Expression profiling reveals differences in immuno-inflammatory gene expression between the two disease forms of sheep paratuberculosis

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Title: Expression profiling reveals differences in immuno-inflammatory gene expression between the two disease forms of sheep paratuberculosis

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Many thanks to the referees for positive and informative comments. We have modified the manuscript in accordance with these comments.

Reviewer #1.

1. The title has now been changed to better reflect the content of the paper.

2. We have amended the Abstract to make unambiguous that we have measured transcripts and not protein.

3. Clinical signs clarified etc. Have explained and referenced IS900

4. We have included descriptions of results for chemokines/receptors/TCR molecules in the Results. I have reformatted Table 1 so that the genes are in strict alphabetic order; it is not possible to arrange them in comparison order as there would be some duplication.

5. The point of the RT-qPCR is validation of the array results – and this it does (section 3.2). The rationale for their selection is now explained in section 3.2.

6. IGFBP6 was represented in the array (see supplementary data Array Express) – but in these experiments it did not show differential expression of >1.5 and p≤0.05 in any comparison. As referenced in the manuscript we had previously identified it in a preliminary series of experiments (Roupaka MSc thesis 2004).

7. There was strong correlation between array and RT-qPCR (section 3.2) but difference in absolute values – this is a common finding and comments/references etc have now been added to the Discussion.

8. MMP9 and TYROBP were not discussed specifically because the purpose of the paper was not to cover all genes exhaustively, but to focus on areas of particular interest. If required I can add a paragraph to the discussion – but it will not add much.

Minor comments…. Asterisks explained in Table 1. SDHA/YWHAZ defined.
Reviewer #2.

1. See comment 1 and 2 (Reviewer #1).
2. Abbreviations now defined.
3. Further detailed choice of housekeeping genes. The two used were selected from six; including ribosomal protein L18, ATPase, GAPDH, \(\beta\) actin as well as SDHA and YWHAZ. I can list these in the text if you wish – but it is not very relevant to the paper.
4. MIQE guidelines followed for abbreviations.
5. Statistics further explained, end section 2.5.
6. References added and clarified. Rather than adding several references for well known facts I have included a reference for an informative review (Campbell \textit{et al.} 2003).
7. Fig 1 legend better described.

Very many thanks,
Expression profiling reveals differences in immuno-inflammatory gene expression between the two disease forms of sheep paratuberculosis

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Abstract

Paratuberculosis is a chronic enteropathy of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP); infection of sheep results in two disease forms – paucibacillary (tuberculoid) and multibacillary (lepromatous) associated with the differential polarization of the immune response. In addition the majority of MAP-infected animals show no pathology and remain asymptomatic. Microarray and real-time RT-qPCR analyses were used to compare gene expression in ileum from sheep with the two disease forms and asymptomatic sheep, to further understand the molecular basis of the pathologies. Microarrays identified 36 genes with fold-change of >1.5 and P ≤ 0.05 in at least one comparison; eight candidates were chosen for RT-qPCR validation. Sequence analysis of two candidates, CXCR4 and IGFBP6, identified three SNPs in each; five were found in all three forms of disease and showed no significant relationship to pathological type. The IGFBP6 G3743 A SNP was not detected in asymptomatic sheep. The data show that the two forms of disease are associated with distinct molecular profiles highlighted by the differential expression of chemokine and chemokine receptor transcripts, the protein products of which might be implicated in the different cell infiltrates of the pathologies. The cells within the lesions also show evidence of abnormal activation; they express high levels of cytokine transcripts but have reduced expression levels of transcripts for T cell receptor associated molecules.

Keywords:
*Mycobacterium avium* subsp. *paratuberculosis*
MAP
Sheep
Chemokines
Genomics
Pathology
1. Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a facultative intracellular bacterium that primarily infects macrophages and is the causative agent of paratuberculosis (Johne's disease), a common enteropathy of ruminants. In cattle an extended preclinical phase is dominated by a type 1 immune response that is followed by progression to fatal granulomatous disease, which is linked to a switch to a type 2 response and the production of non-protective IgG1 antibodies (Koets et al., 2002; Kurade et al., 2004). In sheep both type 1 and type 2 responses give rise to disease states (Clarke, 1997) and there seems to be no progression from the paucibacillary (type 1 or tuberculoid) to the multibacillary (type 2 or lepromatous) disease; both are fatal. As with homologous pathologies in tuberculosis and leprosy, the cytokines that dominate paucibacillary paratuberculosis are IFN and IL-12, while IL-10 dominates multibacillary disease (Smeed et al., 2007). Only a minority of infected (IS900+) sheep progress to disease and the majority remain asymptomatic with no pathology (Koets et al., 2002; Smeed et al., 2007; de Silva et al., 2009).

We hypothesize that the polarization of the immune response is critical to the clinical outcome of paratuberculosis infection. Intestinal tissue damage that results from a type 1 response involves lymphocyte and eosinophil infiltration and is fundamentally different to that caused by a type 2 response, which leads to epithelioid macrophage infiltration and dissemination of infection (Smeed *et al.*, 2007). The related disease leprosy also has this dichotomy of immunology and pathology (Meisner *et al.*, 2001). Furthermore there is a clear relationship between host genetics and disease type (Fortin *et al.*, 2007); many genes have been implicated but one that has received much attention is solute carrier family 11 (SLC11A1, NRAMP-1 or Bcg), which is linked to both tuberculosis susceptibility (Vidal *et al.*, 1993) and discrimination of the two forms of human leprosy (Meisner *et al.*, 2001).
Resistance to MAP infection in mice is also partly linked to this gene, with animals carrying the Bcg' allele (e.g. C3H) being refractory to infection (Veazey et al., 1995).

The principal cell populations associated with mycobacterial infection are cells of the macrophage lineage, a major function of which is the activation of T cells and initiation of the immune response. Antigen-presenting cell subsets play a crucial role in the polarization of responses through the differential expression of IL-10 and IL-12 (Pulendran et al., 1999). They also show differential expression of pattern recognition receptors (PRRs) (Nalubamba et al., 2008), and differential PRR activation can tailor the immune response (Kapsenberg, 2003). Both TLR2 and CARD15 (NOD2) have been shown to be up-regulated in clinical paratuberculosis (Nalubamba et al., 2008) and involved in both recognition (Ferwerda et al., 2007) and response (Weiss et al., 2001) to MAP. Indeed, there is growing evidence for linkage of TLR2 and/or CARD15 mutations and susceptibility to human mycobacterial diseases (Bochud et al., 2003; Ben-Ali et al., 2004) and bovine MAP infection (Mucha et al., 2009).

To begin to unravel the complex interactions between MAP and the immune system that gives rise to bovine paratuberculosis, Coussens and colleagues have used bovine leukocyte microarrays to examine transcript changes in peripheral blood mononuclear cells (PBMC) (Coussens et al., 2002) and macrophages (Murphy et al., 2006). In addition they have examined cattle ileal tissue (Aho et al., 2003) in order to understand the mechanisms that lead to intestinal tissue damage and have identified a number of genes involved in the immunology and pathogenesis of bovine paratuberculosis (including insulin-like growth factor binding protein-6 - IGFBP6). Our own studies (Smeed et al., 2007; Nalubamba et al., 2008) have focussed on ovine paratuberculosis because of the defined pathologies in sheep and exploit an oligonucleotide microarray of ~600 immuno-inflammatory genes (Watkins et al., 2008) to gain insight into the role of these genes in the development of the different pathologies. The defined nature of the disease phenotype in sheep facilitates the identification of genetic markers associated with pathological type, and might also inform studies in cattle.
Competitive hybridization of cDNA isolated from the ileum (the site of paratuberculosis lesions) between the three infected groups was performed to identify changes to transcript expression associated with each disease group and develop molecular signatures of each pathological form. Quantitative real-time RT-PCR (RT-qPCR) on selected candidate genes was used to validate the arrays and the data were analysed to identify and compare physiological pathways associated with the contrasting pathologies. Data sets gathered from such experiments provide the potential to follow pathways of immune reactivity as well as assessing disease states that can then be related to specific gene expression signatures. In addition, as the epidemiology of these mycobacterial diseases suggests a genetic susceptibility (Ben-Ali et al., 2004) we investigated this link, by a preliminary single nucleotide polymorphism (SNP) analysis, between polymorphisms in two of the candidates and pathological form.

2. Materials and Methods

2.1. Experimental animals and tissues

MAP infected animals presented with clinical Johne’s disease (diagnosed by a veterinary surgeon: prolonged weight loss, inappetance and depression, and occasional diarrhoea) were out bred, female sheep (Table S1) of a variety of breeds and ages. All sheep were euthanized and diagnosis was confirmed by histopathology, Ziehl Neelsen (ZN) staining and IS900 real-time quantitative PCR with a sensitivity of 5 genome equivalents (Eishi et al., 2002); sheep of similar ages from the same flocks (Nalubamba et al., 2008), with no signs of clinical Johne’s disease and positive for IS900 DNA were considered asymptomatic (Smeed et al., 2007). IS900 is a specific marker for the precise identification of MAP (Green et al., 1989). Animal procedures were performed under a valid Animals (Scientific Procedures) Act 1986 Project Licence.
2.2. **RNA isolation**

Terminal ileum sections (~0.5 g) were placed in five volumes of RNALater (Qiagen, Crawley, UK), incubated overnight at 4°C and stored at -80°C. Tissues were homogenized in guanidine isothiocyanate lysis buffer (RNeasy, Qiagen) and RNA was isolated using the RNeasy Maxi kit, eluted in 0.8 ml of RNase-free water, precipitated with ethanol and resuspended in 0.3 ml of RNase-free water. Samples were DNase I digested (Qiagen) and genomic DNA contamination assessed by GAPDH PCR and a no reverse transcriptase control. RNA quality was assessed using a RNA 6000 Nano LabChip on the Agilent 2100 bioanalyzer and quantified using a NanoDrop ND-1000 spectrophotometer; all samples had an RNA integrity number >7; n = 6 for the microarray experiments.

2.3. **Preparation of labelled cDNA and microarray hybridization**

cDNA generation and indirect labelling was performed using the Fairplay III Microarray Labelling Kit (Stratagene, Cambridge, UK) incorporating Cy3 and Cy5 dyes (GE Healthcare, Amersham, UK); the DyeEx spin 2.0 kit (Qiagen) removed unincorporated dye. Pre-hybridization and hybridization was carried out using the Pronto!™ Kit (Corning, Loughborough, UK). This analysis utilized the ruminant immuno-inflammatory gene universal array (Watkins et al., 2008); 4,824 spots from 596 genes. A 22x22 mm lifterslip (VWR, VWR, Poole, UK) was then placed over the array and the slide pre-heated to 42°C. Equal dye concentrations from two samples (30 pmols each) were mixed, dried and resuspended in 20 μl cDNA hybridization solution and introduced onto the array. The slides were incubated for 16 - 20 h at 42°C with agitation every 7 s, using a SlideBooster (Implen, Stansted Mountfitchet, UK) and post-hybridization washes of the microarray slides carried out using the Pronto!™ Kit, using an AdvaWash AW400 slide washer (Implen). The slides were scanned using a GenePix Autoloader 4200AL, controlled by GenePix Pro v 6.1 (Molecular Devices, Sunnyvale, CA). In total, we performed 18 microarrays. All groups contained six animals and every sample was analysed on three arrays, with each animal being
compared with an animal in each of the other groups. The identity of the pairings between each group was chosen at random.

Protocols of the experimental procedures, methods of analysis and microarray data are available as supplementary information in the European Bioinformatics Institute’s ArrayExpress database (www.ebi.ac.uk/arrayexpress). Login to www.ebi.ac.uk/aerep/login and query for the accession number of the experiment or array design E-TABM-487.

2.4. Microarray data analysis

Scanned images were aligned to the GenePix Array List (GAL file); background noise subtracted; spot intensity amplitudes generated and quality data extracted using BlueFuse v3.3 (BlueGnome, Cambridge, UK). The log₂ ratios from the two channels for each spot were calculated by Bluefuse. Data generated by Bluefuse were pre-processed using Limma within BioConductor 2.2 (http://www.bioconductor.org). Normalization was carried out using a weighted print-tip Loess method. Spots were weighted by a confidence value provided by BlueFuse. Differentially expressed genes from the different group comparisons (Table 1) were classified based on biological process using GO annotation. GORetriever, GOanna and GoSlimViewer (http://www.agbase.msstate.edu) were used to annotate gene products based on sequence similarity.

2.5. Real time RT-qPCR validation

RT-qPCR was performed using a Corbett Rotor-Gene™ 3000 (Qiagen). First strand cDNA synthesis used 0.5 µg of total RNA and Oligo(dT) and Superscript™ III RT (Invitrogen, Paisley, UK), from all RNA samples used for microarrays with a further two samples from each of the groups (n = 8). Primers for candidate genes were designed (Table S2) and the amplicons cloned using pGEM-T Easy kit (Promega, Southampton, UK). PCR conditions were optimized using a standard curve generated from linearized plasmid. All serial dilutions were generated using a CAS-1200™ Precision Liquid Handling System.
Succinate dehydrogenase complex, subunit A (SDHA) and tyrosine 3/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ); were chosen from a group of six using GeNorm v3.4 (Vandesompele et al., 2002) and NormFinder v 0.953 (Andersen et al., 2004), as the two most stably expressed genes to normalize the RT-qPCR assays. All reactions were 10 µl volumes containing 4 µl of cDNA at the optimum dilution (1:20), 5 µl 2x FastStart SYBR Green Master mix (Roche, Lewes, UK) and 0.5 µl of each primer at optimum concentration (Table S2). PCR conditions were: 94°C for 5 min and 45 cycles of 94°C for 20 s, 60°C for 20 s, 72°C for 20 s, followed by dissociation curve analysis to confirm a single gene product. Each run assayed one gene and contained all samples in duplicate, a cDNA standard curve and a non-template control. This was repeated twice using cDNA from two additional RT reactions, resulting in 6 data points for each gene. The average Cq value for each sample was calculated and converted into relative quantities, taking account of the gene specific efficiency of the reaction and multiple reference gene normalization, using qBase (Hellemans et al., 2007). Statistical analyses were performed on data from individual animals using Kolmogorov-Smirnov to test for normality of distribution and two-tailed Student’s t-test for each of the comparisons.

2.6. **PCR and DNA sequencing**

DNA was extracted from 250 mg ileum using the Qiagen DNEasy Blood and Tissue kit or Qbiogene Fast DNA kit and quantified using the Nanodrop ND-1000 spectrometer. Full length mRNA sequence was obtained for sheep CXCR4 and IGFBP6, and aligned to the bovine genome to identify the coding regions in the genomic sequence. Primers (Table S3) were designed to amplify the whole bovine coding region and the intron / exon boundaries where possible (Fig. S1). Three PCR reactions were performed with each set of primers on ten animals in each infected group (Table S1). The PCR products from each sample were mixed, purified, ligated and cloned into the PGEM-T easy. Three independent sequences were produced for each PCR product and consensus sequences produced using VectorNTI AlignX.
Differences between the genomic sequences were considered to be SNPs when the same change was found consistently in two or more of the consensus sequences. The distribution of alleles and genotypes was compared between the pathological groups using the \( \chi^2 \)-test.

3. Results

3.1. Microarray analysis

Of the 596 genes analysed, 36 fulfilled the criteria of fold-change of \( \geq 1.5 \) and \( P \leq 0.05 \) in at least one comparison (Table 1 and supplementary information). The fold-changes shown are means of all probes for each gene. Intrinsic variation within replicate spots on single arrays (technical) and between arrays (biological) was determined. Technical variation was determined by comparing normalized log\(_2\) ratios of the spots printed in triplicate within each array, for six replicate array experiments of each comparison. The median standard deviation for the 3 comparisons varied from 0.24 (for multi- versus paucibacillary (M v P) to 0.26 (for multi- versus asymptomatic (M v A) with a variance of between 0.06 and 0.07 across the three comparisons. The biological variation was analysed by comparing the log\(_2\) ratios (CH1:CH2) of identical spots between the 6 replicate arrays in each comparison. The median standard deviation for each comparison ranged from 0.37 to 0.42, with a variance between 0.14 and 0.18 across all comparisons, demonstrating that within chip variability (technical variation) was between third and a half of the between chip variation (biological variation).

ITGAL was the only gene significantly affected in all three comparisons (Table 1, Fig. 1) and two other genes (SELL and TFRC) were up-regulated but \( P \geq 0.05 \). Five genes were differentially expressed between the two disease groups and the asymptomatic group (Table 1). Fifteen genes were significantly different in the paucibacillary vs asymptomatic comparison including a 1.52 fold repression of CCL5 and a 2.25 fold increase in CXCL10. Twenty three were significantly different in the multibacillary vs asymptomatic comparison including a 1.64 fold increase in CCL2; 1.54 and 1.82 fold increases in CXCR3 and CXCR4 and 1.59 and 1.67 decreases in CD3E and CD3F. The pauci- vs multibacillary comparison
identified fifteen affected genes, but only four (C9, CLIC5, ITGAM and MMP7) were unique
to that comparison, as the other comparative data was not significant (<1.5 and p>0.05).
Within the limitations of this focussed microarray, it is clear that the differentially expressed
genes associated with each of the paratuberculosis pathologies were connected with a distinct
range of biological processes (Fig. 2). Both clinical diseases showed changes to genes linked
to responses to biotic stimuli, cell differentiation and signal transduction but only in the
multibacillary form were there changes to genes associated with cell communication.

3.2. RT-qPCR validation of microarray results

Of the 36 genes found to be differentially expressed in the microarray experiment,
seven candidates were chosen for RT-qPCR validation of the array; CD63, CXCR4, IGF2R,
ITGB2, MMP9, TLR2, and TYROBP; on the basis that they represent different biological
processes (Fig. 2) and were differentially expressed between pauci- and multibacillary
samples. In addition we identified IGFBP6 as it had been highlighted as being differentially
expressed in bovine paratuberculosis (Coussens et al., 2004) and in a preliminary array
analysis (Roupaka, 2004). Table 2 shows the results expressed as relative fold-changes in the
same comparisons as performed in the microarray. The RT-qPCR analyses for seven original
candidate genes validated the results from the microarray experiment with a correlation
coefficient (r) 0.63 – 0.97, mean of 0.87. Comparison (two-tailed Students t test) of
expression levels of all eight candidates between Blackface x Blue du Maine vs other breeds
in the paucibacillary group and Blackface x Blue du Maine vs other breeds in the
asymptomatic group showed that breed had no significant impact (P > 0.8) on the distribution
of the data. Similar comparisons of the 4-5 year old vs the 2-3 year old Blackface sheep in the
multibacillary group also showed that age also had no effect on the data (P > 0.9).

3.2. Single nucleotide polymorphism (SNP) analysis

Analysis of the consensus CXCR4 sequences revealed the presence of three
synonymous SNPs; in exon 2 a C to T change at position 2080 and a G to A change at 2275
and in exon 3 a G to A change at 3361; the last was 3' of the stop codon. Analysis of IGFBP6 sequence also revealed three SNPs – a non-synonymous C to A change at position 12 in exon 1, which changes a histidine to a glutamine; a synonymous G to A change at 2730 in intron 3 and a G to A change at 3743 in exon 4, 3' of the stop codon at position 3612 (Fig. S1). When these six SNPs were analysed in relation to infection there was no significant relationship between any SNP and disease form (Table 3). Each of the three CXCR4 SNPs and two IGFBP6 SNPs were found in all infected groups. However, IGFBP6 SNP3 (G 3822 A) was only found in clinically diseased animals, and was absent from the asymptomatic group. The animals tested were of a variety of breeds and crosses (Table S1) but there was no discernable, significant relationship between breed and any genotype ($\chi^2$, $P \geq 0.79$).

4. **Discussion**

This is the first microarray analysis of ileal gene expression in sheep with clinical paratuberculosis and directly compares gene expression at the site of infection in each of the pathologies, and has identified novel pathways involved in MAP pathogenesis. Previous work on sheep paratuberculosis has shown that the pauci- and multibacillary pathologies are associated type 1 and type 2 T cell responses respectively, with the differential activation of T cells (Burrells *et al.*, 1998) and the expression of quite distinct panels of both innate receptors (Nalubamba *et al.*, 2008) and cytokines (Smeed *et al.*, 2007). Analysis of the data for the eight candidate genes indicates that it is highly unlikely that these changes are associated with the breed or age (Table S1). Paratuberculosis is characterized by extensive inflammation and cellular infiltration of the terminal ileum; paucibacillary disease is associated with lymphocytes and eosinophils, and multibacillary lesions with epithelioid macrophages (Smeed *et al.*, 2007). These variations in the cellular composition are reflected in the differences in gene expression profiles detected by microarray, with 36 genes found to be differentially expressed (>1.5 fold and $P \leq 0.05$) in at least one comparison (Table 1). The RT-
qPCR data justify our decision to use 1.5 fold (and $p \leq 0.05$) as the cut-off point for our
candidate gene selection as they confirmed the array data in relation to the direction of fold
change. In addition, fold changes of just under 1.5 (but $p \leq 0.05$) were shown to be
significantly different by RT-qPCR, e.g. MMP9. RT-qPCR also confirmed the array data,
both quantitatively and qualitatively, when microarray showed no change ($>–1.5$ to $<1.5$), e.g.
CD63 in the pauci- vs asymptomatic comparison. The correlation coefficient ($r$) between
array and RT-qPCR varied between candidate genes ($0.63 – 0.97$, mean $r = 0.87$) and was
similar to that described in other systems (Dallas et al., 2005), but was much lower for genes
with a fold change of $<1.5$. The magnitude of fold change with RT-qPCR was generally much
higher than with microarray, which is probably due to the different sensitivities of the two
assays (Morey et al., 2006); nevertheless RT-qPCR is the preferred validation method for
microarrays (Dallas et al., 2005).

Within the limitations of the focussed microarray, it is clear that the differentially
expressed genes associated with each of the pathologies were connected with a distinct range
of biological processes (Fig. 2) probably associated with the different cell infiltrates in the
two disease forms. The multibacillary samples displayed a consistent repression of the several
genes encoding elements of the T cell receptor complex - CD3E, CD3G (and CD3Z with –
1.47 fold change and $P \leq 0.05$). This might be related to the fact that the principal cells in
multibacillary ileum were infected epithelioid macrophages and not T cells. It is possible that
this reduction in antigen-receptor expression is a result of infection-associated aberrant
activation and may represent a specific bacterial mechanism for the modification of the host
immune response towards the development of disease as is seen in tuberculosis (Smith, 2003).
Related to this were changes to transcripts of the different immunoglobulin molecules.
IGHA1 and IGHM were raised in both disease forms and IGJ was increased in paucibacillary
lesions. In view of the fact that B cell receptor associated genes were not raised in infected
tissues, these increases in immunoglobulin heavy chain transcripts may be a result of greater
numbers of plasma cells rather than mature B cells. This pattern of immunoglobulin heavy
chain expression does not fit exactly into the Th1/Th2 paradigm, which would predict high levels of IGHA only in the multibacillary (lepromatous) lesions (Mosmann and Coffman, 1989).

Representatives of the β1 and β2 integrins (ITGA4 and ITGAL) were up-regulated in the paucibacillary forms. However, their behaviour was less consistent in the multibacillary form where the β1 integrin ITGA4 is repressed and the β2 integrins ITGAL, ITGAM and ITGB2 were increased. The four transmembrane domain proteins (e.g. CD63) interact with integrins and modulate their function; and the high levels of CD63 in multibacillary lesions may be indicative of different cell populations associated with those lesions. The major cellular ligand for the β2 integrins is ICAM1. This, and the lymphocyte adhesion molecule, SELL (CD62L or L selectin), were high in multibacillary diseased tissues, which reflects major cellular infiltration and also inflammation, as both are up-regulated by inflammatory cytokines (Dustin and Springer, 1991).

The stimulus for leukocyte infiltration into lesions is probably different for each disease form. CCL2 was raised in multibacillary lesions (and 1.65 fold but P ≥ 0.05 in the pauci- vs asymptomatic comparison) and was probably produced by a variety of cells on stimulation with pro-inflammatory cytokines and IFNγ. It is chemotactic for lymphocytes and monocytes but not eosinophils and its role in inflammation is as an activator of neutrophils (Campbell et al., 2003). CCL5 is associated with T cell and dendritic cell recruitment, but it was repressed in the paucibacillary group. CCL5 is a product of activated T cells, but only at the late stage of activation, and is down-regulated by TGFβ and IFNγ both of which were increased in paucibacillary tissues (Smeed et al., 2007). This is additional evidence for the aberrant activation of T cells in the paratuberculosis lesions. The chemokine CXCL10 is associated with chemoattraction of type 1 T cells (Campbell et al., 2003) and was significantly raised in paucibacillary lesions and might explain the T cell infiltration. However its receptor, CXCR3 was up-regulated in multibacillary tissues, probably due to the effect of high levels of TGFβ. The closely related chemokine receptor, CXCR4 is also
responsive to TGFβ and was significantly increased in multibacillary tissues. This is the
receptor for CXCL12 (SDF-1) and one of its major functions is as a chemoattractant for Th2
and regulatory T cells (Campbell et al., 2003).

IGF2R, the receptor for IGF2 and mannose-6-phosphate, was increased in the
multibacillary disease form and plays a role in the activation of TGFβ (Melnick et al., 1998),
a gene previously shown to be up-regulated in these samples (Smeed et al., 2007). IGFBP6
was differentially expressed in sheep paratuberculosis (increased in paucibacillary and
repressed in multibacillary diseased ileum) and it was also up-regulated in cattle PBMC
stimulated by MAP (Coussens et al., 2004). It binds with high affinity to IGF2 and inhibits
many of its functions, including proliferation and differentiation (Kelley et al., 1996). Its
expression has previously been reported to be stimulated by IL-1β and TNFα but inhibited by
the presence of TGFβ (Martin et al., 1994; Liu et al., 1999), and this might explain the
significantly increased levels of IGFBP6 in paucibacillary lesions. In paucibacillary tissues,
high IGFBP6 levels could play a role in controlling cellular proliferation by inhibiting IGF2
and inducing apoptosis. TLR2 was greatly up-regulated in multibacillary lesions, suggesting
that the TLR2 signalling pathway is important in lepromatous pathogenesis. TLR2 is central
to the immune response to mycobacteria, as knockout mice are more susceptible to infections.
In human leprosy TLR2 is linked to differential pathology, as signalling through the mutated
receptor leads to increased IL-10 production and a type 2 immune response (Kang et al.,
2004). Consistent with this are increased levels of IL-10 in multibacillary tissues and raised
IFNγ in paucibacillary lesions (Smeed et al., 2007). Multibacillary tissues show high levels of
both CXCR4 and TLR2 and, interestingly, cross-talk between these has been reported in
*Porphyromonas gingivalis* infection that leads to the undermining of host defence
(Hajishengallis et al., 2008). The data on the linkage between TLR2 and MAP in ruminants is
not clear. Analysis of TLR2 SNPs in sheep paratuberculosis failed to identify any linkage
with pathological form (Nalubamba et al., 2008) although in cattle there was significant
association of a distinct SNP with MAP infection (Mucha et al., 2009).
Due to their differential expression in multibacillary and paucibacillary sheep, CXCR4 and IGFBP6 were selected for preliminary SNP analysis and to assess whether these SNPs were associated with gene expression and pathology. All SNPs except IGFBP6 at 3743 were found in the three infected groups and there was no significant association with any disease form. The IGFBP6 SNP at 3743 is 3’ to the stop codon and was not found in asymptomatic sheep; there was no statistically significant association with disease form possibly because of the small numbers of animals (n=10) in each group.

To summarize, this study identifies novel genes and pathways linked to mycobacterial pathologies. The two disease forms of sheep paratuberculosis are associated with distinct molecular profiles highlighted by the differential expression of chemokines, which are implicated in the different cellular infiltrates of the pathologies and the polarization of the immune response. Cells within the lesions show evidence of abnormal activation, with high levels of cytokine transcripts and a reduction in expression levels of transcripts for lymphocyte receptor molecules. Preliminary analysis could identify no significant link between CXCR4 and IGFBP6 SNPs and paratuberculosis pathologies.

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cells, increased frequency of γδ T cells, and related changes in T-cell function. Infect. Immun. 70, 3856-3864.


averaging of multiple internal control genes. Genome Biology 3, research0034.1-research0034.11.


Figure Legends

**Fig. 1.** Venn diagram showing the relationships between genes differentially expressed in the ileum of sheep infected with MAP; comparisons between the three infected groups; A, asymptomatic; M, multibacillary; P, paucibacillary. The figures are the numbers of differentially expressed genes in the different comparisons with a fold change of ≥1.5 and $P\leq0.05$. Figures in parentheses are the number of genes with fold change of ≥1.5 but $P>0.05$.

**Fig. 2.** Distribution of GO annotations of biological processes terms of genes that were differentially expressed (fold change of ≥1.5 and $P\leq0.05$) in ileum tissue of MAP infected sheep. (a) Comparison of paucibacillary vs asymptomatic. (b) Comparison of multibacillary vs asymptomatic. (c) Comparison of multibacillary vs paucibacillary.

**Fig. S1.** Gene structures of bovine CXCR4 and IGFBP6 illustrating the positions of the cloning fragments and SNPs.
Mean fold–changes of significantly differentially expressed genes.

<table>
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<tr>
<th>NCBI Official Symbol (Gene)</th>
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<th>P v A</th>
<th>M v A</th>
<th>M v P</th>
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<td>CFB (complement Factor B)</td>
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</table>
A – asymptomatic; M – multibacillary; P – paucibacillary.

Bold denotes genes selected for validation by qRT–PCR. Changes less than 1.5 fold (and 
P≤0.05) are in italics to show trends in the data. *Changes not statistically significant, but 
>1.5 fold. The blank cells are fold change <1.5 and P>0.05.
Table 2
Relative fold-change of genes analysed by qRT-PCR.

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<th>M v A</th>
<th>M v P</th>
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<tbody>
<tr>
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<td>3.0 (0.00)</td>
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<td>-1.37 (0.5)</td>
<td>-2.54 (0.02)</td>
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<td>4.2 (0.00)</td>
<td>2.1 (0.00)</td>
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<td>9.4 (0.00)</td>
<td>3.0 (0.00)</td>
</tr>
</tbody>
</table>

\(^a\) mean fold change (P value). Bold, P ≤ 0.05.

A – asymptomatic; M – multibacillary; P – paucibacillary.
### Table 3
Distribution of SNPs

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<tr>
<th>Genotype</th>
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<th>Pauci</th>
<th>Asympto</th>
<th>$\chi^2$</th>
<th>P value</th>
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Fig 2

(a) P vs A

(b) M vs A

(c) M vs P

- Anatomical structure morphogenesis
- Behaviour
- Catabolic process
- Cell communication
- Cell death
- Cell differentiation
- Cell growth
- Cellular homeostasis
- Cell proliferation
- Cell-cell signalling
- Ion transport
- Metabolic process
- Multicellular organismal development
- Organelle organization and biogenesis
- Protein metabolic process
- Protein modification process
- Protein transport
- Reproduction
- Response to abiotic stimulus
- Response to biotic stimulus
- Response to external stimulus
- Response to stress
- Signal transduction
- Transport
Table S1
Pathology, breed and age of sheep

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<td>Texel</td>
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<tr>
<td>Pauci\textsuperscript{b}</td>
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<tr>
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<td>4</td>
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<td>Blackface</td>
<td>3.5</td>
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<tr>
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<td>3</td>
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<td>Multi\textsuperscript{b}</td>
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\textsuperscript{a} animals used in the microarray experiments

\textsuperscript{b} additional animals used in qRT-PCR experiments
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<tr>
<th>Gene</th>
<th>Gene Accession no</th>
<th>Primers</th>
<th>Annealing Temp °C</th>
<th>Product size (bp)</th>
<th>Primer conc (nM)</th>
</tr>
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| CD63 | BC151412.1       | F - GGGCTGTGTTGGAGAAGATTG  
R - GATGAGGGGGCTGAAGAAGAC | 61 | 178 | F: 700  
R: 1000 |
| CXCR4 | AF399642 | F - ACCTCTGTGTTGCTCTACG  
R - AATGCTCCACCTCGCTTG | 62 | 163 | F: 500  
R: 500 |
| IGFBP-6 | NM_001134308 | F - AAGGAGAGTAAGCCCCCAAGC  
R - CGGGAAGGAGTGTTAGAGGT | 60 | 95 | F: 500  
R: 500 |
| ITGB2 | NM_001009485 | F - CTCACCCGACAACCTCAAACA  
R - AAAGTGGAAACCATCGTCTG | 57 | 180 | F: 400  
R: 400 |
| IGF2R | AF353513 | F - GACGACCTGAAGACCCCTGAA  
R - GCAATGAAGCGGATGATG | 60 | 150 | F: 500  
R: 500 |
| MMP9 | X78324 | F - GAGGGTAAGGTGCTGTTC  
R - AAAGTGTGTGCGTGCTAATG | 62 | 133 | F: 400  
R: 500 |
| TLR2 | AM183218 | F - GCACTTCAACCTCCCTTTTA  
R - TCTCCGAAGCACAAGATG | 57 | 125 | F: 500  
R: 500 |
| TYROBP | AJ419228.1 | F - GACCTAGTCTGACCCCTCC  
R - CTGTCTCGGTATGCTGCTG | 56 | 112 | F: 400  
R: 500 |
| SDHA | NM_174178 | F - ACCTGATGCTTTGTGCTCTGC  
R - CCTGGATGGGCTGGAGTAA | 62 | 126 | F: 500  
R: 500 |
| YWHAZ | BC102382 | F - TGTAGGAGCCCGTAGTCTCATC  
R - TCTCTGTATTCTCGAGCCAT | 58 | 101 | F: 500  
R: 500 |
Table S3. Primer sets used to sequence CXCR4 and IGFBP-6

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<sup>a</sup>numbered relative to start codon.