Objective Title:	Objective 4 - Pathobiology
Milestone Number & Title:	4.2 Control of Johne's in deer
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Executive Summary

The field work for this project was completed in the first two years and only the samples from Weeks 3, 12 and 50 from the 2008 study and Week 4 from the 2009 study were sequenced using SOLiD Sage technology. Full sequencing of the 2009 samples should have been completed in the third year of the study but funding for this was withdrawn by JDRC. The fourth year of this study, which would have included validation and further investigation of gene expression of the complete dataset, was not funded by JDRC and the work this year has been to try to salvage as much from the incomplete dataset as possible.

The results from the 2008 study (3R and 3S animals at Weeks 4, 13 and 50) and the 2009 study (3R and 3S animals at Week 4) are exciting and differential up/down regulation of a number of key genes have been identified. One would have expected the results from the full set of 2009 animals would be even more exciting because of the greater separation between R and S outcomes.

The AgResearch Invermay deer MAP challenge model is the only internationally recognized model that can reliably deliver natural Johne's disease outcomes in ruminants of known R and S genotype within a 12 month period.

In this study, a total of 31,500 genes were uniquely mapped and 18,000 genes were annotated against three datasets; the deer and elk transcriptome (in-house), cattle transcriptome (Ensembl cattle genes) and the bovine genome (Btau 4). Of these 18,000 genes, 17,500 were recognized by the Ingenuity Pathway Analysis (IPA) programme. The expression level for the 3 samples at each time point were averaged and an expression value was calculated for each gene and this was expressed as a Log ratio, Fold change and p-value (significance of Fold change). Using IPA, genes were filtered by p-value ($p< 0.05$), organ, tissue type, immune response or inflammation, leaving 238 genes across all the 4 sample groups. The expression of each gene has been compared between various treatments and time points. These results were put onto known mammalian pathways to elucidate the responses in resistant / susceptible deer.

In 2008 and 2009 studies all the animals became infected because MAP was isolated from all the animals at Week 4 and Week 12, which suggests that the innate immune response was not effective at preventing infection. However, it is also clear that in both studies that there were differences in the amount of disease and the number of MAP present in the JJLN at 12 weeks, which shows that R and S animals were starting to head down different pathways in their acquired immune response. There was significantly lower antibody and higher IFNγ in the R than the S animals in this 4-12 week period, suggesting a preponderance of cell-mediated over humoral response in the R animals compared with the S. This should provide clues as to what gene expression pathways are important in these immune responses. However, it appears that the "improvement" in the disease state of the R animals between Week 12 and Week 50 was a long slow process of the host immune mechanisms gaining the upper hand in the fight against MAP, which has the ability to evade destruction in their intracellular niche. Over that time the S animals' disease states either deteriorated or remained the same, showing their ability to eliminate or reduce MAP infestation.

Observations, based on histopathology, culture and immunological readouts, suggest that upregulated genes at Week 4 are likely to be important in identifying key gene differences between R and S animals, which drive the immune response pathways, whereas at Week 12 it appears that there is a much higher level of gene expression, especially in the S animals, which is associated with a higher levels of inflammation and disease in these animals. Excessive inflammation may be part of the pathogenesis of paratuberculosis. There is also a large number of apoptosis and autophagy genes upregulated, especially in R animals at Week 4, which may hold the key to how R animals use cell death to assist in killing intracellular MAP, compared with uncontrolled cell

death and necrosis assisting MAP to evade killing and allow reinfection of other macrophages. There were also 18 genes identified as associated with defence responses to invading pathogens that were highly upregulated (5-33 fold) in R at Week 4 in 2008 and 2009. These may be very significant for elucidating effective killing mechanisms and pathways in R animals. By contrast there were only 2 genes in this class that were moderately upregulated

The analyses are not complete yet, but the results presented here demonstrate the wide range of differences between R and S groups at the 3 time points.

Introduction

Internationally, Johne's disease is recognised as one of the most difficult livestock diseases to control. There is not treatment for infected individuals, there are currently no vaccines that will prevent infection in a contaminated environment, and none of the tests are sensitive enough to eradicate infected animals in a test-and-slaughter programme.
It is crucial that we gain a better understanding of the immunological mechanisms of protection in order to make significant progress in any of these areas of prevention and control against Johne's disease and other similar diseases. Knowledge of these complex mechanisms and targeting key pathways will enhance our ability to improve the efficiency of selection, develop better diagnostic tests, develop better vaccines and discover therapeutics that increase an animal's ability to cope with infection.
Johne's disease affects all ruminants, and in sheep and cattle a small proportion of infected animals develop slowly progressive disease that becomes clinical in 3-4 years. Red deer develop very similar granulomatous enteritis lesions to sheep and cattle, but the disease progresses more quickly and clinical signs often occur in 8-15 month old animals. We have developed an experimental challenge model that reproduces a typical range of infection/disease outcomes within a one year time frame.
Another key advantage with the current deer model is that Professor Frank Griffin and the Disease Research Laboratory have developed a very useful antibody test (Paralisa), which is considerably more sensitive at detecting disease deer than any other antibody test developed for cattle or sheep. The extensive use of this test on farms in NZ has led to the development of an extensive database of results and a number of farms have been using this tests for over 10 years and built of considerable longitudinal datasets. Repeated whole herd testing on one such deer stud (Peel Forest) has led to the identification of breed-lines of deer that are either resistant/resilient or susceptible to Johne's disease. We have access to semen from these animals to breed suitable animals for study. This is quite a unique asset, which is not readily available for other ruminants. However, we believe that the results that we obtain using the deer model will be applicable to cattle and sheep on the basis that the immune pathways related to resistance will be very similar across ruminants.

The objective of this four year study (of which only 3 years has been funded by JDRC) is to identify genes with expression patterns associated with resistance (R) and susceptibility (S) to Johne's disease. It is believed that the immune events quite early in the disease determine the pathway that relatively susceptible or resistant animals take in terms of progressing to disease or recovery. We have also previously observed that infection quickly spreads from the initial sites of invasion and establishment in the intestine, to the draining jejunal and ileocaecal lymph nodes, where the immune response to infection is centred. We therefore chose to surgically biopsy the jejunal lymph nodes at two time points early in the infection viz 4 and 12/13 weeks post challenge (pc), and then at euthanasia in clinically affected animals or at the end of the study in subclinically affected animals viz 49/50 weeks pc.

This study, which was originally funded directly by FRST, started in March 2007 with the breeding of hinds by AI using two unrelated stags. Their offspring were born in November 2007 and they were challenged with MAP in April 2008. On 30 September, 2008 the JDRC took over funding this project.
The field aspects of the 2008 field study were completed in March 2009 with the slaughter of the animals. The diagnostic laboratory aspects of the study were completed in the following 4-5 months.

The 2009 field study started in April 2009 with the challenge of the offspring of two stags R or S to Johne's disease. The field aspects of the 2009 field study were completed in March 2010 with the slaughter of the animals. The diagnostic laboratory aspects of the study were completed in the following 4-5 months.
In the 2010-11 year I selected frozen samples (-80C) from the 3 most resistant and the 3 most susceptible offspring at the 3 time points (4, 12 and 50 weeks pc) from the 2008 and the Week 4 samples from the 3 most resistant and the 3 most susceptible offspring from the 2009 study were processed and submitted for new generation gene expression sequencing at the University of Otago using the Life Technologies SOLiD platform. Three SOLiD plates were divided into 8 segments, which is why we used 24 samples to fill all the plates efficiently (this explains why we added the Week 4 samples to make up the number). The budget was insufficient to include all samples from both studies and also it was considered more sensible to take a staged approach to sequencing and data analysis, so that lessons learnt from the first set could be applied to the second set. Unfortunately JDRC withdrew funding for the sequencing of the 2009 set.

Methodology

MAP experimental challenge

A challenge study was carried out in 2008 and repeated in 2009 with two different stags and the methods and results have been published (Mackintosh et al 2011; Mackintosh et al in press). The studies were approved by the AgResearch Invermay Animal Ethics Committee (AEC 11425 and AEC 11734).
Briefly, in 2008 18 offspring were bred from two unrelated red stags by artificial insemination of unselected red hinds, which were from a herd tested free of TB for 30 years and had a history of freedom from clinical paratuberculosis.. In 2009, semen from two stags designated R or S to paratuberculosis, based on intensive recording of offspring mortality from natural MAP challenge, were used across unselected red hinds to produce nine offspring of each. The deer were bred in a herd tested free of TB for 30 years and had a history of freedom from clinical paratuberculosis. All the hinds and offspring were test negative for paratuberculosis.
In both years the offspring received heavy oral challenge with a bovine strain MAP (4 daily doses of ~10^9 cfu) extracted from jejunal lymph nodes (JJLN) of clinically affected deer. The deer were closely monitored for 49-50 weeks post challenge (pc). Any clinically affected animals were promptly euthanised and necropsied. Surviving animals were killed at 49-50 weeks pc and necropsied. Regular blood samples were taken during the study and tested for interferon –gamma (IFN-γ) and antibody using an IgG1 class specific antibody test (Paralisa™).

In both studies, samples of posterior JJLN were surgically biopsied at Weeks 4 and 12/13 pc and JJLN samples were also collected at euthanasia of clinically affected or at trial end 49/50 weeks pc. These samples were split into three: one sample was cultured for MAP using BACTEC liquid culture; one sample was fixed in 10% formalin for histopathology and one sample was snap-frozen in liquid nitrogen and then stored at -80C.
The disease status for animals in both studies was scaled on clinical outcome, histopathology, culture and serology.

Gene expression profiling

The frozen JJLNs from the 3 least affected and 3 most affected at 3 time points (Weeks 4, 12, 50) in the 2008 study, and from Week 4 in the 2009 study, were processed. The mRNA was extracted and converted into copy DNA (cDNA) by reverse transcriptase. The SAGE (serial analysis of gene expression) method was chosen to identify and quantify which mRNA molecules were transcribed in the transcriptome from the six deer (Velculescu et al 1995). A short sequence at the 3' end of each piece of cDNA was selected and sequenced using "next generation" Life Technologies SOLiD SAGE sequencing. Three plates were each physically subdivided, allowing 18 samples to be sequenced without the need for barcodes.

Bioinformatics

The sequence data first underwent quality control to remove ambiguous and low quality sequences, followed by detailed analyses. The 26-28 base pair (bp) SAGE tags, obtained from the raw sequencing reads, were annotated in order to determine the tag counts per gene. These tag counts were then averaged across the three R animals

and three S animals in order to obtain relative expression levels of genes between R and S animals, given as fold change and a level of significance, with a positive fold change value given if the average R value was greater than S and negative value if S > R.
The fold change values were loaded into the Ingenuity Pathway Analysis (IPA) programme for identifying candidate pathways and genes involved in resistance to Johne's disease.

Results

MAP experimental challenge

In the 2008 study there were no clinical cases of Johne's disease, but the scoring of histopathology and culture of biopsy and necropsy samples showed a range of disease severity from very mild to very severe (Table 1). Although there was no correlation with stag genotype, there was clear separation of the 3 least affected and the 3 most severely affected and they were selected for sequencing.

Table 1. Summary of BACTEC culture results in terms of the number of days to positive (dtp) for jejunal lymph node samples (JJLN) taken at Weeks 4 and 12, and for a pool of three samples of JJLN plus ileocaecal lymph node (ICLN), a pool of three jejunum and ileo-caecal valve samples (JJ+ICV) and faecal samples (FS) at Week 50, compared with lesion severity scores (LSS) at each time point, with the animals sorted by LSS at Week 50.

		Week 4		Week 12		Week 50				
Tag	**Sire**	**LSS**	**JJLN**	**LSS**	**JJLN**	**LSS**	**JJLN+ICLN**	**JJ+ICV**	**FS**	**Affected**
506	TBR	2	36	6	21	3	34	34	Neg	Least
511	TBR	0	36	4	21	4	34	34	Neg	Least
509	TBS	0	36	6	15	5	26	26	Neg	Least
Mean			**36**	5	**19**	**4**	**31**	**31**		
504	TBS	0	32	11	15	5	26	21	Neg	
513	TBR	0	32	11	15	5	18	18	31	
514	TBS	0	28	11	15	5	34	26	Neg	
501	TBS	0	42	11	12	6	18	34	37	
502	TBS	0	36	11	12	6	18	26	Neg	
500	TBS	0	35	6	12	8	21	18	Neg	
517	TBS	0	36	6	12	8	18	26	Neg	
512	TBS	0	36	6	12	9	18	34	31	
Mean			**35**	**9**	**13**	**7**	**21**	**25**	**33**	
510	TBS	2	36	11	12	9	18	26	Neg	Most
508	TBR	0	32	11	15	9	18	26	Neg	Most
507	TBR	0	36	11	12	13	18	18	37	Most
Mean			**35**	11	**13**	**10**	**18**	**23**	**37**	

Key:
Lesion severity scores; LSS of 1 and 2 were regarded as very mild non-specific, 3 as suggestive of very mild paratuberculosis, but no acid fast organisms (AFO) seen, 4-7 as mild, 8-10 as moderate and 11-13 as severe paratuberculosis. Lesions were also described as no AFO, paucibacillary (PB) or multibacillary (MB).

The three worst affected (507, 508, 510) had severe lesions and moderately high numbers of MAP present in JJLN at Week 12 and moderate to severe lesions and still moderate numbers of MAP at Week 50. The three least affected (506, 509, 511) had mild JJLN lesions and moderate numbers of MAP at Week 12, but had improved to very mild to mild lesions and relatively low numbers of MAP at Week 50. Over the course of the 50 week study the three worst affected deer (507, 508, 510) also had the highest antibody levels to a MAP protoplasmic antigen (PPA), while two (506, 509) of the three least affected were seronegative (< 50 EU) for most of the study and 511's titre oscillated from negative (<50 EU) to positive on three occasions (Fig. 1). The IFN-γ of the least affected animals rose earlier and peaked higher than those of the more severely affected animals (Mackintosh et al in press). JJLN from these six animals were selected for gene expression studies.

In the 2009 study, three offspring (2S and R) developed clinical Johne's disease and were euthanised and necropsied. The remaining deer were euthanised 49 weeks pc and necropsied. There was a significant sire effect, with the offspring of the S sire significantly worse affected than the R offspring. The Paralisa antibody

patterns were very different for the R and S animals. All 18 animals were shown to be infected at Week 4 and at Week 13 they had clear signs of mild to severe lesions in the biopsy samples. Subsequently, the S sire had 2 animals clinically affected and overall 7/9 had severe lesions (Table 2). The R sire had one clinical case and overall only 2/9 had severe lesions. One of the R offspring appeared to completely "cure" itself between Weeks 13 and 49, and the remaining 6 R offspring only had very mild disease. This clearly demonstrates that resistance to paratuberculosis is heritable. A paper on this study has recently been published in Veterinary Immunology and Immunopathology (Mackintosh et al 2011).
The six Week 4 lymph nodes samples from 3 least affected and the 3 most severely affected from the 2009 study were added to the full set of 18 samples (6 animals by 3 time points) from the 2008 study for the SOLiD SAGE sequencing to fill all the available channels on 3 plates (each plates was divided into 8 sections).

Table 2.
Histopathological lesion severity scores (LSS) for biopsy samples at Weeks 4 and 13 post challenge (pc) and gross lesion score, LSS, lesion description as multibacillary (MB) or paucibacillary (PB) and Johne's disease (JD) outcome for the offspring of the resistant sire (R), the susceptible sire (S) at euthanasia or slaughter 49 weeks pc.

			Week 4	Week 13			Week 49/euthanasia/death		
Sire	Tag	Sex	LSS	Gross	LSS	AFO	Gross	LSS	AFO
R	92	F	0	0	6	PB	0	0	None
R	91	M	0	0	6	PB	0	2	None
R	88	M	0	1	6	PB	0	5	PB
R	84	M	0	4	11	PB	0	5	PB
R	79	M	0	2	13	PB	0	5	PB
R	94	F	0	0	13	PB	0	5	PB
R	98	M	0	0	11	PB	0	5	PB
R	86	F	0	0	11	PB	0	13	MB
R	82	F	0	1	11	PB	3	13	MB
	Ave		0	0.9	9.8		0.3	5.9	
S	89	M	0	0	11	PB	0	3	None
S	77	M	5	0	13	PB	5	13	MB
S	80	M	2	2	13	MB	0	11	PB
S	81	M	0	1	13	MB	3	13	PB
S	90	M	0	0	6	PB	0	13	MB
S	96	M	0	2	13	PB	5	13	MB
S	87*	F	0	1	13	MB	0	13	MB
S	93	M	0	0	11	MB	5	13	MB
S	85	F	0	0	13	MB	5	13	MB
	Ave		0.8	0.7	11.8		2.9	11.7	

* Animal 87S died 24 hours after Week 13 biopsy surgery, but full necropsy confirmed LSS 13 MB, very severe JD
Key:
Gross Lesion Scores; 0= no visible lesions (NVL), 1= slightly enlarged jejunal lymph nodes (JJLN) and ileocaecal lymph nodes (ICLN), 2= moderately enlarged JJLN and ICLN, 3= very enlarged JJLN and ICLN, 4= enlargement plus a single caseo-granulomatous JJLN or ICLN lesion, 5= multiple JJLN and ICLN lesions.
Lesion severity scores; LSS of 1 and 2 were regarded as very mild non-specific, 3 as suggestive of very mild paratuberculosis, but no acid fast organisms (AFO) seen, 4-7 as mild, 8-10 as moderate and 11-13 as severe paratuberculosis. Lesions were also described as no AFO, paucibacillary (PB) or multibacillary (MB).

SOLiD SAGE Sequencing and Bioinformatics
The SOLiD technology sequencing of 18 samples (3R and 3S at 3 time points) from 2008/9 samples and 6 samples (3R and 3S at 4 weeks pc) was successfully completed at Otago University under the supervision of Dr Jo Stanton. The initial sequence data was analyzed at AgResearch Ruakura by Dr Paul Maclean, under the supervision of Dr Nauman Maqbool, and the analysis of gene expression has been undertaken by Dr Rudiger Brauning, head bioinformatician at AgResearch Invermay.
Ingenuity® Systems Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA) was used to analyse gene expression data. All differentially expressed genes with an adjusted P value < 0.05 were included.

i. Sequencing
   - A total of 566 million reads (24 samples).
   - 15-30 million reads were generated per sample.

ii. Quality control
   - 6.7 million reads (1.2%) were discarded because of ambiguities.
   - No bias for a specific sequence composition was detected.
   - The remaining 560 million reads represent between 9.9 and 19.4 million different sequences per sample point with copy numbers ranging from 1 to 167,389.
   - Interestingly between 93.9% and 96.2% of all different sequences per sample point are present only in a single copy.

iii. Converting raw reads into tags
   - 373 million tags with lengths between 26 and 28 bases were generated.

iv. Converting tags into expression counts per gene
   - Genes were mapped against three datasets; the deer and elk transcriptome (in-house), cattle transcriptome (Ensembl cattle genes) and the bovine genome (Btau 4). Of the 373 million tags, 31,500 genes were uniquely mapped with 18,000 genes also been annotated.

v. Analysis of gene networks
   - Of the above 18,000 genes, 17,500 were recognized by the Ingenuity Pathway Analysis (IPA) programme.
   - The expression level for the 3 samples at each time point were averaged and an expression value was calculated for each gene and this was expressed as a Log ratio, Fold change and p-value (significance of Fold change).
   - Using IPA genes were filtered by p-value ($p < 0.05$), organ, tissue type, immune response or inflammation, leaving 238 genes across all the 4 sample groups
   - The expression of each gene has been compared between various treatments and time points. These results were put onto known mammalian pathways to elucidate the responses in resistant / susceptible deer.

Analyses to date:

Note that in the RS comparison the positive value is up-regulation of the gene in R animals, while a negative value is for up-regulation of the gene in S animals.

Overall Relative Upregulation between R and S

Table 3 summarises the numbers of genes that were filtered out using IPA for each group in a comparison between R and S animals at each time point, including only datapoints that were significantly different between R and S ($p<0.05$). The first 3 groups are 2008 animals at Weeks 4, 12 and 50, and the fourth group comprises the 2009 animals at Week 4. It is interesting to see that in almost all cases the S group had more genes relatively up-regulated in each of the ranges, and in the top range the S group almost always had the higher degree of up-regulation (except 2008 Week 50).

Table 3. Summary of genes, stratified by fold range upregulation, that were filtered out using IPA for each group in a comparison between R and S animals at each time point

Group	S n	S Upregulation Range	R n	R Upregulation Range
2008 Week 4	17	8-33.5	10	8-22.8
	58	4-7.9	20	4-7.9
	60	3-3.9	5	3-3.9
	34	2.5-2.9	5	2.5-2.9
2008 Week 12	110	8-142	26	8-16.9
	142	4-7.9	75	4-7.9
	86	3-3.9	54	3-3.9
	81	2.5-2.9	41	2.5-2.9
2008 Week 50	18	8-25.9	14	8-32.9
	55	4-7.9	22	4-7.9
	52	3-3.9	27	3-3.9
	63	2.5-2.9	21	2.5-2.9
2009 Week 4	89	8-178.3	43	8-34.3
	67	4-7.9	73	4-7.9
	3	3-3.9	61	3-3.9
	0	2.5-2.9	53	2.5-2.9

Candidate genes

There have been a number of recent studies of genes associated with MAP tissue infection, fecal shedding or disease in cattle. Using the Illumina Bovine SNP50 BeadChip, Neibergs et al (Neibergs et al 2010) identified four genes, EDN2, TDGF1, TGFB2, and PIK3R1, which produce proteins with known functions that may be of relevance to MAP tissue infection or Johne's disease. These or very similar genes recorded significant fold change in this study (Table 4).

- EDN1 (but not EDN2) was upregulated 2.75 fold in R animals at 2009 Week 4. EDN2 are genes for member of the endothelin protein family of secretory vasoconstrictive peptides that act at endothelin receptors and initiate intracellular signaling events. Endothelin receptors are widely expressed in all tissues, which is consistent with their physiological role as vasoactive peptides (GeneCards).
- TDGF1 was not significantly upregulated.
- TGFB1/1 (but not TGFB2) was upregulated 3.23 fold in S animals 2008 Week 12. These are genes for members of the transforming growth factor-beta family involved in the regulation of cellular processes, including cell division, differentiation, motility, adhesion and death.
- PIK3R3 (but not PIK3R1) was upregulated 4.81 fold in S animals at 2008 Week 4. These genes code for members of a family of lipid kinases responsible for coordinating a diverse range of cell functions including proliferation, cell survival, degranulation, vesicular trafficking and cell migration (GeneCards).

Pant et al (Pant et al 2010) identified a number of genes associated with resistance to paratuberculosis in cattle, including SLC39A3 and TNFAIP8L1 and the same or similar genes were upregulated in the 2008 and 2009 studies (Table 4).

- SLC39A3 (solute carrier family 39, member 3) was upregulated 12.6 fold in R animals at 2009 Week 4. This gene encodes a member of the ZIP family of metal ion transporters and it functions as a zinc transporter, mediating zinc uptake (GeneCards).

- TNFAIP6 (but not TNFAIP8L1) was upregulated 8.78 fold in S animals at 2008 Week 12. This gene codes for a secretory protein that contains a hyaluronan-binding domain and is involved in extracellular matrix stability and cell migration. It is induced by proinflammatory cytokines such as tumor necrosis factor alpha and interleukin-1 and appears to be involved in cell-cell and cell-matrix interactions during inflammation (GeneCards). Function of TNFAIP8L1 is unknown.

Table 4. Candidate genes identified in other studies associated with MAP infection or disease showing the fold change and p-value in 2008 and 2009 animals.

	Symbol	Fold Change	p-value	Entrez Gene Name
Neibergs et al				
Week 4	PIK3R3	-4.81	0.00972	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
Week 12	TGFB1-I1	-3.23	0.0127	transforming growth factor beta 1 induced transcript 1
2nd Wk 4	EDN1	2.75	0.0445	endothelin 1
	TDGF1	NS	NS	
Pant et al				
Week 12	TNFAIP6	-8.78	0.0000091	tumor necrosis factor, alpha-induced protein 6
2nd Wk 4	SLC39A2	12.61	0.0205	solute carrier family 39 (zinc transporter), member 2

Genes associated with Tb in IPA

A number of genes in the 2008 and 2009 studies were highlighted by IPA as associated with tuberculosis (Tb) (Table 5). These include SLC11A1 (NRAMP) upregulated 12.19 fold and IL8 upregulated 11.18 fold in S animals at 2008 Week 12. However, SLC11A1 was upregulated 2.19 fold and IL8 was upregulated 2.68 fold in R animals at 2008 Week 50. CD209 was upregulated 12 fold in R animals at 2008 Week 12.

SLC11A1 (solute carrier family 11, A1) gene: is primarily expressed in phagosomes and functions as a divalent transition metal (iron and manganese) transporter involved in iron metabolism and host resistance to certain pathogens. A recent review (Purdie et al 2011) states: "The protein exhibits pleiotropic effects on the early innate macrophage response to intracellular bacterial growth (Alter-Koltunoff et al 2008) including regulation of inducible nitric oxide synthase (iNOS) expression in mice (Arias et al 1997).

Several groups have looked at host cellular interactions with mycobacteria and SLC11A1. Strong genetic influences to susceptibility have been established in a mouse model of paratuberculosis (Roupie et al 2012) supported by a relationship between SLC11A1 and inducible nitric oxide synthase (iNOS), expressed at the site of infection in the gut mucosa and lymph nodes, and the pathogenesis of paratuberculosis in cattle (Delgado et al 2010). In mice infected with *Salmonella typhimurium* the formation of IL-10 is suppressed via increased iNOS expression mediated by SLC11A1 (Fritsche et al 2008); this could also apply to the host response to MAP given the role of IL-10 in the pathogenesis of paratuberculosis (de Silva et al 2010).

Reddacliff et al. (Reddacliff et al 2005) identified SLC11A1 polymorphisms in two phenotypically defined Merino flocks with a high prevalence of Johne's disease. The animals were classified as severe, mild or non-diseased based on a combination of clinical signs, serological (agar-gel immunodiffusion test) and histopathological analysis, BACTEC faecal and tissue culture and intradermal testing for delayed type hypersensitivity. The microsatellite markers were chosen based on previously published research in which associations had been reported or polymorphisms within a gene family were implicated in susceptibility to mycobacterial diseases such as tuberculosis, leprosy and M. avium infections in humans. Samples were sourced from two separate flocks and the genotyping results were separately tested for statistical relevance. Associations were thought likely if they were found to be independently significant in both flocks. In a similar study on goats, paratuberculosis susceptibility was significantly associated with a microsatellite in the 3' untranslated region (3'-UTR) of SLC11A1, which may be related to the levels of the gene expressed in response to MAP exposure (Korou et al 2010).

SLC11A1 polymorphisms in cattle were first described by Pinedo et al. (Pinedo et al 2009). Microsatellite markers were used to address polymorphisms of three candidate genes (SLC11A1, interferon-gamma IFN-γ and TLR4) as risk factors for MAP infection in Holstein, Jersey and Brahman-Angus cross (total 431 cattle),

obtained from five dairy herds in the USA. MAP prevalence at the time of sampling was unknown although all herds had a history of clinical MAP infections. An animal was classified as MAP positive if it tested positive in any single diagnostic procedure whereas a MAP negative classification required that an animal test negative for all five diagnostic tests. Microsatellite markers tested in this study were chosen based on previous studies, including that of Reddacliff et al (Reddacliff et al 2005). There were significant differences in the allelic frequencies of two SLC11A1 alleles between MAP positive and negative cattle, even when breed and age were included in the logistic regression model.

In an attempt to analyse the entire functional variability of the SLC11A1 gene in cattle, including all exons and flanking intronic, untranslated and promoter regions, Ruiz-Larranaga et al. (Ruiz-Larranaga et al 2010) carried out a SNP-based candidate gene study. Initially 57 SLC11A1 SNP were identified among 14 bovine breeds; 24 were novel, previously unidentified SNP. The SNP were validated in Holstein-Friesians and 24 were selected for association studies. Two independent subpopulations of Holstein-Friesian cattle were studied; 33 Spanish herds tested once and 8 Dutch farms that had undergone repeat testing. MAP infection status was classified as in the study of Pinedo et al. (Pinedo et al 2009), with positive status assigned if an animal was positive in any individual test. Since paratuberculosis has been present in European herds since 1895, the study assumed that exposure to MAP infection is prevalent in all herds. Although differential significance of SLC11A1 polymorphisms was found between the two cohorts, a joint association analysis combining the populations also identified two minor allele frequency SNP that were significantly associated with susceptibility to paratuberculosis. One of the identified SNP, which leads to an amino acid substitution in the highly conserved transmembrane region 8 of SLC11A1, could be associated with altered stability or secondary structure of the molecule and thus functional impairment.

IL8 gene: The protein encoded by this gene is a member of the CXC chemokine family and is one of the major mediators of the inflammatory response. It functions as a chemoattractant, and its role in the cell includes chemotaxis, activation, migration, recruitment, adhesion, chemoattraction, attraction, transendothelial migration, binding, stimulation and it is also a potent angiogenic factor. This gene and other ten members of the CXC chemokine gene family form a chemokine gene cluster. It regulates: CXCR2 (Table 5), CXCR1, IL8 Receptor, ITGB2, MMP9, Erk1/2, ITGAM, Ca2+, Mapk, MAPK3, Erk, Akt, calcium, ITGAL. It is regulated by: TNF, lipopolysaccharide, IL1B, NFkB, IFNG, SB203580, poly rI:rC-RNA, IL1A, TLR4, phorbol myristate acetate, PD98059, NFKBIA, P38 MAPK, Flagellin, U0126. It binds: CXCR1, CXCR2, RELA, Ap1, AP-1, NFkB, DARC, NFKB1, RNA polymerase II, CEBPB, Histone h3, FOS, JUN, HDAC1n (GeneView). A quantitative immunoassay revealed that IL-8 protein release was significantly elevated in supernatants of macrophages and in lavage fluid obtained from patients with pulmonary tuberculosis compared to normal controls (Zhang et al 1995).

CD209 gene: This gene encodes a transmembrane receptor and is often referred to as DC-SIGN because of its expression on the surface of dendritic cells and macrophages. The encoded protein is involved in the innate immune system and recognizes numerous evolutionarily divergent pathogens ranging from parasites to viruses with a large impact on public health. Molecular functions include receptor activity, sugar binding, mannose binding, peptide antigen binding, virion binding and metal ion binding. Biological processes include endocytosis, cell adhesion, heterophilic cell-cell adhesion, leukocyte cell-cell adhesion, cell-cell recognition, virus-host interaction, virion attachment to host cell surface receptor, viral genome replication, antigen processing and presentation, intracellular signal transduction, interspecies interaction between organisms, innate immune response, intracellular virion transport and peptide antigen transport. Associated with Tb and M.bovis infection (Entrez Gene).

Table 5: IPA Tb Associated genes

	Symbol	**Fold change**	p-value	Entrez Gene Name	Function
Week 4	IFNG	-2.676	0.0461	interferon, gamma	
Week 12	SLC11A1	-12.193	0.000000614	solute carrier family 11, A1	(NRAMP) metal transporter, iron metabolism, host resistance
	IL8	-11.184	0.00000106	interleukin 8	Proinflammatory cytokine, CXC chemokine family
	CCL2	-6.728	0.0000723	chemokine (CC) ligand 2	
	IL6	-5.584	0.048	interleukin 6	
	CXCR2	-4.584	0.0124	chemokine (CXC)receptor 2	
	TLR4	-4.074	0.00273	toll-like receptor 4	
	CD14	-3.295	0.00947	CD14 molecule	
	IFNG	-2.909	0.0203	interferon, gamma	
	CD209	12.002	0.0000925	CD209 molecule	(DC-SIGN) innate immune system and recognizes pathogens
Week 50	GABRA4	-2.875	0.0475	gamma-aminobutyric acid (GABA) A receptor, alpha 4	
	CCR5	-2.798	0.00858	chemokine (CC) receptor 5	
	MC2R	-1.907	0.0497	melanocortin 2 receptor	
	IL16	-1.818	0.0277	interleukin 16	
	SLC11A1	2.193	0.00389	solute carrier family 11, A1	(NRAMP) metal transporter, iron metabolism, host resistance
	IL8	2.681	0.00871	interleukin 8	Proinflammatory cytokine, CXC chemokine family
	SPP1	2.872	0.000138	secreted phosphoprotein 1	
2009 Wk 4	SPP1	-4.056	0.0417	secreted phosphoprotein 1	

Proinflammatory Genes:

There were a number of upregulated genes for proinflammatory cytokines and immune mediators, including C-C and C-X-C motif chemokines and receptors, interferon family proteins, tumour necrosis superfamily members and interleukins IL1A and IL8 (Table 6A and 6B). Notably;

CCRL1 gene (upregulated 13.4 fold and 3.23 fold in S animals in 2008 and 2009 Week 4): a C-C motif receptor-like 1 gene that codes for a receptor for dendritic cell- and T cell-activated C-C type chemokines such as CCL2, CCL8, CCL13, CCL19, CCL21 and CCL25.

IFIT2 gene (upregulated 16.5 fold R animals in 2008 Week 4): IFIT2 belongs to the IFIT gene family whose members function to restrict virus infection (eg West Nile virus) through alteration of cellular protein synthesis. IFIT2 is expressed after virus infection directly upon IRF-3 activation as well as upon IFN signaling because of the presence of both IRF-3 and ISGF3 binding sites in the *Ifit2* promoter (Perwitasari et al 2011).

Table 6A:

	Symbol	Fold Change	p-value	Entrez Gene Name
2009 Week 4	CCRL1	-13.415	0.0283	chemokine (C-C motif) receptor-like 1
2009 Week 4	TNIK	2.881	0.0291	TRAF2 and NCK interacting kinase
2009 Week 4	CCL14	2.945	0.0286	chemokine (C-C motif) ligand 14
2009 Week 4	TNFSF15	4.113	0.0345	tumor necrosis factor (ligand) superfamily, member 15
2008 Week 4	CCL5	-4.691	0.00116	chemokine (C-C motif) ligand 5
2008 Week 4	TNFSF12	-3.527	0.0327	tumor necrosis factor (ligand) superfamily, member 12
2008 Week 4	TNFRSF21	-3.412	0.0267	tumor necrosis factor receptor superfamily, member 21
2008 Week 4	CCRL1	-3.233	0.0355	chemokine (C-C motif) receptor-like 1
2008 Week 4	CXCR6	-2.839	0.0249	chemokine (C-X-C motif) receptor 6
2008 Week 4	IL17RC	-2.826	0.0352	interleukin 17 receptor C
2008 Week 4	IFNG	-2.676	0.0461	interferon, gamma
2008 Week 4	Ifi47	2.483	0.0496	interferon gamma inducible protein 47
2008 Week 4	IFI6	2.753	0.0276	interferon, alpha-inducible protein 6
2008 Week 4	IFI44	2.872	0.0249	interferon-induced protein 44
2008 Week 4	IL1A	4.526	0.00589	interleukin 1, alpha
2008 Week 4	IFIT3	5.41	0.000464	interferon-induced protein with tetratricopeptide repeats 3
2008 Week 4	IFIT2	16.535	0.0000142	interferon-induced protein with tetratricopeptide repeats 2

Table 6B: 2008 Week 12 and 50 proinflammatory cytokines and immune mediators, including C-C and C-X-C motif chemokines and receptors, interferon family proteins, tumour necrosis superfamily members and interleukins IL1A, IL8 and IL17 and receptors.

	Symbol	Fold Change	p-value	Entrez Gene Name
2008 Week 12	CXCL14	-31.951	1.56E-10	chemokine (C-X-C motif) ligand 14
2008 Week 12	CXCL2	-30.567	1.39E-09	chemokine (C-X-C motif) ligand 2
2008 Week 12	CXCL5	-22.261	4.55E-09	chemokine (C-X-C motif) ligand 5
2008 Week 12	IL17F	-15.497	0.00085	interleukin 17F
2008 Week 12	CCL3/MIP-1α	-11.246	2.46E-06	CCL3, aka Macrophage inflammatory protein-1α
2008 Week 12	IL8	-11.184	1.06E-06	interleukin 8
2008 Week 12	CCR10	-8.927	0.0162	chemokine (C-C motif) receptor 10
2008 Week 12	TNFAIP6	-8.778	0.0000091	tumor necrosis factor, alpha-induced protein 6
2008 Week 12	CCL2	-6.728	0.0000723	chemokine (C-C motif) ligand 2
2008 Week 12	TNFRSF12A	-5.616	0.00155	tumor necrosis factor receptor superfamily, member 12A
2008 Week 12	IL17RC	-5.471	0.000383	interleukin 17 receptor C
2008 Week 12	TNFSF10	-4.723	0.00133	tumor necrosis factor (ligand) superfamily, member 10, TRAIL
2008 Week 12	CXCR2	-4.584	0.0124	chemokine (C-X-C motif) receptor 2
2008 Week 12	CXCL2	-3.812	0.00485	chemokine (C-X-C motif) ligand 2
2008 Week 12	IL1A	-3.592	0.00598	interleukin 1, alpha
2008 Week 12	CCL5	-3.057	0.0148	chemokine (C-C motif) ligand 5
2008 Week 12	IFNG	-2.909	0.0203	interferon, gamma
2008 Week 12	TNFRSF17	-2.619	0.0377	tumor necrosis factor receptor superfamily, member 17

2008 Week 12	Ifi47	-2.449	0.0498	interferon gamma inducible protein 47
2008 Week 12	CXCR5	2.803	0.0415	chemokine (C-X-C motif) receptor 5
2008 Week 12	CCL21	3.339	0.00884	chemokine (C-C motif) ligand 21
2008 Week 12	CCL26	5.325	0.000849	chemokine (C-C motif) ligand 26
2008 Week 12	CCL24	5.654	0.0114	chemokine (C-C motif) ligand 24

2008 Week 50	TNFRSF12A	-3.604	0.0223	tumor necrosis factor receptor superfamily, member 12A
2008 Week 50	CCRL1	-2.987	0.00705	chemokine (C-C motif) receptor-like 1
2008 Week 50	CCR5	-2.798	0.00858	chemokine (C-C motif) receptor 5
2008 Week 50	IFNA5	-2.492	0.0217	interferon, alpha 5
2008 Week 50	Ifi47	-2.344	0.00197	interferon gamma inducible protein 47
2008 Week 50	CXCR7	-2.288	0.0237	chemokine (C-X-C motif) receptor 7
2008 Week 50	CXCR6	-2.131	0.00831	chemokine (C-X-C motif) receptor 6
2008 Week 50	IL17RC	-1.749	0.0451	interleukin 17 receptor C
2008 Week 50	CCL20	2.184	0.00672	chemokine (C-C motif) ligand 20
2008 Week 50	CCL21	2.515	0.000604	chemokine (C-C motif) ligand 21
2008 Week 50	IL8	2.681	0.00871	interleukin 8
2008 Week 50	CXCL2	3.04	0.022	chemokine (C-X-C motif) ligand 2
2008 Week 50	CXCL14	3.497	0.000539	chemokine (C-X-C motif) ligand 14
2008 Week 50	CXCL5	8.442	0.000031	chemokine (C-X-C motif) ligand 5

Most notable are the proinflammatory genes upregulated 11.18 to 32.95 x in S animals at Week 12:
CXCL14 gene (upregulated 32 fold): this gene codes for a cytokine involved in the homeostasis of monocyte-derived macrophages rather than in inflammation. It is chemotactic for monocytes and can activate these cells in the presence of an inflammatory mediator called prostaglandin-E2 (PGE2). It is also a potent chemoattractant and activator of dendritic cells, is implicated in homing of these cells, and can stimulate the migration of activated NK cells. It inhibits angiogenesis, possibly as a result of its ability to block endothelial cell chemotaxis.
CXCL2 gene (upregulated 31 fold): Chemokine (C-X-C motif) ligand 2 (CXCL2) is a small cytokine belonging to the CXC chemokine family that is also called macrophage inflammatory protein 2-alpha (MIP2-alpha), Growth-regulated protein beta (Gro-beta) and Gro oncogene-2 (Gro-2). CXCL2 is 90% identical in amino acid sequence as a related chemokine, CXCL1. This chemokine is secreted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells.[1][2][3] The gene for CXCL2 is located on human chromosome 4 in a cluster of other CXC chemokines.[4] CXCL2 mobilize
CXCL5 gene (upregulated 22 fold): The protein encoded by this gene is an inflammatory chemokine involved in neutrophil activation
and is produced concomitantly with interleukin-8 (IL8) in response to stimulation with either IL1 or TNFA. It is produced following stimulation of cells with the inflammatory cytokines interleukin-1 or tumor necrosis factor-alpha.
IL17F gene (upregulated 15.5 fold): codes for a proinflammatory cytokine similar to IL17 that responds to the invasion of the immune system by extracellular pathogens and induces destruction of the pathogen's cellular matrix. Interleukin 17 acts synergistically with TNF and IL1. It is expressed by activated T cells induced by IL23, which can result in destructive tissue damage in delayed-type reactions. It has been shown to stimulate the production of several other cytokines, including IL6, IL8, and CSF2/GM-CSF. This cytokine is also found to inhibit the angiogenesis of endothelial cells and induce endothelial cells to produce IL2, TGFB1/TGFB, and monocyte chemoattractant protein-1 (RefSeq).
CCL3/MIP-1 α gene (upregulated 11.2 fold): Macrophage inflammatory protein-1 (MIP-1), MIP-1α (aka CCL3) and MIP-1β (aka CCL4) are chemokines crucial for immune responses towards infection and

inflammation. In humans, there are two major forms, MIP-1α and MIP-1β, produced by macrophages after they are stimulated with bacterial endotoxins. They activate garnulocytes, which can lead to acute neutrophilic inflammation. They also induce the synthesis and release of other pro-inflammatory cytokines such as IL1, IL6 and TNFα from fibroblasts, macrophages, dendritic cells and lymphocytes.
IL8 gene (upregulated 11.2 fold): (see above).

Table 6C: IPA selected inflammation-related genes in the 2009 Week 4 animals sorted by Fold Change

Symbol	Fold change	p-value	Entrez Gene Name
TOLLIP	-45.07	0.000316	toll interacting protein
VPREB1	-37.17	0.000704	pre-B lymphocyte 1
GRN	-26.447	7.52E-05	granulin
CYP2B6	-12.383	0.00153	cytochrome P450, family 2, subfamily B, polypeptide 6
KAT2B	-11.089	0.0128	K(lysine) acetyltransferase 2B
THRSP	-8.264	0.00574	thyroid hormone responsive
ABCB10	-6.77	0.0278	ATP-binding cassette, sub-family B (MDR/TAP), member 10
RBCK1	-4.778	0.0383	RanBP-type and C3HC4-type zinc finger containing 1
NDFIP1	-4.285	0.034	Nedd4 family interacting protein 1
CISH	2.817	0.0419	cytokine inducible SH2-containing protein
VSIG4	2.978	0.0345	V-set & immunoglobulin domain containing 4
NFKBID	3.107	0.0326	NFKB nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta
TTF1	3.226	0.0495	transcription termination factor, RNA polymerase I
SLC2A6	3.251	0.036	solute carrier family 2 (facilitated glucose transporter), member 6
MYD88	3.581	0.0107	myeloid differentiation primary response gene (88)
SLC11A2	3.658	0.0341	solute carrier family 11 member 2
TNFSF15	4.113	0.0345	tumor necrosis factor (ligand) superfamily, member 15
GATAD2B	4.798	0.0207	GATA zinc finger domain containing 2B
EDAR	5.153	0.0357	ectodysplasin A receptor
RNF41	8.167	0.0374	ring finger protein 41
ADAR	10.509	0.0374	adenosine deaminase, RNA-specific
GAD2	10.509	0.0374	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)
HLA-DRB3	14.207	9.68E-07	HLA class II histocompatibility antigen, DRB1-7 beta chain-like

Inflammation-related genes in the 2009 Week 4 animals selected by the IPA are shown in Table 6C. The majority of these are strongly upregulated in S animals, and the most notable (upregulated 11.09 to 45.07 x) include:

TOLLIP gene (upregulated 45 fold): Codes for an inhibitory adaptor protein within Toll-like receptors (TLR). The TLR pathway is a part of the innate immune system that recognizes structurally conserved molecular patterns of microbial pathogens, leading to an inflammatory immune response.
VPREB1 gene (upregulated 37 fold*):* Codes for pre-B lymphocyte 1 and has also recently been designated CD179A. It is expressed selectively at the early stages of B cell development, in proB and early preB cells.
GRN gene (upregulated 26.4 fold): Codes for the granulin protein, which regulates cell growth. However, different members of the granulin protein family may act as inhibitors, stimulators, or have dual actions on cell growth. Granulin family members are important in normal development, wound healing, and tumorigenesis.
CYP2B6 gene (upregulated 12.4 fold): This gene encodes a member of the cytochrome P450 superfamily of heme-thiolate monooxygenase enzymes, which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.

KAT2B gene (upregulated 11.1 fold): Codes for CBP and p300 are large nuclear proteins that bind to many sequence-specific factors.

Notable genes upregulated in R animals include:
ADAR gene (upregulated 10.5 fold): This gene is upregulated upon inflammation and encodes the enzyme responsible for RNA editing by site-specific deamination of adenosines. This enzyme destabilizes double stranded RNA through conversion of adenosine to inosine. It functions to modify viral RNA genomes and may be responsible for hypermutation of certain negative-stranded viruses. It binds to ILF3/NF90 and up-regulates ILF3-mediated gene expression (GeneCard).
GAD2 gene (upregulated 10.5 fold): This gene encodes one of several forms of glutamic acid decarboxylase, identified as a major autoantigen in insulin-dependent diabetes. The enzyme encoded is responsible for catalyzing the production of gamma-aminobutyric acid from L-glutamic acid. A pathogenic role for this enzyme has been identified in the human pancreas since it has been identified as an autoantibody and an autoreactive T cell target in insulin-dependent diabetes. This gene may also play a role in the stiff man syndrome.
HLA-DRB3 gene (upregulated 14.2 fold): This gene codes for a MHC Class II molecule that plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC, including B lymphocytes, dendritic cells, macrophages), which present them on the cell surface for recognition by the CD4 T-cells. Exogenous antigens that have been endocytosed by the APC are thus readily available for presentation via MHC II molecules, and for this reason this antigen presentation pathway is usually referred to as exogenous. As membrane proteins on their way to degradation in lysosomes as part of their normal turn-over are also contained in the endosomal/lysosomal compartments, exogenous antigens must compete with those derived from endogenous components. Autophagy is also a source of endogenous peptides, autophagosomes constitutively fuse with MHC class II loading compartments.

Defence Response to Bacteria

There were a number of genes associated with defence response to invading pathogens in R and S animals at Week 4 in 2008 and 2009 (Table 7A, 7B and 7C).

Table 7A: 2008 Week4 genes associated with defence against pathogens upregulated in R animals

2008	Fold Change	p-value	Entrez Gene Name
ISG15	22.766	1.86E-09	ISG15 ubiquitin-like modifier
RSAD2	20.025	1.03E-08	radical S-adenosyl methionine domain containing 2
IFIT2	16.535	0.0000142	interferon-induced protein with tetratricopeptide repeats 2
USP18	11.256	0.00000126	ubiquitin specific peptidase 18
OAS1	7.078	0.0000503	2',5'-oligoadenylate synthetase 1, 40/46kDa
IFIT3	5.41	0.000464	interferon-induced protein with tetratricopeptide repeats 3

Table 7B: 2008 Week4 genes associated with defence against pathogens upregulated in S animals

2008	Fold Change	p-value	Entrez Gene Name
NOS2	-7.882	0.0015	nitric oxide synthase 2, inducible
DEFB4	-11.393	0.0195	Beta-defensin 2 (BD-2)

Table 7C: 2009 Week 4 genes associated with defence against pathogens upregulated in R animals

	Fold Change	p-value	Entrez Gene Name
MAP3K11	33.138	0.00000187	mitogen-activated protein kinase kinase kinase 11
CAMP	18.916	0.00401	Cathelicidin 1; cathelicidin antimicrobial peptide
HLA DRB3-Like	14.207	0.000000968	HLA class II histocompatibility antigen, DRB1-7 beta chain-like
EMR3	12.935	0.00000311	egf-like module containing, mucin-like, hormone receptor-like 3
SLC39A2	12.611	0.0205	solute carrier family 39 (zinc transporter), member 2
ADAR	10.509	0.0374	adenosine deaminase, RNA-specific
GZMA	8.167	0.0374	Granzyme A
PTMA	8.167	0.0374	prothymosin, alpha
CEBPE	7.86	0.0000804	CCAAT/enhancer binding protein (C/EBP), epsilon
ALOX5	7.431	0.0286	arachidonate 5-lipoxygenase
IL9	7.237	0.0176	interleukin 9
AMBRA1	6.597	0.000314	autophagy/beclin-1 regulator 1

Apoptosis and autophagy:

A key defence against intracellular parasites (eg MAP) is orderly death of the invaded cell (eg macrophage) by apoptosis or autophagy. This organised cell death allows potentially dangerous cell components to be contained and processed whilst MAP are killed. On the other hand, if the parasite is in control and can block apoptosis and autophagy this can lead to disorganised cell death and release of dangerous components, resulting in inflammation and necrosis and allowing MAP to escape the cell and invade new macrophages. Some of the key pro-apoptosis and autophagy genes were differentially upregulated in R and S animals at different times over the two studies (Table 8). It appears that overall R animals had slightly more, but quite different upregulated genes of this type than S animals, especially 2009 Week 4 animals, although it is noteworthy that S100 calcium binding proteins A8 and A9 were highly upregulated (33.5 and 38.2 fold) in S animals at 2008 Week 4 and 12. Both S100A8 and S100A9 are important for resistance to invasion by pathogenic bacteria (Genecard). They up-

regulates transcription of genes that are under the control of NF-kappa-B, promote phagocyte migration and infiltration of granulocytes at sites of wounding. However they are pro-inflammatory mediators in acute and chronic inflammation and up-regulate the release of IL8 that has been implicated in susceptibility to Tb and paratuberculosis (see above). Their antimicrobial and proapoptotic activity is inhibited by zinc ions.

Table 8: Pro-apoptosis and autophagy genes upregulated.

	Symbol	Fold Change	p-value	Entrez Gene Name
2008 Week 4	S100A8	-33.502	9.79E-11	S100 calcium binding protein A8
2008 Week 4	COX6A1	-4.385	0.0017	cytochrome c oxidase subunit VIa polypeptide 1
2008 Week 4	COX7A2L	-3.252	0.0112	cytochrome c oxidase subunit VIIa polypeptide 2 like
2008 Week 4	CASP9	-2.575	0.0885	caspase 9, apoptosis-related cysteine peptidase
2008 Week 4	CASP8	2.232	0.068	caspase 8, apoptosis-related cysteine peptidase
2008 Week 4	CRADD	3.924	0.0372	CASP2 and RIPK1 domain containing adaptor with death domain
2008 Week 4	MT-CO2	4.184	0.0282	cytochrome c oxidase subunit II
2008 Week 4	OAS1	7.078	0.0000503	2',5'-oligoadenylate synthetase 1, 40/46kDa
2008 Week 12	S100A9	-38.421	1.1E-11	S100 calcium binding protein A9
2008 Week 12	CAPG	-5.86	0.00019	capping protein (actin filament), gelsolin-like
2008 Week 12	TNFSF10	-4.723	0.00133	tumor necrosis factor (ligand) superfamily, member 10, TRAIL
2008 Week 12	CTSZ	-2.724	0.028	cathepsin Z
2008 Week 12	TNFRSF17	-2.619	0.0377	tumor necrosis factor receptor superfamily, member 17, death receptor 6
2008 Week 12	CASP8	5.006	0.000801	caspase 8, apoptosis-related cysteine peptidase
2008 Week 50	CIDEA	-2.643	0.00303	cell death-inducing DFFA-like effector a
2008 Week 50	GSN	-2.372	0.00152	gelsolin
2008 Week 50	CIDEC	-2.331	0.00826	cell death-inducing DFFA-like effector c
2008 Week 50	COX4I2	-2.189	0.0425	cytochrome c oxidase subunit IV isoform 2 (lung)
2008 Week 50	CARD11	-1.743	0.0655	caspase recruitment domain family, member 11
2008 Week 50	BAG5	1.804	0.031	BCL2-associated athanogene 5
2008 Week 50	PIAS4	1.823	0.0305	protein inhibitor of activated STAT, 4
2008 Week 50	CASP8	2.563	0.0011	caspase 8, apoptosis-related cysteine peptidase
2008 Week 50	S100A9	2.914	0.000839	S100 calcium binding protein A9
2008 Week 50	MT-CO2	4.184	0.0282	cytochrome c oxidase subunit II
2008 Week 50	MAPK8IP1	5.334	0.000768	mitogen-activated protein kinase 8 interacting protein 1
2008 Week 50	BOK	5.682	0.0382	BCL2-related ovarian killer
2009 Week 4	DAP	-5.337	0.0191	death-associated protein
2009 Week 4	TNIK	2.881	0.0291	TRAF2 and NCK interacting kinase
2009 Week 4	NFKBID	3.107	0.0326	NFKB nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta
2009 Week 4	S100A9	3.694	0.0452	S100 calcium binding protein A9
2009 Week 4	AMBRA1	6.597	0.000314	autophagy/beclin-1 regulator 1
2009 Week 4	CEBPE	7.86	0.0000804	CCAAT/enhancer binding protein (C/EBP), epsilon
2009 Week 4	RNF41	8.167	0.0374	ring finger protein 41
2009 Week 4	THAP3	8.167	0.0374	THAP domain containing, apoptosis associated protein 3

Comparison between 2008 and 2009 Week 4

There were relatively few genes in common that were significantly upregulated at Week 4 in 2008 and 2009 studies (Table 9). Some were upregulated by different degrees and some switched between R and S animals upregulating.

Table 9: Genes upregulated at Week 4 in 2008 and 2009 studies, ranked by fold change for 2009 and then 2008

2008 Week 4				**2009 Week 4**	
Symbol	**Fold Change**	**p-value**	**Entrez Gene Name**	**Fold Change Sorted 2009**	**p-value**
TCN2	-7.523	0.000048	transcobalamin II	-178.274	0.000000683
CCRL1	-3.233	0.0355	chemokine (C-C motif) receptor-like 1	-13.415	0.0283
ADIPOQ	-5.248	0.0117	adiponectin, C1Q and collagen domain containing	-11.629	0.00169
FAM184B	3.068	0.0181	family with sequence similarity 184, member B	-10.975	0.0021
CISD2	-3.344	0.017	CDGSH iron sulfur domain 2	-7.956	0.0338
PLA2G2A	-3.412	0.00898	phospholipase A2, group IIA (platelets, synovial fluid)	-7.829	0.00719
FAU	-6.859	0.0000633	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	-7.011	0.00654
RAB11FIP2	-3.003	0.0253	RAB11 family interacting protein 2 (class I)	-7.005	0.0289
GGH	-2.539	0.0446	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	-4.758	0.03
ORMDL3	8.114	0.000024	ORM1-like 3 (S. cerevisiae)	3.263	0.0173
PTTG1	-5.63	0.0016	pituitary tumor-transforming 1	3.77	0.0452
C19orf45	-3.831	0.0316	chromosome 19 open reading frame 45	4.888	0.0263
SERPINA5	-2.927	0.0242	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	6.075	0.000483
LOC100507709	-3.233	0.012	HLA class II histocompatibility antigen, DRB1-7 beta chain-like	14.207	0.000000968
	Sorted 2008				
TCN2	-7.523	0.000048	transcobalamin II	-178.274	0.000000683
FAU	-6.859	0.0000633	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	-7.011	0.00654
PTTG1	-5.63	0.0016	pituitary tumor-transforming 1	3.77	0.0452
ADIPOQ	-5.248	0.0117	adiponectin, C1Q and collagen domain containing	-11.629	0.00169
C19orf45	-3.831	0.0316	chromosome 19 open reading frame 45	4.888	0.0263
PLA2G2A	-3.412	0.00898	phospholipase A2, group IIA (platelets, synovial fluid)	-7.829	0.00719
CISD2	-3.344	0.017	CDGSH iron sulfur domain 2	-7.956	0.0338
CCRL1	-3.233	0.0355	chemokine (C-C motif) receptor-like 1	-13.415	0.0283
LOC100507709	-3.233	0.012	HLA class II histocompatibility antigen, DRB1-7 beta chain-like	14.207	0.000000968
RAB11FIP2	-3.003	0.0253	RAB11 family interacting protein 2 (class I)	-7.005	0.0289
SERPINA5	-2.927	0.0242	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	6.075	0.000483
GGH	-2.539	0.0446	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	-4.758	0.03
FAM184B	3.068	0.0181	family with sequence similarity 184, member B	-10.975	0.0021
ORMDL3	8.114	0.000024	ORM1-like 3 (S. cerevisiae)	3.263	0.0173

Interesting genes

There are a number of genes that were upregulated on 3-4 time points (Table 10). Although all three genes were highly upregulated on at least one occasion it is not clear what role they played in R or S to MAP infection, if any. It is possible that they are part of the effect of disease in the S animals in the case of TCN2 and ADIPOQ. The role of ORMDL3 is more intriguing since it has been associated with asthma in genome-wide population studies.

Table 10. Genes that were upregulated on 3-4 time points, including TCN2, which had the highest upregulation (-178.3) in 2009 Week 4 S animals, ADIPOQ and ORMDL3.

Sample	Symbol	Fold Change	p-value	Entrez Gene Name	Function
Week 4	TCN2	-7.5	0.000048	transcobalamin II	cobalamin transport, cell proliferation, mitochondrial dysfunction
Week 12	TCN2	-2.6	0.042	transcobalamin II	"
2009 Wk 4	TCN2	-178.3	0.000000683	transcobalamin II	"
Week 4	ADIPOQ	-5.3	0.0117	adiponectin, C1Q and collagen domain containing	Adiponectin; protein hormone, modulates metabolic processes, including glucose regulation and fatty acid catabolism.
Week 12	ADIPOQ	3.7	0.0322	adiponectin, C1Q and collagen domain containing	"
Week 50	ADIPOQ	-5.2	1.96E-07	adiponectin, C1Q and collagen domain containing	"
2009 Wk 4	ADIPOQ	-11.6	0.00169	adiponectin, C1Q and collagen domain containing	"
Week 4	ORMDL3	8.1	0.000024	ORM1-like 3 (S. cerevisiae)	Associated with risk of asthma and autoimune disease
Week 12	ORMDL3	3.4	0.00889	ORM1-like 3 (S. cerevisiae)	"
Week 50	ORMDL3	32.9	1.54E-26	ORM1-like 3 (S. cerevisiae)	"
2009 Wk 4	ORMDL3	3.3	0.0173	ORM1-like 3 (S. cerevisiae)	"

Discussion

Studies on gene expression rarely give black and white results, but rather shades of grey and they are usually extremely complex. When analysing gene expression levels for deer we are faced with combining these shades of grey at various levels.

1) Generation of sequence tags: The restriction enzymes used are not 100% accurate and the restriction is also not 100% efficient. This results in tags that differ in length and it also results in more than one tag per transcript.
2) Mapping of tags: We want to analyze expression of genes. To do that tags have to be mapped uniquely to a gene. Even if we assume all tags are perfect (which they are not) there are more challenges. Genes can have alternative transcripts with different tags. Here we have to combine all the transcripts and thereby all the tags that belong to a specific gene, carefully trying to avoid combining transcripts of similar genes. Ideally we would have the complete transcriptome of deer available as a reference. In reality we have an incomplete transcriptome available that consists of deer and elk transcripts. To compensate for the incomplete deer and elk transcriptome dataset we used a reference set of cattle genes. This resource can be considered as nearly complete but it is cattle and not deer. To compensate for the incomplete set of cattle genes we used the cattle genome.
3) Annotating genes: Having mapped tags uniquely to a gene, we are interested in the functional annotation of that gene. Annotation is mostly derived from sequence comparisons with annotated genomes of popular species like human. The Bovine Genome Consortium invested considerable effort in annotating the cattle genome. Despite this there remain many genes that do not have enough similarity with other known annotated genes. Finally, there are a large number of genes that have been annotated as genes with unknown function.
4) Examining networks: To look at the interaction of genes we use Ingenuity Pathway Analysis (IPA). IPA collects and curates network information from various databases and from the primary literature. It is human, mouse and rat centric. To analyze our deer genes with IPA they get translated into their human/mouse/rat equivalent. This translation does not work for all genes. The disease examples are also human-centric.

Thus, although the SOLiD SAGE sequencing produced 566 million reads, this came down to 373 million tags, 31,500 uniquely mapped genes, of which 18,000 were annotated and 17,500 were recognized by IPA. Careful filtering brought this down to 200-300 genes up or down regulated across all the time points.

The list of interesting candidates is exciting and will be added to and rationalized in time. The strength of the approach in this study is that it can identify important genes without having any prior knowledge of their function. This is in marked contrast to the candidate gene approach. The most important genes in R / S may be ones for which their function is as yet unknown.

This SOLiD SAGE gene expression study has demonstrated the advantages that new generation sequencing has over micro-array technology because it is not limited to known genes and gene networks and the range of fold change is much greater. There are whole families of genes (eg interferon, S100 calcium binding proteins, solute carrier families etc), which have shown up in these studies and would not have been included in microarrays. However, we are yet to elucidate the true roles of many of these genes. At the very least, this study will considerably broaden our knowledge of the genes involved in host responses to pathogens.

Another issue that may not be resolved in this study relates to “cause or effect”. In other words, which upregulated genes in R and S animals were responsible for driving the immune responses down the R or S pathways versus genes that were turned on as a response to the disease process. It is apparent (Tables 1 and 2) that all the animals became infected because MAP was isolated from all the animals at Week 4 and Week 12, which suggests that the innate immune response was not effective at preventing infection. However, it is also

clear that in both 2008 and 2009 animals there were differences in the amount of disease and the number of MAP present in the JJLN at 12 weeks, which shows that R and S animals were starting to head down different pathways in their acquired immune response. There was significantly lower antibody and higher IFNγ in the R than the S animals in the 4-12 week period suggesting a preponderance of cell-mediated over humoral response in the R animals compared with the S (Mackintosh et al 2011; Mackintosh et al in press). This should provide clues as to what gene expression pathways are important in these immune responses. However, it appears that the "improvement" in the disease state of the R animals between Week 12 and Week 50 was a long slow process of the host immune mechanisms gaining the upper hand in the fight against MAP, which has the ability to evade destruction in their intracellular niche. Over that time the S animals' disease states either deteriorated or remained the same, showing their ability to eliminate or reduce MAP infestation. These observations, based on histopathology, culture and immunological readouts, suggest that upregulated genes at Week 4 are likely to be important in identifying key gene differences between R and S animals, which drive the immune response pathways, whereas at Week 12 it appears that there appears to a much higher level of gene expression, especially in the S animals, which appears to be associated with a higher levels of inflammation and disease in these animals (Tables 6A, 6B and 6C). Excessive inflammation may be part of the pathogenesis of paratuberculosis. There is also a large number of apoptosis and autophagy genes upregulated, especially in R animals at Week 4, which may hold the key to how R animals use cell death to assist in killing intracellular MAP, compared with uncontrolled cell death and necrosis assisting MAP to evade killing and allow reinfection of other macrophages (Table 8).

A review of candidate genes identified in other studies of paratuberculosis did not show any promising lines of investigation in this study apart from SLC39A3 (solute carrier family 39, member 3), which was upregulated 12.6 fold in R animals at 2009 Week 4 (Table 4). Not much is known about it currently other than it is a zinc transporter.

Genes associated with Tb in IPA were assessed and showed a few interesting leads in common. SLC11A1 (NRAMP), which was also identified by a number of other studies as associated with susceptibility to Tb and paratuberculosis, was upregulated 12.19 fold in S animals at 2008 Week 12, but also upregulated 2.19 fold in R animals at 2008 Week 50. Similarly IL8 was upregulated 11.18 fold in S animals at 2008 Week 12 upregulated 2.68 fold in R animals at 2008 Week 50. This suggests that the roles of some of these genes may change with time and the stage of the disease.

There were 18 genes identified as associated with defence responses to invading pathogens that were highly upregulated (5-33 fold) in R at Week 4 in 2008 and 2009 (Table 7A and 7C). These may be very significant for elucidating effective killing mechanisms and pathways in R animals. By contrast there were only 2 genes in this class that were moderately upregulated (Table 7B).

There is still considerable work still required to completely analyse this gene expression dataset and investigate all the relevant pathways. This work is in progress.

Conclusions

These animal experiments produced some exciting and novel results with clear differences in the pathology and immunological responses, which were related to the susceptibility or resistance of the sires. However, the major value of this work required completion of the DNA sequencing and a comprehensive analysis of the data. The withdrawal of funding for sequencing of the samples from 3R and 3S at three time points from the 2009 study during the third year of the planned 4 year study resulted in an incomplete set of data. This drastically reduced the scope and reliability of the final dataset for a number of reasons;

(a) although the 2008 study had satisfactory separation between moderate-severe diseased and minimal disease, the 2009 study had an even better separation;
(b) with only two sires represented in the 2008 dataset it is very desirable to have at least 4 sires in the final dataset for analysis;
(c) the two studies would have been complementary and additive in terms of identifying keys genetic pathways with time.

The withdrawal of funding for the planned programme in the fourth year undermined the principal value of the project because no further investigation or validation of key candidate genes and pathways could be undertaken.

I have done my best to try to salvage as much useful data from this incomplete and non-validated dataset. Papers on the two field studies have been published. While I am currently drafting a paper on the gene expression aspects, the resulting publication will be considerably inferior to that which would have resulted from a complete dataset, full validation, further investigation and more time to concentrate on it. This would have been possible if JDRC had funded the 4th year of this project. Furthermore, the study had the potential to not only offer insights into the mechanisms of resistance/susceptibility but also provide information for industry on the use of genetics for the control of Johne's disease. This potential will not be realized without further funding.

Recommendations

The JDRC reconsider this project for funding to complete the study. The funding requirements to complete the 4th year of this project would be in the order of $250k.

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Appendix:

Genes upregulated 10 fold or greater in the 4 sampling periods.

2008 Week 4:

Symbol	FoldChange	p-value	Entrez Gene Name
S100A8	-33.502	9.79E-11	S100 calcium binding protein A8
GSTM4	-17.278	0.00397	glutathione S-transferase mu 4
LOC100508266	-15.274	0.0073	zinc finger protein 420-like
MICALL2	-13.539	0.01	MICAL-like 2
SAMD13	-13.076	0.000276	sterile alpha motif domain containing 13
AGT	-12.194	0.0195	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
ERBB2	-11.974	0.0195	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
DEFB4_bovin	-11.393	0.0195	Defensin beta 4A
NDUFA5	-11.276	0.0276	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa
LIN37	-11.136	0.00346	lin-37 homolog (C. elegans)
PYCR1	-10.977	0.0000797	pyrroline-5-carboxylate reductase 1
CMBL	-10.846	0.000111	carboxymethylenebutenolidase homolog (Pseudomonas)
SLC22A17	10.467	0.0276	solute carrier family 22, member 17
MX2	10.993	0.0000014	myxovirus (influenza virus) resistance 2 (mouse)
USP18	11.256	0.00000126	ubiquitin specific peptidase 18
IFIT2	16.535	0.0000142	interferon-induced protein with tetratricopeptide repeats 2
RSAD2	20.025	1.03E-08	radical S-adenosyl methionine domain containing 2
ISG15	22.766	1.86E-09	ISG15 ubiquitin-like modifier

2008 Week 12:

Symbol	Fold Change	p-value	Entrez Gene Name
CRABP1	-141.903	8.24E-16	cellular retinoic acid binding protein 1
CRLF1	-62.044	1.02E-11	cytokine receptor-like factor 1
SDS	-54.115	6.14E-10	serine dehydratase
APOBEC3G	-50.784	1.98E-12	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
PYCR1	-39.242	6.87E-10	pyrroline-5-carboxylate reductase 1
S100A9	-38.421	1.1E-11	S100 calcium binding protein A9
CXCL14	-31.951	1.56E-10	chemokine (C-X-C motif) ligand 14
LOX	-31.179	0.000000922	lysyl oxidase
CXCL2	-30.567	1.39E-09	chemokine (C-X-C motif) ligand 2
CYP2B6	-30.52	3.14E-10	cytochrome P450, family 2, subfamily B, polypeptide 6
CHI3L1	-28.969	0.00028	chitinase 3-like 1 (cartilage glycoprotein-39)
COL1A2	-27.653	2.69E-10	collagen, type I, alpha 2
IGFBP6	-25.934	0.0000268	insulin-like growth factor binding protein 6
MMP13	-25.78	4.85E-10	matrix metallopeptidase 13 (collagenase 3)
NDUFA4L2	-25.427	0.000000478	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2
C20orf114	-24.935	0.000000138	chromosome 20 open reading frame 114
FIBIN	-22.849	1.06E-08	fin bud initiation factor homolog (zebrafish)
UPP1	-22.589	5.47E-09	uridine phosphorylase 1

CXCL5	-22.261	4.55E-09	chemokine (C-X-C motif) ligand 5
METRNL	-20.483	0.000000488	meteorin, glial cell differentiation regulator-like
NOS2	-19.529	3.31E-08	nitric oxide synthase 2, inducible
RPL34	-18.654	1.87E-08	ribosomal protein L34
FBLN2	-18.467	1.84E-08	fibulin 2
C1QTNF3	-18.462	1.52E-08	C1q and tumor necrosis factor related protein 3
WFDC2	-17.725	0.000000299	WAP four-disulfide core domain 2
MFAP2	-17.699	0.00000886	microfibrillar-associated protein 2
BMP1	-17.293	0.00000138	bone morphogenetic protein 1
S100A5	-16.74	0.000004	S100 calcium binding protein A5
BGN	-16.619	2.75E-08	biglycan
LUM	-15.944	4.59E-08	lumican
IL17F	-15.497	0.00085	interleukin 17F
VCAN	-14.944	0.000000126	versican
LILRB4	-14.851	0.00106	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4
S100A4	-13.782	0.00000016	S100 calcium binding protein A4
STAR	-12.39	0.0000272	steroidogenic acute regulatory protein
SLC11A1	-12.193	0.000000614	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
RND1	-11.785	0.0000446	Rho family GTPase 1
CRABP2	-11.772	0.00000713	cellular retinoic acid binding protein 2
S100A2	-11.716	0.000000841	S100 calcium binding protein A2
FAM69C	-11.701	0.00108	family with sequence similarity 69, member C
SLMO1	-11.687	0.0273	slowmo homolog 1 (Drosophila)
PXDN	-11.325	0.0000418	peroxidasin homolog (Drosophila)
GPR161	-11.324	0.000113	G protein-coupled receptor 161
LIPG	-11.324	0.0273	lipase, endothelial
FFAR2	-11.194	0.0391	free fatty acid receptor 2
IL8	-11.184	0.00000106	interleukin 8
THBS2	-11.127	0.00000108	thrombospondin 2
SERPINE1	-10.835	0.00000143	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
C11orf83	-10.582	0.0391	chromosome 11 open reading frame 83
SLC2A5	-10.507	0.00000192	solute carrier family 2 (facilitated glucose/fructose transporter), member 5
TPM2	-10.403	0.00718	tropomyosin 2 (beta)
VARS	-10.154	0.0391	valyl-tRNA synthetase
PRRX2	-10.068	0.0000038	paired related homeobox 2
INHBA	-10.048	0.0000107	inhibin, beta A
AURKB	10.055	0.00000312	aurora kinase B
TUBA3C/TUBA3D	10.556	0.0137	tubulin, alpha 3c
C16orf73	10.822	0.0137	chromosome 16 open reading frame 73
DOC2A	10.844	0.0137	double C2-like domains, alpha
SLAIN1	11.014	0.0137	SLAIN motif family, member 1
TUBA1D	11.927	0.0137	**Tubulin alpha-1D chain**
CD209	12.002	0.0000925	CD209 molecule
RABAC1	12.4	0.0287	Rab acceptor 1 (prenylated)
NTRK1	13.157	0.00715	neurotrophic tyrosine kinase, receptor, type 1
THRSP	13.66	0.000001	thyroid hormone responsive
COL4A4	14.699	0.000101	collagen, type IV, alpha 4
FCER2	14.951	0.000744	Fc fragment of IgE, low affinity II, receptor for (CD23)

NUBP2	16.961	0.00718	nucleotide binding protein 2 (MinD homolog, E. coli)

2008 Week 50

Symbol	Fold Change	p-value	Entrez Gene Name
RPL34	-25.437	1.19E-22	ribosomal protein L34
FAM133A	-13.624	0.00182	family with sequence similarity 133, member A
LOC100507686	-12.728	0.0029	HLA class II histocompatibility antigen, DQ alpha 1 chain-like
GLB1L	-10.721	0.00755	galactosidase, beta 1-like
ODZ2	-10.638	0.00755	odz, odd Oz/ten-m homolog 2 (Drosophila)
FCRL2	-10.564	0.00755	Fc receptor-like 2
HAO1	10.194	0.02	hydroxyacid oxidase (glycolate oxidase) 1
SRCIN1	10.263	0.02	SRC kinase signaling inhibitor 1
FUT4	10.33	0.02	fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)
SRCRB4D	10.455	0.02	scavenger receptor cysteine rich domain containing, group B (4 domains)
HOXA10	12.13	0.00755	homeobox A10
ACAN	14.459	0.00467	aggrecan
PMP2	18.662	0.000461	peripheral myelin protein 2
ORMDL3	32.935	1.54E-26	ORM1-like 3 (S. cerevisiae)

2009 Week 4

	Fold Change	p-value	Entrez Gene Name
TCN2	-178.274	0.000000683	transcobalamin II
CYR61	-87.545	0.000018	cysteine-rich, angiogenic inducer, 61
AURKB	-81.772	0.000000407	aurora kinase B
CTSH	-81.654	0.0000016	cathepsin H
C11orf24	-77.484	0.0000298	chromosome 11 open reading frame 24
ITGBL1	-69.554	0.00000804	integrin, beta-like 1 (with EGF-like repeat domains)
URM1	-57.506	0.000117	ubiquitin related modifier 1
TMEM149	-54.009	0.0000236	transmembrane protein 149
USF1	-45.3	0.0000213	upstream transcription factor 1
TOLLIP	-45.07	0.000316	toll interacting protein
ASPM	-42.719	0.000012	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)
RPS9	-42.363	0.00000794	ribosomal protein S9
VPREB1	-37.17	0.000704	pre-B lymphocyte 1
SAMM50	-34.222	0.000018	sorting and assembly machinery component 50 homolog (S. cerevisiae)
FMO1	-33.699	0.00102	flavin containing monooxygenase 1
GOLGA2	-33.108	0.000198	golgin A2
RPS7	-31.269	0.00000912	ribosomal protein S7

HHEX	-30.363	0.00156	hematopoietically expressed homeobox
RNPEPL1	-28.604	0.000146	arginyl aminopeptidase (aminopeptidase B)-like 1
RPL34	-27.497	0.0000202	ribosomal protein L34
???	-27.135	0.0000166	
GRN	-26.447	0.0000752	granulin
CEP57L1	-22.142	0.00408	centrosomal protein 57kDa-like 1
TUSC5	-21.397	0.000125	tumor suppressor candidate 5
MARCKS	-21.229	0.000564	myristoylated alanine-rich protein kinase C substrate
C2orf28	-21.155	0.00111	chromosome 2 open reading frame 28
GLB1	-20.867	0.0000901	galactosidase, beta 1
ZBTB38	-19.398	0.00674	zinc finger and BTB domain containing 38
DCTN1	-18.654	0.00201	dynactin 1
PPP4R4	-18.017	0.000326	protein phosphatase 4, regulatory subunit 4
CXorf38	-18.006	0.0105	chromosome X open reading frame 38
MEMO1 (includes EG:298787)	-17.965	0.000629	mediator of cell motility 1
ASNS	-17.809	0.0105	asparagine synthetase (glutamine-hydrolyzing)
FSIP1	-15.211	0.00102	fibrous sheath interacting protein 1
TOB2	-14.953	0.0197	transducer of ERBB2, 2
ACOT7	-14.567	0.00441	acyl-CoA thioesterase 7
HUS1	-14.425	0.00157	HUS1 checkpoint homolog (S. pombe) EMBL AAI12820.1
RACGAP1	-14.222	0.00124	Rac GTPase activating protein 1
HIG2	-14.169	0.00489	HIG2 protein EMBL AAI46218.1
TKT	-14.107	0.000525	transketolase
AP2M1	-13.642	0.0236	adaptor-related protein complex 2, mu 1 subunit
KIAA0907	-13.451	0.000867	KIAA0907
CCRL1	-13.415	0.0283	chemokine (C-C motif) receptor-like 1
PROCR	-13.088	0.00343	protein C receptor, endothelial
RBMS1	-13.064	0.00233	RNA binding motif, single stranded interacting protein 1
C1orf38	-13.027	0.00651	chromosome 1 open reading frame 38
C1orf156	-12.824	0.0243	chromosome 1 open reading frame 156
SIKE1	-12.824	0.00727	suppressor of IKBKE 1
CYP2B6	-12.383	0.00153	cytochrome P450, family 2, subfamily B, polypeptide 6
C8orf80	-12.375	0.0292	chromosome 8 open reading frame 80
DPF2	-12.155	0.0415	D4, zinc and double PHD fingers family 2
SERPINA3	-12.019	0.00894	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3
SARNP	-11.939	0.0116	SAP domain containing ribonucleoprotein
GPSM3	-11.882	0.00499	G-protein signaling modulator 3
NDRG2	-11.843	0.0353	NDRG family member 2
TCEB2	-11.727	0.000875	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)
ADIPOQ ????????	-11.629	0.00169	adiponectin, C1Q and collagen domain containing
PPIE	-11.575	0.0353	peptidylprolyl isomerase E (cyclophilin E)
HDAC3	-11.54	0.0353	histone deacetylase 3
RET	-11.33	0.043	ret proto-oncogene

FTSJD2	-11.122	0.043	FtsJ methyltransferase domain containing 2
CATSPERG	-11.11	0.043	cation channel, sperm-associated, gamma
KAT2B	-11.089	0.0128	K(lysine) acetyltransferase 2B
	-11.045	0.0033	
ZCCHC24	-11.002	0.043	zinc finger, CCHC domain containing 24
PDXP	-10.998	0.043	pyridoxal (pyridoxine, vitamin B6) phosphatase
FAM184B	-10.975	0.0021	family with sequence similarity 184, member B
KIAA0174	-10.881	0.00118	KIAA0174
BCAM	-10.816	0.00559	basal cell adhesion molecule (Lutheran blood group)
MFGE8	-10.243	0.0164	milk fat globule-EGF factor 8 protein
C15orf17	10.209	0.0205	chromosome 15 open reading frame 17
LIN7B	10.209	0.0205	lin-7 homolog B (C. elegans)
MDC1	10.209	0.0205	mediator of DNA-damage checkpoint 1
PLCD3	10.209	0.0205	phospholipase C, delta 3
TNRC18	10.209	0.0205	trinucleotide repeat containing 18
ADAR	10.509	0.0374	adenosine deaminase, RNA-specific
ATP11C	10.509	0.0374	ATPase, class VI, type 11C
COQ4	10.509	0.0374	coenzyme Q4 homolog (S. cerevisiae)
GAD2	10.509	0.0374	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)
TMEM141	10.509	0.0374	transmembrane protein 141
PFKFB4	12.611	0.0205	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
SLC39A2	12.611	0.0205	solute carrier family 39 (zinc transporter), member 2
EMR3	12.935	0.00000311	egf-like module containing, mucin-like, hormone receptor-like 3
CPO	13.821	0.000067	carboxypeptidase O
ATP5A1	13.851	0.00000193	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
HLA DRB3-LIKE	14.207	0.000000968	HLA class II histocompatibility antigen, DRB1-7 beta chain-like
MEP1B	14.293	0.00673	meprin A, beta
PLA2G4D	14.713	0.0116	phospholipase A2, group IVD (cytosolic)
KCNK7	16.334	0.00401	potassium channel, subfamily K, member 7
CAMP	18.916	0.00401	cathelicidin antimicrobial peptide
LOC619159	24.501	0.00063	leukocyte immunoglobulin-like receptor-like
PLEKHA4	26.543	0.000416	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4
MAP3K11	33.138	0.00000187	mitogen-activated protein kinase kinase kinase 11
OSBPL10	34.271	0.000000233	oxysterol binding protein-like 10