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Microarray analyses of mouse responses to infection by *Neospora caninum* identifies disease associated cellular pathways in the host response

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ABSTRACT

Neospora caninum is a coccidian cyst-forming parasite found in a wide range of host species such as mice, dogs and cattle. The development of methods such as vaccines to prevent abortion and fetal loss due to neosporosis would be greatly assisted by further knowledge on immunity and host responses to infection. In this study we used microarray technology to investigate the protective host responses occurring at 6 h post infection in the spleen of mice infected with a prototype live *N. caninum* vaccine. Naive non-pregnant mice were infected with the NC-Nowra isolate as such infections are known to induce protective host responses that will prevent transplacental transmission of a challenge given using pregnancy. The expression data was analysed by SAM (significance of microarrays), ANOVA and clustering methods. Gene lists were investigated for enrichment of gene ontology terms by functional annotation using hypergeometric tests. The results show that Qs and BALB/c mice infected with NC-Nowra differ in their transcriptional responses to infection and these affect a wide range of biological and molecular processes. Transcriptional changes in the Jak-STAT signaling pathway (as well as Irf and other IFN-γ regulated molecules such as GTPases) confirmed the influence of IFN-γ in the mouse response to *N. caninum*. Gene ontology analyses also assigned some of the molecules involved to well known disease pathways associated with cancer, Parkinson's and Alzheimer's diseases, which were linked to the cell cycle, mitochondrial electron transport chain and coupled proton transport pathways amongst others. Although infection of mice with NC-Nowra causes little or no signs of clinical disease, the molecular functions, processes and pathways identified through these studies clearly warrant further investigation for their role in the development of protective immunity as well as pathogenesis. These studies therefore provide new, exciting leads by which to study neosporosis.

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1. Introduction

The fundamental study of parasitic diseases requires an understanding of the relationship between parasite and host. The dynamics of the host–parasite relationship relies on a number of factors, such as the biology and physiology of the parasite, the location and action of the parasite within the host, the immune response of the host, and the host lifestyle patterns. The key to unravelling many of the disease processes caused by parasitic organisms lies in an understanding of the dynamics of these complex interactions.

Microarrays are a technology which allows the simultaneous measurement of gene expression on an enormous scale, assessing the expression of thousands of genes simultaneously. The potential uses of microarrays are varied and numerous [1,2]. Often the primary objective of microarray experiments is the identification of individual genes, whose transcription is altered (i.e. up regulated or down regulated), in the conditions under study. One such application of microarray technology is the study of host responses to parasite infection [3,4]. A wide range of microarrays are available for many host species, such as humans, mice, rats and chickens. Of the many parasite species for whom the host responses have been investigated, a number have been Apicomplexa. For example, host responses to *Eimeria maxima* and *Eimeria acervulina* infection were studied in order to gain insights into avian coccidiosis, and a number of cytokine and non-cytokine genes were identified which were up regulated and down regulated in a similar manner in both infections [5]. Changes in expression of genes related to the immune response, and other cellular functions such as glucose and mevalonate metabolism, have been noted in *Toxoplasma gondii*

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infected cells [6]. Genes up regulated in *T. gondii* infection and not in *Salmonella typhimurium* and *Chlamydia trachomatis* infection have also been reported [7].

Neospora caninum is a protozoan, cyst-forming coccidian parasite that is now recognised as a common infection of cattle worldwide [8]. The economic importance of this parasite as a cause of abortion in cattle has become the impetus behind research into vaccines that may ultimately reduce the level of fetal loss that occurs in the cattle industry [9]. A number of vaccine strategies have been evaluated in mice and cattle that show promise for the future, but one of the limitations in these studies is the lack of knowledge on the mechanisms of immunity to *N. caninum* [10,11].

In this study the transcriptional changes occurring during the response of BALB/c and Qs mice to infection by *N. caninum* (NC-Nowra) were studied by analyses of gene expression occurring in the spleen using microarray technology. The methods of analysis used to identify differentially expressed genes, included SAM, ANOVA and clustering methods. The choice of the animal models used here was because infection of both these mice types with NC-Nowra results in little or no signs of clinical disease [12,13]. In contrast infections of BALB/c mice with other isolates of *N. caninum* (such as NC-Liverpool) typically result in severe weight loss and death [14]. Non-pregnant mice can also be immunised by infection with NC-Nowra such that transplacental transmission of a challenge given during pregnancy is largely prevented [13]. Such observations have suggested that live vaccines may have a future in the cattle industry for the control of abortion associated with *N. caninum* infections. Studies on other parasitic protozoa have shown that it is possible using microarray technology to identify the host pathways that are turned on or off in response to infection [1,2]. It was anticipated that the study described here would provide a window onto the protective host responses occurring in the mouse in response to infection by NC-Nowra.

2. Materials and methods

2.1. Cell culture and parasite inoculum

The NC-Liverpool [15] and NC-Nowra [12] strains of *N. caninum* were cultured in Vero cells [16] using RPMI medium containing 5% horse serum (CSL, Australia) and 1% Penicillin/Streptomycin. After cell lysis, tachyzoites were purified by extrusion through a 26 gauge needle, washed and suspended in sterile saline. Tachyzoites were counted using a haemocytometer and their viability confirmed with trypan blue staining. Parasites were kept on ice throughout purification and counting, and were injected immediately into mice. The identity of the parasite strains used in this study was verified by PCR of DNA markers as described [17].

2.2. Mouse infection

BALB/c and Qs mice (Gore Hill Laboratories, Australia) were injected with 5×10^6 tachyzoites subcutaneously (sc.) [12,13]. Control groups, injected sc. with 0.9% sterile saline, and no-treatment groups, were included in the experiment. Mice were euthanized at 0 and 6 h post infection (h.p.i.). Spleens were removed and stored in liquid nitrogen prior to RNA isolation.

2.3. Isolation of RNA from samples

RNA was isolated using Trizol (Life Technologies) as follows. Frozen spleen tissue was ground using a mortar and pestle, and resuspended in Trizol reagent (1 ml Trizol per 0.5–1.0 g tissue). The suspension was centrifuged (10,000 × *g*, 10 min at 4 °C) to remove excess fats and polysaccharides, and the supernatant removed and incubated for 5 min at room temperature. Chloroform was then added (0.2 ml per 1 ml Trizol), and the mixture shaken vigorously and incubated (3 min, room temperature). Following incubation, samples were centrifuged (10,000 × *g*, 10 min at 4 °C) and with great care the colourless upper phase (containing RNA) was removed. RNA was then precipitated by addition of isopropanol (equal volume to the volume of the upper phase). Samples were then incubated (10 min, room temperature), centrifuged (10,000 × *g*, 10 min at 4 °C), and the supernatant removed and discarded. The RNA pellet was washed in 1 ml 70% ethanol followed by centrifugation (7500 × *g*, 5 min at 4 °C). Following removal of final traces of ethanol, the RNA was dried under vacuum and then dissolved (with heating) in DEPC-treated H_2O (200–1000 μl depending on pellet size). The RNA was then stored frozen at −80 °C.

2.4. Microarray printing, preparation, hybridisation and scanning

Microarrays were sourced from the Wellcome Trust Sanger Institute (U.K.) and were generated from the NIA 15K Mouse cDNA clone set [18]. The clone set contains ~11,500 unique cDNA clones derived from 52,374 expressed sequence tags (ESTs) of pre- and peri implantation embryos, E12.5 female gonad/mesonephros, and newborn ovary. The clones have an average insert size of ~1.5 kb, and up to 50% are derived from novel genes. The average redundancy of the clone set is 1.3. The identity and details of the genes present on the arrays were sourced from the NIA mouse cDNA project home page (http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html).

A direct experimental design was used for the microarray hybridisations that involved the hybridisation of two experimental RNA samples – one control RNA sample (from a control animal injected with saline) and one infected sample (from a mouse infected with *N. caninum*) – to each array. Control cDNA samples were labelled with Cy3 dye, and cDNA derived from infected animals were labelled with Cy5 dye. cDNA probes were generated by reverse transcription of total cellular RNA using a T_{20}VN anchored primer (Sigma) and amino-allyl dUTP (Sigma) and Superscript II Reverse Transcriptase (Invitrogen). After reverse transcription, cDNA was purified and conjugated to Cy dye in 0.1 M $NaHCO_3$ (pH 9). The labelled cDNA was hybridised to the microarray for 10–16 h at 37 °C using a hybridisation mix containing DIG Easy Hyb (Roche), 2 μl yeast tRNA (11 mg ml^{-1}) and 5 μl calf thymus DNA (9.1 mg ml^{-1}). After hybridisation, arrays were washed three times with 1xSSC, 0.1% SDS at 50 °C and then with 1xSSC (room temperature) and MilliQ H_2O (room temperature).

2.5. Microarray image and data analysis

An overview of the methods used is shown in Fig. 1. Scanned images were analysed using Genepix Pro version 3.0 (Axon, USA). Data transformation and normalisation was performed using the print-tip lowess normalisation algorithm [19]. Normalised data was analysed by Significance Analysis of Microarrays (SAM) for the identification of differentially expressed genes using either a one class or two class (unpaired) response in an analysis of variance [20]. Lists of genes showing significant over or under expression were identified using a false discovery rate (FDR) of ≤5%. The data sets from this study are accessible via the series record GSE23520 in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

K means clustering of the normalised data was performed using Cluster 3.0 and the Euclidian distance [21]. Data was filtered such that genes with a normalised value >±1 were analysed. Clustering was also performed using WinGim and the Gaussian Infinite mixture models [22]. Heatmaps were generated using Java Treeview [23].

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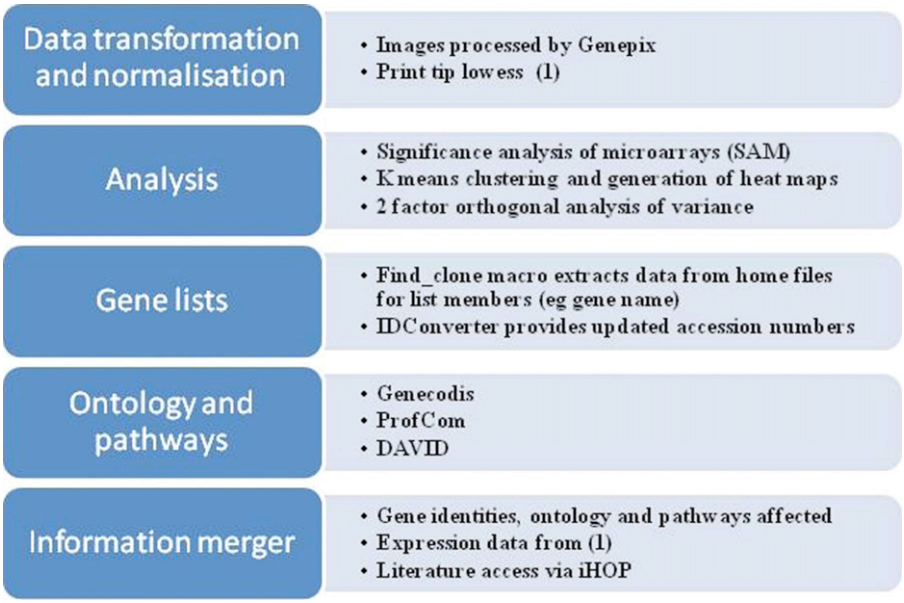

Fig. 1. Summary of the methods used in this study for data analyses. Dual colour microarray data was generated by hybridisation of cDNA from spleen of infected and uninfected mice to the microarrays. The expression data was normalised and analysed by SAM, K means clustering and 2-factor orthogonal analysis of variance to generate lists of genes that were over or under expressed. The lists were investigated for their enrichment of associated gene ontology terms and association with biological processes such as pathways.

A 2-factor orthogonal analysis of variance for each clone on the array, based on the normalised data, was also performed [1]. The two factors were mouse type and parasite strain. The resulting probabilities were then assessed using the false discovery rate [24], based on the q-value method [25]. This was implemented with the QVALUE module for the R statistical package, with a *q*-value cutoff of 0.1 for statistical significance.

Gene information for each clone ID in a list derived from SAM analyses was extracted from the NIA 15K mouse clone set data file (available from http://lgsun.grc.nia.nih.gov/download.html) using an excel macro called Find_Clone; (available from KS on kspringett_677@hotmail.com) written specifically for this project. This macro is incorporated into an Excel spreadsheet (originating spreadsheet) and, when run, takes a selection of clone IDs (or gene names) and searches a master (or database) spreadsheet (target spreadsheet) for matches. All information for those found is shown in the originating spreadsheet. The name of any clone not found is also displayed in the originating spreadsheet. Clone or gene names with no suffix, _1 suffix and blank space suffix are catered for. To carry out a matching search it is only necessary to copy and paste a column of names into the originating spreadsheet, highlight those required for the search and run the macro. In this way gene names and expression data were extracted from their corresponding files for each clone ID in a list derived from any output of SAM.

Automatic functional profiling of gene lists from SAM, ANOVA and clustering was performed using the web tools ProfCom [26], Genecodis [27] or DAVID [28] that use hypergeometric tests to investigate enrichment for gene ontology terms associated with gene lists. The default options were used to analyse lists of genes for gene ontology. Literature on different genes was initially sourced using iHOP [29]. Updated accession numbers and gene names relating to each clone on the microarray were retrieved using IDconverter [30].

3. Results

3.1. Qs response to NC-Nowra at 6 h.p.i.

At an FDR of 3.2%, SAM identified 134 genes as over expressed in the Qs mouse compared to the controls (one class analyses). No genes were under expressed. A hypergeometric test using Genecodis or ProfCom showed enrichment for a variety of gene ontology terms as shown in Tables 1 and 2. Three transcription factors (Hif1, Stat2, and Stat3) feature in the list and along with Irf9, indicate up regulation of the Jak-Stat pathway in the response to infection by *N. caninum*.

3.2. BALB/c response to NC-Nowra at 6 h.p.i.

At an FDR of 3.6%, SAM identified 1263 genes that were up regulated and 529 that were down regulated in response to infection by NC-Nowra (one class analyses). Of the up regulated genes many mapped to pathways associated with well known diseases such as Parkinson's, Alzheimer's and cancer related pathways. A wide range of biological processes were affected (summarised in Fig. 2 and Tables 3 and 4): 59 genes mapped to (GO:0006412: translation), 85 to (GO:0006350: transcription), plus varying numbers of genes to apoptosis, cell division and a wide range of other gene ontologies. Of the down regulated genes 336 were annotated genes and the only pathway detected was (KEGG) 04120: Ubiquitin mediated proteolysis which contained seven genes (Ube2q2, Ppil2, Nedd4, Btrc, Ube2j2, Ube2r2, Ube2d3). Varying numbers of genes also mapped to gene ontology terms for biological processes or molecular functions: 53 to (GO:0046872: metal ion binding), 35 to (GO:0016740: transferase activity), 20 to (GO:0006468: protein amino acid phosphorylation), 41 to (GO:0008270: zinc ion binding), 36 to (GO:0000166: nucleotide binding) amongst others.

3.3. Cluster analysis of Qs and BALB/c response to NC-Nowra at 6 h.p.i.

Two clustering methods were used to identify genes that were up or down regulated in Qs and/or BALB/c mice infected with NC-Nowra. Data from the 6 h.p.i. time point for Qs and BALB/c mice infected with only NC-Nowra was analysed initially by K means clustering. Sixty-nine genes were identified in the data set that met the chosen criteria. Three main clusters of genes were identified (Fig. 3 and Table 5): those genes that were up regulated in both Qs and BALB/c mice (11 genes) and those that were either up or

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Table 1
Summary of KEGG pathways, identified by Genecodis, which were up regulated in the Qs mouse at 6 h.p.i. in response to NC-Nowra infection.

NG[a]	NGR[b]	Hyp[c]	HypC[d]	Genes	Pathway
4	150	0.000924	0.014092	Ccnd2,Stat3,Irf9,Stat2	(KEGG) 04630: Jak-STAT signaling pathway
2	32	0.003798	0.02896	Rars,Got1	(KEGG) 00330: Arginine and proline metabolism
4	188	0.002117	0.021527	Ccnd2,Actb,Fyn,Col4a1	(KEGG) 04510: Focal adhesion
4	102	0.000215	0.004374	Fcgr1,C1r,C1qa,H2-dma	(KEGG) 05322: Systemic lupus erythematosus
4	94	0.000157	0.004791	B2m,Psme1,Hspa5,H2-dma	(KEGG) 04612: Antigen processing and presentation
3	91	0.002295	0.02	Upp1,Polr2g,Pnpt1	(KEGG) 00240: Pyrimidine metabolism
3	74	0.001268	0.015473	Actb,Fyn,Iqgap1	(KEGG) 04520: Adherens junction
3	115	0.00444	0.030094	Cybb,Actb,Msn	(KEGG) 04670: Leukocyte transendothelial migration
4	43	7.11E−06	0.000433	Psma7,Psme1,Psmb6,Psma4	(KEGG) 03050: Proteasome
2	42	0.006472	0.039478	Rars,Wars	(KEGG) 00970: Aminoacyl-tRNA biosynthesis

[a] Number of genes in the input list (derived from the microarray analyses) with the selected annotated category (total number of genes in the input list was 91).
[b] Number of genes in the reference list with the selected annotated category (total number of genes in the reference list was 31,804).
[c] Hypergeometric pValue.
[d] Corrected hypergeometric pValue.

Table 2
Summary of biological processes, identified by Genecodis, which occur in the Qs mouse at 6 h.p.i. in response to NC-Nowra infection.

NG[a]	NGR[b]	Hyp[c]	HypC[d]	Genes	Biological process
8	276	1.33E−06	0.000244	Clec4e,B2m,Gp49a,Fcgr1,H2-dma,Rnf19b,C1qa,C1r	GO:0006955: Immune response
2	61	0.013281	0.048874	Fyn,Katna1	GO:0001764: Neuron migration
6	384	0.000838	0.038545	Casp12,Scotin,Litaf,Nod1,Dnase1l3,Traf1	GO:0006915: Apoptosis
4	101	0.000207	0.012705	Casp12,Hif1a,Nod1,Traf1	GO:0042981: Regulation of apoptosis
2	28	0.002916	0.03577	C1qa,C1r	GO:0006958: Complement activation, classical pathway
3	80	0.001588	0.048689	Fcgr1,C1qa,C1r	GO:0045087: Innate immune response
1	5	0.014226	0.047591	Irf9	GO:0045351: Type I interferon biosynthetic process
2	33	0.004036	0.043679	Hif1a,Stat3	GO:0042593: Glucose homeostasis
2	36	0.004788	0.048949	Wars,Rars	GO:0006418: tRNA aminoacylation for protein translation
2	3	2.42E−05	0.002231	Psme1,Fcgr1	GO:0019884: Antigen processing and presentation of exogenous antigen
3	100	0.002999	0.034486	Psma7,Psma4,Psmb6	GO:0006511: Ubiquitin-dependent protein catabolic process

[a] Number of genes in the input list (derived from the microarray analyses) with the selected annotated category (total number of genes in the input list was 91).
[b] Number of genes in the reference list with the selected annotated category (total number of genes in the reference list was 31,804).
[c] Hypergeometric pValue.
[d] Corrected hypergeometric pValue.

down regulated in BALB/c mice compared to Qs mice. Of those genes that were up regulated in both Qs and BALB/c mice, three were recognised as being under the control of IFN-γ (Igtp, Irf1, Ifitm3, Table 5). These results indicate that the BALB/c mouse changes the transcription of a much larger number of genes in response to NC-Nowra infection, compared to the Qs mouse. This observation was further confirmed by analysis of the expression data at the 6 h.p.i. time point from Qs and BALB/c mice infected with NC-Nowra by

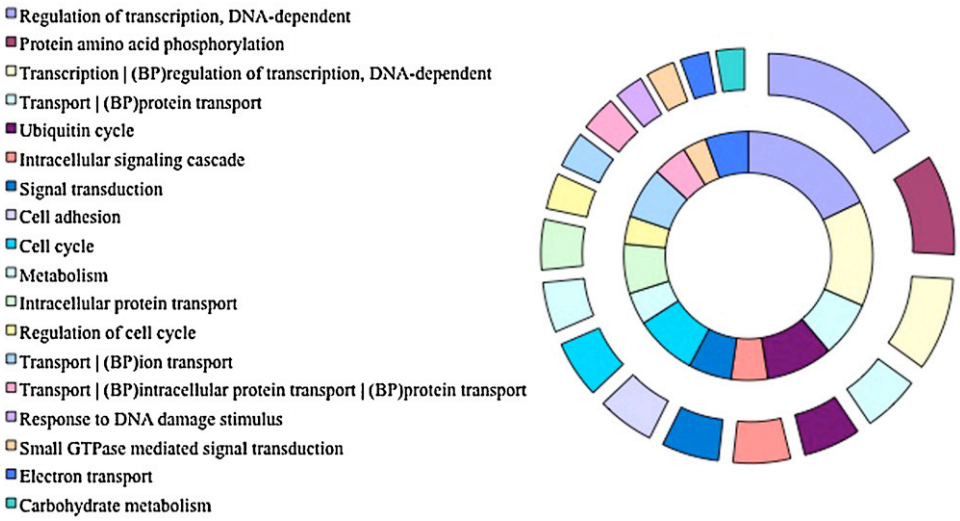

Fig. 2. Example of the biological processes affected at 6 h.p.i. of BALB/c mice with NC-Nowra. The inner circle represents genes up regulated, while the outer ring represents genes down regulated. In this instance the two groups differ in genes associated with carbohydrate metabolism, response to DNA damage, cell adhesion and protein amino acid phosphorylation.

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Table 3
Summary of KEGG pathways, identified by Genecodis, which were up regulated in the BALB/c mouse at 6 h.p.i. in response to NC-Nowra infection.

NG[a]	NGR[b]	Hyp[c]	HypC[d]	Pathway
28	134	6.36E−18	8.97E−16	(KEGG) 00190: Oxidative phosphorylation
29	178	1.81E−15	1.27E−13	(KEGG) 05016: Huntington's disease
25	135	7.41E−15	3.48E−13	(KEGG) 05012: Parkinson's disease
13	42	2.19E−11	6.18E−10	(KEGG) 03010: Ribosome
18	93	2.01E−11	7.10E−10	(KEGG) 00970: Aminoacyl-tRNA biosynthesis
18	109	3.15E−10	7.41E−09	(KEGG) 04110: Cell cycle
12	43	5.04E−10	1.01E−08	(KEGG) 03050: Proteasome
22	186	2.58E−09	4.54E−08	(KEGG) 05010: Alzheimer's disease
16	132	2.69E−07	4.21E−06	(KEGG) 04120: Ubiquitin mediated proteolysis
8	32	1.04E−06	1.46E−05	(KEGG) 00330: Arginine and proline metabolism
22	316	2.33E−05	0.000299	(KEGG) 05200: Pathways in cancer
10	89	8.92E−05	0.001048	(KEGG) 05215: Prostate cancer
15	188	0.000101	0.001099	(KEGG) 04510: Focal adhesion
9	75	0.00012	0.001124	(KEGG) 00260: Glycine, serine and threonine metabolism
7	44	0.000113	0.001141	(KEGG) 05220: Chronic myeloid leukemia
9	83	0.000261	0.002303	(KEGG) 05222: Small cell lung cancer
8	72	0.000482	0.004	(KEGG) 05212: Pancreatic cancer
8	74	0.000581	0.00455	(KEGG) 04520: Adherens junction
5	29	0.000752	0.005303	(KEGG) 04670: Leukocyte transendothelial migration
10	115	0.000725	0.005378	(KEGG) 00252: Alanine and aspartate metabolism
3	9	0.001228	0.007871	(KEGG) 05322: Systemic lupus erythematosus
9	102	0.001189	0.007982	(KEGG) 00400: Phenylalanine, tyrosine and tryptophan biosynthesis
4	20	0.001455	0.008918	(KEGG) 00271: Methionine metabolism
7	67	0.001553	0.009127	(KEGG) 04115: p53 signaling pathway
4	21	0.001761	0.009932	(KEGG) 00360: Phenylalanine metabolism
3	11	0.002322	0.011695	(KEGG) 00240: Pyrimidine metabolism
3	11	0.002322	0.011695	(KEGG) 00290: Valine, leucine and isoleucine biosynthesis
8	91	0.002248	0.012193	(KEGG) 00272: Cysteine metabolism
4	27	0.004577	0.022254	(KEGG) 00220: Urea cycle and metabolism of amino groups
6	64	0.005703	0.026805	(KEGG) 04920: Adipocytokine signaling pathway
2	5	0.006141	0.027058	(KEGG) 00950: Alkaloid biosynthesis I
2	5	0.006141	0.027058	(KEGG) 00750: Vitamin B6 metabolism
6	68	0.007648	0.032678	(KEGG) 05211: Renal cell carcinoma
7	90	0.008103	0.033602	(KEGG) 04912: GnRH signaling pathway
4	34	0.010516	0.042365	(KEGG) 04130: SNARE interactions in vesicular transport
9	146	0.012617	0.049418	(KEGG) 04310: Wnt signaling pathway

[a] Number of genes in the input list (derived from the microarray analyses) with the selected annotated category (total number of genes in the input list was 809).
[b] Number of genes in the reference list with the selected annotated category (total number of genes in the reference list was 31,804).
[c] Hypergeometric pValue.
[d] Corrected hypergeometric pValue.

SAM using a two class (unpaired) response analysis. At an FDR of 3.71%, four genes were identified as down regulated in the BALB/c mouse and 86 up regulated, in comparison to the response seen in the Qs mouse. Sixty-one genes could be mapped to gene ontology terms and automatic functional profiling of the up regulated genes mapped several of them to cell cycle and cancer related pathways (Table 6).

3.4. Differences in expression of the Qs and BALB/c mice

In order to investigate further the hypothesis that transcriptional responses to infection are different in the two mouse types, multivariate analysis of all samples (e.g. all Qs and BALB/c mice infected with *N. caninum*-NC-Nowra or NC-Liverpool) at the 6 h.p.i. time point (a total of 12 arrays) was conducted. A significant difference in gene expression profiles was demonstrated between the BALB/c and Qs mouse types ($P = 0.0019$). The observation of a significant difference between the BALB/c and Qs mouse types indicated that the observed patterns of gene expression in these mice were distinguishably different from each other.

Analyses of all data from mice infected with either NC-Nowra or NC-Liverpool by SAM (two class unpaired using an FDR of 3.83%) identified 268 genes that were highly induced among Qs and BALB/c mice. Of these 151 were annotated genes which

Table 4
Summary of the biological processes, identified by Genecodis, which were up regulated in the BALB/C mouse at 6 h.p.i. in response to NC-Nowra infection.

NG[a]	NGR[b]	Hyp[c]	HypC[d]	Biological process
20	523	4.25E−08	1.83E−05	GO:0006468: Protein amino acid phosphorylation
3	13	0.000175	0.037672	GO:0019827: Stem cell maintenance
9	241	0.000284	0.040769	GO:0007242: Intracellular signaling cascade
7	150	0.00036	0.038648	GO:0016568: Chromatin modification
4	39	0.000364	0.031332	GO:0043687: Post-translational protein modification
4	42	0.000486	0.034811	GO:0051246: Regulation of protein metabolic process
12	451	0.000656	0.040326	GO:0007049: Cell cycle
2	5	0.000743	0.039929	GO:0046686: Response to cadmium ion
12	464	0.00084	0.040117	GO:0015031: Protein transport
2	6	0.001108	0.047639	GO:0045732: Positive regulation of protein catabolic process

[a] Number of genes in the input list (derived from the microarray analyses) with the selected annotated category (total number of genes in the input list was 277).
[b] Number of genes in the reference list with the selected annotated category (total number of genes in the reference list was 31,804).
[c] Hypergeometric pValue.
[d] Corrected hypergeometric pValue.

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Table 5
K means clustering of data from Qs and BALB/c mice infected with NC-Nowra at 6 h.p.i. The table summarises data for three main clusters identified and their fold change in expression. The cluster numbers are those represented in Fig. 3. Cluster 1 represents those genes up regulated in all mice infected with *N. caninum*.

Clone name	Gene	QS[a]	BC[a]	Gene description
1				
H3059F12	Tgtp	4.82	4.79	T-cell specific GTPase
H3092A03		4.79	4.59	
H3004E09		4.29	3.84	
H3157D02	Igtp	3.78	3.76	Interferon gamma induced GTPase
H3085A11	Pbef1	3.36	3.36	Pre-B-cell colony-enhancing factor 1
H3095F01		2.85	3.66	
H3016C07	Marcksl1	2.19	3.41	MARCKS-like 1
H3139C11	Ms4a6d	2.58	3.32	Membrane-spanning 4-domains, subfamily A, member 6D
H3107D05	Ifitm3	2.77	3.48	Interferon induced transmembrane protein 3
H3050H06	Irf1	3.07	3.34	Interferon regulatory factor 1
H3052F12		6.32	10.63	
2				
BALB/c < Qs				
H3156A08		−1.59	−1.85	RIKEN cDNA 9330120H11
H3053F05		−1.21	−1.83	
H3051A01		−1.23	−2.14	
H3052B12		−1.07	−1.77	
H3013B04	Aadacl1	1.07	−1.97	Arylacetamide deacetylase-like 1
H3054E03		−1.39	−1.96	
H3053F04		−1.37	−2.38	
H3052D04		−1.44	−1.77	
H3048H05	Epb4.1l4b	−1.01	−1.92	Erythrocyte protein band 4.1-like 4b
H3055G06	Npc1	1.04	−3.61	Niemann Pick type C1
3				
BALB/c > Qs				
H3091E10	Nupr1	1.25	2.28	Nuclear protein 1
H3013D11	Mt2	1.51	3.14	Metallothionein 2
H3123G07	Zfp804a	−1.11	1.84	Zinc finger protein 804A
H3126B09		−1.09	1.74	
H3005F07		−1.43	1.47	
H3114G05		−1.38	1.58	
H3130H07		−1.30	1.52	
H3118G07	Eef1b2	−1.29	1.48	Translation elongation factor 1 beta 2
H3073G07	Gzmm	−1.35	1.22	Granzyme M (lymphocyte met-ase 1)

[a] Fold change in either Qs or BALB/c mice. A negative sign indicates down regulation of expression.

mapped to gene ontology terms. Most of these genes with altered expression levels were from the BALB/c mouse. Only 23 genes were identified where the expression in Qs mice was significantly upregulated; 18 of these represented annotated genes (Sept6, Naglu, Rnf130, Araf, Klhl5, Jarid1c, Atp8b2, Gm2a, Mum1l1, Thop1, Tppp3, Nr2f2, Tubgcp5, Gpc3, Abi1, Zfp276, Tsen2 and Rab24).

A 2-factor orthogonal analysis of variance for each clone on the array identified 75 sequences that were more highly induced in the BALB/c and Qs mice (Table 7). Sixteen showed increased expression levels in Qs mice (and many of these were the same genes identified by SAM above), while the remainder (59) were highly induced in BALB/c. Five of the genes showing altered expression in the Qs mouse were associated with gene ontology terms associated with transcription and regulation of transcription (Klhdl5, Rere, Jarid1c, Atp8b2, Zfp276; Table 8). In contrast genes showing increased induction in BALB/c mice were associated with functions relating to cell cycle, cell division and nucleotide (ATP) binding (e.g. Ercc3, Jak2, Rad50, Ddx47, Rbm7, Mapk6, Smc4).

Using Cluster 3.0, data from all arrays was filtered for genes showing data >±1 in arrays from all Qs and BALB/c mice. Fourteen genes were identified in a single cluster that were all induced in both mice types (Ly6a, Nulm, Anxa4, Samhd1, Pbef1, Igtp, Irf1, Ifitm3, Ms4a6d, H3092A03, H3095F01 and H3004E09). Clustering was also performed using WinGimm and a cluster containing eight genes was identified that was also up regulated in all groups. This cluster contained genes similar to that identified by K means clustering (Ly6a, Pbef1, Stfa1, Igtp, Ifitm3, Irf1). A second cluster of up regulated genes contained 169 genes that mapped to a wide range of biological processes by functional profiling. It includes several members of the Jak-Stat pathway (Socs3, Stat2, Stat3, Ccnd2), complement cascade (C1qa, C1s, C1r) and the ubiquitin-dependent proteasome (Psma2, Psmb6, Psma4, Psma5, Psma7). Another cluster of genes were up regulated in BALB/c mice and

Table 6
Summary of KEGG Pathways, identified by Genecodis, following a two class (unpaired) SAM analyses of expression data from the Qs and BALB/c mice infected with NC-Nowra.

NG[a]	NGR[b]	Hyp[c]	HypC[d]	Genes	Pathway
4	109	5.86E−05	0.00126	Mcm3,Mad2l1,Ccnb2,Cdk4	(KEGG) 04110: Cell cycle
6	316	3.22E−05	0.001384	Brca2,Cdc42,Aurka,Cdk4,Ctbp1,Lamb1-1	(KEGG) 05200: Pathways in cancer
2	50	0.004178	0.044911	Gpx1,Mgst3	(KEGG) 00480: Glutathione metabolism
3	72	0.000364	0.005223	Brca2,Cdc42,Cdk4	(KEGG) 05212: Pancreatic cancer

[a] Number of genes in the input list (derived from the microarray analyses) with the selected annotated category (total number of genes in the input list was 61).
[b] Number of genes in the reference list with the selected annotated category (total number of genes in the reference list was 31,804).
[c] Hypergeometric pValue.
[d] Corrected hypergeometric pValue.

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Table 7
A summary of the genes identified by a 2-factor orthogonal analysis of variance that differ in expression between the Qs and BALB/c mouse. Genes altered in expression in the different mice types are shown, as well as their mean fold change in expression.

Clone Name	Gene	QS[a]	BC[a]	Gene Description
BALB/c > Qs				
H3013D11	Mt2	1.51	3.14	Metallothionein 2
H3043F05	Dnajb1	0.92	1.12	
H3043F12	Fech	0.85	1.24	
H3051A08	Nploc4	0.84	1.24	Nuclear protein localization 4 homolog (S. cerevisiae)
H3058B10	Cetn4	0.79	1.34	Centrin 4
H3067A04		0.72	1.47	
H3067C09	Smc4	0.77	1.06	Structural maintenance of chromosomes 4
H3067C10		0.80	1.37	
H3067C11		0.71	1.46	
H3067C12	Jak2	0.86	1.34	Janus kinase 2
H3067G06		0.77	1.45	
H3067G12		0.78	1.37	RIKEN cDNA M130001O18
H3069A06	Tacc3	0.83	1.29	Transforming, acidic coiled-coil containing protein 3
H3069C05	Rnf20	0.87	1.35	Ring finger protein 20
H3069D10	Cdca5	0.84	1.29	Cell division cycle associated 5
H3069E07		0.71	1.36	
H3069F05		0.84	1.29	
H3069G09	Foxn2	0.87	1.38	forkhead box N2
H3071D07		0.79	1.36	RIKEN cDNA 9330102E08
H3071E04		0.76	1.41	
H3072C11		0.72	1.45	RIKEN cDNA 4922501C03
H3072D04		0.87	1.25	RIKEN cDNA 4121402D02
H3088D09	Ece1	0.97	1.31	Endothelin converting enzyme 1
H3088H10	Tsfm	0.99	1.20	Ts translation elongation factor, mitochondrial
H3091D11	Dedd	0.97	1.23	Death effector domain-containing
H3091E01		0.90	1.17	
H3094F03	Sfmbt1	0.92	1.24	Scm-like with four mbt domains 1
H3101E03	Pofut2	1.00	1.19	Protein O-fucosyltransferase 2
H3101F12	Atg4b	0.90	1.20	Autophagy-related 4B (yeast)
H3105E09	Ddx47	0.93	1.45	DEAD (Asp-Glu-Ala-Asp) box polypeptide 47
H3106D11	Ift122	0.83	1.39	
H3106F10		0.84	1.41	
H3106G06	Bnip1	0.88	1.34	BCL2/adenovirus E1B interacting protein 1, NIP1
H3108A08	Card10	0.90	1.23	Caspase recruitment domain family, member 10
H3108B08	Rbm7	0.93	1.40	RNA binding motif protein 7
H3108E02	Mapk6	0.91	1.24	Mitogen-activated protein kinase 6
H3108F09	Tom1l2	0.73	1.78	Target of myb1-like 2 (chicken)
H3108F10	Rcc2	0.92	1.31	Regulator of chromosome condensation 2
H3108G09	Ctbp1	0.93	1.31	C-terminal binding protein 1
H3109H03	Ell2	0.81	1.06	Elongation factor RNA polymerase II 2
H3110F03	Dmwd	0.93	1.18	
H3113A06	Gja1	0.86	1.14	Gap junction protein, alpha 1
H3113G01	D12Ertd551e	0.95	1.14	DNA segment, Chr 12, ERATO Doi 551, expressed
H3114G10		0.73	1.29	
H3116G02		0.93	1.17	
H3119E10	Rad50	0.73	1.31	RAD50 homolog (S. cerevisiae)
H3119F04		0.77	1.45	
H3119G11		0.74	1.42	Clone H3119G11, mRNA sequence
H3121B11	Ard1,Arhgap4	0.74	1.11	N-acetyltransferase ARD1 homolog (S. cerevisiae)
H3124C06		0.76	1.56	
H3124C11	Phc2	1.30	1.81	Polyhomeotic-like 2 (Drosophila)
H3130C11	Ube2o	0.92	1.30	Ubiquitin-conjugating enzyme E2O
H3132H05	Ercc3	1.04	1.19	Excision repair cross-complementing rodent repair deficiency, complementation group 3
H3136E12	Ucp2	0.96	1.57	Uncoupling protein 2 (mitochondrial, proton carrier)
H3138G08	Slc25a37	0.74	1.12	Solute carrier family 25, member 37
H3142D06	Pttg1	0.96	1.40	Pituitary tumor-transforming 1
H3150E05	Rai12	0.82	1.45	Retinoic acid induced 12
H3150F08	Thoc5	0.47	1.10	THO complex 5
Qs > BALB/c				
H3017B04	Atp8b2	1.13	0.94	Atpase, class I, type 8B, member 2
H3021G05	Rere	1.21	1.02	Arginine glutamic acid dipeptide (RE) repeats
H3035D04	Gm2a	0.85	0.65	GM2 ganglioside activator protein
H3049A10	Iqsec1	0.94	0.86	IQ motif and Sec7 domain 1
H3049G09	Zfp276	1.03	0.79	Zinc finger protein (C2H2 type) 276
H3051A04	Il16	1.01	0.76	Interleukin 16
H3052B12		0.95	0.55	
H3063C11		1.09	0.64	
H3102C05		1.25	0.92	RIKEN cDNA E030022I16
H3104C04		0.98	0.73	
H3104G11	G6pc3	1.18	0.92	Glucose 6 phosphatase, catalytic, 3

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Table 7 (*Continued*)

Clone Name	Gene	QS[a]	BC[a]	Gene Description
H3107C08	Tppp3	0.98	0.74	Tubulin polymerization-promoting protein family member 3
H3107G10	Naglu	1.02	0.79	Alpha-N-acetylglucosaminidase (Sanfilippo disease IIIB)
H3120G05	Sept6	1.07	0.86	Septin 6
H3132B07	Jarid1c	0.97	0.68	
H3132D08	Klhl5	1.16	0.91	Kelch-like 5 (Drosophila)

[a] Fold change in either Qs or BALB/c mice.

contained 123 genes with functions in the cell cycle (Rad50, Clasp2, 2700078E11RIK), nucleotide binding (Rad50, Rbm7, Ddx47), DNA binding (Aebp2, Runx1t1, Foxn2, Sox11, B020006M18RIK, Obox1, 2700078E11RIK) and regulation of transcription.

Analysis of variance did not identify genes that were uniquely expressed in either mice types in response to infection by either NC-Nowra or NC-Liverpool.

4. Discussion

Microarrays are a popular technology for the study of changes in gene expression and a wide number of applications exist for their use. The identification of specific genes of interest, whose transcription is altered in conditions under study, is the primary objective of most microarray experiments. However, the potential uses of microarrays are varied and numerous. For example, Simon et al. identified three main classes of microarray application; class comparison (contrasting expression profiles of various classes of specimen); prognostic prediction (using expression profiles combined with other factors to predict clinical outcomes); and class discovery (in which important subtypes of specimens are distinguished by expression patterns) [31]. Our experiment applies microarrays to class comparison.

The study of parasites has been aided by the introduction of microarray technology. There are now numerous reports on the use of microarray technology for the study of host responses to Apicomplexa including *Plasmodium* [32–34], *Toxoplasma* [6,35], and *Eimeria* [5,36]. These studies have primarily focussed on understanding events that occur during the interaction between host and parasite as well as the analysis of many elements of the immune response to infection. These advances have improved our knowledge of the mechanisms of pathogenesis as well as aided in the identification of the mechanisms of resistance to parasitic infection.

The immune responses of Swiss Webster and Qs mice to infection by *N. caninum* are characterised by a Th1 response with high levels of IL-12 and IFN-γ [37–43]. An increased IgG2a:IgG1 ratio is

Table 8
Genes identified by 2-factor orthogonal analysis of variance were analysed for enrichment of associated gene ontology terms. Gene lists were analysed using Genecodis.

NG[a]	NGR[b]	Hyp[c]	HypC[d]	Genes	GO	GO description
Qs > BALB/c[a]						
4	2540	0.011924	0.035772	Atp8b2,Jarid1c,Zfp276,Rere	GO:0046872	Metal ion binding
5	3855	0.009897	0.038174	Atp8b2,Jarid1c,Rere,Sept6,Il16	GO:0005515	Protein binding
3	1594	0.019669	0.044255	Jarid1c,Zfp276,Rere	GO:0003677	DNA binding
3	1453	0.015354	0.037688	Atp8b2,Naglu,G6pc3	GO:0016787	Hydrolase activity
1	63	0.023517	0.048844	Jarid1c	GO:0016702	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen
1	13	0.004895	0.022027	Gm2a	GO:0008047	Enzyme activator activity
1	39	0.014619	0.039471	Atp8b2	GO:0016820	Hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
1	29	0.010889	0.036751	Atp8b2	GO:0015662	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism
1	12	0.004519	0.030504	Atp8b2	GO:0004012	Phospholipid-translocating ATPase activity
1	5	0.001885	0.016967	Gm2a	GO:0004563	Beta-N-acetylhexosaminidase activity
1	13	0.004895	0.022027	Iqsec1	GO:0005086	ARF guanyl-nucleotide exchange factor activity
1	3	0.001132	0.015276	G6pc3	GO:0004346	Glucose-6-phosphatase activity
1	1	0.000377	0.010187	Naglu	GO:0004561	Alpha-N-acetylglucosaminidase activity
BALB/c > Qs[b]						
7	1623	0.004994	0.034406	Ercc3,Jak2,Rad50,Ddx47,Rbm7,Mapk6,Smc4	GO:0000166	Nucleotide binding
6	1218	0.005024	0.03115	Ercc3,Jak2,Rad50,Ddx47,Mapk6,Smc4	GO:0005524	ATP binding
2	115	0.010155	0.039351	Ercc3,Ddx47	GO:0004386	Helicase activity
1	2	0.002639	0.023378	Ell2	GO:0008159	Positive transcription elongation factor activity
1	7	0.009208	0.038062	Ctbp1	GO:0048037	Cofactor binding
1	9	0.011824	0.043124	Ercc3	GO:0004003	ATP-dependent DNA helicase activity
1	4	0.005272	0.025144	Jak2	GO:0004718	Janus kinase activity
1	3	0.003957	0.030664	Fech	GO:0030350	Iron-responsive element binding
1	1	0.001321	0.020469	Fech	GO:0046906	Tetrapyrrole binding
1	1	0.001321	0.020469	Fech	GO:0004325	Ferrochelatase activity
1	4	0.005272	0.025144	Gja1	GO:0005243	Gap junction channel activity
1	4	0.005272	0.025144	Slc25a37	GO:0005381	Iron ion transmembrane transporter activity
1	2	0.002639	0.023378	Jak2	GO:0005143	Interleukin-12 receptor binding
1	5	0.006586	0.029166	Cetn4	GO:0031683	G-protein beta/gamma-subunit binding
1	1	0.001321	0.020469	Gja1	GO:0022857	Transmembrane transporter activity
1	2	0.002639	0.023378	Pofut2	GO:0046922	Peptide-O-fucosyltransferase activity

[a] Number of genes in the input list (derived from the microarray analyses) with the selected annotated category (total number of genes in the input list was 12[a] or 42[b]).
[b] Number of genes in the reference list with the selected annotated category (total number of genes in the reference list was 31,804).
[c] Hypergeometric pValue.
[d] Corrected hypergeometric pValue.

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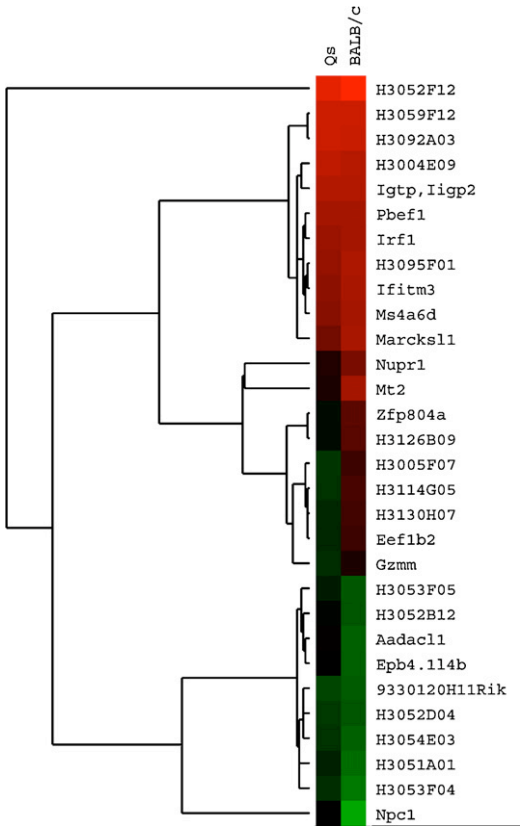

Fig. 3. Heatmap showing the clustering of expression data from BALB/c and Qs mice infected with NC-Nowra. The example (derived from mean expression values) shows three main clusters (numbered 1–3). Cluster 1 contains genes up regulated in both mice types and contains interferon responsive genes; cluster 2 contains genes where the observed expression changes in BALB/c mice > Qs mice; cluster 3 contains genes where expression in BALB/c mice < Qs mice.

also associated with the resistant phenotype, indicating that both Th1 and Th2 responses occur, but the Th1 response predominates [44]. The increased production of Th1 cytokines has been observed very early in infection, with an approximately 150-fold increase in IL-12, and approximately 40-fold increase in IFN-γ observed 6 h after infection in A/J mice [41]. Increases in IFN-γ production in the same study were found to also occur at 10 days post infection [41]. In more susceptible mice strains, such as BALB/c [14], a decreased production of IFN-γ and increased production of IL-4 is seen [42]. In addition, a predominance of IgG1 antibodies, indicative of a Th2 response, is observed [38,40]. Susceptibility is also associated with depletion of IFN-γ or IL-12 [37,39,43], in which increased production of IL-10 (a Th2 cytokine) and a predominance of IgG1 is observed.

In this study we investigated the nature of the transcriptional changes occurring in the mouse in response to infection. Previous studies on other parasitic protozoa have shown that it is possible to identify the host pathways that are turned on or off in response to infection; for example the predominance of interferon-related responses that occur in the mouse brain in response to *Plasmodium berghei* infection [34]. Detecting changed gene expression using microarrays is inherently challenging, and so the approaches adopted in this study used a variety of methods to identify differentially expressed genes, including SAM, ANOVA and clustering methods. Clusters of genes have a strong tendency to be co-expressed and share common roles in biological processes that are changing in response to a stimulus such as an infection [21].

In order to identify the basis of host responses to *N. caninum* infection in the mouse, infections of BALB/c and Qs mice with the NC-Nowra isolate of *N. caninum* was performed. We focussed our efforts on this comparison; however we also performed infections with NC-Liverpool at the same time. Previous studies on experimental mouse infections with NC-Liverpool and NC-Nowra [15,40,42] showed that BALB/c mice were more susceptible to infection with NC-Liverpool, succumbing to infection and dying or requiring euthanasia within 27 days post infection. Infection of BALB/c mice with NC-Nowra, and infection of Qs mice with both NC-Liverpool and NC-Nowra, does not result in similar levels of morbidity and death, but rather in development of resistance to infection and potentially long term chronic infection.

It was predicted that IFN-γ related responses would be identified through the course of this study on spleen expression, and to some extent this hypothesis was proved true. Changes in the Jak-STAT signaling pathway (Stat2 and 3) and the identification of Irf and other IFN-γ regulated molecules such as GTPases show the influence of IFN-γ in the mouse response to *N. caninum*. All these genes were up regulated and in heat maps they clustered together. Such responses stimulated by IFN-γ are also known to occur in the host cell response to infection by *T. gondii* [31,41].

Another conclusion to emerge from this study was that BALB/c and Qs mice differ significantly in their responses to infection by *N. caninum*. Mice were infected with either NC-Nowra or NC-Liverpool so that strain independent responses could be investigated. Several different types of analyses of the data taken at the 6 h.p.i. time point showed that many more genes were either up or down regulated in the BALB/c mouse compared to the Qs mouse, and many more pathways were affected. Such observations were confirmed by a SAM two class (unpaired) analysis, clustering of expression data, and by a 2-factor orthogonal analysis of variance. All analyses yielded gene lists that showed differences in expression amongst Qs and BALB/c mice, as well as a group of genes that were significantly up regulated in both mice types. The biological processes affected by genes that were differentially expressed between the two mouse types were often linked to the cell cycle, cell division or mitosis. Such gene ontology terms of the genes involved were also linked with cancer-associated pathways. We do not suggest for one moment that *N. caninum* infection is responsible for cancer merely that several of the biological processes affected are central to an animal's response to this pathogen and so are common. For example, six genes were linked to regulation of transcription (Trp53, Hif1A, Nfkb1, Smad3, Stat3, Cdtp1) that also share gene ontology terms linked to cancer.

Several genes that were altered in expression also mapped to other gene ontology terms associated with well-known diseases such as ((KEGG) 05012: Parkinson's disease, (KEGG) 05010: Alzheimer's disease, (KEGG) 05322: Systemic lupus erythematosus). With respect to the Parkinson's disease pathway, those genes involved with the host response to *N. caninum* were linked to the mitochondrial electron transport chain (Uqcrc1, Ndufs2, Ufb5, Ufs4, Ufv2, Ufb6, Ufb9, Ufa7, Crb, X7a2) and ATP synthesis coupled proton transport (ATP6v0d1, P5a1, P5c1, P5b, Tp5g3) amongst others. This was also true for the Alzheimer's disease pathway.

The observation that Jak2, which is a central member of the Jak-Stat pathway, differs in expression between the two mouse types suggests a fundamental difference in the response mechanisms involved. A wide range of cytokines, in addition to IFN-γ, and hormone-like cytokines such as prolactin activate Jak2 which plays a central role in numerous cell functions such as production of reactive oxygen species, lipid oxidation, B cell development and erythropoiesis [45]. Stat3 is also known to function in a central role in cellular respiration through control of expression of electron transport complexes [46] as well as regulation of IL-12 and IL-10 expression [47] that may impact on the outcome of immunity to *N.*

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caninum. Stat3 also regulates the expression of molecules, such as cyclin D1 and Bcl that function to control the cell cycle. Hif1a also is known to regulate cell cycle progression as well as IFN-γ production by macrophages during an inflammatory response [48].

The nature of the Qs mouse response to infection by *N. caninum* at the 6 h.p.i. time point is worth further comment. In comparison to the BALB/c mouse relatively few pathways were affected in the Qs mouse by *N. caninum* infection. We therefore speculate that either the absence or the timing of the host response may be linked to the resistance phenotype shown by the Qs mouse. This was a surprising observation, and warrants further investigation in order to understand the basis of host resistance to *N. caninum*. The inclusion of additional time points post infection would appear worthwhile.

Intriguingly, it is possible to speculate that the gene analyses presented here suggest leads for further study into the mechanisms of pathogenesis associated with neosporosis. Although infection of mice with NC-Nowra causes little or no signs of clinical disease, the molecular functions, processes and pathways identified through these studies clearly warrant further investigation for their role in the development of protective immunity as well as pathogenesis. More detailed analyses of mouse infections with NC-Liverpool, which causes severe disease in the BALB/c mouse, is also likely to provide additional support for these and other pathways and their association with the disease neosporosis.

Finally, all the analyses conducted here were completed using a basic laptop computer with Internet access. The rate limiting step in these analyses was the extraction of gene information from the NIA 15K mouse clone set data file (updated using IDconverter), which was solved by the introduction of an excel macro. This macro, which filters data at a rate of approx. 25–30 genes/min, greatly simplifies the data extraction process thereby reducing dependence on the need for more expensive and wider scale computing support.

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