5% CO₂ before washing with warm PBS and replacing the media with 10% deer serum RPMI-1640 with gentamicin. The flasks were incubated for a further 22 hours, media removed and cells lysed with 350 µl of NucleoSpin® RNA lysis buffer (Macherey-Nagel) with 3.5 µl of 2-mercaptoethanol. The flasks were stored at -20°C until RNA extraction.

2.5 Peripheral blood mononuclear cell culture and infection

Due to the laborious nature of MDM culture and the inability to find strong markers for either phenotype, a preliminary PBMC stimulation experiment was performed at 36 and 37 weeks post infection. The purpose of this experiment was to determine the feasibility of such an assay from small volume of blood for future studies and into the diagnostic setting. At these time points, blood was collected into two heparin-coated vacutainers from each animal either as well as, or instead of blood bags. Cultured PBMCs were stimulated with either a combination of Staphylococcal enterotoxin B (SEB) and purified protein derivate johnin (PPDj), or with viable MAP. SEB and PPDj were used in combination as they were expected to non-specifically active the T cells and monocytes, respectively, in the sample. Cultures were also stimulated with MAP for the sake of comparisons, both with the SEB/PPDj stimulated PBMCs and MAP stimulated MDM.
The whole blood from the two vacutainers was pooled and mixed 1:1 with cold citrated-PBS. The blood mixture was carefully layered over 7.5 ml Histopaque 1083 in 50 ml centrifuge tubes and centrifuged for 20 minutes at 600 × g. The PBMCs at the interface were removed using a sterile Pasteur pipette and added to a fresh 50 ml centrifuge tube. Each tube was filled to 40 ml with cold citrated-PBS and centrifuged for 15 minutes at 500 × g. The supernatant was removed and cells resuspended in RPMI-1640 with 4 nM L-glutamine. Cells were counted and concentration adjusted to 1 × 10⁶ cells per ml in at least 3 ml. One ml from each animal was added to separate wells on a 24 well plate in triplicate and incubated for 2 hours at 39°C in 5% CO₂. Deer serum was added to each well to a total of 10%. One well from each animal was stimulated with SEB and PPDj, each at 1 µg/ml, one well stimulated with MAP at a MOI of 10:1, and the third left as an unstimulated control. The plate was incubated at 39°C in 5% CO₂ for 12 hours and centrifuged at 500 × g for 15 minutes. The media was removed and cells lysed with 350 µl of NucleoSpin® RNA lysis buffer supplemented with 3.5 µl of 2-mercaptoethanol. The plates were stored at -20°C until RNA extraction.

2.6 RNA extraction from cultured cells

The NucleoSpin® RNA total RNA isolation kit (Macherey-Nagel) was used for extraction of RNA from lysed cells as per the manufacturer’s instructions. Culture flasks containing cell lysate were thawed at room temperature and lysate transferred to a NucleoSpin® Filter in a 2 ml collection tube. The tubes were centrifuged for 1 minute at 11,000 × g and the filtrate mixed 1:1 with 70% ethanol. The lysate mixture was loaded onto a NucleoSpin® RNA Column and centrifuged for 30 seconds at 11,000 × g. The column was transferred to a new 2 ml collection tube and 350 µl membrane desalting buffer was added. Tubes were centrifuged for 1 minute at 11,000 × g. Ten microlitres of reconstituted rDNase was mixed with 90 µl Reaction Buffer for rDNase and 95 µl of this mixture was loaded directly to the membrane of the column then incubated at room temperature for 15 minutes. Two hundred microlitres of buffer RA2 was added to the column to inactivate the rDNase. Columns were centrifuged for 30 seconds at 11,000 × g and transferred to new 2 ml collection tubes. Six hundred microlitres of buffer RA3 was added and columns centrifuged for 30 seconds at 11,000 × g. The flow through was discarded and column placed back in the collection tube.
A further 250 µl buffer RA3 was added and columns centrifuged for 2 minutes at 11,000 × g. The columns were then placed in labelled nuclease-free microfuge tubes. RNA was eluted in 40 µl of RNase-free H₂O and centrifuged for 1 minute at 11,000 × g. The RNA was then quantified using a NanoDrop 1000.

2.7 Reverse transcription

The SuperScript® III enzyme (Invitrogen) was used to synthesize cDNA from the mRNA in the total RNA samples, as per the manufacturer’s instructions. Briefly, 500 ng of RNA (in a volume of 11 µl), 1 µl oligo(dT)₂₀ (50 µM), and 1 µl dNTP mix (10 mM) were mixed in a 0.2 ml PCR tube. The mixture was incubated at 65°C for 5 minutes and then placed on ice for at least 1 minute. One microlitre each of DDT (0.1 M) and SuperScript® III RT enzyme (200 U/µl), along with 4 µl of 5 × Reaction Buffer were added and mixed. The mixture was incubated at 50°C for 60 minutes then reaction terminated at 70°C for 15 minutes. The cDNA was diluted by adding 180 µl of RNase-free H₂O and stored at -20°C.

2.8 Quantitative-polymerase chain reaction

2.8.1 Protocol

Quantitative-PCR was performed in 384-well plates using a ViiA™ 7 system (Life Technologies) using 10 µl reaction volumes per well. The PCR cycle program used is shown in Table 1. Master mixes were prepared for each gene target to reduce pipetting error. Each reaction consisted of 5 µl Fast SYBR® Green Master Mix (Applied Biosystems®, Life Technologies), appropriate forward and reverse primers at 200 nM (Table 2 and 3), 2 µl of the appropriate cDNA sample, and made up to 10 µl with RNase free water.
Table 1. Temperature cycle program for qPCR

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature/time</th>
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<tbody>
<tr>
<td>Step 1</td>
<td>1 cycle 95°C, 15 min</td>
</tr>
<tr>
<td>Step 2</td>
<td>40 cycles 95°C, 15 sec, 60°C, 1 min</td>
</tr>
<tr>
<td>Step 3</td>
<td>1 cycle 95°C, 15 sec</td>
</tr>
</tbody>
</table>

2.8.2 Data analysis

Quantitative-PCR data was analysed using ViiA™ 7 software (Life Technologies). Relative levels of expression were calculated using the comparative cycle threshold (ΔΔCt) method, where data is presented as a fold change of a target gene expression normalised to a reference gene and relative to an unstimulated control. Normalised Ct (ΔCt) values were calculated for each target gene by calculating the difference in Ct between the target gene of interest and the reference gene, which was beta-2-microglobulin (B2M) in this study. The relative gene expression was then determined by subtracting the unstimulated sample ΔCt, which represents the baseline expression of the gene, from the stimulated sample ΔCt, giving the ΔΔCt. To generate the relative change of gene expression in the ΔΔCt procedure, the negative value of the ΔΔCt was used as an exponent of 2 to reflect the fact that the PCR doubles the amount of product per cycle.

2.8.3 Statistical analysis

Relative gene expression values were tested for statistical significance by performing Mann-Whitney U tests using GraphPad Prism v5.0. Significance was assigned where the calculated p-value was 0.05 or less.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5' - 3')</th>
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</thead>
<tbody>
<tr>
<td>B2M forward</td>
<td>GGCTGCTGTGCTGCTGTCT</td>
</tr>
<tr>
<td>B2M reverse</td>
<td>TCTGGTGGGTTGTCTTTTGAGTAC</td>
</tr>
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</tr>
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Table 3. Lymphocyte specific primers used in qPCR

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</tr>
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</tr>
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<td>IL4 forward</td>
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<td>IL4 reverse</td>
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3 Results

3.1 Phenotype confirmation

Red deer stags displaying heritable R and S phenotypes for JD have been identified previously by the DRL. Using these sires for artificial insemination in crossbred hinds, it was possible to breed progeny that predictably produce either phenotype. Nonetheless, it was necessary to experimentally infect each animal to retrospectively confirm their phenotype before characterizing functional pathways of immunity. The study animals were challenged with MAP, the causative agent of JD, at the beginning of the study and phenotypes were confirmed by diagnostic histopathology at necropsy 10 months later.

Following challenge with MAP, animals developed clinical JD, low grade pathology, or completely cleared the infection. None of the animals from this study group were sufficiently diseased to require elective slaughter before the termination of the study. Following necropsy, histopathology based on the LSS system was analyzed from various sections of the gastrointestinal tract and associated lymphatic system of each animal by Gary Clark as a measure of disease severity. The results from histopathological analysis were used to consign animals into two confirmed phenotype groups: R (resilient) and S (susceptible). A total LSS of 5 or below was considered non-diseased or low grade and therefore R. A LSS of 0 represented an undiseased gut, 1-2 represented a mild non-specific enteric infection and 3-5 represented low grade pathology. An LSS of 6 or above was considered clinically diseased and consequently deemed S, where 6-10 indicates moderate JD and 11-13 severe JD.

Three of the progeny from the R genotype sire had a total LSS of 0 (1209, 1216, and 1229), while a fourth had an LSS of 2 (1230). These animals were confirmed to be of the R phenotype (Table 4). The fifth animal of the R genotype sire (1218) showed an LSS of 6 at necropsy, and was consequently consigned to the S phenotype group (Table 4). Of the progeny from the S genotype sire, one had an LSS of 7 (1220), three had an LSS of 9 (1207, 1214, 1231), and one had an LSS of 10. Therefore, all five S genotype progeny were confirmed to the S phenotype. (Table 4) All six S phenotype animals displayed paucibacillary, as opposed to multibacillary, infections.
Table 4. Summary of study animal phenotype confirmation

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sire genotype</th>
<th>Lesion severity score</th>
<th>Phenotype</th>
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<td>S</td>
<td>9</td>
<td>S</td>
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<td>1220</td>
<td>S</td>
<td>7</td>
<td>S</td>
</tr>
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<td>1223</td>
<td>S</td>
<td>10</td>
<td>S</td>
</tr>
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<td>9</td>
<td>S</td>
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<tr>
<td>1209</td>
<td>R</td>
<td>0</td>
<td>R</td>
</tr>
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<td>0</td>
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<td>R</td>
<td>6</td>
<td>S</td>
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</table>
3.2 Gene expression in monocyte-derived macrophages

The main hypothesis of this study was that a defect in the innate immune system of animals of the S phenotype contributes to an inappropriate gene expression profile in response to MAP. It was expected that these defects could be used as diagnostic markers of susceptibility to JD. To investigate this hypothesis, MDMs were cultured in isolation from blood derived PBMCs at various time points over the course of the study. Following culture, these cells were stimulated with viable MAP to initiate an inflammatory response. RNA was extracted from the cells and reverse transcribed to cDNA which allowed a measurable evaluation of gene expression within the cells using qPCR. The cDNA was used to analyse differential changes in gene expression between R and S animals in response to stimulation with MAP. Data is presented as a relative change in gene expression between the stimulated sample and an unstimulated control. Samples from 2 and 31 weeks post-challenge were excluded from gene expression studies due to a lack of confidence in the culture process and macrophage development.

3.2.1 CCL2

CCL2, also known as monocyte chemotactic protein 1, is a chemokine produced by macrophages to recruit leukocytes to the site of infection. Expression of CCL2, the gene encoding CCL2, remained consistent in both groups over the study period. Aside from 10 weeks post-challenge, at which time this gene was downregulated by each group, CCL2 expression was not altered by stimulation with MAP (Supplementary Figure 1). Consequently, there were no differential expression observed between groups.

3.2.2 CXCL5

CXCL5 is another chemokine which is produced by macrophages following IL1α or TNFα stimulation, and functions as a recruiter of neutrophils to the site of infection. CXCL5 is encoded by the gene CXCL5. This gene was generally upregulated up to 100-fold by MDM in response to stimulation; however, there was no consistent difference in relative quantity of expression between animals with S and R phenotypes (Supplementary Figure 2). Interestingly, a statistically significant difference between the S and R phenotypes was observed at 22 weeks post-challenge (Supplementary Figure 2G). At this time, both groups downregulated CXCL5 expression in response to stimulation, with the R group
downregulating the gene to a greater degree. This was the only time point where CXCL5 was downregulated. Another interesting observation was seen at 14 weeks post-challenge, where one animal from the R group, 1209, showed an upregulation of over 1000-fold, which was a 100-fold greater increase than the other two animals in the group (Supplementary Figure 2E).

3.2.3 IL1A

IL1α, encoded by the gene IL1A, is an important pro-inflammatory cytokine produced by macrophages. This cytokine has a range of functions that ultimately result in generation of inflammation, contributing to pathogen elimination. Therefore, it was expected that expression of this gene would be upregulated by MDM in response to stimulation with MAP. As expected, IL1A was upregulated following stimulation by both the R and S animals (Figure 3). The mean degree of upregulation was greater in animals of the S phenotype across most of the time points (Figure 3). The exceptions to this trend, seen at 14, 18 and 26 weeks post-challenge, all had a single outlier in the R group which raised the mean change in expression above that of the S group (Supplementary Figure 3). However, a similar observation could be observed from 4, 8, and 22 weeks post challenge, where a single outlier in the S groups appeared to increase the mean to above that of the R group (Figure 3A, 3B, 3D). In each case, the outlier at each time represented a different animal; therefore, this could not be explained as an anomaly from a single animal.
Figure 3. IL1A is upregulated to a greater degree in MDM of S animals compared to R animals following MAP stimulation

Relative quantity of expression of the \textit{IL1A} gene in animals with an R or S phenotype at 4 (A), 8 (B), 10 (C), and 22 (D) weeks post-challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.2.4  *IL12A*

*IL12A* is a gene which encodes the p35 subunit of IL12. This gene was included on the panel of markers due to the corresponding cytokine’s ability to promote differentiation and proliferation of Th1 cells, a cell type with a well-documented role in protection against JD. Unexpectedly, *IL12A* was not highly upregulated in either phenotype group in response to MAP stimulation. At 8, 10, 14, and 18 weeks post-challenge, expression of *IL12A* was downregulated by both the R and S groups (Figure 4). At these time points, there was a trend towards a greater degree of downregulation in the R animals compared to the S animals (Figure 4). Mean relative quantity of expression from S animals was slightly downregulated, whereas expression from the R animals was decreased by close to half. At the other time points, 0, 4, 22, and 26 weeks post-challenge, mean expression of *IL12A* was either slightly upregulated or unchanged (Supplementary Figure 4).
Figure 4. *IL12A* is downregulated to a greater degree in MDM of R animals compared to S animals following MAP stimulation

Relative quantity of expression of the *IL1A* gene in animals with an R or S phenotype at 8 (A), 10 (B), 14 (C), and 18 (D) weeks post-challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.2.5 ISG15

ISG15 appeared to be a promising gene target, as expression levels and trends were consistent across the entire study period. This gene, encoding for ISG15, is expressed following Type I interferon signalling. The protein plays roles in labelling of molecules in an ubiquitin like manner, and more recently has been shown to function as a cytokine. ISG15 was upregulated by both phenotypes in response to MAP stimulation at each time point (Figure 5). Furthermore, every time point sampled showed the same trend; the R phenotype upregulated expression of this gene to a greater degree compared to the animals of the S phenotype (Figure 5). This difference was statistically significant at 14 and 18 weeks post-challenge (Figure 5E, 5F). Across most of the study, animals of the S phenotype upregulated ISG15 around 10-fold upon stimulation with MAP, whereas mean relative quantity of expression of animals of the R phenotype reached a 100-fold increase. At 10 and 14 weeks post-challenge, one animal in the R group, 1209, upregulated expression of ISG15 by 750-fold, which represented a 20-70 fold greater increase than the other animals in the same group (Figure 5E).
Figure 5. *ISG15* is upregulated to a greater degree in MDM of R animals compared to S animals following MAP stimulation

Relative quantity of expression of the *ISG15* gene in animals with an R or S phenotype at 0 to 26 weeks post-challenge (A-H). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.2.6 ISG20

Like ISG15, ISG20 is a molecule involved in Type I interferon signalling. This protein is encoded by the ISG20 gene and has been shown to play roles in antiviral immunity through exonuclease activity. ISG20 showed near identical results to ISG15 for expression levels and consistency, and trends of differential expression. ISG20 was highly upregulated across all time points sampled (Figure 6). Most time points showed the same trend as ISG15, where the R phenotype upregulated the gene to a greater degree than the S phenotype (Figure 6). The one exception was the 0 weeks post-challenge sample, which showed highly variable responses to stimulation in both phenotype groups (Figure 6A). Consistent with what was seen in other genes, animal 1209 upregulated ISG20 by over 10-fold greater than the two other animals in the R group at 14 weeks post-challenge (Figure 6E).
Figure 6. *ISG20* is upregulated to a greater degree in MDM of R animals compared to S animals following MAP stimulation

Relative quantity of expression of the *ISG20* gene in animals with an R or S phenotype at 0 to 26 weeks post-challenge (A-H). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.2.7  **MAPK8**

MAPK8 is a secondary messenger protein in the c-Jun N-terminal kinase family, activated by TNFα signaling. The gene encoding for this protein, *MAPK8*, was downregulated by MDM of both phenotypes in response to MAP stimulation. Over the course of the study, *MAPK8* expression trended towards a greater degree of downregulation in the R animals compared to the S animals. While this difference was never significant, it was seen at 4, 8, 10, 14, 18 and 22 weeks post-challenge (Figure 7). At 26 weeks post-challenge, an outlier in the S group which upregulated the expression of this gene dragged the mean change of expression above 1, however the remaining four animals all downregulated expression, so this mean may not accurately reflect the results (Supplementary Figure 5)
Figure 7. MAPK8 is downregulated to a greater degree in MDM of R animals compared to S animals following MAP stimulation.

Relative quantity of expression of the MAPK8 gene in animals with an R or S phenotype at 4 (A), 8 (B), 10 (C), 14 (D), 18 (E), and 22 (F) weeks post-challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.2.8 NOS2

NOS2 is the gene encoding for iNOS, an important molecule produced by macrophages to eliminate phagocytosed bacteria. As expected, NOS2 was upregulated by MDM in response to stimulation across all time points (Figure 8, Supplementary Figure 6). However, the degree of upregulation varied across time points, with mean change in expression ranging from 2-100 fold. A differential upregulation of expression of NOS2 was observed between R and S animals, with R animals upregulating the gene to a greater degree compared to the animals of the S phenotype (Figure 8). This was seen across most of the time points sampled, with the exception of 4 and 8 weeks post-challenge (Supplementary Figure 6). At four weeks, no difference in mean change in expression was observed, while at eight weeks the S animals upregulated the gene to a greater degree compared to the R animals. A significantly greater upregulation of NOS2 in the R group compared to the S group was observed at 18 weeks post-challenge (Figure 8C).
Figure 8. NOS2 is upregulated to a greater degree in MDM of R animals compared to S animals following MAP stimulation

Relative quantity of expression of the NOS2 gene in animals with an R or s phenotype at 10 (A), 14 (B), 18 (C), 22 (D), and 26 (E) weeks post-challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
PKLR, the gene encoding pyruvate kinase, was added to the panel of targets as a novel gene based on previous transcriptomics data which indicated its potential for differential expression between R and S animals. From 0-18 weeks post-challenge, PKLR was downregulated in response to infection (Figure 9). Differences in expression were observed between the two groups at these time points (excluding 0 weeks post-challenge), where the animals of the R phenotype downregulated expression to a greater degree than those of the S phenotype (Figure 9). S animals tended to downregulate PKLR expression only slightly, while animals of the R phenotype downregulated expression by over half. This difference was statistically significant at 10 and 18 weeks post-challenge (Figure 9C, 9E). At 22 and 26 weeks post-challenge, mean PKLR expression was either slightly upregulated or unchanged; however no differences were observed between groups (Supplementary Figure 7).
Figure 9. *PKLR* is downregulated to a greater degree in MDM of R animals compared to S animals following MAP stimulation

Relative quantity of expression of the *PKLR* gene in animals with an R or S phenotype at 4 (A), 8 (B), 10 (C), 14 (D), and 18 (E) weeks post-challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.2.10 *SOCS3*

SOCS3, encoded by the *SOCS3* gene, is a protein which plays roles in the negative regulation of Type II interferon signalling. *SOCS3* expression was extremely consistent in both phenotype groups upon stimulation with MAP. The mean change in expression for both groups was extremely close to 1 at each time point, representing neither up nor downregulation (Supplementary Figure 8). No differential trends were uncovered for this gene.

3.2.11 *SOCS4*

Like SOCS3, SOCS4 is a protein involved in regulation of interferon signalling, however it is relatively unstudied. This protein is encoded by the gene *SOCS4*. *SOCS4* expression was downregulated by animals of both phenotypes across most time points in response to MAP stimulation (Supplementary Figure 9). At 0 and 8 weeks post-challenge, *SOCS4* was downregulated by the S phenotype but upregulated by the R phenotype (Supplementary Figure 9A, 9C). This difference was significant in the 0 weeks post-challenge sample. At 4 weeks post-challenge, *SOCS4* was upregulated by both groups (Supplementary Figure 9B). At the remaining time points, 10, 14, 18, 22, and 26 weeks post-challenge, *SOCS4* was downregulated by both groups, with a trend towards greater downregulation in the R group compared to the S group in all but 10 weeks (Supplementary Figure 9). The inconsistency in change in gene expression following MAP stimulation meant that no noteworthy differential expression trends were observed.

3.2.12 *STAT3*

STAT3, encoded by the *STAT3* gene, is a major transcription factor of the immune system, and has been shown to be important in the differentiation of Th17 cells. *STAT3* was either downregulated or unchanged by MDM of animals from both phenotypes when stimulated (Figure 10. Supplementary Figure 10). At animal challenge, *STAT3* was downregulated to a greater degree in the S animals compared to the R animals (Supplementary Figure 10A). However, from 4 to 18 weeks post-challenge, a trend developed in the opposite direction, with R animals downregulating the gene to a greater degree than S animals (Figure 10). At 22 and 26 weeks post-challenge, mean relative quantity of expression of *STAT3* remained at a value of one, representing no change in expression following stimulation.
Figure 10. STAT3 is downregulated to a greater degree in MDM of R animals compared to S animals following MAP stimulation

Relative quantity of expression of the STAT3 gene in animals with an R or S phenotype at 4 (A), 8 (B), 10 (C), 14 (D), and 18 (E) weeks post-challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.2.13 **TLR2**

*TLR2* encodes TLR2, an important pathogen recognition receptor in mycobacterial recognition. Unfortunately, results for *TLR2* were confounded by poor levels and consistency of expression. Of the time points where *TLR2* was expressed at detectable levels, a differential trend in expression was observed at 10 and 18 weeks post-challenge, where S animals upregulated the gene to a greater degree compared to R animals (Supplementary Figure 11C, 11D). The other detectable time points showed no considerable difference between the expression levels of the two phenotypes (Supplementary Figure 11).

3.2.14 **TLR9**

TLR9 is a pathogen recognition receptor which acts through detection of unmethylated CpG sequences found in microbial DNA. This TLR is encoded by the *TLR9* gene. *TLR9* showed a varied trend of expression changes when MDM were stimulated with MAP. The animals of the S phenotype showed downregulated expression of this gene across all time points (Supplementary Figure 12), excluding 4 weeks post-challenge where the S animals upregulated expression by 2-fold (Supplementary Figure 12B). The animals of the R phenotype downregulated expression before animal challenge and 22 weeks post-challenge, did not alter expression at 4, 14, and 18 weeks post-challenge, and upregulated expression at 8 and 10 weeks post-challenge (Supplementary Figure 12). This varied expression of *TLR9* following MAP stimulation meant that no consistent differential trends of expression were evident.
3.3 Gene expression in peripheral blood mononuclear cells

Due to the laborious nature of cervine macrophage culture, making it impractical for use in the diagnostic setting, and the well documented importance of T cells in the immunology of JD, differential gene expression was analysed from PBMC cultures at 36 and 37 weeks post infection. Culture of PBMCs was achieved using a much smaller volume of blood compared to macrophage culture (20 ml vs. 200 ml) to better replicate practical diagnostic testing. Following culture in 24 well plates, PBMCs from each animal were stimulated with either viable MAP organisms, or SEB and PPDj in combination, with one well left unstimulated as a control.

3.3.1 36 weeks post-challenge

PBMC culture was first trialled at 36 weeks post-challenge. In general, expression of the marker genes was upregulated in response to stimulation with either SEB/PPDj or MAP (Figure 11, Supplementary Figure 13). The one exception was IL6, which was downregulated when cultures were stimulated with SEB/PPDj (Figure 11C). Three of the four MAP stimulated R animals also downregulated IL6 expression. Both IL2 and IL4 displayed a greater upregulation of expression in response to the SEB/PPDj stimulation compared to the MAP stimulation (Figure 11A, 11B). However, no differences were observed between the R and S groups for either stimulation. Similar to the gene expression from MDM, ISG20 was upregulated to a greater degree in R animals compared to S animals in the PBMC samples (Figure 11E). This was observed for both MAP and SEB/PPDj treatment. IL1A, IL1B, and IL8 trended towards a higher upregulation of expression from MAP stimulated cells compared to SEB/PPDj stimulated; however there were no differences observed between the R and S groups for either stimulation (Supplementary Figure 13).
Figure 11. Differential gene expression from PBMCs of R and S animals 36 weeks post-challenge

Relative quantity of expression of IL2 (A), IL4 (B), IL6 (C), IL8 (D), and ISG20 (E) in animals with an R or S phenotype. Relative quantity of expression (mean ± SEM) is defined as gene expression upon stimulation with MAP (M group) or SEB + PPDj (S group) compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
3.3.2 37 weeks post-challenge

At 37 weeks post-challenge, all genes analysed from PBMC cultures were upregulated or unchanged in response to stimulation with either MAP or SEB/PPDj (Figure 12, Supplementary Figure 14). *IL1A* displayed a trend of greater upregulation of expression in the S animals compared to the R animals from both stimulation groups (Figure 12A), a trend that was replicated by *IL2* (Figure 12B). While expression of *IL1A* trended towards a higher upregulation of expression from MAP stimulated PBMCs compared to SEB/PPDj, *IL2* was upregulated to a greater degree in the SEB/PPDj stimulated cells. *IL4* was also expressed at higher levels in cells stimulated with SEB/PPDj compared to MAP, however no difference was observed between the R and S groups for either stimulation (Figure 12C). *IL6* expression was differentially expressed from MAP stimulated PBMCs, where R animals upregulated the gene to a greater degree than S animals (Figure 12D), however no difference was observed in SEB/PPDj stimulated cells. *IL1B*, *ISG15*, *ISG20*, *IL18*, and *IL17A* did not display differences between either the animal groups, or stimulation treatments.
Figure 12. Differential gene expression from PBMCs of R and S animals 37 weeks post-challenge

Relative quantity of expression of *IL1A* (A), *IL2* (B), *IL4* (C), and *IL6* (D) in animals with an R or S phenotype. Relative quantity of expression (mean ± SEM) is defined as gene expression upon stimulation with MAP (M group) or SEB + PPDj (S group) compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
4 Discussion

Despite an abundance of research into mycobacterial infections spanning decades, it is still unclear how a fraction of infected individuals succumb to clinical disease while the vast majority successfully contain infection. This phenomenon is seen in many mycobacterial species, including MAP infection leading to JD in ruminants such as red deer. Previous work by the DRL has led to the hypothesis that the individuals that succumb to JD do so due to their susceptible phenotype, which is brought about by a genetic imbalance of the innate response to infection. The primary aim of this study was to address this hypothesis by utilizing cervine MDM culture and qPCR to determine how ex vivo responses of macrophages differ between genetically R and S red deer in response to MAP stimulation. This knowledge is to be used to explore differential gene expression as markers for proactive diagnosis of the R or S phenotype. The study was also extended to include a preliminary study to assess the feasibility of investigating differential gene expression from PBMC cultures, which is more amenable for use in diagnostic assays.

Quantitative-PCR was chosen as the primary method of investigating the immune response due to the difficulty in performing immunological studies in a cervine model; lack of cervine antibodies limits the effectiveness of immunological assays such as ELISA and flow cytometry. Furthermore, qPCR presents a more flexible platform for diagnostic tests than more commonly used immunoassays. The ΔΔCt method of data analysis was employed; qPCR data is presented as a fold change of the target gene expression, normalised to a reference gene and relative to an unstimulated control. The purpose of a reference gene is to standardise the target gene Ct values to account for variations in RNA levels added for reverse transcription. Consequently a gene was selected which exhibits consistent levels of expression, including following stimulation. The reference gene used in this study was B2M, the gene encoding for B2M, a component of the MHC-I molecule. MHC-I is constitutively expressed by all nucleated cells and therefore expression of its components was not expected to be altered in response to infection. This particular gene was selected as it was compatible with previous studies in the DRL, where it has been confirmed as a suitable normalisation gene in the cervine model. A second consideration for studies using qPCR, particularly when using the ΔΔCT method, is that this method assumes that the amplification efficiencies of
the normalisation gene and the target genes of interest are approximately equal. The primers used in this study were verified as such in previous experiments.

### 4.1 Study animals

Animals were challenged with MAP in order to confirm their R/S phenotypes at necropsy. None of the study animals were seen to exhibit clinical disease requiring elective slaughter before the completion of this study. This is inconsistent with what has been seen in previous challenge experiments, where animals have begun to lose sufficient weight to require euthanasia from 14 weeks post-challenge. This was likely due to a reduced number of viable MAP organisms in the challenge inoculum compared to previous years ($10^8$ vs. $10^9$), and it is thought that extension of the study period would have led to a heavier disease state in the S animals. The infectious dose of a study of this type is somewhat of a balancing act; it must be high enough to cause disease in the S animals within the study period, but not too high as to cause infection in animals that would be resilient to natural infection. Using too low a dose could cause S animals to appear R, while too high a dose could cause R animals to appear S. Infected animals can shed up to $10^8$ organisms per gram of faeces, leading to heavy environmental contamination and high natural exposure to the infectious agent. Regardless, all five of the progeny from the suspected S sire had evidence of pathology compatible with JD at necropsy and were confirmed to be of the S phenotype. One of the progeny from the suspected R sire exhibited clinical JD and consequently was consigned to the S group, whereas the remaining four were confirmed to be of the R group. This is consistent with previous observations by the DRL, in which R or S phenotypes have a paternal heritability of 80%. Furthermore, this confirms the predicted phenotypes of the two sires, suggesting the potential for continuation of immunological studies using progeny from these animals.

### 4.2 Monocyte-derived macrophage gene expression

Macrophages were cultured from the blood of the study animals at various time points over the course of infection. However, at 2 and 31 weeks post-challenge there was a lack of confidence in the culture process leading to the exclusion of these time points from further analysis. It is thought that human error led to an incorrect culture process, resulting in macrophages that appeared unhealthy when morphology was observed after seven days of culture. These cells were consequently excluded from analysis to ensure consistency of data,
as gene expression responses from unhealthy cells would undoubtedly differ from healthy cells.

To address the hypothesis that a genetic imbalance in the innate immune system of S animals contributes to their phenotype, differential gene expression between R and S animals was analysed from MDM over the course of infection. MDM were cultured in isolation to directly address the hypothesis without interference from other cell types, such as lymphocytes. The gene targets to be investigated were determined from roughly 60 targets identified for their potential from previous work in the laboratory. These targets were condensed to a workable panel based on a variety of factors, including biological relevance, consistency of expression, and association with a range of immunological pathways. Stimulation of MDM with MAP led to a consistent differential expression profile for the genes \( IL1A \), \( IL12A \), \( ISG15 \), \( ISG20 \), \( MAPK8 \), \( NOS2 \), \( STAT3 \), and \( PKLR \).

The gene targets investigated that were considered to have the most potential were the pro-inflammatory genes, \( IL1A \), \( IL12A \), and \( NOS2 \). This was due to both their importance in direct control of pathogens, and their ability to stimulate further pathways in the defence against mycobacteria. Intuition dictates that these genes would be upregulated in response to MAP infection, with R animals upregulating the genes to a greater degree than S animals. However, previous results from the laboratory by Dobson et al. indicate that in fact the opposite is true, where S animals upregulate these genes to a greater degree than R animals\(^{104}\). It is likely that the increased upregulation of pro-inflammatory genes by S animals is excessive, leading to a dysfunctional response to the pathogen which is incompatible with protection. Along with the obvious susceptibility associated with lack of cytokine production, there is also documented examples of excessive cytokine production leading to susceptibility to infection\(^{105}\). The excess in pro-inflammatory cytokine production seen in S animals could also be a direct result of impaired bacterial clearance seen in MAP susceptibility, rather than the initial cause. Defects in clearance of the infectious mycobacteria in S animals may lead to compensatory yet excessive cytokine production, which further contributes to the impaired clearance and worsens the disease state. Conversely, R animals exhibit a comparatively greater intrinsic expression of inflammatory cytokines. This is suggested to represent a state of “readiness”, which is adequate in
controlling infection without significant compensatory upregulation. A similar idea was suggested by Orlova et al., who observed that resilience or susceptibility of mice to M. tb was dependent on intrinsic gene expression levels of alveolar macrophages. An interesting investigation for future studies of the cervine response to MAP could be to analyse the absolute instead of relative gene expression in the unstimulated samples as an indication of intrinsic gene expression and its effect on host susceptibility.

Consistent with Dobson et al., the present study found that IL1A, the gene encoding for IL1α, showed a trend towards greater upregulation from MDM of S animals compared to animals of the R phenotype (Figure 3). This is also consistent with the widely reported observation that clinically diseased animals express greater levels of IL1A in the gut and associated lymphatic compared to undiseased animals. IL1α is a general pro-inflammatory cytokine, and it is believed that this increased IL1α is excessive, playing a pathologic role within the host. Specifically, it is thought that IL1α excess induces necrosis, rather than apoptosis, in infected macrophages, resulting in impaired destruction of internalized MAP and the subsequent release of bacilli from the cell. This impaired clearance leads to further IL1α expression which compounds the disease. A similar mechanism has been suggested in susceptibility to tuberculosis associated with excess TNFα production in both zebrafish and humans. Moreover, IL1α has been implicated in the recruitment of leukocytes for granuloma formation in mycobacterial infections. Granuloma formation, while historically considered to protect against mycobacteria, is now known to be a site of expansion and subsequent spreading of bacilli, and its excess in S animals contributes to the greater gross pathology observed compared to R animals.

Unexpectedly, expression of NOS2 did not follow the same trend. Expression of NOS2, the gene encoding iNOS, was upregulated to a greater degree in the R animals compared to the S animals (Figure 8). While this is not consistent with what was seen in previous studies, it is a more intuitive response representative of the R animals enhanced ability to eliminate invading bacteria. Mycobacterium have been shown to prevent iNOS recruitment to macrophage phagosomes during infection, one of the strategies these bacteria use to subvert the immune response. It is possible that macrophages from R animals are able to prevent this block in recruitment via enhanced early responses, which necessitates a greater
expression of NOS2. It is unclear why this is inconsistent with results from previous studies into the R and S phenotypes; however it may be an anomaly of the novel genotypes of the sires used in the present study.

Dobson et al. found that there was a greater rate of apoptosis in MDM of the R animals compared to the S animals following MAP infection, assessed by DNA fragmentation. Apoptosis is the controlled death of a cell during which the integrity of the cellular membrane remains intact. This is in contrast to necrosis, which is an uncontrolled cell death characterized by disruption of the plasma membrane. Consequently, apoptosis leads to destruction of internalized bacteria, while necrosis can result in release of viable bacteria into the extracellular milieu. Apoptosis is one of the key mechanisms by which a macrophage can eliminate phagocytosed bacteria, and the finding by Dobson et al. suggests the specific importance in controlling MAP infection. This is supported by the present study’s finding that R animals have a greater capacity to upregulate NOS2 compared to S animals, as NO has been shown to directly induce apoptosis during infection. Furthermore, diseased animals have been shown to upregulate TNF receptor associated factor 1 (TRAF1), a strong inhibitor of TNF induced apoptosis. Taken together, this could infer the importance of apoptosis in protection against JD and may suggest the potential for investigating apoptotic pathways in more depth for further candidate gene expression studies.

Another unexpected result was that of IL12A, which was consistently downregulated in response to MAP stimulation by animals of both the R and S phenotype (Figure 4). IL12A encodes for the p35 subunit of IL12, a key cytokine in the differentiation of Th1 cells and is therefore critical in the defence against intracellular pathogens such as MAP. Therefore, it was expected that MAP stimulation would cause the MDM to upregulate the expression of both subunits of IL12. This result may be explained by a study by Gerosa et al. investigating IL12 and IL23 cytokine expression in human monocyte-derived DCs. When interpreting the findings from these studies, it is important to note that IL12 is made up of subunits IL12 p35 and p40, and IL23 is made up of subunit IL23 p19 and the p40 subunit of IL12. Gerosa et al. found that infecting DCs with M. tb, or mimicking infection via stimulation with NOD2 and TLR2 ligands, preferentially induced IL23 but not IL12 cytokine expression. In fact, stimulation with the TLR2 ligand was shown to inhibit IL12 production.
concurrent stimulation of DC with IFNγ greatly increased the production of IL12 and inhibited IL23. These patterns of IL23 and IL12 production were mirrored in the mRNA levels of IL23 p19 and IL12 p35, respectively. This data indicates that the p40, but not p35, subunit of IL12 is expressed by the innate immune system following bacterial recognition via PRRs, whereas the p35 subunit requires further stimulation by the adaptive immune system. When considering the data presented in this study, it is possible that a similar trend is occurring. The MDM cultures investigated here lack involvement from T cells, including the necessary cytokine for p35 production, IFNγ. While macrophages are capable of producing IFNγ, it is not produced in considerable levels able to exert a significant effect. Furthermore, interaction of MAP with TLR2 may actually inhibit p35 production. The trend of a greater decrease in expression by the R animals compared to the S animals may be a result of an enhanced sensitivity of TLR signaling in the R animals, leading to an enhanced inhibition of IL12 p35 expression. These results suggest that the p40, not the p35, subunit of IL12 may have been the more informative biomarker for use within MDM cultures. Conversely, IL12 p35 may be a good marker in PBMC cultures due to the inclusion of T cells, and consequent IFNγ production.

The role ISG15 plays in mycobacterial disease has been well studied in recent times. This protein, which is expressed through Type I interferon signalling or directly expressed following TLR9 stimulation, has been suggested to play a role as a cytokine, promoting IFNγ production from T cells. Through this pathway, ISG15 has been shown to play an essential role in mycobacterial immunity. Furthermore, ISG15 deficiency has been proposed as the molecular basis of Mendelian susceptibility to mycobacterial disease (MSMD), an extremely rare disorder in which host individuals have a susceptibility to clinical disease caused by typically avirulent mycobacterial species, such as the Bacille Calmette-Guerin (BCG) vaccine. In a preliminary study by Bogunovic et al., young children with MSMD who developed disease following BCG vaccination had mutations in the ISG15 gene, which led to a loss of expression of the corresponding protein. The well documented importance of IFNγ in mycobacterial immunity and the more recent evidence of ISG15 stimulating IFNγ production indicate a clear causal link between the ISG15 mutations and MSMD.
This may explain the results of the present study, in which ISG15 was upregulated to a greater degree in animals displaying an R phenotype to MAP infection compared to those displaying an S phenotype (Figure 5). Similar to the case of MSMD, animals of the S phenotype may have defects or mutations leading to a comparative reduction in the expression of the ISG15 gene and ultimately an impaired IFNγ response. Like all mycobacterial diseases, an impaired IFNγ response towards MAP infection is associated with development of clinical disease113. Not only does this finding allude to the potential of ISG15 expression as a diagnostic marker for the R and S phenotypes of JD, it also adds support to the hypothetical importance of ISG15 in general mycobacterial immunity.

Another Type I interferon signalling gene, ISG20, was also consistently upregulated to a greater degree in the R animals compared to the S animals (Figure 6). While ISG20 has well known roles in antiviral immunity, the effect it may have on mycobacterial species is less clear. This protein has exonuclease activity, with a specificity for single stranded RNA, thus it has been demonstrated to help protect against viruses with RNA genomes, such as human immunodeficiency virus and influenza114. ISG20 may play an as yet undiscovered role in cell signalling, similar to ISG15, during bacterial infection. Conversely, the increased expression observed may be redundant in mycobacterial immunity, rather representing a non-specific result of pathogen recognition receptor and interferon signalling. Regardless of its function, ISG20 expression was consistently higher in animals of the R phenotype, indicating the potential of the gene to contribute to a diagnostic on the proposed platform.

Expression of MAPK8 was downregulated in response to bacterial stimulation, with a trend towards a greater degree of downregulation in the R animals compared to the S animals (Figure 7). MAPK8 is a secondary messenger activated by TNFα signalling, which is critical for TNF induced apoptosis in macrophages. It has been shown that pathogenic mycobacteria are able to manipulate the activation of the MAPK pathway, diminishing the level of inflammatory mediators produced by that cell115. While it is not clear whether this reduced activation extends to alteration of expression, this could be the cause of the observed decreased expression. It is also unclear why a greater reduction in expression was observed in the R animals compared to the S animals; however this may be related to the R animals ability to protect themselves from MAP induced pathogenesis. It is thought that TNFα is in
part responsible for the pathogenesis caused by JD\textsuperscript{116}. Thus a reduction in MAPK8 would result in a reduction in TNFα signalling and ultimately the reduced pathogenesis observed in R animals. However, this finding is somewhat inconsistent with the previously mentioned hypothesis that effective apoptosis is critical for resisting MAP infection, as a reduction in MAPK8 should theoretically lead to a reduced ability to induce apoptosis due to its importance in TNF induced apoptosis. This is likely an observation based on a balance in the immune system, where expression changes compensate for each other to prevent excessive or imbalanced reactions.

\textit{STAT3} was also downregulated by animals of both the R and S phenotypes following MAP stimulation. A trend was observed of greater downregulation in the R animals compared to the S animals (Figure 10). While the most notable role of STAT3 is as an important regulator of Th17 differentiation, the present study examined \textit{STAT3} expression from macrophages and thus this role was irrelevant. However, it has also been observed in macrophages that STAT3 is required to mediate the anti-inflammatory activity of the cytokine IL10\textsuperscript{117}. The observed reduction of \textit{STAT3} expression in macrophages following stimulation could be a mechanism to decrease IL10 responsiveness following infection, which would reduce the anti-inflammatory effects of the cytokine. Similar to what was seen in \textit{MAPK8} expression, the response to infection of \textit{STAT3} expression is likely about maintaining immune balance, where \textit{STAT3} was downregulated to outweigh the effects of increased IL10 production. The greater downregulation in R animals compared to S animals could be a result of a higher intrinsic expression of \textit{STAT3} in the R animals, which is expected due to their controlled response to MAP infection. Expression of \textit{STAT3} in this case must reduce by greater levels in the R animals compared to the S animals to effectively reduce the anti-inflammatory effects of IL10. This further suggests that the functional significance of this study could be improved by observing absolute rather than relative gene expression.

Along with the genes important for immunity, \textit{PKLR} was investigated as a novel biomarker of the resilient and susceptible phenotypes based on preliminary transcriptome data. A trend was observed of a greater downregulation of \textit{PKLR} in animals of an R phenotype compared to those of the S phenotype, which was generally unchanged (Figure 9). \textit{PKLR} is the gene for pyruvate kinase, an enzyme which contributes to glycolysis by catalysing the transfer of
a phosphate group from phosphoenolpyruvate to ADP, producing pyruvate and ATP. When considering how this enzyme plays a role in the immune response, it is important to consider both the M1-M2 macrophage paradigm and the mechanisms of macrophage metabolism. The dependence of macrophages on anaerobic glycolysis has been known for almost a century. More recent analysis suggests while this is true of the M1-type macrophages, M2 macrophages are capable of utilising oxidative metabolism. This disparity is related to the greater NO production on activation of M1 macrophages compared to M2 macrophages, which inhibits mitochondrial respiration, forcing the M1 macrophage to switch to glycolysis. The differences between the R and S animals levels of PKLR expression is potentially due to differences in M1 and M2 populations; S animals succumb to disease and produce predominantly M1 cells, whilst R animals produce a higher proportion of M2 cells that are likely an effect of the successful clearance of the pathogen. The lack in M1 cells in R animals would lead to a relative reduction of macrophage glycolysis and therefore a decrease in the requirement and therefore expression of pyruvate kinase. Supporting this is the observation that animals of the R phenotype upregulated NOS2, the gene leading to respiration inhibiting NO production, to a greater degree than S animals. While the aforementioned disparity between M1 and M2 populations is the most likely cause of the greater decrease in PKLR expression from R animals, it could alternatively be an active mechanism for control of infection, whereby the host macrophage tries to reduce the availability of pyruvate as a component of MAP metabolism and thus growth. A third possibility is that the R macrophages actively downregulate PKLR as a survival mechanism. Metabolic glycolysis leads to the generation of an acidic environment, which could have negative effects on cell stability and survival during infection.

The remaining genes, CCL2, CXCL5, SOCS3, SOCS4, TLR2, and TLR9 either showed no change in expression following MAP stimulation, or variable change with no differential trends present. In some cases this may be because the gene and resulting molecule plays no role during the immune response to MAP. Conversely, it may be that these molecules primarily exert their effect through activation instead of increased expression. Therefore, the host relies on the intrinsic expression of these genes. This theory could be investigated following the development of cervine monoclonal antibodies, where antibodies detecting the level of activation (through phosphorylation or other mechanisms) are employed for
immunoassays. Regardless, we believe that these genes have little potential for use in diagnostics for differentiation of the R and S phenotypes, and their inclusion on the panel of gene targets for investigation should be discontinued.

While trends in differential gene expression were uncovered, this study was unable to find markers able to solely identify either phenotype. This was not unexpected in this observational study, the small sample size restricts the generation of statistical significance and intensifies outlier variation. Furthermore, the complexity and polygenic nature of susceptibility to infectious disease likely necessitates the use of biomarkers in combination for phenotypic diagnosis. However, the results suggest that the pathways responsible for apoptosis and Type I interferon signalling are the most important of those investigated in discriminating between the R and S phenotypes. The literature suggests TNFα excess is also involved in the S phenotype through granuloma formation and initiation of necrosis. Consequently, based on this study and the literature, it is suggested that further research in this field significantly weights investigation towards the pathways involved in apoptosis, Type I interferon signalling, and granuloma formation. A model of how these pathways contribute to the R and S phenotypes is proposed in Figure 13. Resilient animals produce strong apoptotic responses to successfully eliminate invading MAP. These animals also exhibit a strong Type I Interferon response which contributes to effective recruitment and activation of Th1 cells. Conversely, excess pro-inflammatory cytokines produced by susceptible animals leads to necrosis of infected macrophages, resulting in release of viable MAP into the extracellular environment. It is unclear whether this cytokine excess is the initial cause or a direct result of impaired pathogen elimination. Regardless, the increased bacterial burden compared to resilient animals exacerbates the excess pro-inflammatory cytokine production. To compensate for increased bacterial burden, granuloma form in an attempt to wall off MAP, however provide an environment for MAP to multiply.
Figure 13. Model of immune response to MAP infection leading to resilience or susceptibility

A proposed model of immune responses contributing to the R and S phenotypes to JD, based on observations from this study and the literature. Resilient animals appear protected through potent Th1 and apoptotic responses. Conversely, susceptible animals produce excess pro-inflammatory cytokines leading to necrosis of infected macrophages, impaired pathogen elimination, and pathology due to granuloma formation.
At some time points, individual animals showed a much higher change in expression across many gene targets compared to the other animals in the group. This was true of the R animal 1209 at 14 weeks post-challenge, where the genes $CXCL5$, $CCL2$, $IL1A$, $ISG15$, $ISG20$, and $SOCS3$ were upregulated to a much greater degree than the other animals in the same group. It is unclear why this anomaly was observed, however it may have occurred due to stress on the animal at the time of blood sampling, perhaps as a direct result of the MAP challenge, as the animal may have been at a critical time for eradication of the infection. Conversely, the stress may have come from infection with an environmental pathogen, such as a parasite. Regardless, this indicates the potentially unpredictable nature of the assay, where the end result is confounded by the unnoticed ill health of the animal.

Presentation of the data in its current form can be difficult to interpret, as the large number of variables means that consistent differences in the response patterns are hard to observe. Consequently, principal component analysis (PCA) was applied to the MDM expression data in an attempt to simplify the analysis. PCA is a form of multivariate analysis which aims to identify hidden patterns in a set of variables by reducing the number of dimensions of the data. The essence of PCA is that variables are condensed to “principal components”, which explain a certain amount of the variability observed from the data. When principal components are observed in graphical form, patterns of similarity between observations are uncovered. It was thought that trends may be uncovered in this study concerning the balancing nature of the immune system, where gene expression is altered to compensate for change in expression of other genes. Unfortunately, this was not the case, likely due to the small sample size. Only three animals of the R group meant that any variation, such as what was seen by animal 1209, played a big role in the observation of trends. It is thought that increasing the sample size would reduce the effect of individual variation, thus PCA remains a viable alternative to observe trends within similar studies.

### 4.3 Peripheral blood mononuclear cell gene expression

Analysis of differential gene expression from MDM can be laborious in nature due to the considerable volume of blood required and seven day culture period. For a diagnostic test to be feasible, it would need to be achieved from a small volume of blood in a relatively short time frame. Consequently, PBMC cultures were investigated as an initial determination of
their feasibility as a diagnostic assay. Along with the smaller volumes of blood and significantly decreased culture duration, PBMC cultures are advantageous due to the inclusion of lymphocytes, expanding the scope of potential biomarkers. This would likely better represent the range of biomarkers contributing to the complex R and S phenotypes.

The stimulation method of PBMC cultures was an important consideration, as viable MAP is unlikely to evoke a substantial response in lymphocyte cells over the short stimulation period. As a solution to this, PBMCs were stimulated with a combination of SEB and PPDj. SEB, a polyclonal T cell activator, was used as a non-specific stimulant of lymphocytes as it was expected to significantly intensify the expression response compared to MAP stimulated cells. PPDj was used in conjunction with SEB to stimulate activation of the innate immune cells. Indeed, SEB/PPDj stimulated samples were able to upregulate expression of the lymphocyte specific genes \( IL2 \) and \( IL4 \) to greater degree than MAP stimulated cells. However, \( IL1A, IL1B, IL6 \) and \( IL8 \), genes generally considered to be macrophage specific, were upregulated to a greater degree in the MAP stimulated samples. This was likely a result of the enhanced ability of whole organisms to stimulate a macrophage response, compared to derivatives of an organism (PPDj). This means that the selection of markers for diagnostic use could be somewhat dependent on the method of stimulation used.

No strong markers of either phenotype were uncovered in the PBMC cultures. MAP stimulated expression of \( IL1A \) was greater in the R animals compared to the S animals at both 36 and 37 weeks post-challenge; however this was the only trend that was consistent between time points. The vast majority of investigated biomarkers showed no differential expression between respective animals of the R and S phenotypes. However, despite the lack of differences between phenotypes, PBMC culture remains a viable diagnostic alternative to MDM culture. Collection of a small volume of blood was sufficient to isolate ample cell counts for detection of gene expression. Moreover, all genes were upregulated following stimulation to a level of consistency not observed from MDM cultures, which were highly variable. The lack of consistent differences is likely due to the investigated markers themselves, where alternate markers may be more appropriate. The transcriptomics study undertaken by Marfell et al., which was used for the selection of biomarkers for analysis by this study, was performed in MDM and therefore was of little use in the selection of markers.
for PBMC analysis. Therefore, more work must be done to determine an appropriate panel of genes if this assay is to be used in the diagnostic setting.

### 4.4 General discussion

The data presented in this study, be it significant or not, must be considered carefully due to the relatively small sample size. The MDM simulation study consisted of only eight animals, while the PBMC study consisted of ten. This was a consequence of the difficulty in sampling from large animals such as red deer. Increasing the sample size of each would have led to severe limitations in terms of cost, sampling duration, processing time, and equipment availability. This is why it is important to consider the presented data with that from other studies in the context of the overall project to gain a meaningful understanding.

This study was a continuation of work presented by Marfell et al., and Dobson et al. Marfell et al., reported a transcriptomics approach to exploration of signature molecules linked to the S and R phenotypes. This was expanded on by Dobson et al., who used qPCR to examine the contribution made by specific inflammatory markers to either phenotype. These studies found many of the pro-inflammatory genes were upregulated significantly higher in MDM of S animals compared to R animals in response to infection, whereas the trends presented in this study are opposite or null across many genes. However it is important to note that the animals used by Dobson et al., were defined as R or S based on their genotype instead of phenotype; consequently the animals were not challenged with MAP. This represents one of the possible limitations of this study: the infection status of the study animals. In order to retrospectively confirm the R and S disease phenotypes, the animals had to be infected with viable MAP. However, it is unclear what effect this may have had on the current study. It may have been the case that the infection confounded the results by systemic effects of the infection altering the physiology of the cells isolated for culture. Differential gene expression would have been particularly confounded late in infection, as although both groups were infected with MAP, the S animals were clinically diseased whereas the R animals were able to successfully contain or eliminate the infection. Therefore the differential results observed may have been a difference in “diseased vs. undiseased” animals instead of the desired “resilient vs. susceptible” comparisons and, consequently, it is unclear how these results would relate to naïve animals.
In opposition to this idea is the extraordinary plasticity of macrophages; they have a well-documented ability to alter their physiology based on their environment. It is likely that this plasticity would negate the infection status once the cells have been taken from the animal, as they would quickly revert to their natural state. On the contrary, differences have been observed in gene expression of cultured cells between diseased and healthy animals\textsuperscript{21,119,120}, suggesting infection status does affect subsequent gene expression. However, it is unclear whether observations from these studies were confounded by the susceptibility phenotype of the animals. One would assume that the diseased animals of these studies had an S phenotype, whereas the healthy animals could be of intermediate or R phenotype. This could result in the observed differential gene expression being due to inherent differences between R and S macrophages instead of disease status.

Regardless of whether or not infection status affects the differential gene expression, a more effective method of study would be to analyse the gene expression in a large group of young, MAP negative, purebred red deer and monitor these over a long period of time to identify those of the R and S phenotypes. However, this type of study would need to be on farms with a history of clinical JD where each animal is likely to encounter infection. Furthermore, the scope of the project would be huge as a very large group of deer would need to be monitored (>100) over a period of years. Assumptions would need to be made about each animal encountering sufficient MAP to cause infection in S animals. Due to the economic and time demands of an investigation of this nature, it was well beyond the scope of the current study and should be employed in the trial phase of a newly developed diagnostic assay.

As has been alluded to previously, it is possible that inconsistencies between the present study and previous studies are manifestations of the different genotypes of the animals sampled. The present study was the first time investigating the sires used; previous studies used progeny of different R and S sires. It is not unexpected that gene expression would differ between sires despite consistent phenotypes. As is the complexity of infectious disease, susceptibility to JD is not dependent on a single factor, rather caused by the accumulative effect of many immune defects of limited penetrance. While the same factors are heritable through the paternal lineage, it is expected that animals of different lineages
may produce similar phenotypes due to different deficiencies. Consequently, although
different genetic deficiencies may lead to susceptibility through the same biological
pathways, animals used across studies may differ in responses to MAP while displaying the
same phenotype. This adds further support to why the investigation of many animals of each
phenotype is important in order to observe the range of markers associated with either
phenotype, and why the data presented here must be considered along with data from other
studies. Differential sire expression does, however, raise a question over animal 1218. While
this animal was the progeny of the R sire, it developed sufficiently severe disease to be
consigned to the S group. Considering the differing parental lineage to the rest of the S group,
this animal could be expected to exhibit a different gene expression profile following MAP
stimulation. While this animal was an outlier in the S group for some genes, falling into the
range of the R group, it was not consistent enough across the time points to draw meaningful
conclusions. Further research into animals with phenotypes deviating from their parental
genotype would provide a better understanding of the range of possible variables in the
diagnostic setting.

A limitation of this study was the single stimulation end point. The MDM and PBMC
cultures were stimulated for 24 and 12 hours respectively, periods that were optimized in
previous studies. Following stimulation, cells were lysed and RNA extracted to give a
snapshot of the expression profile at that time, meaning temporal changes in gene expression
were not accounted for. A previous study has observed that in cervine PBMCs stimulated
with PPD-bovis, an 8 hour stimulation is optimal for IL2 expression, whereas 48 hours is
necessary for considerable IL4 expression. It is possible that some genes are upregulated
eyli in stimulation but return to normal levels by the end point of this study, incorrectly
inferring that the genes were not altered following stimulation. This could also be the reason
for the observed differences between R and S animals. One group may have a substantial
initial response that is reduced to normal by the stimulation endpoint, whereas the other
group has a persistent expression. Further research into the temporal expression of genes
following stimulation could elucidate important differences between the R and S animals
that are important for their respective phenotypes.
Temporal changes within the stimulation period may also account for the downregulation observed in many genes. Many genes within the immune system exhibit a negative feedback type regulation to prevent excess expression. An example is in the regulation of IL12, where stimuli leading to IL12 production concurrently stimulates activation of phosphoinositide 3-kinase\(^1\). This protein leads to negative regulation of IL12 expression to prevent excessive Th1 polarisation. This may be occurring in the genes observed to be downregulated in response to MAP stimulation; the initial upregulation of expression leads to a negative feedback to decrease the expression, and thus levels of mRNA fall to below intrinsic levels by the completion of the assay despite a considerable upregulation in protein expression.

Whilst the data presented in this study is useful to identify putative markers for the R and S phenotypes in red deer, it is important to reflect that they are not a direct representation of what is occurring in the gut of diseased animals. The current study was an investigation into the intrinsic capacity of immune cells to alter relevant gene expression following MAP infection. While this may give a good initial idea of what is occurring in JD, and general interpretations of the trends seen in this study are offered as assumptions of inherent cellular function, more work must be done to elucidate the significance of each finding. Furthermore, this work found associations between gene expression and the R and S phenotypes, whereas causality is more difficult to determine. However, future investigations are limited by the inability to perform immunoassays from the cervine species. There are multiple reasons why this data may not be a direct representation of what is occurring in natural infection.

Firstly, as is the nature of qPCR, it cannot be assumed that the increase in gene expression equates to an equivalent increase in protein expression without further experimentation. Therefore, the biological relevance based solely on the increased gene expression is questionable. It is commonly regarded that there is a positive correlation between the abundance of mRNA and the abundance of protein in eukaryotic cells; however this correlation is often weak at best\(^1\). As the cervine model is relatively novel and exotic, the efficiency of this correlation must be investigated before conclusions are made. In a study of human circulating monocytes, the correlation coefficient (\(r\)) between mRNA and protein concentrations ranged from 0 to 0.832 dependent on the gene of interest\(^1\). In another study investigating the correlation between mRNA and protein abundance of cytokines from
PBMCs of women post HPV vaccination, IFNγ was seen to have strong correlation \((r = 0.69)\) while very low correlations were seen in the expression of IL1α, IL10, and IL17 \((r > 0.20)\). Taken together, it is clear that the correlation between mRNA and protein levels is variable, not only between genes but most likely between individuals. However it is also important to note that the aforementioned studies utilised microarray data for mRNA levels; qPCR is likely to be more specific and thus higher correlations may be seen using this technique.

Secondly, the functional relevance of the presented data cannot be inferred due to the source of the cells under investigation. This study analysed cells isolated from the blood; however the gut is the natural site of infection in JD. As the resident gut immune cells encounter a range of foreign antigens ranging from normal commensal bacteria to food antigens, the gut must provide an immunotolerant environment. For example, it is likely that there are a higher proportion of M2-like macrophages and T_{REG} cells compared to other environments, such as the blood. Furthermore, in keeping with the immunotolerant environment, gut immune cells do not express some of the PRRs or associated molecules necessary to induce immune responses to foreign antigen. For example, it has been shown that human intestinal macrophages lack CD14, a co-receptor for LPS, thus they have dampened responses towards LPS and LPS containing bacteria\(^{125}\). Therefore the responses from blood derived immune cells are likely to be altered compared to responses seen in the gut and associated tissues.

4.5 Use of a diagnostic assay

The current model of JD control revolves around a “test-slaughter” method, where animals that show signs of JD are culled. Not only does this prevent animal decline and suffering, it also prevents spread of the infectious organism through the herd. However this method is somewhat ineffective as MAP is ubiquitous throughout the environment, meaning livestock can acquire infection though other reservoirs. Furthermore, subclinically infected animals can show no outward symptoms but still shed viable organisms into the environment. The most effective model of control of JD is prevention of infection, which has been achieved only to limited degrees of success with vaccination. The present study was performed on the premise that a more effective mechanism of JD control is to genetically select livestock to produce a naturally resilient herd. As JD becomes more of a concern worldwide, economic
and zoonotic pressures may force cervine breeders to consider such genetic manipulation of herds, which would require identification of animals with R and S phenotypes.

At present, the only way to determine whether a red deer stag and its progeny are R or S to JD is through experimental inoculation. For the goal of producing a genetically R herd to be achieved, a faster, more effective method of phenotype diagnosis is crucial. Analysis of an animals ex vivo innate response to MAP has exhibited its potential as such an assay. Because of the strong paternal heritability, where 80% of progeny exhibit the paternal phenotype, the obvious use of the proposed diagnostic would be selection of R sires with strong production traits for breeding programs, while excluding high risk S sires, with the ultimate aim of reducing production losses on infected farms. Not only would this directly provide protection to R progeny but also an indirect level of herd immunity, decreasing the possibility of spread throughout the herd. Consequently, candidate gene expression was investigated to identify biomarkers with the ability to distinguish the R and S phenotypes. This study has indicated the potential for multiple genes, particularly those involved in apoptosis and Type I interferon signalling pathways, to be useful for phenotypic diagnosis.

As has been mentioned previously, deer of separate lineages but consistent phenotypes likely have different factors contributing to resilience and susceptibility. To account for such a phenomenon and the complex nature of disease susceptibility, the proposed diagnostic test must incorporate as many markers as relevant and feasible to be sensitive enough for routine use. This will allow differentiation of a range of R and S animals irrespective of genotypic differences amongst phenotypic groups. Values or scores would be assigned to each marker, culminating into an overall score of susceptibility based on expression patterns. This approach could also define the spectrum of disease that is exists in reality; while the present study uses the terms resilient and susceptible as phenotypic descriptors, such absolute classifications are somewhat unrealistic. This is especially true of the crossbred farmed animals, which most often do not exhibit such polar phenotypes.

Another consideration is that red deer show an age related susceptibility to JD; young animals are particularly susceptible but become resilient with age. It has been suggested that this is in part due to the immature immune system of young animals responding to MAP infection in an inappropriate manner\(^2\). The maturation of the immune system to become less
erratic and more effective has been documented in humans\textsuperscript{126}, mice\textsuperscript{127}, cattle\textsuperscript{128}, and deer\textsuperscript{21}. This has important implications on the effectiveness of the proposed diagnostic. The R and S phenotypes are under investigation from young animals, due to their susceptibility to disease compared to older animals. Consequently, a diagnostic assay developed based on these investigations may have little meaning for older animals, due to the more mature immune response in these animals. This disparity between young and old animals may, however, be a useful tool for determining what constitutes a protective response.

### 4.6 Conclusions

This study investigated the hypothesis that a dysfunction in the innate immune response to MAP causes susceptibility leading to clinical JD in red deer. Furthermore, it was thought that this dysfunction could be identified through differential gene expression between R and S animals, and used to proactively diagnose either phenotype. The hypothesis was supported through the identification of innate biomarkers differentially expressed between macrophages isolated from animals of R and S phenotypes. Specifically, \textit{ISG15} and \textit{ISG20} showed great promise for contribution to the diagnosis of either phenotype, while results from \textit{IL1A}, \textit{IL12A}, \textit{MAPK8}, \textit{NOS2}, \textit{STAT3}, and \textit{PKLR} suggest further research could unearth their potential for the same. This study also determined that diagnostic analysis of differential gene expression from PBMC cultures was feasible, and presented favourably in terms of blood volume required and culture duration compared to MDM culture. Development of a diagnostic assay for identifying animals of the R and S phenotypes could lead to selection of R sires for breeding, resulting in a genetically resilient herd and significant reduction in production losses on affected farms. Furthermore, it is possible that this assay could be extended to cattle for use in dairy and beef farming.

Despite the positive findings of this study, there are still questions that must be answered before the proposed diagnostic assay becomes a reality. Did the infection status of the animals affect the results of the present study? What effect does the age of the animal have on differential gene expression, and therefore, will the diagnostic assay be applicable to mature animals? What genes are suitable for use as diagnostic markers from PBMC cultures? Ongoing research from the DRL aims to answer such questions in due course. Future research should attempt to find suitable genetic markers from PBMC cultures, as this appears
the more suitable assay for routine diagnostic use. Utilization of RNA-Seq to compare the transcriptome of PBMCs from R and S animals following stimulation, as was performed in MDMs, could be a useful tool for initial identification of potential markers.
References


Appendix 1. Recipes

10 × P.B.S Stock

160 g NaCl
4 g KCl
22.7 g Na₂HPO₄
4 g KH₂PO₄

Make up to 2L with deionized water

Citrated PBS

7.66 g Na₃C₆H₅O₇

Add to 2L of 1 × PBS

RBC Lysis Buffer

8.29 g NH₄Cl (0.15 M)
1.0 g KHCO₃ (0.1 M)
37.2 mg Na₂EDTA (0.1 M)

Make up to 800 ml in distilled H₂O, adjust pH to 7.2 - 7.4 and autoclave.
Appendix 2: Supplementary figures
Supplementary Figure 1. Expression of CCL2 from MDM of R and S animals

Relative quantity of expression of the CCL2 gene in animals with an S or R phenotype at 0 to 26 weeks post animal challenge (A-H). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.

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Supplementary Figure 2. Expression of CXCL5 from MDM of R and S animals

Relative quantity of expression of the CXCL5 gene in animals with an S or R phenotype at 0 to 26 weeks post animal challenge (A-H). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 3. Expression of *IL1A* from MDM of R and S animals

Relative quantity of expression of the *IL1A* gene in animals with an S or R phenotype at zero (A), 14 (B), 18 (C), and 26 (D) weeks post animal challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 4. Expression of IL12A from MDM of R and S animals

Relative quantity of expression of the IL12A gene in animals with an S or R phenotype at zero (A), 4 (B), 22 (C), and 26 (D) weeks post animal challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 5. Expression of MAPK8 from MDM of R and S animals

Relative quantity of expression of the MAPK8 gene in animals with an S or R phenotype at zero (A), and 26 (B) weeks post animal challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 6. Expression of NOS2 from MDM of R and S animals

Relative quantity of expression of the NOS2 gene in animals with an S or R phenotype at zero (A), four (B), and eight (C) weeks post animal challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 7. Expression of PKLR from MDM of R and S animals

Relative quantity of expression of the PKLR gene in animals with an S or R phenotype at 0 (A), 22 (B), and 26 (C) weeks post animal challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 8. Expression of SOCS3 from MDM of R and S animals

Relative quantity of expression of the SOCS3 gene in animals with an S or R phenotype at 0 to 26 weeks post animal challenge (A-H). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 9. Expression of SOCS4 from MDM of R and S animals

Relative quantity of expression of the SOCS4 gene in animals with an S or R phenotype at 0 to 26 weeks post animal challenge (A-H). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 10. Expression of STAT3 from MDM of R and S animals

Relative quantity of expression of the STAT3 gene in animals with an S or R phenotype at 0 (A), 22 (B), and 26 (C) weeks post animal challenge. Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 11. Expression of TLR2 from MDM of R and S animals

Relative quantity of expression of the TLR2 gene in animals with an S or R phenotype at 0 to 26 weeks post animal challenge (A-F). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 12. Expression of TLR9 from MDM of R and S animals

Relative quantity of expression of the TLR9 gene in animals with an S or R phenotype at 0 to 26 weeks post animal challenge (A-H). Relative quantity of expression (mean ± SEM) is defined as expression upon stimulation with MAP compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 13. Gene expression from PBMCs of R and S animals 36 weeks post-infection

Relative quantity of expression of *IL1A* (A), *IL1B* (B), *IL17A* (C), and *ISG15* (D) in animals with an S or R phenotype. Relative quantity of expression (mean ± SEM) is defined as gene expression upon stimulation with MAP (M group) or SEB + PPDj (S group) compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.
Supplementary Figure 14. Gene expression from PBMCs of R and S animals 37 weeks post-infection

Relative quantity of expression of IL1B (A), IL8 (B), IL17A (C), ISG15 (D), and ISG20 (E) in animals with an S or R phenotype. Relative quantity of expression (mean ± SEM) is defined as gene expression upon stimulation with MAP (M group) or SEB + PPDj (S group) compared to an unstimulated control. Statistical significance determined by using the Mann-Whitney test; * p < 0.05.

Appendix 2:
Appendix 3. Primer dissociation curves

- B2M
- CCL2
- CXCL5
- IL1A
- IL12A
- ISG15