Gene expression profiling of mesenteric lymph nodes in red deer (*Cervus elaphus*) resilient or susceptible to Johne’s disease

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Abstract

Johne’s disease is a chronic wasting granulomatous enteritis of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Previous research has largely focused on the immune-associated gene expression profiles of the peripheral blood as a measure of the complex immunological response to MAP, with less information on the immune response occurring at the site of infection; the mesenteric lymph nodes. The DRL has in previous work established breed lines of deer that naturally express paternally heritable resilient (R) or susceptible (S) phenotypes to Johne’s disease. Differential expression levels of specific immune genes have been linked to R/S phenotypes which could ultimately be used in the diagnosis of S animals, and the selective breeding of R animals.

In this present study, samples from the posterior jejunal lymph nodes of 20 red deer (*Cervus elaphus*) experimentally challenged with MAP were taken at 13 weeks post-infection, and at 40 weeks following slaughter. Quantitative-PCR was utilised to determine expression profiles of candidate innate and adaptive immunity-associated genes. Aside from increased IFNG in intermediate (I) and S animals at 40 weeks p.i., no significant differential expression was observed between R, I and S animals in the expression of pro-inflammatory Th1, Th17 and anti-inflammatory Th2 and Treg candidate genes at 13 and 40 weeks p.i.. While this study failed to identify biomarkers associated with R/S phenotypes, a dramatic differential expression of the candidate genes between 13 and 40 weeks was observed, which may provide insights into the role and progression of immune parameters as the diseases progresses over time.
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1 Introduction

1.1 Chronic Infectious disease: Mycobacteria

Chronic disease is a disease of long duration that does not resolve itself and is generally not curable\(^1\). Mycobacteria are a genus of bacteria that are made up of many saprophytic species and a small number of species that are pathogenic. Infections caused by virulent mycobacteria and a small group of opportunistic pathogens can cause life-threatening chronic disease in susceptible individuals. Disease manifestation and disease severity depends on the interaction between the specific mycobacteria and the hosts immune system\(^2\).

Mycobacteria are environmentally ubiquitous, acid-fast and facultative intracellular bacilli. They are commonly characterised by a slow growth in culture, the presence of a lipid-rich waxy cell wall and other innate mechanisms that make them resistant to clearance by the immune system and able to withstand harsh conditions in the environment\(^3\)–\(^6\).

Infections caused by mycobacteria are difficult to treat with conventional drug therapies due to their intracellular growth and robust cell wall that differs in structure from other bacteria\(^5\),\(^7\). A hallmark trait of mycobacteria is their propensity to infect the phagocytic immune cells, macrophages, and form persistent infections\(^5\),\(^8\). The most common chronic diseases reportedly caused by a mycobacterial infection in both humans and animal species have been associated with the mycobacterial species: *Mycobacterium tuberculosis*, *M. bovis*, *M. avium* subsp. *paratuberculosis*, *M. leprae*, *M. ulcerans* and *M. avium*\(^4\),\(^9\).

1.2 Johne’s disease: An Inflammatory Bowel Disease of Animals

Johne’s disease (JD) was first described in 1895 by Johne and Frothingham as a peculiar case of a tuberculosis-like enteritis found in cattle\(^3\),\(^10\). Johne’s disease is now widely recognised as a chronic granulomatous enteritis affecting ruminant species, particularly cattle, sheep and deer\(^4\),\(^11\). The disease is caused by infection with the mycobacteria *Mycobacterium avium* subsp. *paratuberculosis* (MAP)\(^5\). Red deer (*Cervus elaphus*) are natural hosts of MAP and are particularly prone to developing JD
from a young age (8-12 months), in contrast to other ruminant species where disease is only manifest in older animals (>3 year old)\textsuperscript{12}.

Clinically diseased animals typically display granulomatous lesions of the small intestines, an enlargement of the mesenteric lymph nodes, intestinal tissue thickening and damage, and diarrhoea and emaciation due to an inability to absorb nutrients which ultimately results in the animals’ death\textsuperscript{5}.

Subclinically infected animals lack obvious precise symptoms and for this reason are major sources for the spreading of MAP among herds or farms, particularly through faecal shedding. This issue, in combination with poor production and reproduction prefaces the need to identify and remove subclinically infected individuals as well as severely diseased animals, to prevent further spread and economic losses\textsuperscript{5,8,13}.

Johne’s disease is responsible for significant economic losses worldwide in the farming of ruminants\textsuperscript{6}. The disease is endemic to many countries including the United States and New Zealand, resulting in constraints for the export and import of animals and animal products. Statistics have stated losses as high as $18.9 million for the dairy industry, $9.9 million for sheep and $340,000 per year in NZ alone due to JD\textsuperscript{14}. Previous statistics published have declared an even greater financial loss of up to $220 million per year in the US dairy industry\textsuperscript{5,6,10}.

Any of the above figures are likely to be a gross underestimate of the true burden of JD both in NZ and worldwide as they are not current\textsuperscript{8,13}. Estimation of true infection and disease levels are difficult due to a combination of insufficient diagnostic testing and an unclear definition of subclinical and clinical disease states between different infected species\textsuperscript{9,14–16}. Clearly, an in depth knowledge of the ruminant immune response to infection could improve diagnostics and preventative strategies to minimise future economic loss to the agricultural and farming industry.

As with tuberculosis, JD conforms to the “Iceberg” phenomenon of infection: whereby of all the animals exposed to MAP, most develop subclinical infection rather than clinical disease – ‘The iceberg above the water’ (Figure 1). When infection leads to clinical disease, it is typically characterised by a long latency period with clinical disease developing some period of time later in only a small proportion of animals. Disease severity and progression is shaped by a unique combination of factors
involving the infectious agent, the host, and the environment. Together they lead to a dysregulation or breakdown in protective immunity and the consequent development of pathological disease or clinical JD\textsuperscript{6,12,17}.

\textit{Mycobacterium avium} subsp. \textit{paratuberculosis} has been shown to be transmitted ‘vertically’ through mother to offspring (\textit{in utero}), but is primarily transmitted via the faecal-oral route through the ingestion of infected feed, water and pastures\textsuperscript{4,8,11,18,19}. \textit{Mycobacterium avium} subsp. \textit{paratuberculosis} has a relatively broad host range and infections have been reported not only in ruminant species, but also in primates, swine, rabbits and foxes, indicating that non-ruminants act as natural reservoirs for MAP infection\textsuperscript{4,20}.

![The Iceberg Model of Infection](image)

\textbf{Figure 1: The Iceberg Model of Infection.} The outbreak of disease in a group of animals can be divided into a large proportion of exposed and unaffected animals (white); a smaller group of subclinically infected animals (blue); and few severely diseased, clinical cases (red).

There is a large amount of controversy whether MAP may also be a causative agent in the pathogenesis of human Inflammatory Bowel Disease (IBD), namely Crohn’s disease (CD)\textsuperscript{21}. While CD is considered to be autoimmune in its origin, evidence has increasingly emerged which suggests an additional infectious aetiology, with a mycobacterial infection being a possible cause due to its environmentally ubiquitous nature\textsuperscript{22,23}. The suggestion that there is an association between the two is based on the observation that intestinal lesions formed in both CD-affected patients and clinical JD
animals are similar\textsuperscript{17}. While MAP has been isolated from adults and children with CD, it has also been found in healthy individuals or in patients suffering from other disease\textsuperscript{5}. While no causal link has been established between MAP infection and human CD, there is a clear association between the two disease syndromes. Considering that the pathology of both diseases is almost identical, it is conceivable that MAP infection which causes JD may be used as a model to study the pathogenesis and treatment of CD\textsuperscript{17,21,24}.

1.3 \textit{Mycobacterium avium} subsp. \textit{paratuberculosis} Invasion of the Host

From the lumen of the intestine, MAP may enter the intestinal tissue through either direct adsorption by the gut epithelia, antigen presenting cells or goblet cells. It is thought, however that intestinal microfold or M cells are most likely the primary source of entry for MAP into the intestinal mucosa and the gut-associated immune tissues. Microfold cells are located in the Peyer’s patches in the ileum or jejunum of the small intestine in ruminants and it is by uptake by these cells that the mycobacteria are able to make contact with host immune cells and resident macrophages\textsuperscript{5,25,26}. Preferential attachment and internalisation of MAP and other mycobacteria into M cells may be dependent on the interaction between Fibronectin attachment proteins (FAPs) and Fibronectin (FN), which bind \(\beta1\)-integrins on host M cells\textsuperscript{8,25}.

Various other receptors have also been suggested as a potential means of entry for MAP and other mycobacteria into macrophages, including the mannose receptor and the complement receptor 3, among others\textsuperscript{6}. Intracellular survival within host macrophages seems to be an essential feature of all pathogenic mycobacteria suggesting a common mechanism to establish infection\textsuperscript{8}.

As with other mycobacterial infections, MAP is very well-adapted to intracellular survival in its host, despite the aggressive immune response which has evolved to remove such chronic infections. \textit{Mycobacterium avium} subsp. \textit{paratuberculosis} affects the phagosome of macrophages preventing the formation and maturation of phagolysosomes. Additionally, MAP inhibits the production of macrophage reactive oxygen and nitrogen metabolites that are critical in efficiently destroying internalised bacteria, as well as preventing apoptosis of infected cells\textsuperscript{27–29}. Macrophages play a
central role in determining the overall quality of the innate and adaptive immune response through their bactericidal activity and antigen presenting ability; by targeting macrophages, MAP essentially destroys a body’s normal natural first line of defence against an invading pathogen and permits its own survival and replication in the cell\(^5,6,8,21,30\). Since dendritic cells (DC) are the only antigen presenting cells with migratory properties, infection of these cells may additionally contribute to induction of the MAP-specific immune response in the peripherally and localised gut associated mesenteric lymph nodes\(^5\).

Bacilli successfully engulfed via receptor-mediated endocytosis are able to migrate around the body via the infected macrophages and dendritic cells to the local mesenteric lymphatic tissue, namely the ileal, jejunal and ileocaecal lymph nodes where they closely encounter and activate lymphocytes, initiating the adaptive arm of immunity. When infection is not controlled through protective pathways of immunity, alternative immunopathological responses may cause granulomatous lesions or ulcerations in the gut mucosae and lymphatics\(^5,26,27\).

1.4 The Immunological Response to MAP and the Development of Immunopathology

1.4.1 Innate and Adaptive Immunity

Innate immunity defines the early response of the host to an infection. Innate immunity is non-specific, fast-acting and recognises unique but shared motifs on pathogenic bacteria. Professional antigen presenting cells such as DC and macrophages mediate the innate immune response and are essential in contributing to activation of the crucial adaptive immune response as well as aiding in removing the specific pathogen that is targeted by the adaptive immune response\(^31\).

Unlike the innate immune response, the adaptive response is pathogen-specific and provides long-lasting protection; however these two arms of the immune system are dependent on each other to successfully eliminate a foreign invader. T and B cells are the fundamental immune cells of the adaptive response that mediate cell-mediated and humoral immunity respectively. Cell-mediated immunity involves a response where antigen-specific T cells are activated to carry out various functions including the activation of macrophages via “help” and killing of infected cells. When B cells are
activated they secrete antibody (immunoglobulins) which interact with foreign pathogens, thus aiding their destruction and removal by the phagocytic cells (macrophages) of the innate system.\textsuperscript{31,32}

1.4.2 **The CD4\(^+\) T helper Response**

After activation via antigen presentation on major histocompatibility complex proteins (MHC-II), naïve CD4\(^+\) T cells become activated and differentiate into one of multiple effector CD4\(^+\) T cell subsets largely dependent on the cytokine milieu of the microenvironment (Figure 2). These subsets include, but are not limited to: Th1, Th2, Th17 and T regulatory (Treg) cells, each of which has a distinct biological function. Differentiation of the appropriate T helper (Th) subset is under the control of a ‘master regulator’ or transcription factors that polarise naïve T cells towards a specific phenotype or lineage, e.g. T-bet, Gata-3, FoxP3 and RORγt driving the differentiation of Th1, Th2, Treg and Th17 subsets, respectively.\textsuperscript{33}

The Th1 response is essential in any intracellular infection and is characterised by the production of classic Th1-associated cytokines like IFN-γ and IL-12. Interferon-gamma is a potent pro-inflammatory cytokine that activates the bactericidal activity of macrophages and is known to have a role in granuloma formation.\textsuperscript{34} Activated macrophages secrete IL-1\(\alpha\) and IL-1\(\beta\) along with other chemotactic factors and cytokines, including IL-12 and TNF-α, which recruit more immune cells to the site of infection. Activation of recruited MAP-specific CD4\(^+\) T cells leads to the release of more pro-inflammatory and macrophage-activating cytokines for e.g., IFN-γ, TNF-α, and the T cell stimulatory cytokine, IL-2, which induces the proliferation and survival of all T cells.\textsuperscript{8,10,33,35}

The Th17 response has a role in promoting a pro-inflammatory response during extracellular bacterial and fungal infections. Classic Th17 cytokines include IL-17, TGF-β and IL-23 which are integral to the function of Th17 cells, and IL-6 which is produced by macrophages, among other cells, and may be required in the activation of Th17 cells. T helper 17 cells and cytokines appear to function in parallel to the Th1 response to upregulate or induce the production of more pro-inflammatory cytokines.\textsuperscript{12,33,36}
T helper 2 cells mount an immune response to extracellular parasites. The key Th2-associated cytokines include IL-4 and IL-5. Interleukin-4 is a key regulator in humoral immunity by inducing the differentiation of B cells into antibody producing plasma cells and its activity has been linked to tissue and wound repair. Interleukin-4 is known to induce the differentiation of Th2 cells which upon activation secrete more IL-4 that promotes an anti-inflammatory response.

T regulatory cells include more than one cell types that are essential in the maintenance of peripheral tolerance and down-regulating inflammatory responses. The mechanisms in which Tregs exert their immunosuppressive function are not well understood but likely involve the anti-inflammatory cytokines IL-10, TGF-β and IL-35. Interleukin-10 can also be produced by Th2 cells and is a multi-functional, potent, anti-inflammatory cytokine that interferes with the function and expression of pro-inflammatory genes such as IFN-γ and IL-2. Treg-associated cytokine mediated suppression may also include the down-regulation of MHC-II and co-stimulatory molecules on macrophages that are required for macrophage and T cell activation.

1.4.3 The CD4⁺ T helper Response Relevant to JD

Johne’s disease is relatively well characterised in bovine and ovine models but the immunological response is less understood in cervine species. In terms of the pathology observed, clinical JD is similar between ruminant species, thus, a model of infection is likely to apply to all ruminants. Large gaps still remain in the understanding of the cervine immunological response to infection, the development of immunopathology, and the uncertainty in what constitutes a ‘protective’ vs. a ‘non-protective’ response, due in part to the lack of relevant experimental models.

It is generally accepted across all ruminant species that the early subclinical stages following infection are dominated by a protective, pro-inflammatory Th1 response, while the progression to clinical disease is associated with a transition to a strong antibody-producing anti-inflammatory Th2 response. This model is likely to be a significant oversimplification of the response since Th17 and Treg cell types are known to have contributing roles, though their roles are less well understood.

The Th1 response is responsible for restricting MAP proliferation during early infection and is largely characterised by the production of IFN-γ. The precise role of IFN-γ is
conflicting; some studies have reported an upregulation in subclinically diseased animals, while others have reported higher endogenous IFN-γ in severely diseased animals\textsuperscript{28,39}. In susceptible animals, MAP may block the ability of host cells to appropriately respond to IFN-γ\textsuperscript{28}.

As the Th1 response begins to decline, bacterial proliferation and release from granulomas increases and the bacteria spread to new sites encouraging a ‘switch’ to a Th2 antibody-producing response\textsuperscript{6,8}. This late humoral response is characterised by the production of IgG1 and classic Th2 cytokines, for e.g., IL-4 and IL-10\textsuperscript{8,15}. A significant decrease in the expression of IL-4 has been observed in severely diseased animals which may account for the extensive tissue damage and exacerbated inflammatory response\textsuperscript{39}. Unfortunately, antibody production is largely regarded as ineffective and does little to fight the infection as bacteria contained in dense granulomas are shielded from the antibody’s effects. Antibody production may actually exacerbate inflammation by promoting the uptake of opsonised bacteria by macrophages\textsuperscript{6}.

T helper 17 cells have been shown to have contributing roles in human IBD and mycobacterial diseases and recently, a proposed role in JD\textsuperscript{43}. T helper 17 cytokines may either help to clear a MAP infection, or exacerbate the infection through the production of Th17-specific cytokines that work synergistically alongside the Th1 response. Indeed, elevated expression of IL-17 transcripts have been observed in the lymphatic tissues of severely diseased animals; the expression of other Th17 associated genes (IL-23, RORγt etc.) have mostly failed to reach the same significant differential expression\textsuperscript{33,39}.

To prevent extreme tissue damage through excessive inflammation, a population of immunosuppressive and anti-inflammatory Tregs dampen the inflammatory response which may account for the decline in the Th1/Th17 responses and the subsequent transition to a Th2 response during chronic infection\textsuperscript{8,44}. The Treg-associated cytokine, TGF-β is known to negatively regulate IFN-γ levels. Increased TGF-β likely decreases the bactericidal activity of macrophages and promotes a more anti-inflammatory, suppressive phenotype\textsuperscript{38}. Decreased TGF-β has been seen in the lymphatic tissues of clinically diseased animals\textsuperscript{39}. 
Because MAP actively prevents phagolysosomal maturation, this essentially removes a key defence step in what would normally clear a bacterial infection. It has therefore been proposed that the activation of a CD8\(^+\) cytotoxic effector T cell response is also necessary to lyse infected macrophages since IFN-\(\gamma\) and TNF-\(\alpha\) may do little to cure persistently infected macrophages. A proposed theory for the activation of a cytolytic CD8\(^+\) response involves MAP antigen leaking from the cytosol of infected macrophages where it is cross presented on MHC-I to CD8\(^+\) T cells\(^6\).

The characteristic immunopathology that we observe in JD is a result of a chronic inflammation of the intestinal tissues, mediated by a complex network of interacting cytokines and immune cells.
Figure 2: The Interactions between MAP-infected Macrophages and CD4⁺ T helper cells. A representation of the activation and differentiation of Naïve Th0 cells following antigen presentation by MAP infected macrophages. Specific cytokines and transcription factors drive the polarisation of T helper cells which each have a specific purpose and desired outcome in an immunological response. Figure adapted from Sethi et al. (2013).
1.4.4 Granuloma Formation

Histological severity and scoring of the granulomatous lesions formed in a MAP infection vary widely and are graded similar to the classification system for the grading of lesions seen in leprosy and tuberculosis\textsuperscript{18,46,47}. Current literature seems to report similar observations in respect to the gross appearance of granulomas in the gut-associated lymphatics of JD affected animals\textsuperscript{46–48}. Granulomas occur as a result of the mycobacteria’s facultative intracellular nature inducing the production of inflammatory cytokines and the activation of immune cells. While granuloma formation is an attempt of the immune system to limit, contain and clear an infection, they provide an ideal environment for the pathogen to both evade and redirect the immune response mounted against it\textsuperscript{4,8}. In JD, granulomas form central sites of infection in the intestinal tissue and the associated lymph nodes\textsuperscript{29}.

Granuloma type and morphology can be classed based on disease stage and severity of the animal i.e. whether the animal is in a state of subclinical or clinical infection. Lesions first begin to develop during the subclinical phase and are usually confined to the ileum and the ileoceleal valve region of the small intestine\textsuperscript{6}. Granulomas of the subclinical nature or paucibacillary granulomas are characterised by a small size, the presence of macrophages, epitheloid cells, giant multinucleated cells, lymphocytes and \( \gamma\delta \) T-cells, and a low bacterial burden\textsuperscript{47}. Paucibacillary granulomas are presumed to be associated with a strong cell-mediated response and may show necrosis or mineralisation as they function to contain and control bacterial proliferation\textsuperscript{18}. In contrast, lesions of the clinical kind (or multibacillary) are diffuse and associated with a weak cell-mediated immune response, extensive bacterial proliferation and the progression of the disease into severe enteritis\textsuperscript{18,47,49}. Multibacillary granulomas show a lack of organisation, significantly higher numbers of macrophage and bacteria and a disorganised infiltrate of lymphocytes, leading overall, to poor granuloma function in terms of isolating the infection and preventing bacterial dissemination\textsuperscript{8,18,47,48}.

An understanding of granuloma morphology is important because it is known that cytokines that are associated with a Th1-type immune response drive the development of initial paucibacillary granulomas, and the fine balance between Th1 and Th2 responses directly influences both granuloma morphology and the progression from subclinical to clinical disease\textsuperscript{10,48}.
1.4.5 **The Role of the Gut-associated Lymphatic Tissues**

The overall quality of the immune response is dictated by each compartment of the immune system and how these interact together. The immune response against MAP in the periphery differs to that of the gut lymphatics and similarly, the immune response occurring in the mesenteric lymph nodes will differ to the response of the rest of the lymphatic tissues in the body. Dissemination of MAP to the mesenteric lymph nodes during an infection is a common feature observed in JD\(^5,15\).

The lymph nodes are relatively small immune-associated organs that have a key role in initiating an adaptive immune response. Immune cells such as DC and macrophages constantly travel to and from the LNs where they encounter T and B cells and present antigen, thus, initiating protective cell-mediated and humoral responses. The mesenteric lymph nodes play a pivotal role in immune anatomy as they both form a boundary between mucosal immunity and the remainder of the immune system, and drain the affected tissues\(^50\). In the case of JD, MAP is frequently orally ingested and as a consequence, has exclusive access to the mesenteric lymph nodes and the ability to breach protective mucosal immunity.

To date, most research carried out on JD has focused primarily on the immune response and gene expression profiles in the peripheral blood and PBMCs with less focus on the gut lymph-associated tissues. Analysis of the blood is useful for diagnostic purposes but provides little information of what is occurring at the site of infection; the mesenteric lymph nodes\(^5,39\). Because the enteric lymphatic tissue is the primary site of infection, and the infection is primarily restricted to the gut, the mesenteric lymph nodes are the most probable site of MAP antigen presentation and induction of a protective adaptive response; so, sampling the mesenteric lymph nodes could provide an insightful representation of the mucosal lymphatic response\(^5,51\).

1.5 **Resilience and Susceptibility**

1.5.1 **Resilient/Susceptible Phenotypes and Genotypes in Red Deer**

Susceptibility to infectious diseases rarely follows a simple Mendelian mode of inheritance\(^52\); most pathways of infection involve a complex pattern of inheritance involving a range of genes which in combination with host and environmental factors,
influence the outcome of clinical disease. Resistance and susceptibility have been observed in both human and animal populations to a range of infectious diseases, including mycobacterial tuberculosis and leprosy. Of particular importance, variability in susceptibility to bacterial and parasitic infections has been observed in previous studies in ruminants, particularly cattle. More recently, experimental models have implicated the role of host genetics in susceptibility to JD.

A genetic role first became apparent in studies in which experimental MAP inoculation in animals raised in the same conditions produced a spectrum of infection and disease over time, suggesting that some animals were better able to clear an infection or perhaps, were more resistant to developing clinical disease.

Resistant (R) or Susceptible (S) phenotypes to MAP in red deer were observed by Mackintosh et al. (2011). Progeny from two sires known to produce animals naturally resilient or susceptible to MAP were challenged with MAP and their patterns of immune response and immunopathology were monitored over the course of a year. A striking observation that perhaps best demonstrates the R phenotype in red deer was shown in one animal which exhibited mild lesions in tissue biopsy samples from 13 weeks post-challenge, but which had completely resolved the lesions and tested MAP negative at 49 weeks. This observation suggests that resilient animals do not resist infection, but rather, resolve and recover from an established infection, which is why they are termed ‘resilient’ rather than resistant. This finding parallels results seen in other studies using cattle and sheep where individual animals are able to clear a MAP infection following experimental challenge.

Purebred progeny tend to show the same response to MAP challenge as their parents, most notably from their sire, suggesting a strong role for heritability in resilience and susceptibility. While, not unexpectedly, heritability of the parental phenotype in progeny is strong it is not totally dominant where roughly 80% of progeny display the paternal phenotype. In contrast, a spectrum of disease severity ranging from phenotypically resilient to susceptible can be seen in crossbred deer with only a small proportion (5%) displaying either extreme R or S phenotype.

Interestingly, resilience to developing disease seems to be acquired with increasing age. A study by Mackintosh et al. (2010) used three different aged groups: weaners,
yearlings and mixed aged hinds; upon challenge, each of these groups of deer showed remarkable differences in both their disease severity and progression. Clinical JD was observed exclusively in the weaner group of deer as well as a significantly larger proportion of subclinical cases with more severe lesions compared to the older aged groups. Additionally, young red deer exhibit a much shorter time course in the progression to clinical disease (~1 year) which makes them a suitable and perhaps more informative experimental model to study immunological responses to MAP infection.

1.5.2 **Immune Biomarkers in Deer associated with Resilience/Susceptibility**

Research into genetic variation between species has begun to identify key immune-related gene markers associated with R and S that have remarkable differences in their expression across different immune cells. The use of biomarkers could improve diagnosis and prognosis of the disease and offer a more reliable method of screening since the use of biomarkers are already proposed to have a role in diagnostics for other chronic diseases, for e.g. tuberculosis and cancer.

Robinson et al. (2011) demonstrated differential gene expression profiles in R/S red deer by measuring a range of genes associated with Th1, Th2, Th17 and Treg immunity rather than focusing on singular genes thought to be prototypic for different states of infection and disease. A significant difference was observed in gene expression between control, minimally diseased and severely diseased animals. Severely diseased animals (S) displayed an up-regulation of pro-inflammatory genes, for e.g. *IFNG, IL1A* and *IL17A*; with a concomitant down-regulation of anti-inflammatory genes including *IL4, FOXP3* and *TGFB1* also seen. This study highlighted the complex polygenic nature of responses to MAP infection that involve a vast array of innate and adaptive genes from all arms of the immune system that may contribute to susceptibility and immunopathology.

Similarly, macrophages from genetically S animals have been shown in multiple studies to up-regulate the expression of inflammatory markers like *IL1A, NOS2* and *TNFA* compared to macrophage cultures from genetically resilient animals.

It is proposed that these differences in gene expression result in a variable response of the immune system to efficiently handle or clear MAP. An excessive pro-inflammatory response may lead to a dysfunctional innate system, which along with a lack of a
protective adaptive response, may define the S phenotype. Conversely, R animals may be characterised by both a tightly controlled inflammatory response and the initiation of a protective adaptive response that is sufficient to control and eliminate MAP infection\textsuperscript{12}.

Efforts into controlling the spread of MAP have been severely hindered due to poor diagnostic tests and the lack of an effective vaccine. A general lack of understanding of how MAP evades the innate and adaptive immune responses and what constitutes protection has made any development in this area extremely difficult. Additionally, most of the research into vaccine advancement has primarily used mice models of infection whose relevance to ruminant models is very limited. A vaccine is available at present which limits the severity of immunopathology and the progression to clinical disease, but is insufficient to prevent infection or control the spread of MAP\textsuperscript{6,35,39}. Selective breeding using resilient sires and dams could provide a further strategy for the control of JD within herds or flocks of domestic livestock. However, this would require the \textit{in vitro} identification of R- and S associated immune biomarkers to select appropriate animals for breeding (R) or exclusion from breeding (S)\textsuperscript{12}.

1.5.3 \textbf{Assays to Investigate Resilience and Susceptibility}

Investigating gene expression profiling is the only flexible technique currently available to study the host response to MAP infection in deer, particularly the differential immune response between R and S animals. Limited access to biological reagents and monoclonal antibodies specific to study the immune biology of deer make it impossible to develop a bioassay platform for this exotic species of ruminant.

The transcription of specific genes into coding mRNAs is an essential prerequisite for the generation of functional proteins and the first step in any given biological or physiological process. Gene expression is a complex process that adapts rapidly to exogenous stimuli or changes in biological/physiological conditions. Thus, the transcriptome in its entirety will include all mRNA transcripts that reflect the current physiological state of a tissue, organ or even a single cell\textsuperscript{60}. In terms of measuring the immune response to MAP, gene expression profiling has provided valuable insights into the biological or physiological state of individual immune cells and the directly affected tissues in response to MAP\textsuperscript{39,57}.
Due to the complexity of the immune pathways involved with JD, no single gene or biomarker will be indicative of susceptibility to disease; it is more likely that multiple genes that together have small contributing effects on the manifestation of disease are required to distinguish between R and S and different disease states; hence a ‘profile’ of gene expression is needed. Cognisance must be given to the fact that gene expression profiling does not take into account post-translation modifications which can significantly alter the function of a protein. In addition, the initial amount of mRNA transcribed may not be an indication of the actual amount of protein present in a cell which could give misleading results. Accepting the possible disconnect between gene expression levels and functional pathways involving bioactive molecules, it is possible that specific gene expression may provide real or surrogate markers in animals with either an R or S phenotype or genotype, by their differential expression in response to MAP challenge.

1.6 Aims & Hypothesis

Quantitative-PCR (q-PCR) based methods for gene expression have been demonstrated to be informative in previous work from the Disease Research Laboratory. The DRL have established a model of infection using red deer which display a spectrum of disease states in a condensed time frame of 1 year. Highly selected deer breeds have been selected that display extreme levels of resilience or susceptibility to JD, confirmed by necropsy following experimental infection with MAP. The integrity of these phenotypes has been confirmed in four successive breeding studies carried out over the past 4 years using progeny from R or S sires derived from AI breeding programmes involving crossbred females. These young animals have provided an ideal platform to investigate the genetic basis for resilience or susceptibility in deer experimentally infected with MAP.

This study aims to characterise the differential gene expression profiles from the gut-associated lymphoid tissue of farmed red deer (*Cervus elaphus*) which are either genetically resilient (R) or susceptible (S) to Johne’s disease by measuring the expression of 12 immune related genes (Table 1). The rationale for picking these genes is on the basis of their proposed contributing roles in human Inflammatory Bowel Disease and mycobacterial disease, and from previous work investigating differential gene expression in transcriptomes of peripheral blood and lymphoid tissues.
More specifically, the aims of this project were:

1) To determine if gene expression levels in lymph node biopsy samples taken surgically from animals (at 13 weeks post-infection), are similar to necropsy samples taken at elective slaughter (40 weeks post-infection).

2) To compare gene expression levels in lymph node samples from animals with either an R or S phenotype, confirmed following slaughter.

Based on current knowledge, our working hypothesis was that a differential profile of gene expression could be observed between R and S animals. An excessive up regulation of pro-inflammatory genes and a downregulation of the anti-inflammatory genes may define the S phenotype, correlating directly with the presentation of severe clinical pathology. Conversely, R animals may show a profile of expression representing a more controlled inflammatory response and the initiation of an adaptive response that is sufficient to clear MAP infection. There is likely to be a significant difference in the profiles of expression between 13 weeks and 40 weeks post-infection as biopsy samples were taken early in the course of infection when animals are likely to have latent or subclinical infection compared to samples taken at slaughter where the disease will have progressed.

Extensive studies of natural MAP infection within a large deer stud over the past decade have identified deer breeds that express extreme R or S phenotypes. These animals which express highly predictable phenotypes are an important resource to identify biomarkers for these phenotypes which could ultimately be used diagnostically to identify R or S animals unrelated in herds that have not been infected with MAP. Selection of animals with resilient phenotypes for breeding could make important contributions to protocols designed to control JD in farmed red deer. This knowledge could conceivably be translated to other farmed ruminants for the identification of R animals for breeding. The identification of R and S associated biomarkers could also advance future JD diagnostics (responses in S animals) and candidate markers (responses in R animals) for protection to enhance development of more efficacious vaccines.
Table 1: Gene expression profiling – candidate biomarkers and their function

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>IL-6</td>
<td>Cytokine</td>
</tr>
<tr>
<td>IL4</td>
<td>IL-4</td>
<td>Cytokine</td>
</tr>
<tr>
<td>IL10</td>
<td>IL-10</td>
<td>Cytokine</td>
</tr>
<tr>
<td>IL12A</td>
<td>IL-12p35</td>
<td>Cytokine</td>
</tr>
<tr>
<td>IL17A</td>
<td>IL-17</td>
<td>Cytokine</td>
</tr>
<tr>
<td>TBX21</td>
<td>T-bet</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGFB1</td>
<td>TGF-β</td>
<td>Cytokine</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA-3</td>
<td>Transcription factor</td>
</tr>
<tr>
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<td>IFN-γ</td>
<td>Cytokine</td>
</tr>
<tr>
<td>IL1B</td>
<td>IL-1β</td>
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</tr>
<tr>
<td>IL2</td>
<td>IL-2</td>
<td>Cytokine</td>
</tr>
<tr>
<td>FOXP3</td>
<td>FOXP3</td>
<td>Transcription factor</td>
</tr>
</tbody>
</table>
2 Methodology

2.1 Deer

Progeny were bred by artificial insemination using frozen semen originating from breeding stags of a resilient (R) or susceptible (S) genotype, from which 20 mixed gender weaner animals were selected for this study (Table 2). For the duration of the study the animals were housed at AgResearch (Invermay). All experimental manipulations performed on the animals were approved from the Invermay AgResearch Animal Ethics Committee (AEC 12928).

Four month old deer were challenged via oral gavage with $3 \times 10^8$ of MAP each day, for 4 consecutive days. The health of all animals was routinely monitored over the period of the study and any animal that presented with severe clinical disease, as defined by a loss of 10% of their body weight over a 2 week period, were euthanised and necropsied. At 13 weeks post infection, the animals were placed under general anaesthetic and biopsy samples of the posterior jejunal lymph nodes (PJLN) were surgically removed and immediately snap frozen in liquid nitrogen to maintain mRNA integrity. At 40 weeks post infection, the study was terminated and any remaining deer were euthanised and sections of the intestinal tissues were harvested, including the anterior jejunum and associated lymph nodes; the mid jejunum and associated lymph nodes; the posterior jejunum and associated lymph nodes; and the ileocaecal valve and associated lymph nodes. Samples of the PJLN were again collected at this time point and immediately snap frozen in liquid nitrogen specifically for this study. Following histopathological staining and analysis each animal was then assigned a Lesion Severity Score (LSS) based on the disease severity of each of the affected tissues and LN to retrospectively determine the phenotype of each animal.
Table 2: Summary of study animals and genotype by sire.

<table>
<thead>
<tr>
<th>Animal Tag Number</th>
<th>Assigned number</th>
<th>Genotype of Sire</th>
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</tr>
<tr>
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<td>S</td>
</tr>
<tr>
<td>1215</td>
<td>19</td>
<td>S</td>
</tr>
<tr>
<td>1214</td>
<td>20</td>
<td>S</td>
</tr>
</tbody>
</table>
2.2 RNA Extraction from Lymphoid Tissues

Biopsy and necropsy posterior jejunal lymph node samples were trimmed of the connective tissues and roughly a pea-sized amount of each tissue sample was added to 2 mL of TRIzol (Life Technologies) in 5 mL centrifuge tubes. Samples were homogenised using a rotor-stator blender to disrupt and lyse cellular material while maintaining RNA integrity. Two-hundred microlitres of chloroform was added to 1 mL of homogenate which was then agitated vigorously for 15 seconds and incubated at room temperature for 3 minutes. Samples were centrifuged for 15 minutes at 12,000 x g at 4°C. Centrifugation separated the homogenates into 3 distinct layers, with the upper clear/straw coloured aqueous layer containing the desired RNA. The aqueous layer of each sample was carefully removed and pipetted into fresh microcentrifuge tubes. Isopropyl alcohol was added at 0.5 mL per mL of TRIzol and the samples were shaken and incubated for 10 minutes at room temperature, then further spun for 10 minutes at 12,000 x g at 4°C. After centrifugation, the pellet was present as an obvious white/grey mass at the bottom of the microcentrifuge tube. The supernatant from each tube was carefully removed, so as not to disturb the pellet and 75% ethanol (made with diethylpyrocarbonate (DEPC) treated RNase-free water) was added at 1 mL and the samples were further spun at 7,500 x g for 5 minutes at 4°C to wash the pellet. Again, the supernatant was carefully removed and the samples were re-spun for 10 seconds at 7,500 x g at 4°C with any excess liquid removed carefully via pipette. The pellets were left at room temperature to air dry for 10 minutes. Each sample was made up to 100 µL by re-suspending the pellets in DEPC-treated RNase-free water by passing through a pipette tip.

2.3 RNA Quantitation

The approximate yield of RNA in each sample was quantified by UV spectrophotometry using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies) which gave an indication of the quantity and purity of the RNA. The absorbance readings were used to calculate how much RNA would be required (in ng/µL) to purify 30 µg of RNA for further work.
2.4 RNA isolation/purification from crude RNA

The NucleoSpin® RNA kit (Machery-Nagel) was used for the further purification of RNA from the lymphoid tissues according to the manufacturer’s instructions. Firstly, a Buffer RA1 – ethanol mix at a 1:1 ratio was made by mixing 300 µL of Buffer RA1 to 300 µL of ethanol (100% EtOH) per each 100 µL RNA sample. To each 100 µL sample, 600 µL of Buffer RA1 – ethanol premix was added and mixed by vortexing. Each sample was loaded onto a NucleoSpin® RNA binding column in a 2 mL collection tube and centrifuged for 30 s at 11,000 x g. The columns containing the bound nucleic acid were removed and placed into new 2 mL collection tubes. Three-hundred-and-fifty microlitres of Membrane Desalting Buffer (MBD) was added to the columns and centrifuged further at 11,000 x g for 1 minute to dry the membrane. Ninety-five microlitres of DNase reaction mixture (consisting of 10 µL reconstituted rDNase + 90 µL rDNase Reaction Buffer) was pipetted directly onto the silica membrane of the column and the column was incubated at room temperature for 15 minutes. Next, 200 µL of Buffer RAW2 was added and the columns centrifuged for 30 s at 11,000 x g to inactivate and remove the rDNase. Following centrifugation, the columns were placed into new 2 mL collection tubes and a second wash was carried out by adding 600 µL of Buffer RA3 to the columns; these were again spun for 30s at 11,000 x g in the centrifuge. The flow-through was discarded and the columns were placed back in the same collection tube and 250 µL of Buffer RA3 was again added to the columns and centrifuged for 2 minutes at 11,000 x g to completely dry the silica membrane. Columns were placed into new 1.5 mL nuclease-free collection tubes and loaded with 60 µL of RNase-free water and spun at 11,000 x g for 1 minute to elute the RNA. The eluted RNA was quantified by UV spectrophotometry.

2.5 RNA Quality

The assessment of RNA quality for gene expression work was determined by RNA integrity. RNA Integrity Numbers (RIN) values were determined by running 1 µL of each RNA sample on an RNA LabChip® on an Agilent 2100 bioanalyzer (Agilent Technologies) following the protocol recommended by the manufacturer. A RIN value above 7 indicates high quality, intact RNA. RNA Integrity Numbers for each RNA sample are given in Appendix I.
2.6 cDNA synthesis

The Tetro cDNA Synthesis Kit (Bioline) was used to reverse transcribe mRNA to cDNA as per the manufacturer’s protocol. Briefly, priming premixes were prepared by the addition of: 1 µL of Oligo (dT)₁₈ primer, 1 µL of 10 mM dNTP mix, 4 µL of 5x RT Buffer, 1 µL of Ribosafe RNase Inhibitor, 1 µL Tetro Reverse Transcriptase (200u/µL) and total RNA. The amount of RNA (µL) to efficiently reverse transcribe 1 µg was calculated for each RNA sample and added to the priming premix. Each priming premix was made up to 20 µL with DEPC-treated RNase-free water. Samples were incubated at 45°C for 30 minutes and then at 85°C to terminate the reaction. Samples were stored at -20°C until further use for quantitative-PCR work.

2.7 Quantitative-polymerase chain reaction

2.7.1 Protocol

Quantitative-PCR was carried out in 96-well plates in a ViiA™ 7 system (Life Technologies) using 20 µL reaction volumes per well. Master mixes were prepared for each gene to minimize pipetting error. Each reaction included 2 µL of cDNA representing each study animal; appropriate forward and reverse primers at a final concentration of 100 nM and 10 µL of Fast SYBR Green mix (Applied Biosystems). Reaction volumes were made up to a final volume of 20 µL with Milli-Q water. Each reaction was run in duplicate and negative controls for each gene consisted of a no template control that contained no cDNA. Beta-2-microglobulin (B2M) was used as the normalising gene in all q-PCRs carried out which has been determined to be an optimal normalization gene in previous work from the DRL. The protocol for the PCR thermal cycling programme is demonstrated in Table 3. All primer sequences for target genes can be seen in Table 4. Primer sequences were previously designed and synthesised (Sigma-Aldrich) via comparison against the annotated bovine genome. Melt curves were analysed following each q-PCR run to determine the specificity of the primers and the products produced after each reaction (Appendix II)
2.7.2 **Data analysis**

All q-PCR data was analysed using ViiA™ software (Life Technologies) using the comparative cycle threshold (ΔΔCt) method. This method involves comparing the Ct values of samples of interest with a calibrator sample to determine the relative quantity of expression. The Ct values of both the calibrator and the sample of interest are normalised to the endogenous expression of a reference or normalising gene (e.g. *B2M*).

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature/Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>95°C for 15 minutes</td>
</tr>
<tr>
<td>Step 2</td>
<td>95°C for 15 seconds; 60°C for 1 minute; 95°C for 15 seconds</td>
</tr>
<tr>
<td>Step 3</td>
<td>60°C for 1 minute; 95°C for 15 seconds; 60°C for 15 seconds</td>
</tr>
</tbody>
</table>

**Table 3: PCR cycle programme**

2.7.3 **Statistical analysis**

Statistical significance was determined on all relative gene expression values by a two-tailed Mann-Whitney U test using GraphPad Prism v5.0. Statistical significance was assigned where the calculated *p*-value was less than 0.05 (*); 0.01(**); or 0.001(***).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M forward</td>
<td>GGCTGCTGTCGCTGCTGCT</td>
</tr>
<tr>
<td>B2M reverse</td>
<td>TCTGGTGAGTGGTCTTGAGTAC</td>
</tr>
<tr>
<td>IL12A forward</td>
<td>GCCTCAACTACTCCCCAAAACCT</td>
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<tr>
<td>IL12A reverse</td>
<td>GCAGGAGTAAAATTTCTAGGGTTTTCG</td>
</tr>
<tr>
<td>IL2 forward</td>
<td>CAAGCTCTCCAGATGCATACATT</td>
</tr>
<tr>
<td>IL2 reverse</td>
<td>GAGTTCTTCTAGTAGACAGTGAAGTGTCTTTCAA</td>
</tr>
<tr>
<td>IL4 forward</td>
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</tr>
<tr>
<td>IL17A forward</td>
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<tr>
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</tr>
<tr>
<td>IFNG forward</td>
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</tr>
<tr>
<td>IFNG reverse</td>
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<tr>
<td>GATA3 forward</td>
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<tr>
<td>GATA3 reverse</td>
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<tr>
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<td>FOXP3 reverse</td>
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<tr>
<td>IL1B reverse</td>
<td>CTGCTTGCACAGAAGCTCATG</td>
</tr>
</tbody>
</table>
3 Results

3.1 Confirmation of Phenotype at Necropsy

The phenotypes of each of the 20 study animals was confirmed retrospectively by diagnostic histopathological examination of the affected gut tissues and associated lymph nodes, carried out by veterinary pathologist, Gary Clark (Wanaka, NZ). Nineteen animals were classified as R or S at 40 weeks post-infection at the termination of the study. Diagnosis by necropsy was performed on 1 S animal that was electively slaughtered due to severe disease before completion of the study. Lymph node samples representative of the 40 week time point were not available for this animal and consequently, no data was available for analysis at this time point. The designated calibrator animal for q-PCR analysis (Animal 1) was removed from all graphical representation and analysis.

Following experimental MAP challenge, animals developed either clinical JD, low grade pathology, or presented with a minimal enteric infection. The assignment of Lesion Severity Scores (LSS)\textsuperscript{61} by histopathological examination was carried out blinded and was based upon the severity of disease presented in each animal. This was used to classify each animal as either phenotypically R (resilient), S (susceptible), or an intermediate phenotype (I). The posterior jejunum, mid jejunum, anterior jejunum, ileocaecal valve and associated lymph nodes were graded separately, and together, gave a total LSS, represented in Table 5. Non-diseased animals or those showing low grade pathology were assigned a total LSS of between 0-5, with a LSS of 0 representing a non-diseased or infected gut. Animals assigned a LSS of between 0 and 4 were deemed R by phenotype. Animals showing moderate disease to clinical disease were given an LSS greater than 6, with a LSS of 14 indicating severe pathology and clinical disease. The intermediate phenotype was defined by animals that presented with mild-moderate pathology and a LSS of 5-8. Animals that displayed severe pathology and an LSS above 9 were classified as S.

During the course of the study only 1 animal (animal 14) presented with severe clinical disease, as shown by a LSS of 14, and was electively slaughtered at 18 weeks. Throughout the course of the whole study, no animals displayed a non-diseased gut or lymphatic tissues. Few animals (animals 2, 4, 13, and 15) exhibited a mild enteric
infection, as indicated by a LSS of 1-2 which along with animals 6, 8, 11, and 16, which exhibited mild pathology, these 8 animals were therefore deemed R which correlated with the R genotype of their sire. An intermediate phenotype was observed in animals 1, 3, 10, 12, 17 and 19, where the progeny were derived from sires that had an R or S genotype. Six animals (animals 5, 7, 9, 14, 18 and 20) presented with an S phenotype and with significant pathological changes in the gut mucosae and associated lymphatics at the completion of the study. Aside from animal 14 which displayed multibacillary granulomas of the intestinal and lymphatic tissues, all other animals largely showed paucibacillary granulomas upon histopathological examination (data not shown). This was not unexpected as these animals were challenged with a relatively low dose ($10^8$ vs $10^9$ colony forming units [cfu]) of MAP compared with previous experimental infection studies.
Table 5: Summary of Lesion Severity Scores (LSS) and confirmation of phenotype at necropsy.

<table>
<thead>
<tr>
<th>Animal Tag No.</th>
<th>Animal No.</th>
<th>Sire No.</th>
<th>Sire genotype</th>
<th>Total LSS</th>
<th>Phenotype</th>
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Table 6: Binary classification of resilient and susceptible animals by genotype and phenotype.

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3.2 Gene expression of Pro- and Anti-inflammatory Genes by Genotype versus Phenotype

Following the methods as outlined in Chapter 2, gene expression was determined relative to a calibrator animal (Animal 1; expression = 1.00). The study group of animals came from 4 sires of either a susceptible or resilient genotype (Table 6). The relative expression of the 12 target markers was first sorted by genotype of sire compared to the phenotype at necropsy. From initial analysis all 20 animals were first grouped into 1 of 2 phenotypes: R or S, based on disease severity and scoring following a lesion scoring system as in past work. Figure 3 demonstrates the relative expression of IFNG, a gene representative of the pro-inflammatory response (Fig.3A, B), and a prototypical anti-inflammatory gene IL10 (Fig.3C, D). Interferon-gamma expression at 40 weeks post-infection was the only target gene that displayed significant differential expression between R and S animals by genotype ($p<0.05$) and phenotype ($p<0.01$), though no difference was seen between genotype and phenotype by R and S animals alone at both time points (Fig.3A). The mean expression of IL10 was similar between genotype and phenotype for R animals alone at 13 weeks post-infection; for S animals alone at 40 weeks post-infection; and between R versus S animals by both genotype and phenotype (Fig.3C, D). A large scatter in expression was seen in both genotypes and phenotypes, and expression appeared clustered into a higher expressing and a lower expressing group by each R and S phenotype and genotype. These observations were also seen in the other 10 pro-inflammatory and anti-inflammatory markers of interest (Supplementary Figures 1, 2, 3 & 4). These results indicate that measuring gene expression by genotype of sire as a prediction of the immune response between different animals is relatively uninformative for this study and for this reason; measuring expression by phenotype became the focus for the remainder of the results.

Additional to a large scatter and variation in expression, and the clustering of values within a single phenotype, it was also noted in these animals that those that presented with a mild non-specific infection only came from animals of an R genotype and no animal that presented with severe pathology was of the R genotype; the animals that displayed a mixed genotype and phenotype were the only animals to present with moderate symptoms which identified an interesting intermediate group of animals that expressed a phenotype intermediary between the R and S phenotype i.e. moderate pathology. The identification of this novel phenotypic group resulted in an adjustment
of the initial classification of animals into 3 phenotypes: R, I or S (Table 5), (rather than R or S as was initially done [Table 6]) that more accurately represented their disease severity at necropsy.
Figure 3: Relative Expression of IFNG and IL10 by Genotype and Phenotype in R and S animals at 13 weeks and 40 weeks post-MAP Infection. Relative expression of (A) IFNG at 13 weeks p.i.; (B) IFNG at 40 weeks p.i.; (C) IL10 at 13 weeks p.i.; (D) IL10 at 40 weeks p.i. were measured by genotype and phenotype from PJJLN samples taken at biopsy (13 weeks) and necropsy (40 weeks) from animals of a resilient or susceptible genotype via q-PCR using the ∆∆Ct method. All gene expression was normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was carried out by a two-tailed Mann-Whitney U test. Horizontal bars represent the mean expression. Statistical significance is denoted as: * = p<0.05; ** = p<0.01.
3.3 Gene Expression of R, I and S Animals over the Course of Infection

3.3.1 Differential expression of Pro-inflammatory Genes

The relative expression levels of the pro-inflammatory genes *IL12A*, *TBX21*, *IL1B* and *IFNG* are shown in Figure 4. Interleukin-12A, which encodes for the p35 subunit of the cytokine IL-12, was expressed at relatively similar low levels between the three phenotypic groups at 13 weeks post-infection. At this time point, *IL12A* expression was tightly grouped for each distinct phenotype, particularly in the intermediate group. By 40 weeks, *IL12A* expression had significantly increased by 10-fold in the R animals (*p*<0.001), I animals (*p*<0.01) and the S animals (*p*<0.01) (Fig.4A). T-box 21 or *TBX21* encodes for the Th1-specific transcription factor T-bet. At 13 weeks post-infection, *TBX21* expression tended to be lower in the R animals and higher in the I animals; the expression levels were scattered in each phenotypic group of animals, as indicated by a wide variation in the mean expression and large error bars. Interestingly, as the disease progressed in time, *TBX21* expression decreased in all 3 groups, though only to significant levels in the intermediate animals (*p*<0.01) (Fig.4B). At 13 weeks post-infection, *IL1B* was expressed at lower levels in the intermediate animals compared to R and S and displayed a large scatter in the R animals. By 40 weeks post-infection, *IL1B* was significantly decreased in the resilient animals (*p*<0.01); however expression of *IL1B* was decreased overall in the I and S animals which is shown by fewer data points on the plot (Fig.4C). Interferon-gamma expression was scattered in the R and S groups at early infection (13 weeks p.i.) but was grouped more tightly at 40 weeks post-infection with less variability in the mean expression levels. Between 13 weeks and 40 weeks, *IFNG* expression remained relatively even in R animals but was significantly increased in the I and S animals (*p*<0.05) (Fig.4D).
Figure 4: Relative Expression of Pro-inflammatory IL12A, TBX21, IL1B and IFNG at weeks 13 and 40 post-MAP Infection. Relative expression of (A) IL12A; (B) TBX21; (C) IL1B; (D) IFNG were measured by phenotype from PJILN samples taken at 13 weeks post-infection (biopsy) and 40 weeks post-infection (necropsy) from animals of a resilient or susceptible genotype using q-PCR via the ∆∆Ct method. All gene expression was normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was carried out by a two-tailed Mann-Whitney U test. Horizontal bars represent the mean expression. Statistical significance is denoted as: * = p<0.05; ** = p<0.01; *** = p<0.001.
Differential expression of the pro-inflammatory genes $IL6$ and $IL17A$ over the course of infection was also observed. At 13 weeks post infection, $IL6$ expression was relatively even among the 3 groups of animals, though marginally decreased in the intermediate animals. At 40 weeks post infection $IL6$ expression was marginally increased in all phenotypes; however this increase only reached statistical significance in the I group, with a $p$-value of less than 0.05 (Fig.5A). Relative expression of the Th17 gene $IL17A$ was at minimal levels early in infection (13 weeks post-infection) with the highest value in each phenotypic group approximately 1, and the lowest being 0.02 seen in the R group. At 40 weeks, expression of $IL17A$ increased 10-fold in all three groups reaching a statistical significance of $p<0.01$ in R animals; $p<0.05$ in I animals; and $p<0.01$ in S animals (Fig.5C). The mean expression of the T cell stimulatory cytokine $IL2$ remained largely even across the two time points of sampling (Fig.5B). Interleukin-2 relative expression had a large scatter at 13 weeks post-infection which was observed by all 3 phenotypes. At 40 weeks post-infection, $IL2$ expression was marginally increased in the I group of animals compared to the R and S animals. The S group of animals displayed a much tighter grouping of $IL2$ expression compared to R and I animals; a trend which was similarly observed by $IL6$ in S animals (Fig.5A). Overall, the data from Figures 4 and 5 indicate a general increase in pro-inflammatory cytokines at 40 weeks post-infection.
Figure 5: Relative Expression of Pro-inflammatory *IL6*, *IL17A* and *IL2* at weeks 13 and 40 post-MAP Infection. Relative expression of (A) *IL6*; (B) *IL2*; (C) *IL17A* were measured by phenotype from PJILN samples taken at 13 weeks post-infection (biopsy) and 40 weeks post-infection (necropsy) from animals of a resilient or susceptible genotype using q-PCR via the ∆∆Ct method. All gene expression was normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was carried out by a two-tailed Mann-Whitney U test. Horizontal bars represent the mean expression. Statistical significance is denoted as: * = p<0.05; ** = p<0.01.
3.3.2 Differential expression of Anti-inflammatory Genes

The relative expression levels of genes associated with an anti-inflammatory response are depicted in Figures 6 and 7. At 13 weeks post-infection, expression of the Treg-associated transcription factor FOXP3 was similar for R and S animals and marginally increased in the I group of animals. Relative expressed of FOXP3 decreased significantly by over 10-fold in all groups of animals at 40 weeks post-infection. The decrease of FOXP3 reached statistical significance in all 3 groups with a p-value of less than 0.001 in the R group of animals and a p-value of less than 0.01 in both I and S animals. Compared to the R and S animals at both time points, FOXP3 expression was clustered very tightly in the I group (Fig.6A). Interleukin-10 expression was scattered largely for all 3 phenotypes at 13 weeks post-infection, with some animals showing a relative expression of 1-fold and others as high as 20-fold. Relative expression of IL10 was significantly decreased by 40 weeks in all 3 groups, though only reaching statistical significance in the R (p<0.05) and S animals (p<0.05). Similar to FOXP3 expression in I animals at 40 weeks post-infection, IL10 expression was also clustered tightly for the I animals at this time point (Fig.6B). No differential expression of TGFB1 was seen throughout the course of infection as the relative mean expression levels were similar at both time points, though the I group appeared to be marginally increased at 40 weeks post infection (Figure 6C).
Figure 6: Relative Expression of Anti-inflammatory FOXP3, IL10 and TGFβ1 at weeks 13 and 40 post-MAP Infection. Relative expression of (A) FOXP3; (B) IL10; (C) TGFβ1 were measured by phenotype from PJJLN samples taken at 13 weeks post infection (biopsy) and 40 weeks post infection (necropsy) from animals of a resilient or susceptible genotype using q-PCR via the ∆ΔCt method. All gene expression was normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was carried out by a two-tailed Mann-Whitney U test. Horizontal bars represent the mean expression. Statistical significance is denoted as: * = p<0.05; ** = p<0.01; *** = p<0.001.
Relative expression of GATA3, the Th2-specific transcription factor, showed a reduction between 13 and 40 weeks post-infection (Fig. 7A). At 13 weeks, the expression of GATA3 was scattered and most highly expressed in the I animals followed by the S then R animals. By 40 weeks post-infection, GATA3 was decreased in all groups and expression was both grouped more tightly, as indicated by smaller error bars, and the mean expression was more even among the 3 groups. The relative expression of IL4 showed a similar pattern to GATA3 (Fig. 7B). Interleukin-4 expression was most variably expressed in the I group of animals during the early phase of infection, compared to R and S groups. The expression of IL4 was decreased approximately 10-fold in the R (p<0.001) and S (p<0.01) animals by 40 weeks post-infection, which overall, Figures 6 and 7 indicate a decrease in anti-inflammatory genes over a course of infection.
Figure 7: Relative Expression of Anti-inflammatory GATA3 and IL4 at weeks 13 and 40 post-MAP Infection. Relative expression of (A) GATA3 and (B) IL4 were measured by phenotype from PJJLN samples taken at 13 weeks post-infection (biopsy) and 40 weeks post infection (necropsy) from animals using q-PCR via the ∆∆Ct method. All gene expression was normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was carried out by a two-tailed Mann-Whitney U test. Horizontal bars represent the mean expression. Statistical significance is denoted as: ** = p<0.01; *** = p<0.001.
4 Discussion

It has been well established that while innate immunity plays a central role in mycobacterial infections, alone it is insufficient without the adaptive arm of immunity, especially the T helper (Th) cells, since both the innate and adaptive immune responses are required to eliminate a pathogen. Macrophages are pivotal innate effector cells that are required both for the killing and removal of bacteria, and the presentation of antigen to the CD4+ and CD8+ T cells required for effector responses in adaptive immunity. The global CD4+ T cell response is characterised by subsets of different cells that each have a pre-defined role in defining qualitative aspects of the immune response, most importantly T helper cell function. It is widely accepted that the Th1 response influences the activation of macrophages through the production of cytokines or “help”. Similarly, Th2, Th17 and Treg cells influence the outcome of an immune response by the nature of their interactions with other immune cells, and the action of the cytokines that they specifically produce. Any attempt to understand the progression of Johne’s disease must consider the central role of Th1 and Th2 responses. Recent findings however infer that the Treg and Th17 responses may also play important roles in the outcome of MAP infection and the onset of JD. Rather than focusing solely on the immunology of subclinical versus clinical disease, the current research has been designed to investigate how animals that display heritable resilience and susceptibility to JD can be used to explore the functionality of the immune response in animals which display disparate phenotypes following exposure to MAP infection. A study of R animals should represent protective immune phenotypes while S animals should display an immune phenotype associated with disease diagnosis or a pathological phenotype. Previous in vivo and ex vivo immunological studies in the host laboratory suggest that susceptibility to JD is associated with an exacerbated pro-inflammatory innate response and the lack of an efficacious adaptive response. It remains to be determined whether upregulation of pro-inflammatory genes in S animals is a compensatory mechanism as a result of impaired MAP clearance, or it represents dysfunctional clearance of MAP or inappropriate antigen presentation to induce a protective adaptive cellular immune response. Other experimental infection studies show that resilient animals do clear MAP infection through innate immune pathways, but also control the infection and eventually recover by generating adaptive immune
responses resulting in protection from the development of clinical disease. As a consequence the present study examines pathways of innate and adaptive immunity in animals that are resilient to experimental infection or develop pathology following infection. While these responses may be reflected in peripheral blood mononuclear cells it is possible that immune cell activation within the gut mucosae or localised lymphatics may provide immune signatures that may better reflect functional immunity in different facets of protective immunity or immune pathology following MAP infection.

The primary focus of this study was to investigate gene expression profiles *ex vivo* from the mesenteric lymphatics (posterior jejunal lymph nodes) of animals selected because they were either resilient or susceptible to JD. Previous studies in a large deer stud had identified different breeds of deer that display extreme phenotypes for resilience (R) or susceptibility (S) to natural or experimental MAP infection. The paternal genotype appears to have a major influence on the phenotype of offspring where the use of semen from R or S sires for AI matings with crossbred females produces progeny where 80% express the R or S phenotype of the paternal breed. The custom bred progeny (fawns) have been used in successive breeding programmes over the past three years to study the immune biology of resilience or susceptibility to MAP infection. The current project studied immune gene expression profiles in mesenteric lymph node tissues from R or S progeny that were challenged experimentally with MAP infection and samples obtained at 13 weeks post-infection (by laproscopic surgery) or at 40 weeks post-infection by elective necropsy. The candidate genes studied involve endogenous expression of 7 pro-inflammatory and 5 anti-inflammatory genes related to innate and adaptive immunity. The immune pathways targeted were the Th1, Th2, Th17 and Treg responses which were measured via quantitative-PCR using specific primers for individual genes. Beta-2-microglobulin (*B2M*) was used as a normalising gene as this has been determined to be an accurate housekeeping gene for the quantification of expression of immune genes in deer cells. While q-PCR is a sensitive and accurate tool to detect and quantify gene expression, it is important to note that using q-PCR as a measure of differential gene expression is influenced by the amount of cDNA in the initial PCR reaction, which is calibrated to be the same for each reaction. The use of a normalising gene such as *B2M* accounts for the inherent variation of RNA between samples that invariably occurs between animals.
4.1 Confirming Phenotype following Experimental MAP Challenge

Throughout the course of the study only 1 of 20 animals bred from an S sire displayed symptoms of extreme clinical disease and required elective slaughter at 18 weeks. This differed from previous experimental MAP challenge studies performed in the DRL, where clinical JD can be observed in 10-20% of animals relatively early (12-20 weeks) following infection. The relative lack of severe disease observed in the current study may relate to the lower challenge dose. In previous experimental challenges animals have usually received doses containing $10^9$ virulent MAP organisms in contrast to the 3 $\times$ $10^8$ MAP challenge inoculum that was given in this study. Had this study been extended longer, perhaps animals that exhibited moderate pathology (i.e. the intermediate animals) may have progressed from a mild subclinical infection to obvious clinical disease, and the animals deemed S may also have developed greater disease pathology. Nonetheless, animals that were of an S genotype exhibited more severe disease upon histopathological examination. No animal with R genotype presented with severe clinical disease or a LSS above 7 (Table 5). On the other hand, no R animals were completely disease or MAP-free (LSS<1) indicating that all animals had received an adequate dose that constituted a true infectious challenge. Previous experimental challenge studies$^{64}$ show that the onset and severity of disease is influenced by the dose and virulence of the challenge inoculum. While the development of pathology was less severe in the current study, the lower challenge may have prolonged the natural progression of events, both in terms of the course of infection and the progression of the immune response, thus, delaying the complete recovery of the animal. Had these animals survived past 40 weeks post-infection, perhaps either a complete, or a better resolution of infection would have been seen, ergo enforcing resilient as a more appropriate term for the aforementioned phenotype, rather than resistant.

All progeny (5/5) from one sire with an S genotype produced progeny (Sire number 4; Table 5) that were classified as S by phenotype. All 3 other sires produced 4/5 (80%) progeny with a phenotype that mirrored their paternal genotype (Tables 5 & 6). These findings were consistent with previous studies carried out by the DRL where R and S phenotypes of progeny demonstrate 80/20 heritability of the paternal phenotype$^{41}$. This infers that the challenge inoculum used in the current study was sufficient to disclose both R and S phenotypes.
Despite the fact that 20% of the progeny from a known R breeding stag appear relatively susceptible to MAP infection, these animals represent a more attenuated or intermediate (I) phenotype rather than producing an extreme S response. The same held true for the 20% proportion of relatively resilient R progeny bred from S sires. In terms of production, reproduction and economic value, these intermediate animals are still of greater value than S animals because they display minimal to mild pathology; a delayed progression to clinical JD; and represent a relatively resilient population. A study of animals with an I phenotype will be valuable in future immunological studies that attempt to map immune markers linked with a resilient (R) or susceptible (S) phenotype for JD.

4.2 Comparing Gene expression Profiles based on Genotype or Phenotype

As the phenotype of these animals largely conformed to the 80/20 rule of heritability that has been described in previous experimental work, the relative expression of the 12 candidate markers were first initially analysed by grouping the animals on the basis of their known genotype by sire and the phenotype confirmed after necropsy. This comparison was to determine whether a significant difference in the level of expression of these genes could be seen in the animals that had been grouped phenotypically rather than by their known genotype.

Prior to establishing a group with an intermediate (I) phenotype, the animals were grouped on a binary scale as either R or S based on a different lesion severity scoring system (Table 6). The only gene that showed differential expression in R and S groups was *IFNG* at 40 weeks post-infection. The expression levels for this gene was significantly lower for animals with R genotype and phenotype compared with equivalent S groups (Figure 3). No significant differential expression was observed between genotype and phenotype for R and S animals for the 11 other genes of interest at either time point of sampling. This is surprising considering that for the phenotypic grouping 2 animals with a R genotype were reclassified as S and 3 animals with a S genotype were reclassified as R. Together the findings suggest that none of the biomarkers tested, with the exception of *IFNG* are likely to distinguish between R or S animals. Unless the gene expression levels in an animal are skewed in one direction (significantly increased or decreased) there is likely to be no obvious significant change
to the mean expression of the gene between R or S groups irrespective of whether they are grouped by genotype or phenotype. Because the initial phenotypic separation of animals into two groups (R or S) may have obscured more subtle differences between animals with polarised phenotypes it was decided to create an intermediate group (LSS 5-8). This was done so that smaller groups of animals with extreme phenotypes for R or S could be compared directly.

4.3 Separate Assignment of Animals with an Intermediate Phenotype

Bimodal gene expression of animals into a high or low expressing group was observed by most genes. This trend was particularly evident in *IFNG* at 13 weeks, *IL10* at 13 weeks, *IL17A* at 40 weeks, and *TGFB1* at 13 weeks, where the animals can be divided into two distinct groups of differential expression within a single phenotype or genotype. All three animals that had a confirmed phenotype different to their known genotype all displayed a moderate form of pathology rather than distinctly R or S. This finding, along with the aforementioned observations, identified a group of animals that appeared to manifest a phenotype that was an intermediate of R and S, thus the identification of an intermediate (I) group. The assignment of animals to the I group required an alteration to the previous classification of disease severity and phenotype of animals. A small group of animals that were previously deemed R or S were consigned as I for further analysis.

The intermediate phenotype is realistic if we consider both the contribution of maternal genetics to the makeup of a progeny’s immune system which in this case, were crossbred females that often do not display polarised R and S phenotypes, and the notion that under natural circumstances, a spectrum of states exists between the two traits, with absolute R and S representing the two extremes. As previously alluded to, susceptibility to most common infectious diseases rarely follow a simple Mendelian mode of transmission; only few exceptional cases of this actually occurring in nature have been documented. Susceptibility to infectious disease, including susceptibility to JD results from an accumulation of multiple disease associated variants or mutations that individually have a limited penetrance. Animals that exhibit a more polarised S phenotype represent the additive effect of a large number of defective immune genes and hence, more severe disease, while the I phenotype may be associated with the
accumulation of fewer of these defective genes and consequently, a milder form of the disease.

### 4.4 The Role of Pro-inflammatory Genes in Disease Progression

When investigating the expression profiles of genes associated with a pro-inflammatory immune response, significant increase were seen in *IL12A*, *IFNG*, *IL17A* and *IL6* at 40 weeks post-infection were seen (Figures 4 & 6). Interleukin-12A, which encodes for the p35 subunit of the cytokine IL-12, is a cytokine that is produced by the macrophage and is associated with the activation of pro-inflammatory Th1 cells\(^{33}\). We could postulate that for JD, the expression of *IL12A* would be greater in S animals and to a lesser extent the I animals to correlate with the observation that S animals exhibit a greater upregulation of pro-inflammatory genes; however, this was not the case. All three phenotypic groups of animals increased *IL12A* at 40 weeks to relatively similar levels of expression. No significant differential expression of *IL12A* was observed between R, I and S animals at 13 and 40 weeks, and this trend was observed in every pro-inflammatory gene of interest. It is tenable that IL-12 may play a dual role either in protective immunity involving Th1 cells or as a mixed Th1/Th2 response in disease\(^{39}\).

Alongside IL-12, activated macrophages also secrete IL-1, consisting of both IL-1\(\alpha\) and IL-1\(\beta\) which function to promote the activation and differentiation of Th1 cells and are essential for the effective removal of intracellular pathogens\(^{33}\), including MAP. The expression of *IL1B* was significantly reduced in the R group of animals at 40 weeks post-infection. While the I and S animals displayed an increased expression of *IL1B*, this was actually represented by fewer animals and a greater variation in expression; many of these animals may have expressed *IL1B* at a level below detection using qPCR. Findings from Robinson *et al.* (2011) infer that *IL1B* may not actually be involved in the pathogenesis and pathology of JD, whereby *IL1B* was found to be expressed highly similarly in the lymphatic tissues between control and diseased animals\(^{39}\).

Interferon-gamma activates the bactericidal activity of macrophages, thus enabling phagocytosis of the target pathogen\(^{35}\). A model has been published\(^{6}\) that proposes a protective role for IFN-\(\gamma\), whereby IFN-\(\gamma\) producing Th1 cells are required early in infection to restrict bacterial proliferation via their interaction with the phagocytic
macrophages. An increase in IFN-γ production late in infection may negate the protective capabilities and consequently contribute to the pathology that is observed in late or clinical infection in the S animals. In contrast, upregulation of IFNG during early infection in the R animals may reflect a protective response. Indeed, expression of the potent pro-inflammatory cytokine, IFNG, was found to be significantly increased in the I and S groups at 40 weeks post-infection. These results corroborate Robinson et al. (2011) findings that endogenous IFNG expression is significantly upregulated in the lymph node tissue of severely diseased animals.

A dysfunctional IFN-γ response and the development of clinical disease is not unique to MAP; this response is common to most, if not all mycobacterial diseases due to their intracellular nature. While an IFN-γ directed Th1 response is successful in limiting bacterial multiplication, an excess of IFN-γ significantly contributes to the development of immunopathology; and this is a hypothesis that has been widely studied in tuberculosis. Interferon-gamma has a widely recognised role in the formation of granulomatous lesions due its macrophage-activating properties. While granuloma production is largely linked to disease, granulomas share the same common function that they result in the containment and clearance of an infection. Inadvertently, in some circumstances granulomas provide an ideal microenvironment for the protection and proliferation of bacteria such as MAP. In the context of an animal susceptible to JD, an increase of IFN-γ induces phagolysosomal maturation in the macrophage. Where MAP is able to inhibit phagolysosomal maturation and bacterial killing by the phagolysosome, granulomatous lesions form. Disseminated multibacillary granulomas are found in the tissues of clinically diseased animals, such as was observed in animal 14 (data not shown).

Interestingly, while IFNG expression was increased in I and S animals, an unexpected finding was the marginal reduction at 40 weeks post-infection in the mean expression of the gene encoding the Th1-specific transcription factor, TBX21, in all 3 groups of animals, though only to significant levels in the I group. In contrast, no difference in expression of T-bet (TBX21) between control, minimally diseased, and severely diseased animals was found in the lymphoid tissue as reported by Robinson et al. (2011). Intuitively, an increase in TBX21 and Th1 cells should correlate with an
increase of Th1-type pro-inflammatory genes, though this was not observed in this case.

At 40 weeks post-infection, IL6 expression was increased significantly in the I group of animals and marginally decreased in the S animals. Interleukin-6 is a potent pleiotropic cytokine that has been implicated in the activation of pro-inflammatory Th17 cells[43]. This finding is unexpected as an increase of IL6 in S animals may be expected due to its pro-inflammatory nature. An excess of IL-6 in the tissues has indeed been linked to chronic inflammation, including ulcerative colitis, pancreatitis and hepatitis, to name a few[67]. As was the case for IL6, no significant differential expression of IL2 was observed between the two time points of sampling. This observation contrasts to the findings of Robinson et al. (2011) where a marked decrease of IL2 was observed between control and minimally diseased animals, and a further reduction of IL2 was seen in the lymphatic tissues of severely diseased group of animals[39].

Th17 cells have a role in protective immunity against extracellular bacterial and fungal infections; however they have also been implicated in the onset of autoimmune diseases including rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease (IBD)[43]. It is conceivable that the Th17 response plays either or both, a protective and pathological role in JD considering the link between Th17 responses and IBD. Interleukin-17 may have a role in the inhibition of the proliferation of intestinal cells which may contribute to the maintenance of chronic pathology during IBD[43]. The role of the Th17-specific cytokine IL-17 in JD is not yet fully understood, though an upregulation of IL17A has been observed in severely diseased animals[39]. An interesting finding from this project was the observation that IL17A was increased similarly in all 3 phenotypic groups at 40 weeks post-infection, rather than the S animals alone.

Because the lymphatic tissues contain a multitude of cell types it is difficult to directly infer or correlate the expression of a gene with a specific cell type in many cases. For instance, in a subset of Th17 cells, IFN-γ and IL-17 may be co-expressed together[68]. While this makes sense as the Th17 response is known to upregulate inflammation alongside the Th1 response[43], it may also imply the Th1 response is not involved in clinical disease, but rather it is the role of the Th17 response. This hypothesis is compatible with the current data since IL17A and IFNG were increased while TBX21 was decreased.
4.5 The Role of Anti-inflammatory Genes in Disease Progression

The role of Treg immunity in mycobacterial tuberculosis has been widely studied; however only recently has the Treg response been suggested to play a role in the immunology of JD. Regulatory T cell responses appear to be pivotal for the prevention of autoimmunity and regulating pro-inflammatory immune responses by the down-regulation of effector CD4+ and CD8+ T cells\(^3\). A study by Chen et al. (2007) found that the proportion of FOXP3\(^+\) Tregs increases in the periphery and at the site of infection in patients with active tuberculosis. This expansion of Tregs suppresses TB-specific immunity and therefore contributes to the pathogenesis and pathology of TB in humans\(^6\). The expansion of Tregs during TB was similarly demonstrated in a study by Guyot-Reval et al. (2005)\(^6\). Historically, the clinical manifestation of TB has been known to be determined to a large extent by a fine balance between protective and pathological Th1 responses\(^6\) and this notion is likely applicable to JD. To prevent excessive tissue damage and exacerbated pro-inflammatory Th1 and Th17 responses, the Treg response is activated to downregulate or suppresses the pro-inflammatory response through the downregulation of effector cytokines such as IFN-\(\gamma\) and IL-2\(^3,6\). T regulatory cells likely exert their immunosuppressive function through the production of TGF-\(\beta\) and IL-10. Indeed, TGF-\(\beta\) and IL-10 have been implicated in suppressive immune responses in active TB\(^6\).

In the context of JD, the immunosuppressive Treg response is thought to have a role during the clinical stage where they suppress the “protective” Th1 cells and thus bacterial proliferation and dissemination ensues\(^3\). The innate response and macrophages consequently produce pro-inflammatory cytokines like IL-1\(\alpha\) and IL-1\(\beta\), perhaps as a compensatory mechanism, in response to the increase of MAP proliferation; it is for this reason that we observe extensive pathology during clinical JD in S animals\(^3\).

Interestingly, the findings from the present study saw a significant decrease at 40 weeks post-infection in the Treg-specific transcription factor, FOXP3, and IL10; the gene encoding for IL-10. A decrease in these two genes was observed by all three phenotypic groups. Of interest, gene expression was very tightly grouped in the I group compared to R and S and this finding may require further investigation. No significant differential expression was observed in TGFB1 between 13 and 40 weeks post-infection. The
significant decrease in *FOXP3* mirrors results seen by Robinson *et al.* (2011); however that study also reported a significant decrease of *TGFB1* in severely diseased animals and no change in expression of *IL10* between control and diseased animals

Interleukin-10 expression is not just restricted only to Treg cells and has been additionally investigated as a biomarker associated with Th2 immunity. T helper 2 (Th2) immunity normally occurs after Th1 immunity has been activated and the transition between these two responses may account for the progression to clinical JD in S animals. Numerous studies have found antibody production can occur early in infection and animals can co-express IFN-γ and antibody late in chronic infections, such as TB and JD. While the Th2 antibody-associated response is largely regarded as ineffective in protection against MAP, Th2 immunity may not have an effector role in clinical disease. It is possible that immunosuppression of the Th1 response by the Treg response and the consequent increase in bacterial proliferation accounts for the activation of the Th2 response. Due to the role of Th2 cells in tissue repair it is plausible that a lack of Th2 responses in S animals contributes to extensive tissue damage during JD. The findings of this study in part corroborate with the findings of Robinson *et al.* which showed a downregulation in the lymph nodes of *IL4* and *GATA3*. At 40 weeks-post infection, a substantial decrease in *GATA3* and a significant decrease in *IL4* expression was observed by R, I and S groups of animals. As *GATA3* is the master regulator of Th2 differentiation it is reasonable that a decrease in the Th2-specific cytokine gene *IL4* was observed in the present study.

It was surprising that, like the expression of pro-inflammatory genes, the expression of anti-inflammatory genes was similar for R, I and S groups so it cannot be inferred that these genes play an important role in either the R or S phenotype. The fact that R animals showed a decrease in Th2 as well as Treg genes could suggest that these responses were no longer required as MAP infection was beginning to resolve, although this was also seen in the I and S groups. Because this study was investigating resilience and susceptibility to JD rather than infected versus uninfected, it would be of more biological relevance to investigate a larger group of animals that exhibited a resolved infection and thus a resilient phenotype, and susceptible animals that were truly clinically diseased. Because this specific study used a lower dose of viable MAP organisms for experimental challenge we were not able to see these precise phenotypes.
throughout the course of the study, so the results of this study may not be truly representative of the complex and multifaceted immune response that occurs naturally.

An obvious limitation to this study is that the animals used came from 4 different sires that were genetically unrelated. Intrinsic differences in gene expression and the functionality of the immune response will invariably be observed between animals that have come from a different genetic background; however, this model is still of biological relevance.

4.6 Conclusions & Future Directions

While profiling the levels of expression of candidate genes in the mesenteric lymphatic tissues obtained from experimentally infected animals did not identify consistent differences that could potentially be associated with resilient or susceptible phenotypes, there was however an observed dramatic differential expression between 13 weeks and 40 weeks post-infection in a large number of the candidate genes investigated. This observation may provide insights into the progression of immune parameters as the disease is progressing in time from early to late infection. A proposed model for the role of CD4$^+$ helper cells in disease progression is given in Figure 8. As previously alluded to, the lymphatic tissue is extremely complex and forms a site for the interaction of many different immune cell types. It is conceivable that the interactions seen in the lymph nodes provide such dynamic responses that the identification of differentially expressed immune markers is confounded and thus, unlikely to be informative in the diagnosis of resilience and susceptibility to JD. The fact that the present study used a lower challenge inoculum than would have been used previously resulted in less extreme pathology which may have obscured differences in gene expression between R and S animals. Aside from the increase in IFNG in I and S animals this study overall largely failed to identify a panel of biomarkers that could be of use in the future to identify and diagnose R and S phenotypes by the profiles of expression in the lymphatic tissue; however, if this study was repeated with a higher challenge dose perhaps more specific R and S phenotypes would be observed. To gain more insight into the progression of disease that is occurring between R, I and S animals it would be of more use to obtain lymphatic tissue biopsy samples at multiple points throughout the course of infection rather than just at 13 weeks, and at elective slaughter. Thirteen weeks after challenge is a relatively long period of time and critical
changes or interactions (particularly involving the early innate response) may be occurring very early on in infection (before 13 weeks) that are being missed by neglecting this time point. Much like putting the pieces of a puzzle together, profiles of expression from samples taken at many time points may build up a clearer and more specific picture of how the various arms of the immune response are changing and interacting and how this correlates to the progression from subclinical to clinical disease in S animals. Similarly, how changes in the immune response constitute protection in the R animals, and at what point post-infection has an animal completely resolved the infection may be identifiable. The present study did not use non-infected control animals or biopsy samples that were taken pre-infection which may be of use in future studies to determine a baseline or “normal” expression levels for comparison of the candidate genes to the expression of the same genes in the infected state.
Figure 8: Proposed Model for the Role of CD4+ T helper Cells in the Progression of Disease. Based on observations from current literature and this study, a model can be proposed for the role of the T helper cells in the local mucosal immune response to MAP and the progression of disease in R and S animals. Resilient animals appear to clear an infection relatively early through protective innate and adaptive responses involving the efficient phagocytosis of MAP and the suppression of pro-inflammatory Th1 and Th17 responses to prevent pathology. Susceptible animals may bypass immunoregulation or display a decreased Treg response, which when coupled with defective MAP clearance by the macrophage, bacterial proliferation increases and activates the Th2 response. Macrophages produce excess pro-inflammatory cytokines in response to impaired MAP clearance which further induces IFN-γ production. An exacerbated mixed Th1/Th2/Th17 response confounds the clearance of MAP and results in tissue damage and the progression to clinical disease in S animals.
References


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Supplementary Figures

**Supplementary Figure 1:** Relative expression of pro-inflammatory *IL12A*, *IL1B* and *TBX21* between genotype and phenotype in/between R and S animals. Relative expression of (A) *IL12A* at 13 weeks; (B) *IL12A* at 40 weeks; (C) *IL1B* at 13 weeks; (D) *IL1B* at 40 weeks; (E) *TBX21* at 13 weeks; (F) *TBX21* at 40 weeks between resilient (R) and susceptible (S) animals by genotype of sire and phenotype at slaughter. Expression of each gene was measured from PJILN samples taken at 13 weeks post MAP infection (biopsy) and 40 weeks post MAP infection (necropsy) via q-PCR using the ΔΔCt method. Gene expression is normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was determined by a two-tailed Mann-Whitney U test. Horizontal bars represent mean expression.
Supplementary Figure 2: Relative expression of pro-inflammatory IL2, IL17A and IL6 between genotype and phenotype in/between R and S animals. Relative expression of (A) IL2 at 13 weeks; (B) IL2 at 40 weeks; (C) IL17A at 13 weeks; (D) IL17A at 40 weeks; (E) IL6 at 13 weeks; (F) IL6 at 40 weeks between resilient (R) and susceptible (S) animals by genotype of sire and phenotype at slaughter. Expression of each gene was measured from PJILN samples taken at 13 week post MAP infection (biopsy) and 40 weeks post MAP infection (necropsy) via q-PCR using the ∆∆Ct method. Gene expression is normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was determined by a two-tailed Mann-Whitney U test. Horizontal bars represent mean expression.
Supplementary Figure 3: Relative expression of the Th2-associated transcription factor GATA3, and IL4 between genotype and phenotype in/between R and S animals. Relative expression of (A) IL4 at 13 weeks; (B) IL4 at 40 weeks; (C) GATA3 at 13 weeks; (D) GATA3 at 40 weeks between resilient (R) and susceptible (S) animals by genotype of sire and phenotype at slaughter. Expression of each gene was measured from PJJLN samples at 13 weeks post MAP infection (biopsy) and 40 weeks post MAP infection (necropsy) via q-PCR using the ∆ΔCt method. Gene expression is normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was determined by a two-tailed Mann-Whitney U test. Horizontal bars represent mean expression.
Supplementary Figure 4: Relative expression of anti-inflammatory genes FOXP3 and TGFB1 between genotype and phenotype in/between R and S animals. Relative expression of (A) FOXP3 at 13 weeks; (B) FOXP3 at 40 weeks; (C) TGFB1 at 13 weeks; (D) TGFB1 at 40 weeks between resilient (R) and susceptible (S) animals by genotype of sire and phenotype at slaughter. Expression of each gene was measured from PJJLN samples at 13 weeks post MAP infection (biopsy) and 40 weeks post MAP infection (necropsy) via q-PCR using the ∆∆Ct method. Gene expression is normalised to β2-microglobulin expression and relative to a calibrator animal (Animal 1). All statistical analysis was determined by a two-tailed Mann-Whitney U test. Horizontal bars represent the mean expression.
Appendices

Appendix I: RIN values

Animal 1

RIN = 9.1

Animal 2

RIN = 9.3

Animal 3

RIN = 9.4

Animal 4

RIN = N/A

Animal 5

RIN = 9.4

Animal 6

RIN = 9.3
Animal 7

RIN = 9.3

Animal 8

RIN = 9.7

Animal 9

RIN = N/A

Animal 10

RIN = 7.7

Animal 11

RIN = N/A

Animal 12

RIN = 7.7
Animal 13: RIN = 8.5
Animal 14: RIN = 8.3
Animal 15: RIN = 5.8
Animal 16: RIN = 8.4
Animal 17: RIN = 8.7
Animal 18: RIN = 6.8
Animal 19

RIN = 8.2

Animal 20

RIN = N/A

Ladder
Appendix II: Primer Melt Curves

Cervine *B2M*

Cervine *FOXP3*

Cervine *GATA3*

Cervine *IFNG*

Cervine *IL1B*

Cervine *IL2*
Melt Curve Plot

Cervine *TGFβ1*