

Published in final edited form as:

*Res Vet Sci.* 2011 August ; 91(1): 40–51. doi:10.1016/j.rvsc.2010.09.002.

## Comparative analysis of the early transcriptome of *Brucella abortus* - infected monocyte-derived macrophages from cattle naturally resistant or susceptible to brucellosis

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### Abstract

Brucellosis is a worldwide zoonotic infectious disease that has a significant economic impact on animal production and human public health. We characterized the gene expression profile of *B. abortus*-infected monocyte-derived macrophages (MDMs) from naïve cattle naturally resistant (R) or susceptible (S) to brucellosis using a cDNA microarray technology. Our data indicate that 1) *B. abortus* induced a slightly increased genome activation in R MDMs and a down-regulated transcriptome in S MDMs, during the onset of infection, 2) R MDMs had the ability to mount a type 1 immune response against *B. abortus* infection which was impaired in S cells, and 3) the host cell activity was not altered after 12h post-*B. abortus* infection in R MDMs while the cell cycle was largely arrested in infected S MDMs at 12h p.i. These results contribute to understand of how host responses may be manipulated to prevent infection by brucellae.

### Keywords

*Brucella*; macrophages; cattle; resistance; microarray

### 1.4 Introduction

Infectious diseases are usually controlled by traditional interventions such as antibiotics or vaccines. However, these interventions are not completely effective, as diseases persist in animal populations. Repeated observations over time in domestic livestock have

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### Conflict of interest

Authors declare no conflict of interest.

demonstrated that clinical manifestations of infectious disease rarely occur in all members of the population exposed to the same pathogen under similar conditions. Genetic implications of these observations were initially ignored until association of natural resistance to pathogens with genetic markers in animal species, breeds or families was established (Carmichael, 1941; Cameron et al., 1942; Bumstead and Barrow, 1993; Xu et al., 1993). The genetic regulation of natural resistance to infectious disease is variable and usually complex, and includes both immune and non-immune mechanisms, although sometimes expression of an allele at one locus can significantly modify the disease pathogenesis in individuals (Adams and Templeton, 1998).

Brucellae are the etiological agents of brucellosis, a worldwide zoonotic infectious disease that has a significant economic impact on animal production and human public health (Corbel, 1997). Among animal species, most mammals are susceptible to brucellosis. Bovine brucellosis is mainly caused by *Brucella abortus* which is clinically characterized by abortion and infertility in cows, and orchitis and inflammation of the accessory sex organs in bulls (Enright, 1990). Natural *B. abortus* infection in cattle occurs primarily through penetration of the mucosa membrane of the oropharynx followed by uptake by macrophages (MØ) and transport to the regional lymph nodes (Adams, 2002; Olsen et al., 2004). Successful initial establishment is due to the stealthy strategy employed by *Brucella* to modulate activation of the innate immune system, while persistent infection resides in the ability of the pathogen to modify trafficking to survive and replicate inside MØ by overcoming bactericidal mechanisms (Roop II et al., 2004; Barquero-Calvo et al., 2007).

The presence of invading microbes is detected by sentinel cells such as MØ and dendritic cells (DC). After contact with the pathogen, sentinel cells secrete a mixture of cytokines and process and link the exogenous antigen to MHC-II molecules to activate T-helper (Th0) cells in secondary lymphoid organs. According to the stimulus received, Th0 cells differentiate into Th1 and Th2 subsets, which polarize the immune response (Salyers and Whitt, 2002). Th1 subset of cells develop in response of Th0 to IL-12, inducing a Th1-oriented immune response, mostly involved in protection against intracellular pathogens through cell-mediated immunity and characterized preferentially by secretion of interferon-gamma (IFN- $\gamma$ ) and interleukin 2 (IL-2) cytokines. On the other hand, sentinel cells that secrete IL-4 induce a Th2 subset of cells development and a Th2-oriented immune response. Th2 immunity is characterized by secretion of IL-4, IL-5, IL-10 and IL-13 and is mainly responsible for protection against extracellular pathogens by mediating antibody production (Tizard, 2004).

Previous studies have reported that Th1 immune response is particularly involved in host protection against *Brucella* infection through cell-mediated immunity (Oliveira et al., 2002). When *Brucella* invade naïve hosts non-activated professional phagocytes uptake the pathogen and release interleukin-12 (IL-12). Subsequently, IL-12 induce Th0 cells to differentiate into IFN $\gamma$ -secreting Th1 cells that are capable of activating MØ for increased anti-microbial mechanisms, and thus promote clearance of the bacteria (Zaitseva et al., 1995; Dornand et al., 2002). However, virulent *Brucella* have developed active strategies to interfere with innate immunity and consequently avoid being eliminated. For instance, *Brucella* impair apoptosis in human MØ (Gross et al., 2000; Fernandez Prada et al., 2003) and inhibit or delay dendritic cells maturation and antigen presentation (Billard et al., 2008). Moreover, *Brucella* alter the production and secretion of cytokines of infected host cells (Caron et al., 1994), modify the intracellular trafficking (Rittig et al., 2003), inhibit degranulation of neutrophils (Bertram et al., 1986; Orduna et al., 1991), and impair NK cell activity (Salmeron et al., 1992).

Previously, our laboratory identified cattle naturally resistant (R) and susceptible (S) to *B. abortus* infection (Harmon et al., 1985; Templeton et al., 1988). In these studies, the R cattle developed low transient serologic titers and were negative for *Brucella* isolation, while S infected cows developed high titers, aborted and *Brucella* was isolated from secretions. Later experiments focused on innate immunity found that mammary gland MØ from R cows produced significantly higher oxidative burst activity and had significantly greater *in vitro* bacteriostatic activity than MØ from S cows, when both were stimulated with opsonized *B. abortus* (Harmon et al., 1989). Furthermore, *B. abortus* were demonstrated to bind differentially to the peripheral blood monocyte-derived MØ (MDMs) from R or S cattle and also the cells from R animals were significantly superior in their ability to control the *in vitro* intracellular replication of *B. abortus* than those derived from S cattle (Price et al., 1990; Campbell and Adams, 1992; Campbell et al., 1994; Qureshi et al., 1996). These findings further substantiate the importance of the mononuclear phagocyte system in natural resistance to bovine brucellosis. In order to associate natural resistance with genetic markers, later studies identified the bovine *SLC11A1* gene (formerly *NRAMP1*) as one of the major elements in controlling of intracellular replication of *B. abortus* in MØ (Feng et al., 1996; Adams and Templeton, 1998; Barthel et al., 2001). To better understand the differences in the phenotype and identifying novel cattle candidate genes and pathways involved in innate resistance to brucellosis, we characterized the expression profile of *B. abortus*-infected MDMs from naïve cattle naturally R or S to brucellosis using a cDNA microarray technology. In concordance with previous knowledge, our results demonstrated that R MDMs were superior controlling *B. abortus* infection due to the ability to polarize an immune response toward Th1, while the innate immune system of S MDMs failed to generate appropriate signals to mount an effective immune response against invading bacteria.

## 1.5 Materials and Methods

### Bacterial strain, media and culture conditions

The smooth virulent *Brucella abortus* S2308 (gift of Dr. Billy Devoe, USDA, Agricultural Research Service, National Animal Disease Center, Ames, IA) was maintained as frozen glycerol stocks and resuspended in fresh complete RPMI (C-RPMI) 1640 medium (RPMI 1640 medium supplemented with 4mM L-glutamine, 1mM non-essential amino acids, 1mM sodium pyruvate and 2.9mM 7.5% sodium bicarbonate) (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (American Type Culture Collection – ATCC, Manassas, VA) at  $1 \times 10^7$  CFU/ml and incubated at 38°C for 1 h prior to use in assays. The CFU inoculated was corroborated retrospectively by serial dilution plating the inoculum on TSA plate.

### Isolation of peripheral blood monocytes

Blood was collected from a clone of a bull naturally R to *B. abortus* S2308 infection (Westhusin et al., 2007) and a progeny from the pedigreed family of cattle previously characterized as S to conjunctival challenge with live *B. abortus* (Price et al., 1990). The cattle were unvaccinated and serologically negative to brucellosis by the card test and complement serological assays. Blood was extracted and processed as previously explained (Campbell and Adams, 1992). Briefly, 50 ml of blood was collected by aseptic venipuncture of the jugular vein into 7.5 ml of acid-citrate-dextrose (ACD) and diluted in an equal volume of phosphate-buffered saline containing citrate (PBS-C). Diluted blood was carefully poured on the top of Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at 1000X g for 30 min. The interface cells were washed 3 times in cold PBS-C, resuspended in C-RPMI 1640 medium supplemented with 4% of autologous serum and transferred to Teflon Erlenmeyer flasks. After 38°C incubation overnight in humidified atmosphere with 5% CO<sub>2</sub>, non-

adherent cells were removed and fresh media added to each flask before replacing them in the incubator. The cells were kept in culture for 21 days with medium changes every 7 days to let monocytes mature to MØ before using them in assays.

### Infection assay

Twenty-four hours prior to infection, MDMs were harvested by chilling the Teflon flasks on ice for 15 min, enumerated and subcultured in 25cm<sup>2</sup> cell culture flasks (Corning, Corning, NY) at a concentration of  $2 \times 10^5$  MDMs/flask and replaced to the incubator. Infection with *B. abortus* S2308 was performed at the multiplicity of infection (MOI) of 5:1 (bacteria:MDMs) by centrifuging bacteria onto cells at 800X g for 10 min. After 30 min incubation to allow bacteria:MDMs interaction, extracellular bacteria were killed by replacing the culture media on each flask by C-RPMI medium supplemented with 50µg/ml of gentamicin solution (Sigma, St. Louis, MO). After 1 hour incubation, all flasks were washed 3 times with PBS to eliminate antibiotic residue and 8 ml of fresh C-RPMI 1640 medium supplemented with 10% HI-FBS was added to every culture before replacing them to the incubator. Sham inoculated MDMs were used as controls. To determine the number of intracellularly viable *B. abortus* S2308 at T0 and T12, MDMs were plated in triplicate in 24 well plates at  $2 \times 10^5$  MDMs/well and infected at MOI 5:1. At T0 (i.e., immediately after cells were washed to eliminate antibiotic residue) and T12 (i.e., 12 h post infection, -p.i.-), infected cells were lysed with 0.5% Tween-20 (Sigma) in sterile distilled water, lysates, serially diluted and 100µl of dilutions cultured on TSA plates for quantification of CFU. Triplicate wells containing bacterial suspensions alone were plated in each well and used as a control for the adequacy of killing of extracellular bacteria and for the control of bacterial growth.

### Isolation of total RNA from MDMs

Total RNA from infected and control MDMs from cattle R or S to *B. abortus* infection was isolated at 12 h p.i. At this time point, supernatants from the cultures were harvested and the cells were rinsed once with cold PBS. One ml of Tri-reagent® (Ambion, Austin, TX) was added to each flask and RNA extracted using the protocol recommended by the manufacturer. The resultant RNA pellets were re-suspended in DEPC-treated water (Ambion) with 1% RNase inhibitor (Promega, Madison, WI). Genomic DNA was removed by RNase free - DNase I treatment (Ambion) according to the manufacturer's instructions, and samples were stored at -80°C until used. RNA concentration was quantified by measuring absorbance at λ260nm using a NanoDrop® ND-1000 (NanoDrop, Wilmington, DE), and the quality of the RNA determined using a Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

### Preparation of bovine reference RNA

Based on previous references that demonstrated no statistical difference between genomic DNA and reference RNA for standardizing spotted microarray data (Weil et al., 2002), we prepared bovine RNA common reference set for standardizing our array experiments (Pollack, 2002). Total RNA was isolated from Madin-Darby bovine kidney (MDBK) and bovine B lymphocyte (BL-3) cell lines (ATCC) and fresh bovine brain, as explained above. Cell lines were grown in 150 cm<sup>2</sup> cell culture flasks with minimum essential medium Eagle (MEME) (ATCC) supplemented with 10% HI-FBS. Bovine brain was harvested from cortex and cerebellum of a Holstein male calf immediately after euthanasia. The tissue was homogenized in ice-cold Tri-reagent® (Ambion). RNA concentration from each sample was quantified and bioanalyzed before and after pooling the samples. Total RNA isolated from three samples was pooled together in equal amounts, aliquoted and stored at -80°C until needed.

## Construction of 13K bovine cDNA microarrays and annotation

A custom bovine cDNA library consisting of unique 70-mer oligonucleotides representing 13,257 unique oligos of 12,220 cattle ORFs obtained from normalized and subtracted cattle cDNA libraries was printed in 150 mM phosphate buffer at 20  $\mu$ M concentration in duplicate on aminosilane-coated glass slides at the W. M. Keck Center (University of Illinois at Urbana-Champaign). The oligos were annotated based on the GenBank accession number, when available. Additional description of the array design, construction and annotation has been previously published (Everts et al., 2005; Loor et al., 2007). Sequence annotation of the differentially expressed spots was manually improved by blast searches on public databases (GenBank, UniGene).

## Sample preparation and slide hybridization

Three biological replicates of *B. abortus*-infected and non-infected MDMs from cattle naturally R or S to brucellosis at 12 h p.i. (n = 12) were analyzed. The labeling and hybridization procedures were adapted from the protocol described previously (Rossetti et al., 2009). Briefly, 10 $\mu$ g of experimental and reference total RNA were reverse transcribed overnight to amino-allyl cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Cy3 and Cy5 dye esters (Amersham Pharmacia Biosciences, Piscataway, NJ) were covalently linked to the amino-allyl group by incubating the samples with the dye esters in 0.1M sodium carbonate buffer (pH 9.0). After one hour incubation in the dark, uncoupled dyes were removed and dye incorporation calculated based on NanoDrop<sup>®</sup> analysis. Dry, labeled experimental cDNA samples were resuspended in 20 $\mu$ l of nuclease – free water (Ambion) and reference samples in 20 $\mu$ l of bovine competitor DNA (Applied Genetics Laboratories, Melbourne, FL) and combined. Mixed samples were heated at 95°C for 5 min, annealed at 60°C for 10 min and kept at room temperature for another 10 min. Samples were kept at 42°C until hybridization. Forty  $\mu$ l of 2X formamide-based hybridization buffer [50% formamide; 10X SSC; 0.2% SDS] was added to pre-annealed samples, mixed well and applied to the 13.2K bovine oligo-slides. Prior to hybridization, the microarrays were denatured by exposing to steam from boiling water, UV cross-linked and immersed in prehybridization buffer [5X sodium chloride, sodium citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS) (Ambion); 1% bovine serum albumin (BSA)] at 42°C for a minimum of 45 min followed by four washes in RNase-DNase free, distilled water, immersion in 100% isopropanol for 10 seconds, and dried by centrifugation. Slides were hybridized at 42°C for approximately 40 h in a dark humid hybridization chamber (Corning) and washed for 10 min at 42°C with low stringency buffer [1X SSC, 0.2% SDS] followed by two 5-min washes in a higher stringency buffers [0.1X SSC, 0.2% SDS and 0.1X SSC] at room temperature with agitation. Slides were drying by centrifugation at 800X g for 2 min and immediately scanned.

## Data acquisition and microarray data analysis

Hybridized slides were scanned by a commercial laser scanner (GenePix 4100; Axon Instruments, Inc., Foster City, CA). Scans were performed using the autoscan feature with the percent saturated pixels set at 0.03%. The spots representing genes on the arrays were adjusted for background and normalized to internal controls using image analysis software (GenePix Pro 6.0; Axon Instruments, Inc.). Spots with fluorescent signal values below background were disregarded in all analyses. Initially, arrays were normalized against bovine reference RNA signals across slides and within each slide (across the duplicate spots). Data were analyzed using GeneSifter (VizX Labs, Seattle, WA) and using a combinatorial statistical approach as follows: data were normalized by mean and then combined, Student's t test with Benjamini-Hochberg correction was performed with a cutoff value of 0.05, and pairwise comparisons were made with a fold-change cutoff of 2-fold. Genes were considered as significantly altered if the average fold-change was at least 2, the



adjusted  $p$  value was less than 0.05 and the alteration was reproducible across replicates. Microarray data are deposited in GEO database at NCBI (Accession # GSE16112).

### Microarray results validation

Ten selected genes with differential expression by microarray results (i.e., 5 differentially expressed genes in S MDMs and 5 in R MDMs at 12 h p.i. compared to uninfected cells) were analyzed by quantitative RT-PCR (qRT-PCR). Two micrograms of RNA were reverse transcribed using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). For relative quantitation of target cDNA, samples were run in individual tubes in SmartCycler II (Cepheid, Sunnyvale, CA). One SmartMix bead (Cepheid) was used for 2 – 25  $\mu$ l PCR reactions along with 20ng of cDNA, 0.2X SYBR Green I dye (Invitrogen) and 0.3 $\mu$ M forward and reverse primers (Sigma Genosys) designed by Primer Express Software v2.0 (Applied Biosystems) (Table 1). For each gene tested, the individual calculated threshold cycles (Ct) were averaged among each condition and normalized to the Ct of the GAPDH from the same cDNA samples before calculating the fold change using the  $\Delta\Delta C_t$  method (Livak and Schmittgen, 2001). For each primer pair, a negative control (water) and an RNA sample without reverse transcriptase (to determine genomic DNA contamination) were included as controls during cDNA quantitation. Array data were considered valid if the fold-change of each gene tested by qRT-PCR was  $>2.0$  and in the same direction as determined by microarray analysis.

## 2 Results

### Invasion and intracellular growth of *B. abortus* S2308 revealed different patterns in MDMs from R and S cattle

At T0, the CFU of *Brucella* recovered from wells with R MDMs was significantly lower ( $P < 0.05$ ) than those from wells with S MDMs. At 12 h p.i. (T12), the number of intracellular *B. abortus* was 18% lower in MDMs from R animal, and 27% higher in MDMs from S animal, compared to their own T0 value (in both cases  $P < 0.05$ ) (Fig. 1). The number of *B. abortus* S2308 CFU present in growth control wells increased more than 1 log in the first 12 h p.i. compared with the original inoculum and the number of bacteria present in growth control wells after antibiotic treatment was reduced to zero. Altogether, these results indicate that *B. abortus* attach and internalize less efficiently in R than S MDMs and furthermore R MDMs kill more efficiently and inhibit *B. abortus* intracellular replication in the first 12 h p.i. than S MDMs.

### Reliability of array data

Twelve RNA experimental samples representing 4 different scenarios (triplicate biological replicas of infected and uninfected MDMs from R and S animals at 12 h post-treatment) were manually co-hybridized against a common bovine reference RNA on custom two-color DNA microarrays, each representing ~13,000 bovine ESTs, spotted in duplicate. The RNA analyzed from all samples was of good to excellent quality (RIN  $\geq 9.0$ , 28S/18S ratio  $\geq 1.6$ , OD<sub>260/280</sub>  $\geq 2.0$ , OD<sub>260/230</sub>  $> 1.8$  for experimental samples; and RIN = 9.7, 28S/18S ratio = 2.1, OD<sub>260/280</sub> = 2.01, OD<sub>260/230</sub> = 1.85 for reference RNA). The reference RNA generated readable signal intensities for more than 85% of the genes on the microarray (SNR  $> 3SD$  above background), and co-hybridization with experimental samples provided comparisons of MDMs gene expression profiles across all treatments and time points. For each of these samples, the spots represented on the arrays were adjusted for background and normalized to internal controls using GenePix Pro 6.0 software. Linear regression analysis of reference signal values (before normalization) from each array yielded an average R-squared value of 0.784 (minimum of 0.632 and maximum of 0.874), indicating that labeling procedures were

consistent across all arrays. A comparison of like-experimental samples (e.g., control replicate 1 versus control replicate 2) yielded similarly consistent results (Fig. 2).

To confirm the microarray results, we randomly selected 10 differentially expressed genes at 12 h p.i. (five from each condition, i.e. R and S) and performed qRT-PCR. Based on qRT-PCR results, transcript levels of 9 of 10 genes were altered greater than 2.0-fold and in the same direction as was determined by microarray analysis. The other gene (OSMR) was determined to be differentially expressed and in the same direction of microarray analysis, but the fold change was lower than 2 (Fig. 3A and B).

### Uninfected R and S MDMs displayed different transcriptional profiles

In order to identify candidate molecular markers or pathways relevant for *B. abortus* innate resistance, we compared the transcriptomes of uninfected S and R MDMs to identify infection-independent differences between the cells. Microarray data analysis revealed that 135 genes (80 with known function and 55 without functional characterization) were significantly altered (at least 2-fold and *P* value less than 0.05) between uninfected R and S MDMs under the same condition (Table 2). More specifically our data indicate that 123 (90%) of the differentially expressed genes were down-regulated and only 12 genes were up-regulated in R MDM cells versus S MDM cells. Differentially expressed genes with known or inferred function (80 genes) were grouped in terms of the associated biological processes attributed to each of their products (<http://www.amigo.geneontology.org>). The pie graph in Fig. 4 provides an overview of the groups sorted by biological processes of differentially expressed genes by uninfected R and S MDMs. Down-regulation expression of immune-related genes such as IL-18bp, C1RL, FCAR, ALOX5AP, PF4 and CCL2 may enhance Th1 immune response and reduce humoral and inflammatory reaction. Simultaneously, the reduction of cell proliferation activity is reflected by the down regulation of the 39 of 41 of the genes differentially expressed related with cell growth, differentiation and proliferation. The most highly and the lowest expressed genes in R MDMs compared to S were 2 loci without functional characterization (19.5 and -47 fold). Summarizing, the general functional trend suggests that the MDMs-resistant phenotype presents a basal enhanced Th1 immune response and a reduced expression of humoral and inflammatory factors and cell proliferation activity as well, compared to gene expression profiles of S MDMs.

### *Brucella*-infected S MDMs had a down-regulated transcriptome at 12h post-*B. abortus* infection

*B. abortus* S2308 infection induced alteration in the signal intensity values of 241 different genes (46 up- and 195 down-regulated) in S MDMs at 12h p.i. compare to uninfected cells (Table 3). One hundred forty-four (60%) of these 241 genes have known function or blast searches revealed high similarity to genes with known functions, with 22 (15%) of them up- and 116 (85%) down-regulated. Up-regulated genes with known function were randomly distributed among different functional categories, with higher proportions in cell adhesion (4/6) and immune response (3/6) clusters. Conversely, the majority of characterized down-regulated genes at 12h p.i. had some bias to some specific functions such as cell development, growth and differentiation (30/33), protein biosynthesis, folding and catabolism (24/27), general metabolism (16/20), signal transduction (11/13), intracellular trafficking (6/6), transcription regulation and mRNA processing (9/9), transport (8/9) and DNA replication and repair (7/9). Analysis of individual immune and inflammatory-associated genes differentially expressed reveals that *B. abortus* induce an up-regulation in IL-1A, CCL2, CCL5 and RANTES and a down-regulation of HSPA14, TCIRG1 and C1QBP genes during the first 12 h p.i. infection. Altogether, these data indicate that *B.*

*abortus* induce a down-regulation of Th1 subset of immune response and arrest the cell cycle in infected S MDMs at 12h p.i.

### ***B. abortus* infection stimulates modest alteration of transcript levels in R MDMs at 12h post-infection**

The infection assays disclosed that the number of intracellular *B. abortus* in R MDMs was 18% lower at T12 (12h p.i.) than at T0 ( $P < 0.05$ ) (Fig. 1). Microarray analysis identified *B. abortus* infection induced alteration in the signal intensity values of 56 different genes in R MDMs at 12h post-infection compared to uninfected controls (Table 4). Of these 56 genes, 31 were up- and 25 were down-regulated, but only 21 of these 56 genes (37.5%) had assigned functions (11 up- and 10 down-regulated). Individual analysis of differentially expressed genes with known function revealed that R MDMs mounted a type 1 immune response to *B. abortus* infection, which is the opposite of what was observed in S MDMs. These inferences correlate with the up-regulation of CCL4 (or MIP-1b) and the reduced expression of the activator of B-lineage gene expression (EBF1) and oncostatin M-specific receptor beta (OSMR-b). Also contrary to what was observed in infected S MDMs, the analysis of microarray data indicates that cell proliferation is active in infected R MDMs. Altogether these data indicate that *B. abortus* modestly induce a genome activation in bovine R MDMs during the first 12h p.i. infection, with bias to type 1 immune response and no host cell activity modification.

## **Discussion**

Our initial results indicate that *B. abortus* attach and internalize less efficiently in R than S MDMs. This result is in line with those obtained by Campbell *et al.* (1994), who found that MDMs from R cattle were less permissive to invasion by *B. abortus* 2308 than MDMs from S animals. These authors also found that the pathogen was bound to different surface's molecules on R or S MDMs, influencing the intracellular fate of *Brucella*. It is well known that *Brucella* is an intracellular pathogen that has the ability of survive and replicate inside professional phagocytic cells (Roop II *et al.*, 2004); therefore, the lower internalization of *Brucella* in R MDMs is integral to the host innate response to reduce *Brucella*'s opportunities to establish an intracellular niche for replication. Also in concordance with Campbell and Adams (1992), we observed that the number of intracellular *Brucella* at 12h p.i. increased in S MDMs but was decreased in R MDMs, which indicates that R MDMs kill more efficiently and inhibit *B. abortus* intracellular replication in the first 12 h p.i than S MDMs.

Immune response mechanisms are required by hosts to protect themselves from microbial invaders. The innate immune response is not only the first barrier of defense but also induces and modulates the acquired immune response, a more powerful and prolonged specific response. Previous studies have shown that Th1 cellular immune response is effective to promote *Brucella* clearance from the host (Dornand *et al.*, 2002; Rolán and Tsolis, 2008), while Th2 is detrimental for controlling *B. abortus* infection (Fernandez and Baldwin, 1995). To identify infection-independent molecular differences in *B. abortus* innate resistance, we compared the transcriptomes of uninfected S and R MDMs. Our data revealed that uninfected R MDMs had enhanced Th1 expression and reduced Th2-immune response-related genes, compared to gene expression profiles of S MDMs. The enhancement of type 1 cytokine response in uninfected R MDMs was correlated with the down-regulated expression of IL18BP, an inhibitor of the IL18- induced IFN $\gamma$  production (Novick *et al.*, 1999). IFN $\gamma$  was demonstrated to be crucial to inhibit *B. abortus* intracellular growth (Murphy *et al.*, 2001), and IL18BP down-regulation may be related to mechanisms that stimulate the early Th1-host protected immune response. The C1RL gene encodes the first component of the classical complement pathway that it is activated by the antigen-antibody



complex, and FCAR gene encodes the Fc receptor for IgA. The lower number of transcripts from C1RL and FCAR genes detected in R MDMs may correlate with the weak contribution that antibodies play in the role protecting against *Brucella* infections (Harmon et al., 1985; Baldwin and Goenka, 2006). In addition, the lower number of Fc receptors on R MØ may significantly reduce the opportunity for *B. abortus* to attach and invade mononuclear phagocytes (Campbell et al., 1994). Genes encoding chemoattractant products to polymorphonuclear (PMN) neutrophilic leukocytes and mononuclear phagocytic cells such as ALOX5AP, PF4 (also called CXCL4) and CCL2 (or MCP1) (Deuel et al., 1981; Meter et al., 2005) also had lower expression in uninfected R than in S MDMs. These data suggest that *Brucella* may take advantage of the higher predisposition of S animals to recruit phagocytic cells to hide inside and thus avoid the more deleterious extracellular environment. Simultaneously, PF4 has an anti-apoptotic activity in monocytes (Scheuerer et al., 2005), which is also enhanced by *Brucella* after invasion (Gross et al., 2000). Collectively, these observations may indicate that *Brucella* infection is enhanced by the more prolonged half life in S MDMs, which may be advantageous to the intracellular life style of the pathogen. In parallel, the observed down-regulation of CCL2 transcription would be expected to contribute to the Th1 immune response polarization (Omata et al., 2002; Del Corno et al., 2009) of R MDMs. On the other hand, no apparent association could be established between R phenotype to brucellosis and the down-regulated expression of HLA-A (or BoLA) gene. HLA-A encodes a heavy chain of the MHC-I. Foreign peptides derived from intracellular synthesized antigens (mainly virus) are presented in association with MHC-I and recognized by CTLs-CD8<sup>+</sup>. However, this mechanism does not seem to be relevant during *Brucella* infection, as bovine lymphocytes expressing CD8<sup>+</sup> molecules do not react with *B. abortus* and MHC-I – knockout mice controlled and cleared *B. abortus* infection as well as wild type mice (Smith III et al., 1990; Baldwin and Parent, 2002).

Two non-immune related genes differentially expressed between uninfected R and S MDMs that could influence the natural resistance to brucellosis in cattle were transferrin (TR) and tumor rejection antigen (TRA1). TR is a serum protein that transports iron from storage sites to all proliferating cells in the body. Paradoxically, iron is not only an essential element to promote bacteria growth (Finlay and Falkow, 1997) but is also involved in anti-microbial mechanisms (Babior, 1984). In concordance with the observation that increased levels of iron correlate with increased susceptibility to infectious disease (Ampel et al., 1989), the lower expression of TR gene in R animal may, in part, explain the restricted intracellular growth of *B. abortus* in R MDMs. TRA1 (also known as GP96/GRP94) encodes a molecular chaperone of the endoplasmic reticulum. In addition to the role in refolding denatured proteins after stress, the product of this gene is involved in a cross-priming process, which includes the uptake, processing and presentation of antigens by professional APCs (Warger et al., 2006). Simultaneously, it was also shown that GP96 gene product binds bacterial LPS and augments its biological activity. It is well known that *Brucella* presents an unconventional LPS that allows the pathogen to evade innate immunity (Lapaque et al., 2005). Collectively, these data indicate that R MDMs may have the ability to amplify its innate immune response to *Brucella* infection due to the higher number of GP96 molecules that would attach to *B. abortus* LPS and augment its biological activity. It is not immediately clear how several other differentially expressed genes between uninfected R and S MDMs might contribute to susceptibility or resistance of cattle to *B. abortus* infection; it is possible that some of the differences in transcript levels observed might be individual variation however others are related to resistance / susceptibility to *B. abortus* infection.

The analysis of microarray performed with RNA extracted from *B. abortus*-infected S MDMs revealed up-regulation in some inflammation-associated host genes during *B. abortus* infection, such as IL-1A, CCL2 and CCL5. IL-1 is a potent pro-inflammatory cytokine with pleiotropic effects. However, this interleukin has not been found to have an

effect on intracellular *Brucella* growth (Jiang and Baldwin, 1993), although it does play a critical role in the development of Th2 cell cytokine production (Manetti et al., 1994). CCL2 (also called MCP-1) and CCL5 (or RANTES) are chemotactic cytokines for monocytes to the inflammatory site. The up-regulation of these genes after *B. abortus* infection may indicate that the pathogen enhances the influx of the mononuclear phagocytic cells to the already predisposed host to the site only to infect the MDMs and remain protected from external stimulus. Simultaneously, CCL2 modulates incoming monocytes differentiation into dendritic cells and inhibits Th1 cell development (Omata et al., 2002). In parallel, abortion is the main clinical symptom of brucellosis in cattle, but little is known about the molecular mechanism. In a recent publication, it was demonstrated that the increased expression of RANTES contributes to abortion in *B. abortus*-infected pregnant mice (Watanabe et al., 2008). This finding enables speculation that the increase rate of abortion induced in S cows after challenging with *B. abortus* (Harmon et al., 1985) could be in part due to the increased expression of RANTES gene. Also among immune-related genes, microarray analysis displayed decreased expression of HSPA14, TCIRG1 and C1QBP genes at 12 h post-*B. abortus* infection in S MDMs. HSPA14 encodes for a heat shock protein from the Hsp70 family. Proteins from this family were demonstrated to have potent effects polarizing immune responses toward Th1 (Wan et al., 2009), while the product of TCIRG1 gene induces T cell activation, IL-2 secretion and IFN $\gamma$  expression (Utku et al., 1998), all elements that contribute to eliminate *Brucella* from the host. Altogether, these data indicate that *B. abortus* induces a down-regulation of Th1 subset of immune response in S MDMs, which would be expected to contribute to chronic brucellosis.

Another interesting finding from the infected S MDMs gene expression analysis indicates that the cell cycle is largely arrested in infected cells at 12h p.i. (Table 3). The down-regulation of the great majority of genes clustered in DNA replication, transcription regulation and cell growth and proliferation groups are in concordance with the lower number of transcripts from protein metabolism-encoding genes detected in infected compared to uninfected MDMs. Similar results were observed in mouse MDMs infected with *B. abortus* at earlier time point (Eskra et al., 2003; He et al., 2006). Perhaps, reduced host metabolism is necessary for *B. abortus* to survive and replicate inside MDMs. Considering the numbers and types of genes with decreased expression in S MDMs at 12 h p.i., it is possible that susceptibility results from the inability of MDMs to mount appropriate immune responses against invading *Brucella* as well as dampening of required general cell physiology and metabolism.

On the other hand, the analysis of microarray results revealed that R MDMs mounted a type 1 immune response to *B. abortus* infection. These inferences correlate with the up-regulation of CCL4 (or MIP-1b) (Schrum et al., 1996) and the reduced expression of EBF1, an activator of B-lineage gene expression (Roessler et al., 2007). Transcription and secretion of CCL4 by mononuclear phagocytes is induced by IFN $\gamma$  (Hariharan et al., 1999), and its product is chemotactic for T helper1 cells (Siveke and Hamann, 1998) and a co-activator of MDMs (Dorner et al., 2002). OSMR encodes for an oncostatin M-specific receptor beta that is specifically activated by oncostatin M (OSM) (Mosley et al., 1996). Deficient expression of this gene facilitates an enhanced influx of monocytic cell trafficking into the site of inflammation with NF-kB activation (Hams et al., 2008). Normally, *B. abortus* infection induces minimal levels of cytokines and does not generate an inflammatory response (Barquero-Calvo et al., 2007) therefore the lower expression of OSMR gene could be part of the more active innate immune response in R MDMs that enhances control of *B. abortus* replication. Also contrary to what was observed in infected S MDMs, the analysis of microarray data indicates that cell proliferation is active in infected R MDMs. This fact is supported by the increased transcription of cell proliferation-induced genes such as PDGFC (Platelet Derived Growth Factor C) and MARCKSL1, the anti-apoptotic gene BCL2A1 and

the up-regulation of the DNA-replicative gene KCTD13 (or PDIP1) (He et al., 2001) as well. The up-regulation of these cell cycle progression-induced genes and the sub-expression of RAD51 gene, which encodes the catalytic component in homologous recombination (Lundin et al., 2003), suggest that the intracellular presence of *Brucella* does not significantly interfere with the cell physiological processes, and the pathogen does not generate host DNA damage in R MDMs in the first 12h p.i. On the other hand, the up-regulation of RAD18, another DNA damage repair gene, is somewhat contradictory. The product of RAD18 gene accumulates in nuclei after UV irradiation and double-strand breakage in cells entering S-phase and acts as a proximal signal to recruit Y-family polymerases to bypass damaged DNA (Kakar et al., 2008). These data are in concordance with the active, continuous proliferative capability of infected R MDMs. Recently, it was revealed that *B. abortus* uptake choline from the host to elaborate their own phosphatidylcholine (PC), which is necessary to sustain a chronic infection process (Comerci et al., 2006). The product of the locus LOC619625 shows high similarity to the protein encoded by the *S. cerevisiae* SEC14 gene, which regulates the phosphatidylcholine (PC) homeostasis and the intracellular trafficking of vesicles from the trans-Golgi export pathways in yeast (Curwin et al., 2009). A down-regulation of the SEC14 implicates higher levels of host PC that negatively influences Golgi vesicle generation (Howe and McMaster, 2006). In the view of our results, we propose that the down-regulation of the LOC619625 locus stimulates increased concentrations of PC in R MDMs as a mechanism to sequester choline from the pathogen and simultaneously modulate intracellular trafficking and interfere with the establishment of the *B. abortus* intracellular niche. The contribution of the other differentially expressed genes in R MDMs inhibiting intracellular *B. abortus* survival and replication in the first 12h p.i. is challenging; however, our data clearly indicate that 1) *B. abortus* modestly induces a genome activation in bovine R MDMs during the onset of infection, 2) R cattle have the ability to mount a type 1 immune response against *B. abortus* infection, and 3) the host cell activity is not altered significantly after 12h post-*B. abortus* infection.

In conclusion, the data from our experiments reveal that R but not S MDMs inhibit *B. abortus* intracellular growth in the first 12h p.i. Gene expression analyses of both uninfected and *B. abortus* infected MDMs 12h p.i identified completely different sets of expressed genes, a phenomenon that was observed for both susceptible and resistant MDMs. The data highlighted that R MDMs have the ability to mount a type 1 immune response against *B. abortus* infection, which was impaired in S MDMs. Also, there was a general trend for greater numbers of gene expression alterations (mainly down-regulation) to occur in *B. abortus* infected S MDMs, as opposed much lower numbers of gene expression changes in infected R MDMs, and especially when compared to uninfected cells. Similarly, a higher number of transcriptional alterations was observed in uninfected S compared to R uninfected MDMs. We propose that a reduced extended response to *B. abortus* infection facilitates natural resistance to the bacteria.

## Acknowledgments

Mr. Alan Patranella for taking care of the animals and blood recollection, and Mrs. Roberta Pugh and Mrs. Doris Hunter for their technical assistance. This study was supported by U.S. Department of Homeland Security – National Center of Excellence for Foreign Animal and Zoonotic Disease (FAZD) Defense grant ONR-N00014-04-1-0 and a NIH grant 2U54AI057156-06. CAR was sponsored by Fulbright-INTA scholarship from Argentina.

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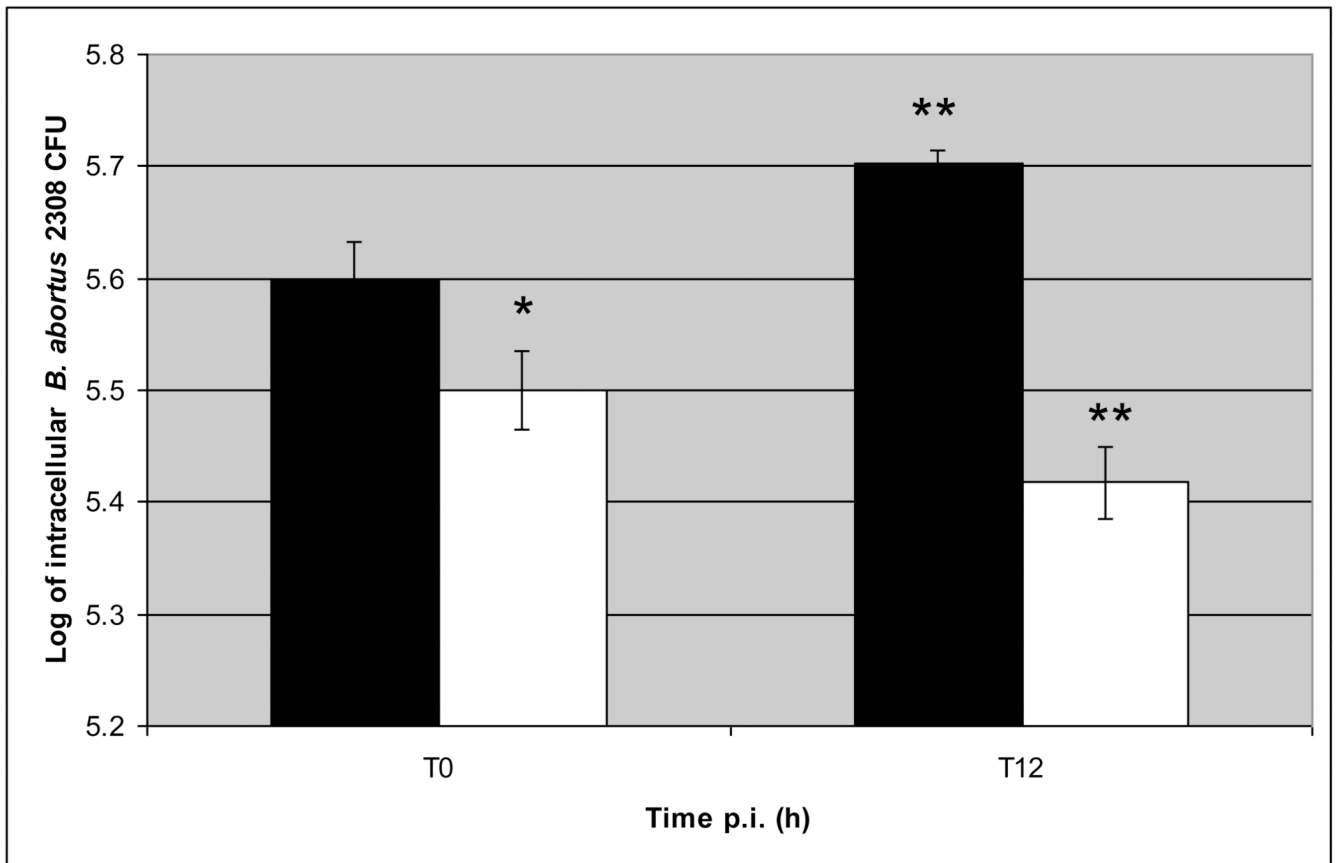
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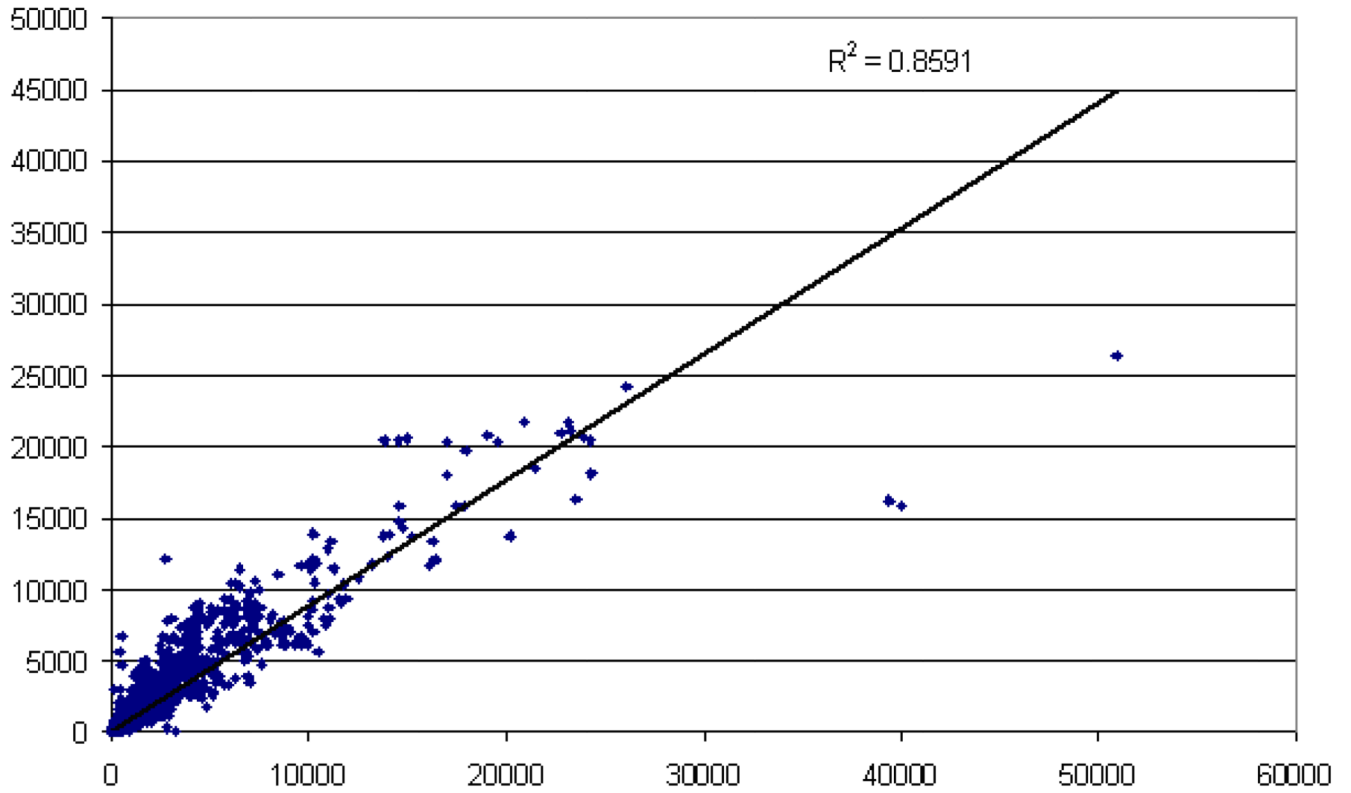
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**Figure 1. Kinetics of *B. abortus* S2308 intracellular growth in bovine monocytes-derived macrophages from cattle naturally resistant (R) or susceptible (S) to brucellosis**  
 Monocyte-derived macrophages (MDMs) were plated in triplicate in 24 well plates at  $2 \times 10^5$  cells/well and infected with *B. abortus* S2308 at a MOI 5:1. After 30 min – interaction, extracellular bacteria were killed by co-incubation with gentamycin for 1 h, and then washed 3 times with PBS. At 0 (T0) and 12 (T12) h post-infection, cells were lysed and serial dilutions cultured on TSA plates for quantification of CFU. The intracellular number of *B. abortus* S2308 was significantly different ( $P < 0.05$ ) in MDMs from R and S cattle at T0 (\*) and at T12 from R or S MDMs compared to their own T0 value (\*\*). Means  $\pm$  SD (bars) of 3 independent assays done in triplicate are shown. Solid bars indicate intracellular *B. abortus* CFU from S MDMs, open bars indicate intracellular *B. abortus* CFU from R MDMs.

**Reference Control 1 vs Reference Control 2**

**Figure 2. Expression profile chart for genes from bovine reference RNA samples**

An expression profile chart of two identical reference samples with linear trend line and R-squared values is shown. The ordinate represents non-normalized signal intensity values for each spot (gene) from one randomly chosen reference sample, and a second reference sample is indicated on the abscissa.



FIG. 3A

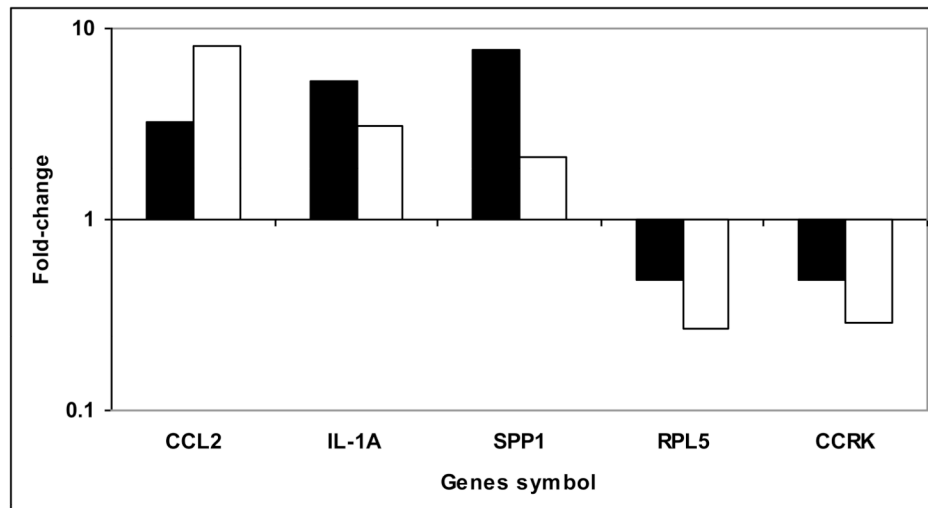
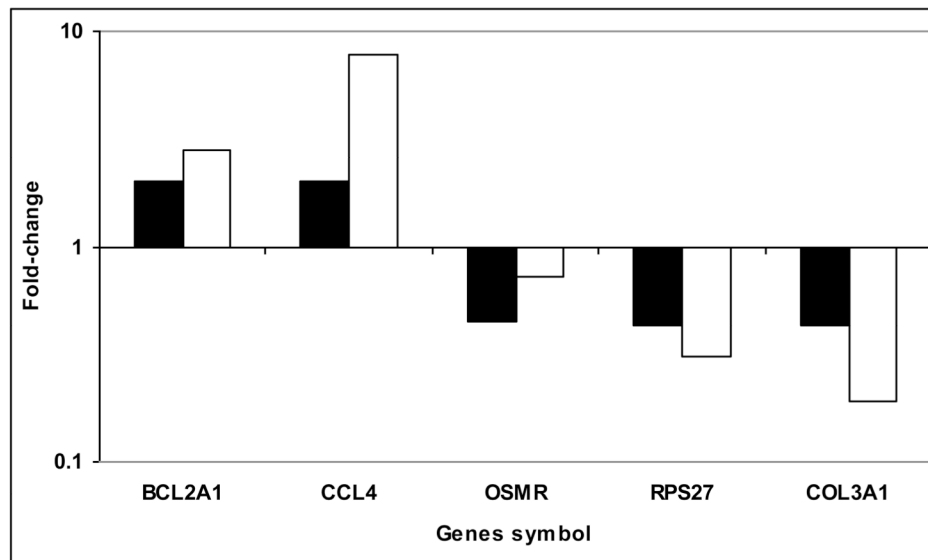
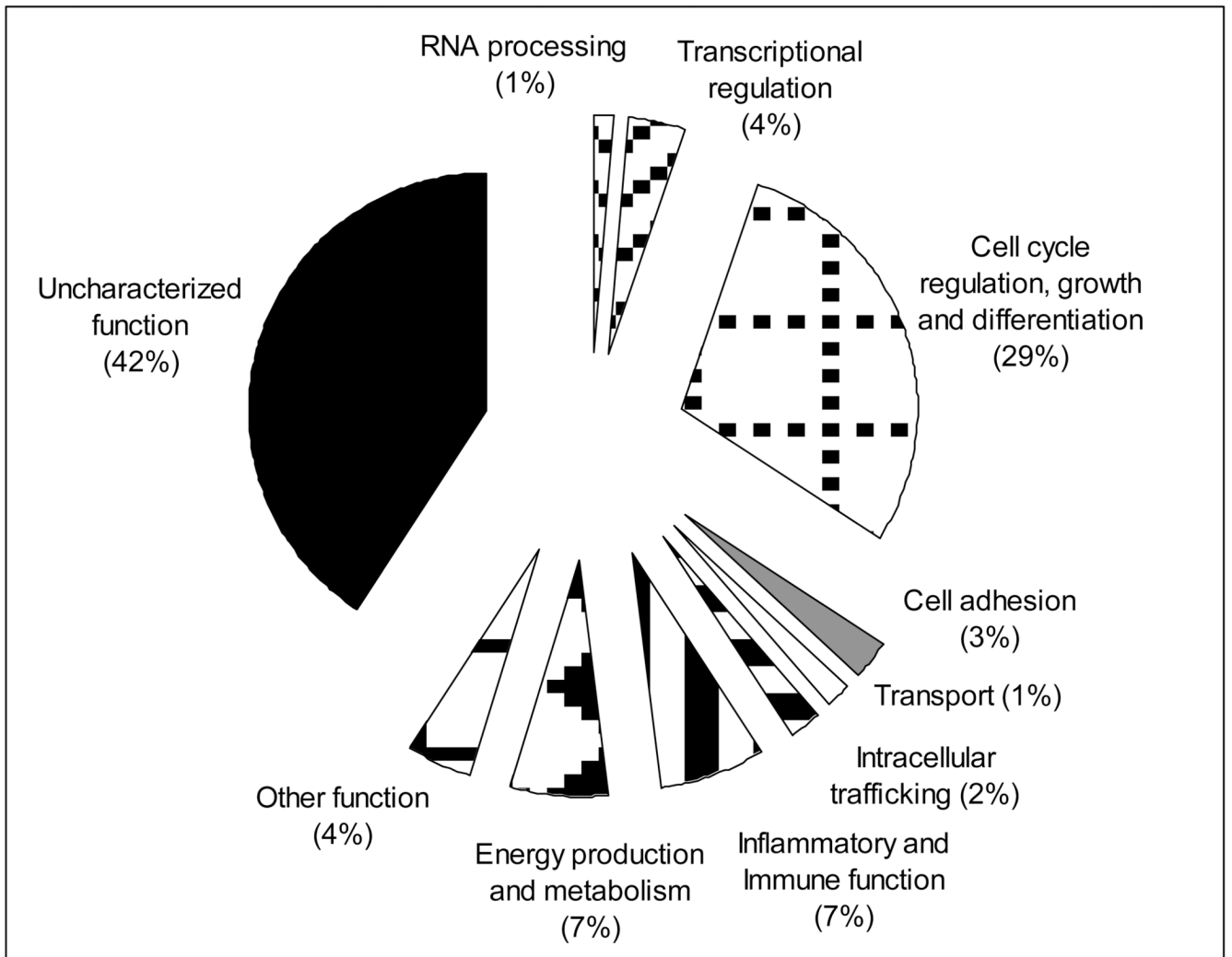


FIG. 3B



**Figure 3. Validation of microarray results by quantitative Real Time - PCR**

cDNA was synthesized from the same RNA samples used for microarray hybridization. Ten selected genes that were differentially expressed based on microarray analysis between *B. abortus*-infected susceptible bovine MDMs (A) and *B. abortus*-infected resistant bovine MDMs (B) at 12h p.i. as compared with non-infected cells (control), were validated by quantitative RT-PCR. Fold-change was normalized to the expression of GAPDH and calculated using the  $\Delta\Delta C_t$  method. All the genes tested had fold-changes in the same direction by both methodologies and 9 of 10 were also altered greater than 2-fold. Solid bars indicate microarray fold-change, open bars indicate qRT-PCR fold-change.



**Figure 4. Proportional representation of the biological processes groups of differentially expressed genes between uninfected bovine R and S monocyte-derived macrophages. Detailed information is presented in Table 2**

**Table 1**

Primers for Real Time – PCR evaluated genes of bovine monocyte- derived macrophages.

GeneBank accession #	Gene symbol	Gene name	Forward primers (5'-3')	Reverse primers (5'-3')
NM_174006	CCL2	Chemokine (C-C motif) ligand 2	TCCTAAAGAGGCTGTGATTTTCAA	AGGGAAAGCCGGAAGAACAC
NM_174092	IL-1A	Interleukin 1, $\alpha$	GCCACAAAGCAAGAAAAATTGG	ACATGCTCAGCGAGTGACTAACA
NM_174187	SPP1	Secreted phosphoprotein 1	TGCCACAGAGGAGGACTTCAC	CTTGTCTTATCCTTGCCCTTTG
NM_001035306	RPL5	Ribosomal protein L5	GCCAGAACTACTACCGGAATAAA	CCATGATGTGCTTTCGGTGTA
NM_001098933	CCRK	Cell cycle related kinase	GCTGTGAGCTTCCAATTTTGTG	CCCTGCCTGGTGGAAATCC
NM_001037100	BCL2A1	BCL2 related protein A1	GCCAGAACAATATTCACCAAGTG	TGATGAACTCCGCCACAAAG
NM_001075147	CCL4	Chemokine (C-C motif) ligand 4	AGCTGTGGTATTCCAGACCAAAA	GCATGGAGAGGGTGCATCTC
NM_001080272	OSMR	Oncostatin M receptor	GTTGTTACAGCCACGCTTCT	GGAGGTAAGCTCCTTGGCATT
NM_001098135	RPS27	Ribosomal protein S27	TGCAGAGCCCAATTCCTAT	GCCAACACACAAGACTACTGTTGT
NM_001076831	COL3A1	Collagen, type III, alpha 1	CACTCCATATGTTCTTTTGTCTAATC	CACTCCATATGTTCTTTTGTCTAATC
NM_001034034	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TTCTGGCAAAGTGGACATCG	GCCTTGACTGTGCCGTTGA

**Table 2**

Comparison of genes differentially expressed in uninfected monocyte-derived macrophages from cattle resistant to susceptible to brucellosis.

<b>Sequence Identifier</b>	<b>Gene symbol</b>	<b>Fold-change</b>
<b>RNA processing</b>		
CR452407	PSRC2	-2.6
NM_174594	RNASE6	-5.9
<b>Transcription regulation</b>		
CN437874	LBH	2
CN442084	SFMBT2	-2
CN437084	NR2F1	-3.1
BF041696	PMF1	-3.2
CN436867	TRIP13	-3.5
<b>Cell cycle regulation/growth/differentiation</b>		
CR452577	Transcribed locus	3
NM_174264	CCNB2	-2
CR454106	CABLES1	-2.1
CR453022	MCM2	-2.1
BM364143	ARL6IP1	-2.3
CN437507	TCFL5	-2.5
CN434361	TYMS	-2.6
CR455944	CCNA2	-2.7
CN436077	FANCD2	-2.8
CR454614	HELLS	-2.9
AW357584	CDC6	-3
CR452898	SPBC25	-3.2
BM364726	RBL2	-3.2
CN438866	ALX1	-3.3
CR550984	SMC2	-3.3
BF040210	DAB2	-3.3
CR453527	RRM2	-3.4
CN440981	CEP72	-3.4
CN437405	DZIP1	-3.7
BF045590	RYBP	-3.7
CN433387	CDCA3	-4
CR456279	CENPF	-4.2
CN441415	SPAG5	-4.3
CK773173	CDC45L	-4.5
BP100977	ASF1B	-4.9
CN433213	CKAP2	-5
CN433471	TPX2	-5.1
CN432347	CTTNBP2	-5.1

Sequence Identifier	Gene symbol	Fold-change
CN440096	STMN1	-5.2
DR749363	CCNF	-5.6
CR455093	KNTC2	-5.6
DR749295	CENPA	-6.1
BF043168	NEK2	-6.2
AW461931	IQGAP3	-7
CR452593	TMSL8	-7.8
CN440232	KIF11	-8.1
CN433506	KIAA0101	-9.4
CN433360	TOP2A	-12.2
CN434640	DLG7	-16.8
<b>Cell adhesion</b>		
CN437044	CDH15	2.9
CR454464	VCL	2.7
CN442238	TMEM204	-3.5
CN435183	THBS2	-4.9
<b>Transport</b>		
CR455643	TF	-2.7
NM_174782	SLC12A2	-5
<b>Intracellular trafficking</b>		
CR453444	AP3D1	2.2
BM251684	SYTL1	-2
CR453562	KIF20A	-3.5
<b>Inflammatory &amp; immune functions</b>		
NM_173982	AGER	2.8
BF044563	ALOX5AP	-2
BF041442	ELMOD3	-2
BF040320	C1RL	-2.9
BF042057	IL18BP	-3
AY247821	FCAR	-3.6
BF440567	HLA-A1	-3.7
BF044309	LILRB6	-4
CN440842	PF4	-5.2
BF043950	CCL2	-8.6
<b>Energy production and metabolism</b>		
AW464548	CTSK	3.1
CR452848	TRA1	2.4
BF440264	ZDHHC2	-2
CN440619	NAAA	-2
AW464985	USP9X	-2.2
CR453258	ATP5G1	-2.3



Sequence Identifier	Gene symbol	Fold-change
CK728068	ACOX3	-2.4
AW464210	DPP4	-2.4
BF041253	FAR2	-3.3
<b>Other functions</b>		
NM_174566	OPN1LW	-2
CR455862	MBP	-2.1
CR552253	CALD1	-2.2
BF440451	CLEC2D	-2.5
BF440280	ANXA3	-3.4
CR452494	COL1A2	-5.3
<b>Uncharacterized</b>		
BM361913	mRNA sequence	19.5
DR697542	mRNA sequence	7.6
BF042955	mRNA sequence	3.1
BF046062	DBNDD1	2.4
CN433011	mRNA sequence	-2
AY563829	Transcribed locus	-2.1
AW465803	Transcribed locus	-2.1
CN437617	NKD2	-2.1
CN437228	mRNA sequence	-2.2
CN440381	Transcribed locus	-2.2
AW463556	mRNA sequence	-2.2
DR749282	Transcribed locus	-2.2
BF043021	mRNA sequence	-2.3
CR454941	Transcribed locus	-2.3
CN432553	NRM	-2.3
CN440892	Transcribed locus	-2.3
BF040477	mRNA sequence	-2.3
BF040697	mRNA sequence	-2.4
CN440892	Transcribed locus	-2.4
CN435160	Transcribed locus	-2.5
CN437807	mRNA sequence	-2.5
BF440307	mRNA sequence	-2.7
CN438535	mRNA sequence	-2.8
BF440292	mRNA sequence	-2.8
CR454384	GPATCH8	-2.8
BM362405	SUSD3	-2.9
TC222073	mRNA sequence	-3
CN435160	Transcribed locus	-3
BF044422	Transcribed locus	-3.1
CB423716	Transcribed locus	-3.2

Sequence Identifier	Gene symbol	Fold-change
BM363262	LOC513111	-3.2
CN432697	Transcribed locus	-3.2
CR452791	mRNA sequence	-3.4
AW463283	Transcribed locus	-3.4
AW461570	C23H6orf129	-3.5
BF043154	Transcribed locus	-3.6
CN438539	mRNA sequence	-3.6
BM363349	mRNA sequence	-3.8
CN438081	Transcribed locus	-3.8
BM366640	Transcribed locus	-3.9
CR553166	mRNA sequence	-4.2
BM364214	LOC618541	-4.3
CN441931	Transcribed locus	-4.4
CN436436	Transcribed locus	-4.6
CN440116	mRNA sequence	-4.6
CN436425	Transcribed locus	-5.1
CN436345	Transcribed locus	-5.3
CN433474	mRNA sequence	-5.4
CR453971	Transcribed locus	-5.4
BM366656	Transcribed locus	-5.8
CN436426	mRNA sequence	-6.4
CN441242	mRNA sequence	-6.9
CN435532	Transcribed locus	-8.8
CR453356	mRNA sequence	-12.5
CR553566	Transcribed locus	-47

Negative sign (-) before the number indicates down-regulation of the gene.

**Table 3**

Genes significantly up - regulated in *B. abortus* – infected monocyte-derived macrophages from bovine naturally susceptible to brucellosis 12 h post-infection, compared to uninfected cells.

Sequence identifier	Gene symbol	Fold-change	P value
<b>DNA replication and repair</b>			
CN436535	RAD51AP1	2	0.012798
CN440965	MEN1	2	0.033644
BM363362	HP1BP3	-2.1	0.014615
CR451784	SSRP1	-2.1	0.001088
BF042701	XRCC5	-2.2	0.028438
AW463670	CHD3	-2.3	0.010785
CN433291	H2AFX	-2.3	0.00807
CR452840	ATIC	-2.3	0.03416
BM366355	CHRAC1	-2.5	0.020614
<b>Transcription regulation and RNA processing</b>			
CN434076	RBM23	-2	0.001493
BF044611	SKIV2L2	-2	0.019285
CN439289	ZNF211	-2	0.04369
CN435437	SNRPG	-2.1	0.02193
BF440611	QKI	-2.1	0.028399
CR453755	TINP1	-2.2	0.002914
CR454921	TAF11	-2.4	0.048223
BF440603	DNTTIP2	-2.4	0.010831
DR697555	IGF2BP3	-3.7	0.033597
<b>Cell development, growth and differentiation</b>			
CK846466	CBLN1	4.2	0.010537
AW466205	CIDEA	2.2	0.030579
NM_205787	REG3A	2.2	0.006797
BF045587	AATF	-2	0.014579
CR451700	MLF2	-2	0.043153
CN438718	SKIL	-2	0.035406
CN434165	PIGU	-2	0.01877
CK959975	ANGPT4	-2	0.047019
BF045982	EPB41L2	-2	0.044067
AW462514	GSPT1	-2	0.007692
AW461998	CSRP1	-2	0.029829
CN440711	OPTN	-2.1	0.048056
BF039833	CCRK	-2.1	0.035498
AW463034	AKAP12	-2.1	0.041319
CN437378	ARPC1B	-2.2	0.002672
CN436304	SMC4	-2.2	0.040856
BF440228	TGFBR3	-2.2	0.022062

Sequence identifier	Gene symbol	Fold-change	P value
BM363735	AIF1	-2.3	0.003817
NM_174005	CCIN	-2.3	0.004703
CR454883	MEST	-2.3	0.019538
CR451635	GTSE1	-2.3	0.003521
CN440112	MINA	-2.3	0.00067
CK943499	TACC2	-2.3	0.04264
CR452742	ENC1	-2.3	0.007579
BF440347	FGD3	-2.3	0.021089
AW463810	EAPP	-2.3	0.006253
NM_174209	UACA	-2.4	0.012996
BF045016	ZMYM3	-2.4	0.02123
AW462369	RARRES1	-2.4	0.001494
CB421393	BBS7	-2.6	0.014228
AW462158	DDX28	-2.6	0.015702
BM365145	TUSC4	-2.8	0.01158
AW463470	NCAPD2	-3	0.015283
<b>Transport</b>			
NM_174071	GLRB	2.6	0.04727
CR454289	TNPO3	-2	0.038832
BF046658	TIMM50	-2	0.030007
CR455688	FDX1L	-2	0.006493
CN437583	CLNS1A	-2.1	0.001805
BF040012	SLC19A2	-2.1	0.010527
BM363775	CLIC1	-2.2	0.011465
NM_174371	KCNA4	-2.5	0.01934
BF043853	SYT4	-2.7	0.015922
<b>Metabolism</b>			
CB446606	CA8	4	0.011733
BF039557	LOC785314	2.6	0.002684
NM_201527	SOD2	2.6	0.040638
BF043599	ATAD4	2.1	0.01223
CR455483	CRYZ	-2	0.023141
CR452141	NAT12	-2	0.035025
CN437401	DPM1	-2	0.038338
BF044835	DIP2B	-2	0.038872
BF043099	GSTK1	-2	0.024588
CN435685	RGN	-2	0.002805
AW464776	TXN2	-2.1	0.025333
AW462172	ARSB	-2.1	0.038167
CN437243	ISCU	-2.2	0.002229
CN432284	PIK3C3	-2.2	0.019429

Sequence identifier	Gene symbol	Fold-change	P value
DR697431	LOC782666	-2.3	0.005903
AW461872	MSR1	-2.3	0.006326
CN434081	PCCB	-2.4	0.009599
AW261169	PCCA	-2.5	0.003671
AW266859	MSRB2	-2.7	0.008601
BF041637	GNPAT	-3.6	0.024213
<b>Protein processing</b>			
BF040619	NEDD4L	2.8	0.027674
BM431662	SPINK1	2.2	0.04782
NM_181008	CSN2	2	0.038672
CR454222	MRPL3	-2	0.029686
CO895992	ASPA	-2	0.001912
BF046090	FBXO11	-2	0.022913
BF042334	RPS6KC1	-2	0.016106
CR553712	OS9	-2	0.006614
BF044848	UBE2V2	-2	0.011541
CR553513	UCHL5	-2.1	0.01485
CR552645	DUSP12	-2.1	0.00738
CR454607	MRPL13	-2.1	0.021589
CR453519	RPL35A	-2.1	0.006397
CR452490	RPL5	-2.1	0.01003
BM364981	PFDN5	-2.1	0.00125
BF046118	PSMD1	-2.1	0.010022
BF045731	VBP1	-2.1	0.004496
CR453689	RPL22	-2.2	0.015299
CR453525	RPS6	-2.2	0.043312
BF045932	MRPS11	-2.2	0.002001
BF045297	RPL10	-2.2	0.048448
AW465874	ARIH1	-2.2	0.027491
CR454940	ST3GAL6	-2.2	0.001991
BM366593	DNAJA3	-2.2	0.01866
BF042101	SHMT2	-2.3	0.013722
CN434588	FKBP3	-2.8	0.008926
CN441491	SIAH1	-3	0.007214
<b>Signal transduction</b>			
M_001001149	CALCB	2.7	0.001067
NM_174406	NXPH2	2.6	0.042586
NM_174236	AKAP5	-2	0.001748
BF043621	PYGO2	-2	0.018663
BM363977	TBC1D9	-2	0.008686
BF440275	TNIK	-2	0.016256



Sequence identifier	Gene symbol	Fold-change	P value
AU279004	TM2D3	-2	0.00015
CN436332	CNIH4	-2.1	0.007068
CN437637	CALR	-2.2	0.012659
CN440743	GKAP1	-2.2	0.020772
CR456175	S100A10	-2.3	0.004438
CN438280	GNB5	-2.7	0.045939
CR453647	AMFR	-2.9	0.018384
<b>Intracellular trafficking</b>			
DR697451	ENAH	-2	0.014658
AW465699	ARF6	-2	0.010549
CF613563	ATG5	-2	0.033296
BF040141	RAB24	-2.2	0.001572
BF039386	COG2	-2.4	0.047562
BM362494	GBF1	-2.5	0.002042
<b>Cell adhesion</b>			
CN435936	SPP1	7.8	0.006672
BF043281	GJA3	2.4	0.010211
NM_174280	CNTN1	2.2	0.031111
L27869	NRXN3	2.2	0.035758
BF043702	CEACAM8	-2.2	0.004872
CN439886	GPM6B	-2.2	0.021116
<b>Inflammatory and immune response</b>			
X12497	IL1A	5.3	0.029947
BF043950	CCL2	3.2	0.04788
BM363499	CCL5	2.8	0.04067
CR553339	HSPA14	-2	0.007621
AW462950	TCIRG1	-2.2	0.026645
CN433781	C1QBP	-2.3	0.00346
<b>Unknown function</b>			
CK394086	GRAMD1C	4.7	0.005583
CR453360	mRNA seq	3.6	0.028089
CN434319	Transcr locus	3.5	0.003021
CR552017	INDOL1	3.2	0.005439
BF042120	mRNA seq	2.9	0.011408
BF040440	Transcr locus	2.9	0.013091
BM364050	mRNA seq	2.8	0.045414
CK394163	Transcr locus	2.6	0.038814
AW464659	mRNA seq	2.6	0.021362
BF042444	Transcr locus	2.6	0.030362
AY563735	mRNA sequ	2.6	0.017374
CN438563	mRNA	2.5	0.038378

Sequence identifier	Gene symbol	Fold-change	P value
DR749459	mRNA seq	2.4	0.024572
CR454841	mRNA seq	2.2	0.005294
CN435906	mRNA seq Hypothetical	2.1	0.04738
CK394134	LOC100174927	2.1	0.012865
BM363550	mRNA seq	2.1	0.010831
AW463075	mRNA sequ	2.1	0.011975
AW656826	Transcr locus	2.1	0.0332
DR749406	ALKBH4	2	0.037488
CN441759	KIAA0406	2	0.020521
CN436628	Transcr locus	2	0.025941
CR454029	Transcr locus	2	0.037561
CN438621	mRNA sequ	2	0.044158
CV798869	Transcr locus	-2	0.005075
CR553807	MORN2	-2	0.008144
CR553297	DONSON	-2	0.016478
CR552451	Transcr locus	-2	0.016826
CR550949	ENKUR	-2	0.039676
CR452826	TMCC3	-2	0.043398
CN441384	mRNA seq	-2	0.013879
CN437904	Transcr locus	-2	0.02252
CN433518	Transcr locus	-2	0.011052
CN433286	Transcr locus	-2	0.026338
BM364672	C21orf59	-2	0.008
BM362937	mRNA seq	-2	0.016551
BF440346	CCDC125	-2	0.031793
BF046723	mRNA seq	-2	0.033449
BF041501	LOC515651	-2	0.015499
AW461946	MGC128747	-2	0.001249
AW465670	mRNA sequ	-2	0.032058
CN440220	LOC617694	-2	0.021621
BE722725	Transcr locus	-2	0.03681
BF044805	Transcr locus	-2	0.014413
AW465807	Transcr locus	-2	0.003764
CR452356	LOC506074	-2.1	0.004625
CN437533	Transcr locus	-2.1	0.010029
CN437200	C1orf52	-2.1	0.029358
CN436474	MGC148355	-2.1	0.042662
CN435422	mRNA seq	-2.1	0.004848
BM364118	mRNA seq	-2.1	0.034525
BM363762	Transcr locus	-2.1	0.024959
BF041485	Transcr locus	-2.1	0.001438

Sequence identifier	Gene symbol	Fold-change	P value
BF040156	FAM96A	-2.1	0.02247
AY563713	Transcr locus	-2.1	0.024982
AW463226	C7H5orf15	-2.1	0.002516
CN433567	MGC139126	-2.1	0.003927
BF440393	Transcr locus	-2.1	0.029318
DR697587	Transcr locus	-2.2	0.017607
CN441772	CCDC90A	-2.2	0.019245
CN439397	Transcr locus	-2.2	0.044367
CN438023	Transcr locus	-2.2	0.008942
BM364157	mRNA seq	-2.2	0.020096
BF440257	Transcr locus	-2.2	0.024855
BF045639	Transcr locus	-2.2	0.043847
BF044066	YIPF2	-2.2	0.047593
AW463110	LOC514162	-2.2	0.00779
AW461934	TMEM165	-2.2	0.008286
CN439481	Transcr locus	-2.2	0.018453
BM363176	WDR54	-2.2	0.045691
CR454316	TCTEX1D2	-2.3	0.019889
BM363007	mRNA seq	-2.3	0.030154
BM362963	TMEM183A	-2.3	0.004398
BF045994	mRNA seq	-2.3	0.001834
BF040988	Transcr locus	-2.3	0.004564
CR453653	Transcr locus	-2.3	0.044028
CR455800	Transcr locus	-2.4	0.018999
CR453901	RSRC2	-2.4	0.044236
CR451871	Transcr locus	-2.4	0.007411
BM364112	mRNA seq	-2.4	0.018626
BF043474	LOC507724	-2.4	0.029765
AY563886	mRNA seq	-2.4	0.011197
CN438652	Transcr locus	-2.4	0.045309
BM364672	C1H21ORF59	-2.4	0.010719
CN436507	LENG8	-2.4	0.026297
CN434826	mRNA seq	-2.5	0.010429
BF440233	mRNA seq	-2.5	0.031531
BF044267	ZC3H11A	-2.5	0.005648
DR749252	mRNA seq	-2.6	0.007047
CN434082	Transcr locus	-2.6	0.003073
BF440521	mRNA seq	-2.6	0.026848
BF043361	NIPSNAP1	-2.6	0.018738
AW464396	TMCC1	-2.6	0.032342
CN439836	Transcr locus	-2.6	0.016679

Sequence identifier	Gene symbol	Fold-change	P value
BF044820	DCTN6	-2.6	0.049392
BF440233	mRNA sequ	-2.6	0.045429
CN432742	C3H1orf50	-2.7	0.029089
BF039679	mRNA seq	-2.7	0.005038
DR749422	LOC513273	-2.8	0.01908
DR697523	Transcr locus	-2.8	0.039045
CR550825	Transcr locus	-2.9	0.037771
AW266853	mRNA seq	-3	0.003122
BF041611	Transcr locus	-8	0.046242

Negative sign (-) before the number indicates down-regulation of the gene.

**Table 4**

Genes significantly altered in *B. abortus* – infected monocyte-derived macrophages from bovine naturally resistant to brucellosis 12h post-infection, compared to uninfected cells.

Sequence identifier	Gene symbol	Fold-change	P value
<b>DNA replication &amp; repair</b>			
CK776451	RAD18	2	0.005927
CK770264	RAD51L1	-2.2	0.002847
<b>Transcription regulation</b>			
CR454810	COMMD7	2.2	0.048429
AW465701	RCOR1	2	0.03685
CR452102	RMP	2	0.000759
CR453476	EBF1	-2.1	0.01349
<b>Cell proliferation</b>			
BF045152	PDGFC	2.3	0.029767
CN439003	MARCKSL1	2.1	0.029038
BF440414	BCL2A1	2	0.027301
<b>Immune response</b>			
BM364954	CCL4L1	2	0.023221
AW463280	OSMR	-2.2	0.035044
<b>Metabolism</b>			
CK981355	CA8	2.5	0.013259
CN435598	LRRC47	-2.2	0.022079
CR453715	RPS27	-2.3	0.047924
CN442150	FBXL12	-3.3	0.004795
<b>Transport</b>			
CN437407	KCTD13	2.1	0.023717
<b>Intracellular transport</b>			
BF042139	LOC619125	-2.6	0.008686
<b>Cell adhesion</b>			
CR552676	COL3A1	-2.3	0.012799
DR697491	CRISPLD2	-2.8	0.033894
<b>Signal transduction</b>			
CR452358	GPR155	2	0.02611
CN437082	REM2	-2.2	0.031759
<b>Unknown function</b>			
CR551153	LOC785824	5.8	0.031443
DR749367	Trcr locus	5.2	0.016925
NG010005B14F03	mRNA sequence	3.5	0.02925
CK394002	mRNA sequence	2.4	0.015736
BF039815	mRNA sequence	2.4	0.039986
CN437846	Trcr locus	2.3	0.009568
AW462726	Trcr locus	2.2	0.030233

Sequence identifier	Gene symbol	Fold-change	P value
CN441215	Trcr locus	2.1	0.02319
AW463633	PTPLB	2.1	0.028519
DR697311	TM9SF4	2.1	0.045581
BF042044	mRNA sequence	2.1	0.041983
CN439741	Trcr locus	2.1	0.044695
CN434587	Trcr locus	2.1	0.031319
BF045605	Trcr locus	2	0.039534
CR452655	Trcr locus	2	0.034942
TC225637	mRNA sequence	2	0.036141
BM362622	KIAA1797	2	0.014768
CV798711	mRNA sequence	2	0.002806
CN440913	mRNA sequence	2	0.018244
CV798772	CCDC62	2	0.043505
AW461867	TMEM86A	-2	0.028343
CN439630	Trcr locus	-2	0.046217
CN441187	Trcr locus	-2	0.006074
DR749216	mRNA sequ	-2	0.03689
CN434275	Trcr locus	-2.1	0.034322
CR551689	GALNTL1	-2.2	0.042343
AW464049	Trcr locus	-2.3	0.03765
NM_174508	BTN1A1	-2.3	0.047924
CN441319	mRNA sequ	-2.3	0.040534
BF039032	Trcr locus	-2.4	0.017324
CR452929	LOC513129	-2.5	0.034724
BM364151	mRNA sequ	-2.6	0.008686
BM364245	mRNA sequ	-2.6	0.001497
X95395	mRNA sequ	-2.8	0.004258
CR454451	Trcr locus	-2.8	0.002231

Negative sign (-) before the number indicates down-regulation of the gene.