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Brucella melitensis, B. neotomae and *B. ovis* Elicit Common and Distinctive Macrophage Defense Transcriptional Responses

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Abstract

Brucella spp. establish an intracellular replicative niche in macrophages, while macrophages attempt to eliminate the bacteria by innate defense mechanisms. *Brucella* spp. possess similar genomes yet exhibit different macrophage infections. Few *B. melitensis* and *B. neotomae* enter macrophages with intracellular adaptation occurring over 4–8 hr. Conversely, *B. ovis* are readily ingested by macrophages and exhibit a persistent plateau of infection. Evaluating early macrophage interaction with *Brucella* spp. allows discovery of host entry and intracellular translocation mechanisms. Microarray analysis of macrophage transcriptional response following a 4 hr infection by different *Brucella* spp. revealed common macrophage genes altered in expression compared to uninfected macrophages. Macrophage infection with three different *Brucella* spp. provokes a common innate immune theme with increased transcript levels of chemokines and defense response genes and decreased transcript levels of GTPase signaling and cytoskeletal function that may affect trafficking of *Brucella* containing vesicles. For example, transcript levels of genes associated with chemotaxis (*IL-1*β, *MIP-1*α), cytokine regulation (*Socs3*) and defense (*Fas*, *Tnf*) were increased, while transcript levels of genes associated with vesicular trafficking (*Rab3d*) and lysosomal associated enzymes (*prosaposin*) were decreased. Genes with altered macrophage transcript levels among *Brucella* spp. infections may correlate with species specific host defenses and intracellular survival strategies. Depending on the infecting *Brucella* species, gene ontology categorization identified genes differentially involved in cell growth and maintenance, endopeptidase inhibitor activity and G-protein mediated signaling. Examples of decreased gene expression in *B. melitensis* infection but not other *Brucella* spp. were growth arrest (*Gas2*), immunoglobulin receptor (*FcγrI*) and chemokine receptor (*Cxcr4*) genes, suggesting opposing effects on intracellular functions.

Keywords

Brucella infection; macrophages; transcriptome; *Brucella melitensis*; *Brucella ovis*; *Brucella neotomae*

Introduction

Brucella species (spp.) are zoonotic pathogens able to infect humans and cause abortion in domestic animals. Human infection generally requires contact with a limited number of organisms (infectious dose estimated as less than 100 organisms) (1), progresses with

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inconsistent and persistent flu-like symptoms from 2–6 weeks post-inoculation and, if left untreated, develops into chronic brucellosis. Macrophages phagocytose *Brucella* spp. and initiate an innate immune response, while *Brucella* subvert the host antimicrobial defense mechanisms to establish an intracellular replicative niche (2). Once resident within the macrophage, *Brucella* avoid exposure and killing by the humoral immune response.

Host preference and virulence among species are unaccounted when comparing the few differences between genomic sequences of *B. melitensis*, *B. abortus* and *B. suis* (3). When comparing six historically identified *Brucella* spp., only 217 open reading frames present in *B. melitensis* were absent in the other species (4). Ultimately, *Brucella* research has revealed a limited number of factors that significantly alter host specificity by *Brucella* spp. Human infections with *B. melitensis* are severe in pathogenesis and are widely reported; conversely, neither *B. ovis* nor *B. neotomae* have been reported to cause human infection, and pathogenesis is at most limited. Investigating the murine macrophage response to highly similar *Brucella* spp. may provide additional understanding regarding the ability of *Brucella* spp. to establish and maintain infections.

Although transcriptional profiles of murine macrophages infected with *B. abortus* have been studied (7), no studies have compared host response among infections of differing *Brucella* spp. Altered host transcriptional response among *Brucella* spp. infections may identify not only common responses to infection, but also distinguish genes and pathways specific to each *Brucella* spp. infection. Identifying alterations in the macrophage transcriptome may provide greater understanding of host mechanisms involved in pathogen killing and bacterial regulation that limit damage to host cells during infection.

Murine macrophages are frequently used to investigate *Brucella* infection. The transcription profile after 4 hr of infection would evaluate general as well as specific response to different but genetically similar *Brucella* spp. Bacteria enter host cells and translocate to an endoplasmic reticulum containing a replicative niche within a few hours post infection; concurrently, a portion of the bacteria die by phagosome-lysosome fusion (8). The majority of host transcriptional response occurs during this early time (9). Examining an early time, such as 4 hr post infection, permits discovery of potential mechanisms of entry and intracellular translocation that take place before bacterial replication becomes evident after 8 hr (2,10–12). The present microarray analyses evaluate macrophage response to *Brucella* spp. infection by testing 6 hypotheses and focusing on analogous and distinct transcriptional responses elicited by *B. melitensis*, *B. neotomae* and *B. ovis*.

Materials and Methods

Bacteria and Cell Lines

B. melitensis, *B. neotomae* and *B. ovis* were grown in 12- by 75-mm tubes on a shaker platform in BBL Brucella broth (BD Biosciences, Franklin Lakes, NJ) or on Brucella broth plates containing 1.5% agar. *B. melitensis*, *B. neotomae* and *B. ovis* were transformed with pBBR1MCS/GFP$_{uv}$ containing green fluorescent protein (*gfp$_{uv}$*) under a constitutive Tac promoter and with chloramphenicol resistance (13). *Brucella* spp. for infections were grown in broth with or without chloramphenicol at 37°C for 1–2 days and colony forming units (CFUs) determined by plating on agar and incubating 3 days at 37°C with 5% CO_2.

RAW 264.7 (TIB-71, ATCC) and J774A.1 (TIB-67, ATCC) mouse macrophage cell lines were maintained at 37°C with 5% CO_2 in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 0.2 mM L-glutamine, antibiotic-antimycotic (100 U/mL penicillin G, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, Gibco), 1 mM sodium pyruvate (SAFC Biosciences, St. Louis, MO) and MEM amino acids (Hyclone, Logan, UT).

Intracellular Survival of *Brucella* spp. in Macrophages

Macrophages ($0.5–1 \times 10^6$/well) were plated in 6-well plates 2–12 hr prior to infection in medium without antibiotics. *Brucella* spp. were grown to stationary phase in Brucella broth and then serially diluted and plated on Brucella agar to estimate CFU/mL. Macrophages were infected at a multiplicity of infection (MOI, bacteria to macrophage) of 1000:1 for 90 min at 37°C with 5% CO_2. Extracellular bacteria were removed using three PBS washes followed by 30 μg/mL gentamicin (MP Biomedicals, Inc., Irvine, CA) in RPMI. After 30 min, macrophages were washed three times with PBS. RPMI supplemented with 2 μg/mL gentamicin was added to cultures after 2 hr. At 4 hr, cultures were washed, lysed with 0.1% Triton X-100, serially diluted and plated twice on Brucella agar to determine bacterial CFUs. Experiments were repeated independently a minimum of three times.

Flow Cytometry of *Brucella*-GFP_{uv} Infected Macrophages

Macrophages ($5 \times 10^7/T_{25}$ flask) were plated 2–12 hr prior to infection in medium without antibiotics. *Brucella* spp. were grown to stationary phase, and macrophages were infected at a multiplicity of infection (MOI, bacteria to macrophage) of 1000:1 for 90 min at 37°C with 5% CO_2 followed by gentamicin treatment to remove extracellular bacteria as described above. After 4 hr of incubation, cells were washed three times with PBS, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hat-field, PA) for 30 min and observed by fluorescence microscopy (Carl Zeiss, Thornwood, NY). Using a FACScan (Becton Dickinson, Palo Alto, CA), ten thousand events were collected. Debris and dead cells were eliminated from the analysis on the basis of side scatter and forward scatter. Cells infected with *B. melitensis*, *B. neotomae* and *B. ovis* strains containing pBBR1MCS/GFP_{uv}, were analyzed by flow cytometry to determine percentage of RAW 264.7 or J774A.1 cells infected at 4 hr.

Macrophage Infection for RNA Isolation

RAW 264.7 cells for microarray and RAW 264.7 and J774A.1 cells for RT-PCR were plated at 5×10^6 cells/T_{75} flask 12–24 hr prior to infection, in supplemented RPMI 1640 without antibiotics. Macrophages were infected with 1 mL of a stationary phase *Brucella* spp. culture (MOI 1000:1). Infected macrophages were incubated for 4 hr at 37°C with 5% CO_2, washed once with PBS and then lysed for RNA collection (RNeasy, Qiagen, Germantown, MD).

Target Preparation for Microarray Hybridization

Total RNA was isolated from macrophage cultures (RNeasy, Qiagen) with lysate centrifugation to remove intact bacterial cells. DNase treated RNA from two independent infections was pooled for each target preparation. Target RNA was prepared according to the manufacturer's protocols (Affymetrix, Santa Clara, CA). Briefly, RNA was converted to double stranded cDNA (Invitrogen) except that T-7-(dT)24 oligomer (Genset Corp., San Diego, CA) was used. Double stranded cDNA was isolated using GeneChip® Sample cleanup and *in vitro* synthesis of biotin-labeled cRNA was completed with Enzo BioArray High-Yield RNA Transcript Labeling (Affymetrix). Labeled cRNA mixed with fragmentation buffer was incubated at 94°C for 35 min and was confirmed by agarose gel electrophoresis. Final RNA concentration ranged from 0.5–1.1 μg/μL.

Microarray Hybridization and Analysis

Labeled cRNA was hybridized to GeneChip® Test3 arrays and Murine Genome U74Av2 microarrays (Affymetrix). Eleven MG_U74Av2 GeneChip® microarrays were independently hybridized with cRNA from uninfected macrophage samples (2 independent samples) and each of three *Brucella* spp. infected macrophage samples (3 independent samples for each *Brucella* species infection). GeneChip® washing, hybridization and scanning was performed by the University of WI Biotechnology Center, Gene Expression Center (University of WI-

Madison) utilizing Affymetrix protocols and procedures. Affymetrix *.CHP, *.CEL and spreadsheets of signal output are available through NCBI Gene Expression Omnibus database at the time of publication, Series accession number GSE8385.

mRNA from uninfected RAW 246.7 macrophages was compared to macrophages infected for 4 hr with *B. melitensis*, *B. ovis* or *B. neotomae*. All genes were subjected to analysis by EBarrays (14,15), a statistical analysis package in the comprehensive R archive network (http://cran.r-project.org/). Data conformity to the statistical assumptions was checked (coefficient of variation and log-normal normal model) and best fit models were used. Six biologically relevant hypotheses of altered transcript levels were tested (Mφ designates RAW 264.7 macrophages):

$$H_0: M\varphi = M\varphi + B.\,melitensis = M\varphi + B.\,neotomae = M\varphi + B.\,ovis$$

Infected and uninfected macrophages have similar transcription.

$$H_1: M\varphi \neq M\varphi + B.\,melitensis = M\varphi + B.\,neotomae = M\varphi + B.\,ovis$$

Brucella spp. infected macrophages express mRNA different from uninfected macrophages.

$$H_2: M\varphi \neq M\varphi + B.\,melitensis \neq M\varphi + B.\,neotomae = M\varphi + B.\,ovis$$

B. ovis and *B. neotomae* infected macrophages express mRNA different from *B. melitensis* or uninfected macrophages.

$$H_3: M\varphi \neq M\varphi + B.\,melitensis = M\varphi + B.\,neotomae \neq M\varphi + B.\,ovis$$

B. melitensis and *B. neotomae* infected macrophages express mRNA different from *B. ovis* or uninfected macrophages.

$$H_4: M\varphi \neq M\varphi + B.\,melitensis = M\varphi + B.\,ovis \neq M\varphi + B.\,neotomae$$

B. melitensis and *B. ovis* infected macrophages express mRNA different from *B. neotomae* or uninfected macrophages.

$$H_5: M\varphi \neq M\varphi + B.\,melitensis \neq M\varphi + B.\,neotomae \neq M\varphi + B.\,ovis$$

Each macrophage culture expresses distinct mRNA.

Empirical Bayesian statistics designated a posterior probability (EBarrays Probability) specific to each gene, evidence that a given gene follows the transcription pattern designated by each of the hypotheses. The probability of random gene assignment to any hypothesis was 0.167, while posterior probabilities ≥ 0.2 were considered significant. Genes were sorted within each experimental hypothesis, and each gene was allocated only to the hypothesis with the largest posterior probability. The signal logarithm ratio (SLR) was calculated as the logarithm, base 2, of the ratio between infected (experimental) signal and uninfected (control) signal. Thus, the experimental:control ratio was transformed to zero for no change between conditions and the equivalent of a two-fold increase or decrease becomes 1 or −1 SLR, respectively.

Additional data analysis was conducted utilizing TM4 microarray analysis tools ANOVA (16) and SAM (17,18) contained within TIGR MultiExperiment Viewer (MeV) (http://www.tm4.org/) (19,20). Genes were annotated utilizing Affymetrix's NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) and DAVID Bio-informatics Resources (http://david.abcc.ncifcrf.gov/). Gene ontology (GO) categorization of genes identified with altered transcription between uninfected and *Brucella* spp. infected macrophages were completed with EASE (http://david.abcc.ncifcrf.gov/). Genes were iteratively subjected to GO biological processes categorization and, while genes may be categorized in several of the GO categories, each gene was listed once.

Real Time RT-PCR (RT-PCR)

Total RNA was isolated from macrophage cultures (RNeasy, Qiagen) according to manufacturer's protocol with DNase treatment. Macrophage RNA (2–4 μg) was primed with oligo$(dT)_{20}$, reverse transcribed with SuperScript™ II or III (Invitrogen), and resultant cDNA diluted 1:5. Primers (listed in Text S1) and double-dye oligonucleotide probes for PCR were developed to amplify mRNA fragments 80–130 basepairs in length using Primer3 (21). RT-PCR was performed on cDNA samples utilizing qPCR Mastermix (Eurogentec, San Diego, CA) or iQ supermix (Bio-Rad, Hercules, CA) with dual labeled probes and iQ SYBR® green Supermix (Bio-Rad), respectively, to quantify relative transcript levels. The PCR was optimized on the iCycler (Bio-Rad) with primer concentrations ranging from 300–500 nM and probe concentrations at 125 nM. Amplification cycles were 95°C for 15 sec followed by 60 sec at 60°C with fluorescence detected during the extension phase. Relative gene mRNA quantities were quantified by the standard curve method using a housekeeping gene as a reference gene. SLR was calculated with these normalized transcription values.

ELISA Measurement of TNF Activity

BMDM cells were flushed from the femurs and tibiae of 10–12 week-old wild-type C57BL/6 mice. Cells were grown for 5–8 days in RPMI 1640 (Gibco, Grand Island, NY), 10% FBS (Equitech-Bio Inc., Kerrville, TX), 25 μg/mL gentamicin (MP Biomedicals, Inc., Solon, OH) and 20–30 ng/mL M-CSF (R&D Systems, Minneapolis, MN). BMDM cells plated at 1×10^6 cells/well in 6-well plates were infected with *B. melitensis* at an MOI 100 and incubated at 37°C. Supernatants were collected at 12 hr from cells infected with *B. melitensis* or medium. Supernatants were filtered through a 0.22 micron PES Millipore filter (Millipore, Billerica, MA) and assayed for TNFα using a Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).

Additional Calculations and Statistics

Error bars throughout indicate the standard error of the mean (SEM). A Student's *t* test determined if there was significant difference between two independent samples using a pooled estimate of variation.

Results

Brucella spp. Infection of RAW 264.7 and J774A.1 Macrophages Assessed by Colony Forming Units (CFUs) and Flow Cytometry Analysis of *Brucella*-GFP_{uv}

RAW 264.7 macrophage infections with *B. melitensis* and *B. neotomae* were similar, with low numbers of these bacteria phagocytosed compared to the number of bacteria introduced (Fig. 1, panel A). Macrophage uptake of *B. ovis*, at 4 hr in both RAW 264.7 and J774A.1 cells was nearly two logarithmic units higher than *B. melitensis* or *B. neotomae*, paralleling previous observations (6,22).

Similar to CFU results, flow cytometry analysis of RAW 264.7 macrophages infected for 4 hr with *Brucella* spp. expressing GFP_{uv} had low levels of *B. melitensis* and *B. neotomae*, compared to *B. ovis* (Fig. 1, panel B). This observation was further supported by fluorescent microscopy (Fig. 1, panel C). At this early time, 20–30 percent of macrophages were infected with *B. melitensis* or *B. neotomae*, while greater than 95 percent of macrophages were infected with *B. ovis* (Fig. 1, panel B) consistent with uptake for smooth and rough *Brucella* (22). Interestingly, the numbers of *Brucella* phagocytosed at these levels did not correlate in a linear or proportional manner to levels of transcriptional response, as changes in macrophage mRNA amounts following *B. ovis* infection were often similar to *B. melitensis* and *B. neotomae* infections.

Because of the difference in macrophage infection by *B. melitensis* and *B. neotomae* compared to *B. ovis*, macrophage viability was examined to determine if infection levels would influence macrophage survival. RAW 264.7 and J774A.1 macrophages were continuously infected with *Brucella* spp. for 4 hr without antibiotics and viability determined. No significant macrophage death was observed (data not shown). Therefore, host transcription with *Brucella* spp. infection at 4 hr was not biased toward cell death pathways.

Microarray Analysis of RAW 264.7 Macrophages Infected with Each of Three *Brucella* spp

Genes Identified for Housekeeping Controls with No Transcriptome Change Across Three *Brucella* spp. Infection Conditions—A series of commonly used housekeeping genes were evaluated for potential as control genes in downstream analysis, and changes in transcript levels between *Brucella* spp. infected and uninfected macrophages were compared (Table 1). Genes with high posterior probabilities and low signal log ratio (SLR) with small variability appeared as the best control genes for *Brucella* spp. infections. *Hypoxanthine guanine phosphoribosyl transferase 1* (*Hprt1*) and the *TATA box binding protein* (*Tbp*), which function in glycolysis and transcription, respectively (23), were transcribed at a moderate level (signal strength on microarray between 1×10^4 and 1×10^2) and had SLR near zero with small variability. *Hprt1* and *Tbp* were unaltered by *Brucella* spp. infections and were therefore utilized as control housekeeping genes in downstream analysis of the transcriptome. The transcript levels of β-*actin*, β-*glucuronidase*, *transferrin receptor* and *glyceraldehyde-3-phosphate dehydrogenase* were minimally altered (−0.6 to 0.1 SLR) by infection, but each had a pronounced standard error and were considered not optimal for use as control genes.

Genes Identified With Increased Transcription Following *Brucella* spp. Infections—Seventy-two genes were identified under H_1 (transcription from macrophages infected by three *Brucella* spp. differs from uninfected macrophages) with SLR increases between 2.0 and 6.8 following infection with any *Brucella* spp. (Table 2). Defense and chemotactic response, both related to a common inflammatory response, encompass the largest groups of genes with increased transcript levels during infection. *Interleukin-1*β, *tumor necrosis factor*, macrophage inflammatory protein genes (*MIP-1*α, *MIP-1*β, *MIP-2*α), colony

stimulating factor genes (*Csf2* and *Csf3*) and *Fas* had increased transcription similar to reports with a variety of infectious agents (7,24). Also, increased transcription was observed for cytokine regulation, anti-inflammatory and/or anti-apoptotic response genes (*Socs3*, *Slfn2*, *IL-1rn*, *Gadd45b* and *Tnfaip3*). These genes may prevent commitment to an inflammatory response pathway camouflaging immune recognition and providing a safe environment for bacterial survival. Although macrophages were infected with a greater number of *B. ovis* compared to *B. melitensis* and *B. neotomae*, Figure 1, no statistical difference was observed in the SLR between the 72 macrophage genes of Table 2 when infected with *B. ovis* compared to *B. melitensis* or *B. neotomae*.

Genes Identified with Decreased Transcription Following *Brucella* spp. Infections—Fifty-eight genes were identified with SLR decreases between −2.0 and −3.4 following infection with any *Brucella* spp. (Table 3). GO categories for genes with repressed transcript levels represented several biological functions, ranging from small GTPase mediated signal transduction and carboxylic acid metabolism to cell proliferation and lysosomal proteins. Small GTPase mediated signaling may be altered in response to the engulfment and association of *Brucella* spp. with the membrane of macrophages; included among this group are *Rab3d*, *Gna12*, *Cfl2* and *Iqgap1*. Small GTPases are key regulators associated with trafficking of *Brucella* containing vesicles to the endoplasmic reticulum (25), a crucial step in the establishment of a replication niche. In contrast to the inflammatory response genes commonly increased following infection, there were also genes categorized as response to external stimulus with decreased transcription. *Mr1*, *Abhd8* and *IL17a* may provide insight into mechanisms that the macrophage does not use in the response against this intracellular bacterium. Also, a decrease in *prosaposin* transcript levels was observed, a lysosomal enzyme that catabolizes glycosphingolipids that may enhance intracellular survival of *Brucella* spp.

Genes Identified with Altered Transcription Among *Brucella* spp. Infections—Thirty-three genes (Table 4) were identified under hypotheses that transcription among *Brucella* spp. infections was different from each other and uninfected macrophages ($H_{2–4}$). Of the 33 genes with altered expression among *Brucella* spp. infections, 21 genes had decreased transcript levels, 10 genes had increased transcript levels and, for two genes, the direction of change in expression was dependent on the species used for infection. Generally, the direction of change in transcription was uniform across the species tested. Twelve genes were altered based on the virulence descriptions of *Brucella* spp. (Table 4, part A), as *B. melitensis* was compared to *B. neotomae* and *B. ovis* infections (H_2). GO biological and molecular function categorization unveiled genes altered according to H_2 are involved in cell growth and maintenance, endopeptidase inhibitor activity, response to external stimuli and G-protein mediated signaling. When the infections were grouped according to LPS phenotype of the *Brucella*, i.e., smooth *B. melitensis* and *B. neotomae* versus rough *B. ovis*, eleven genes were identified with altered transcription (H_3) (Table 4, part B). Although smooth *Brucella* enter macrophages via lipid rafts (26), the mechanism of *B. ovis* entry is unknown. If different entry mechanisms occur for *B. ovis*, a distinct group of host genes may be altered compared to smooth *Brucella* spp. However, few macrophage genes were altered between rough and smooth *Brucella* spp. infections, and there was little GO commonality. Ten genes had altered transcript levels when infection with *B. melitensis* and *B. ovis* were considered similar and distinct from *B. neotomae* infection (H_4) (Table 4, part C). All three *Brucella* spp. infections increased *interleukin 1 alpha* gene expression, but levels were higher in *B. melitensis* and *B. ovis* infections. Genes identified according to this pattern (H_4) were categorized in GO biological functions of cell growth and maintenance and signal transduction. Lastly, no genes were identified with each of the four conditions, uninfected and three *Brucella* spp. infections, having distinct transcription (H_5).

Confirmation of Microarray Data by Quantitative Real Time-PCR (qRT-PCR)

To verify the micro-array changes in transcript levels, eight genes were analyzed by qRT-PCR using the infection conditions as for microarray analysis. Housekeeping genes, *Hprt* and *Tbp*, identified via this microarray analysis and previously tested (23,27,28) were used to normalize cDNA levels and resulted in similar patterns and levels of gene transcription (data not shown). qRT-PCR confirmed that the eight genes tested (*Socs3*, *Tnf*, *IL-1α*, *IL-1β*, *FcγRI*, *Mef2c*, *Cxcr4* and *Abcd2*) had altered expression in RAW 264.7 macrophages following *Brucella* spp. infection in the same direction (increased or decreased) indicated by microarray analysis (Fig. 2, panels A and B). Further, J774A.1 and RAW 264.7 macrophage cell lines were used in parallel to verify that qRT-PCR results found in RAW 264.7 were applicable to J774A.1 cells. RAW 264.7 gene transcription altered among *Brucella* spp. infections was similarly changed (increased or decreased) during J774A.1 infection (Fig. 2, panels C and D) with the direction of altered transcription paralleling microarray results.

TNFα Production by Macrophages

To confirm the microarray change in TNFα transcription, bone marrow derived macrophages were infected with *B. melitensis* and supernatant analyzed by ELISA. Figure 3 illustrates that infected macrophages produced greater than 15-fold more TNFα than non-infected macrophages. These results together with the microarray findings suggest that *B. melitensis* activates TNFα production by macrophages.

Discussion

Murine macrophages infected with *Brucella* strains were profiled by microarray analysis. Interestingly, *B. melitensis* causes severe human infection, but neither *B. ovis* nor *B. neotomae* cause human pathogenesis. However, *in vitro* growth of *B. melitensis* and *B. neotomae* is similar in murine macrophages in contrast to *in vivo* human infection where *B. neotomae* is unable to initiate or maintain infection (29,30). The IRF-$1^{-/-}$ mouse model can distinguish degrees of *Brucella* virulence (5), where *B. melitensis* and *B. neotomae* induce death by 9–12 days (6), while *B. ovis* does not (E. Petersen, Personal Communication). The differing responses between rough (*B. ovis*) and smooth (*B. melitensis* and *B. neotomae*) *Brucella* in mice led us to explore transcription profiles of murine macrophages following infection by these three *Brucella* strains. A comparison of host response among infections by differing *Brucella* spp. may identify common responses to infection, as well as distinguish genes and pathways specific to each *Brucella* spp. infection. Identifying alterations in the macrophage transcriptome may provide greater understanding of host mechanisms involved in pathogen killing and bacterial regulation.

Common Macrophage Transcriptional Response by *B. melitensis*, *B. neotomae* and *B. ovis* Infections

At 4 hr, low numbers of intracellular smooth *B. melitensis* and *B. neotomae* were observed, as reported by others, prior to *Brucella* reaching its replicative niche (22,31,32). However, RAW 264.7 macrophage uptake of rough *B. ovis* after 4 hr of infection was almost two logarithmic units higher than smooth *B. melitensis* or *B. neotomae*, perhaps from non-lipid raft mediated phagocytosis, although this is untested for rough *B. ovis* (22,31,32). Yet, microarray analysis of rough *B. ovis*-infected macrophages was qualitatively similar to those from smooth *B. melitensis* and *B. neotomae* infections. This similarity suggests that macrophages sense and respond to different *Brucella* species by a common profile of transcriptional changes.

The majority of these common response genes were inflammatory related suggesting similar activating and/or regulating effects of *Brucella* on the macrophage regardless of the *Brucella* species. Host response to *Brucella* spp. encompassed pro-inflammation independent of

infection differences between rough and smooth *Brucella* spp. In comparing the common macrophage transcription profile to differing *Brucella* spp., we hypothesized that a large number of host genes with overlapping gene ontology function would be identified similar to that observed in wild-type and mutant *Salmonella* (33). Others have reported that LPS and CpG DNA can activate TLR signaling activating NFκB and AP-1 pathways inducing innate immune mechanisms (34). Adaptive immune mechanisms may be activated by Schlafen 2 (*Slfn2*) expression that regulates T cell activity as well as innate immunity (34). Pro-inflammatory gene expression may reflect a host protective response as many genes were chemotaxis related, potentially recruiting additional macrophages and immune cells to the site of *Brucella* spp. infected macrophages.

Genes that could serve to subdue or counteract inflammatory response genes, however, were also observed as upregulated in macrophages. For example, increased expression of *Socs3* was observed. *Socs3* participates in a negative feedback loop of cytokine signaling limiting the extent of cytokine in innate and adaptive immune responses (35). Also, *IL-13R* and *Gadd45b* were increased in transcription suggesting regulation of activated macrophages. Transcription of *Bcl2*, *Bat4* and *Phlda1* that participate in blocking apoptotic mechanisms was upregulated. In agreement with this finding, smooth *Brucella* spp. can protect against apoptosis (36), while engineered rough *Brucella* spp. can induce apoptosis (37). Consequently, the upregulation of genes that block apoptotic mechanisms may oppose upregulated inflammatory genes to camouflage immune recognition and produce a protective environment for *Brucella* replication. Also, decreased transcript levels of *Psap* (lysosomal catabolic enzyme), *Sgip3* (clathrin mediated endocytosis) and *Mpeg1* (a perforin like protein) would suggest that *Brucella* induces evasion strategies to protect its intracellular niche. Others have suggested evasion strategies such as *Brucella* altering host cell responses by shielding PAMPs from Toll-like receptors (38) and LPS masking of MHC molecules (39).

Of the macrophage genes commonly upregulated by all *Brucella* species tested, first, eicosanoids are important in mammalian repair (40). In macrophages infected with *Brucella* we observed induction of eiconosoid *Ptgs2*, also termed *Cox2*, encoding the cyclooxygenase 2 enzyme. Eicosanoids are considered important in antigen presentation and in macrophage effector functions, and play an important role in contributing to inflammation (40).

A second common inflammatory signal elicited by *Brucella* infection of macrophages is *Gadd45*. Gadd45A is an 18 kDa acidic nuclear protein involved in maintenance of genomic stability, DNA repair, cell cycle checkpoints and suppression of cell growth. *Gadd45* is also a known stress response gene with the ability to regulate MAP-kinase signaling (41). Regulation of MAP-kinase signaling by Gadd45 can affect response to extracellular stimuli and regulate gene expression, mitosis, differentiation and cell survival/apoptosis. GADD45A may therefore be expressed as a stress response protein to inhibit macrophage growth during *Brucella* infection.

A third common inflammatory signal elicited by *Brucella* infection is an odorant binding protein. Odorant binding proteins (OBPs) belong to the lipocalin family of proteins involved in extracellular transport of hydrophobic ligands. OBPs were originally identified in nasal mucus and mucosa of insects and were proposed as molecular shuttles between the air mucus interface and the olfactory receptor binding sites. Their function in mammals is unknown, but OBPs can bind odorants of diverse chemical structures with a higher affinity for aldehydes and large fatty acids (42). In macrophages, OBPs may serve as chaperones for signaling molecules.

Macrophage Transcriptome Response to *B. melitensis* Differ from *B. neotomae* and *B. ovis*

Macrophages infected with *B. melitensis* elicited a specific response with altered transcription in twelve genes compared to *B. neotomae* and *B. ovis* (H_2) (Table 4, part A). These genes

provide insight into macrophage mechanisms used by the various *Brucella* species to establish a replicative niche. For example, two genes with established function during inflammation, *FcγRI* (43) and *Cxcr4* (44), have decreased expression in *B. melitensis* infected macrophages compared to *B. neotomae* and *B. ovis* infections. *FcγRI* (*CD64*) may be involved in regulating antigen presentation by limiting additional phagocytosis and committing the cell to present antigens of engulfed pathogens (45). Decreased levels of *FcγRI* may be advantageous for bacteria by reducing downstream signaling initiation, including antibody dependent cell mediated cytotoxicity (ADCC) (46), production of reactive oxygen and nitrogen species (47, 48), and increased phagocytosis and cytokine secretion (49). Downregulation of *Cxcr4* transcription in combination with *Socs3* upregulation may reduce macrophage chemotaxis, adhesion and other inflammatory responses (50, 51). Suppressing several immune pathways initiated at the cell membrane, virulent *Brucella* may secure an intracellular replicative niche.

Three other genes with decreased expression in macrophages infected with *B. melitensis* compared to *B. neotomae* and *B. ovis* (H_2 hypothesis) are endopeptidase inhibitors, *cystatin C* and *serpina3c* and the actin associated protein encoded by *Gas2*. These natural peptidase inhibitors may influence cell migration, chemotaxis, proliferation, phagocytosis and respiratory burst (52–55). Interestingly, the treatment of mice harboring *Leishmania* with cystatin and a sub-optimal dose of IFN-γ led to parasite clearance and shifted the non-productive Th2 (type 2, humoral) response towards a Th1 response (56). Nitric oxide (NO) production (57) and phagosomal processing (55) are mechanisms where these endopeptidase inhibitors act and modulate intracellular infection. These same mechanisms are vital to progression of *Brucella* spp. infection (58–60) and exogenous modulation may provide the immunologic boost necessary for pathogen clearance. *Brucella* spp. may specifically alter the protease-inhibitor balance by bacterial production of extracellular proteases and cleavage of host endopeptidase inhibitors, similar to how *Staphylococcus aureus* functions in accessing nutrients and facilitating dissemination (61). Lastly, *Gas2* was specifically down-regulated in *B. melitensis*. GAS2-like protein 1 is an actin-associated protein expressed at high levels in growth-arrested macrophages (62). Inhibition of *Gas2* transcription may increase macrophage survival and growth, an advantage for *Brucella* spp. survival within an intracellular niche.

Eleven genes were altered in expression (Table 4, part B) by *B. melitensis* and *B. neotomae* but not by *B. ovis* (H_3). These genes could not be grouped according to function, but did categorize the *Brucella* spp. according to LPS type, rough or smooth. While *Brucella* spp. uptake based on smooth or rough phenotype did not affect transcript levels of many inflammatory-related genes in our study (H_1), LPS may subtly affect transcription when comparing *B. melitensis* and *B. neotomae* to *B. ovis*. Microarray analyses of macrophages responding to various bacteria indicate that an early response to infection parallels the response to LPS alone and is conserved across bacterial genera (63–65). For example, macrophage response to *Salmonella* versus purified *Salmonella* LPS is similar (64). However, *Brucella* LPS induces a 100-fold less stimulation of macrophages than enterobacterial LPS (2, 66), and may have less effect on macrophage transcription.

Cytochrome P450 4a10 (*Cyp4a10*) and *protein kinase C-α (Prkca)* may provide insight into intracellular pathways that differ between *Brucella* species (H_3) (Table 4, part B). A wide variety of bacterial and parasitic infections depress expression of *cytochrome P450* (67), a gene important in detoxification (68). *Cyp4a10* can enhance oxidative stress, through production of reactive oxygen species and reactive nitrogen species (69); therefore, decreased transcription induced by bacteria with smooth LPS could protect intracellular *Brucella* from innate killing mechanisms. Another gene with oxidative related functions, *Prkca*, has decreased transcript levels in the infected macrophage. *Prkca* is important in regulating phagosome-lysosome fusion (70), intracellular vesicle regulation (71) and generation of reactive oxygen species (72), events that may be regulated differently by diverse *Brucella* spp. Constitutive knock down

of *Pkrca* during intracellular infection with *Legionella*, *Leishmania* and *Salmonella* (48, 73) leads to increased pathogen survival, likely through suppression of host respiratory burst. Downregulation of *Cyp4a10* and *Prkca* pathways may prevent additional oxidative stress in the macrophage during smooth *Brucella* spp. infection, while *Brucella* spp. production of Cu, Zn superoxide dismutase (SodC) protects *Brucella* from oxidative damage (74). These results suggest that down-regulation of *Cyp4a10* and *Pkrca* may shift the macrophage toward a less hostile intracellular environment for *Brucella*.

Finally altered transcription was identified when *B. melitensis* and *B. ovis* were considered dissimilar from *B. neotomae* (H_4). Nine of ten genes had decreased transcript levels in *B. neotomae* infection compared to the other *Brucella* spp., the most important examples being *IL-1a*, *Abcd2* and *mef2c*. *B. neotomae* may moderate the IL-1α transcription earlier or through alternative stimulation. *Abcd2* knock down has been correlated with oxidative stress or tissue damage (75) and abnormalities in mitochondria, Golgi and endoplasmic reticulum of host cells (76). The importance of Abcd transporters is reinforced by decreased transcript levels of other *Abcd* family members during *Brucella* infection (9,77). Lastly, the alteration of *mef2c* expression suggests that *B. neotomae* infection may render RAW 264.7 macrophages more susceptible to activation-induced cell death (78) than *B. melitensis* or *B. ovis* infections. When compared to *B. melitensis* and *B. ovis*, *B. neotomae* infected macrophages appeared to have decreased immuno-stimulatory capability resulting in reduced oxidative stress and increased susceptibility to cell death.

Brucella infection of RAW 264.7 and J774A.1 macrophage cell lines resulted in similar results. While RAW 264.7 and J774A.1 cells are macrophage-like in lysozyme secretion (79), phagocytosis of particles, response to LPS stimulus (80) and activation state (81), differences do exist in the abilities of these cells to mediate cytolytic activity (81), synthesize nitrite and nitrate (80), and potentially other macrophage trafficking and defense mechanisms. Our microarray results were supported by similar transcription profiles between these *Brucella* infected macrophage cell lines. However, internalization of smooth *Brucella* by phagocytic macrophages occurs faster than by nonphagocytic cells such as trophoblasts (82) and may contribute to differences in microarray results between phagocytic and nonphagocytic cells. Ultimately, individual genes of interest must be further investigated for influence on *Brucella* spp. infections. In conclusion, the present study evaluated the macrophage transcriptome altered during *B. melitensis*, *B. neotomae* and *B. ovis* infections. Common changes in gene expression were observed among *Brucella* spp. infected macrophages suggesting similar innate immune mechanisms by macrophages when responding to different *Brucella* species. These changes were related to increased chemokine, alteration in cytokine signals and defense responses. Also, these three *Brucella* spp. induced decreased transcription of select genes, suggesting that repressed transcription may result from pathogen-specific manipulations. Further, a subset of macrophage genes was differentially altered based on a particular *Brucella* species that may reflect the differing intracellular survival abilities of each *Brucella* species. Microarray analysis of host transcription provides a foundation to understand variations in *Brucella* spp. infection of mice. Future studies will reveal variations in the intracellular survival strategies used by different *Brucella* spp. by evaluating the contribution of individual host genes during infection.

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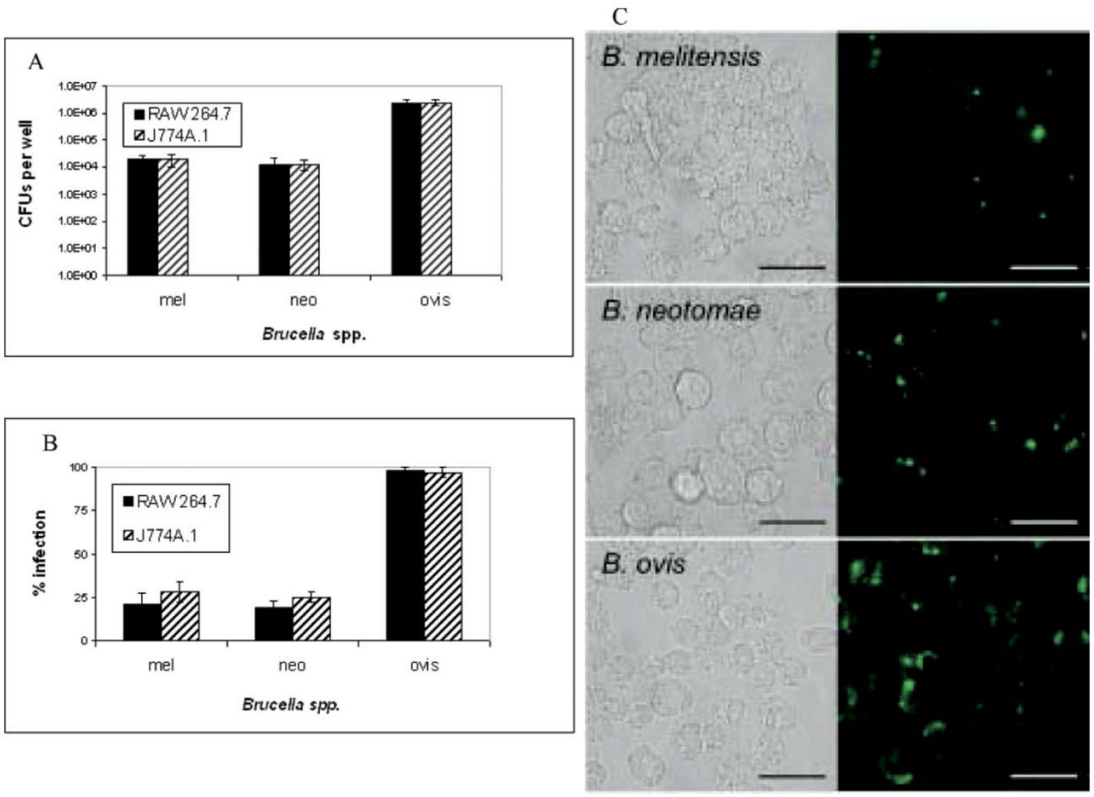

Figure 1.

Intracellular survival of *Brucella* spp. in RAW 264.7 and J774A.1 macrophages. Panel A. Intracellular *Brucella* spp. were isolated from RAW 264.7 or J774A.1 macrophages and enumerated at 4 hr. Macrophages were infected with 1000:1 MOI of each *Brucella* spp. and allowed to infect for 90 min followed by gentamicin treatment for 30 min. At 4 hr, macrophages were washed then lysed with 0.1% Triton X-100. Intracellular *Brucella* were enumerated by plating serial dilutions on agar. Four independent experiments were conducted, and the number of *Brucella* spp. isolated per well was averaged with error bars representing the SEM. Comparing *B. melitensis* or *B. neotomae* to *B. ovis* infection is significantly different with $P \leq 0.005$. Panel B. Flow cytometry analysis of *Brucella*-GFP_{uv} infection in RAW 264.7 and J774A.1 macrophages at 4 hr. RAW 264.7 or J774A.1 macrophages were infected for 90 min with *Brucella* spp. containing GFP_{uv} followed by gentamicin treatment to remove extracellular bacteria. After 4 hr, RAW 264.7 or J774A.1 cells were fixed in 4% paraformaldehyde, and the percent of infected cells determined by flow cytometry analysis. Comparing *B. melitensis* or *B. neotomae* to *B. ovis* infection is significantly different with $P \leq 0.005$. Panel C. RAW 264.7 macrophages were infected continuously for 4 hr with *Brucella* spp. containing GFP_{uv}. Cells were washed to remove extracellular *Brucella* and fixed in 4% paraformaldehyde. Matched bright field and fluorescence images were digitally captured at ×63 oil immersion magnification. Bar equals 20 μm. A color version of this figure is available in the online journal.

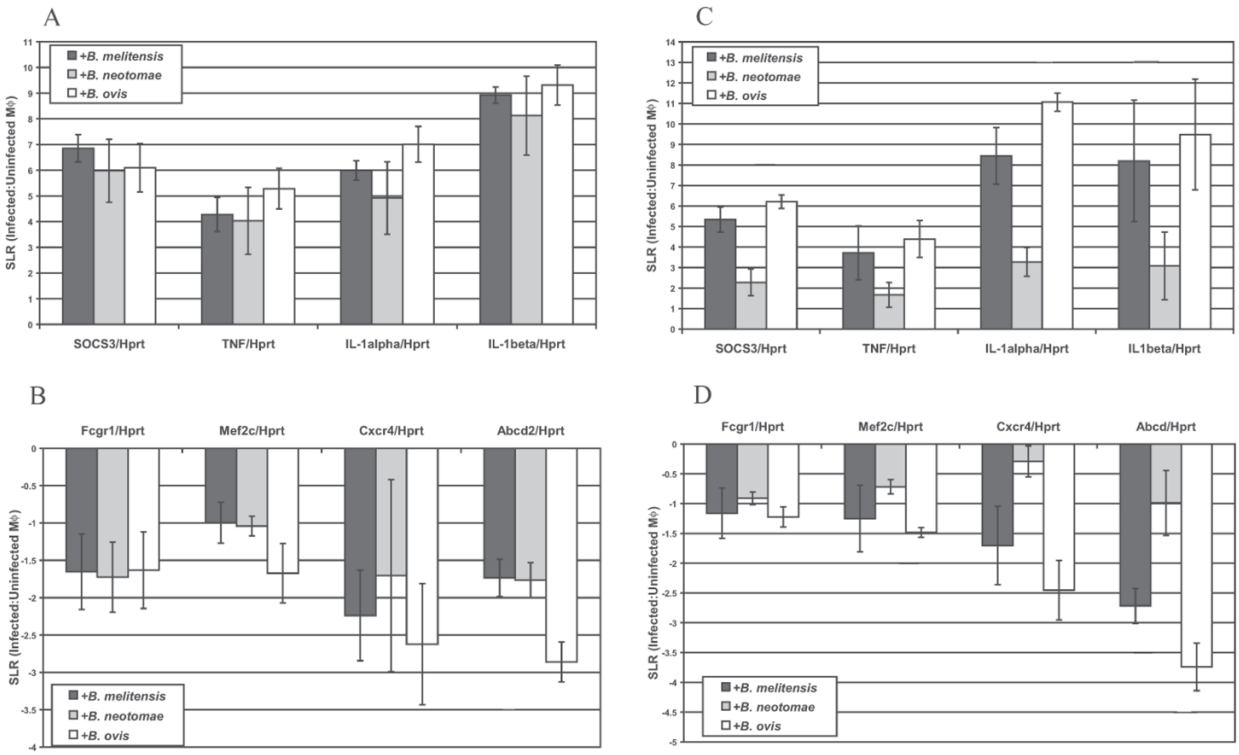

Figure 2.

Transcript level changes in select gens following *Brucella* spp. infection of RAW 264.7 (Panel A) or J774A.1 (Panel B) macrophages. RT-PCR results confirm genes with increased A or C or decreased B or D transcript levels following infection of RAW 264.7 or J774A.1 cells, respectively. Each RNA sample was normalized using *Hprt* transcript levels. Signal log ratios (SLR), a comparison of each infected sample to uninfected control RAW 264.7 samples, were averaged from four independent RNA isolations and error bars represent the SEM.

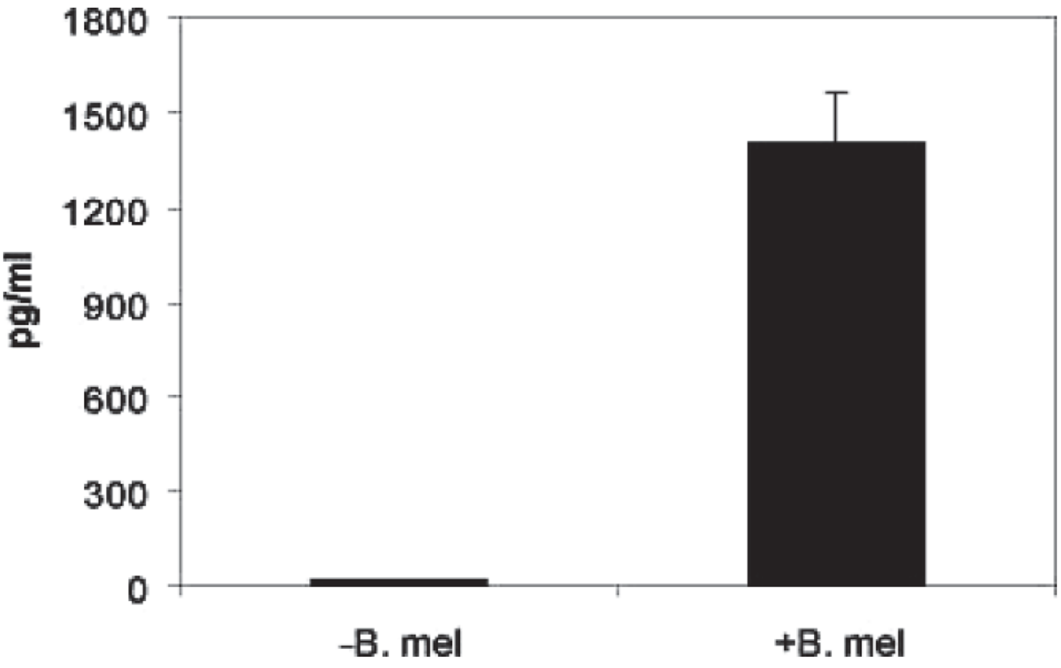

Figure 3.
Induction of TNFα by the *B. melitensis* in BMDMs from C57BL/6 mice. Cytokine levels were assayed from supernatants of *B. melitensis* 16M infected or medium alone bone-marrow derived macrophages (BMDMs). BMDMs were cultured 5–7 days pre-infection. Cytokines were measured in pg/mL by ELISA at 12 hr post-infection. Data are from 7 mice/experiment.

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Table 1

RAW 264.7 Macrophage Transcription of Common Housekeeping Genes Following *Brucella* spp. Infection

H_0: Mφ = Mφ + *B. melitensis* = Mφ + *B. neotomae* = Mφ + *B. ovis*						
Probe set	**Entrez gene**	**Average signal**	**SLR[a]**	**EBarrays probability**	**Gene symbol**	**Gene product**
101213_at	11837	5.7×10^4	−0.2 ± 0.06	1.000	Arbp	Acidic ribosomal phosphoprotein PO
101578_f_at	11461	2.2×10^4	−0.6 ± 0.6	0.870	Actb	β-actin
101214_f_at 162210_r_at 97751_f_at	14433	2.5×10^4	0.1 ± 0.2	0.894	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
93346_at	18655	2.0×10^4	0.2 ± 0.1	1.000	Pgk1	Phosphoglyerate kinase 1
93088_at	12010	1.6×10^4	0.2 ± 0.2	1.000	b2m	β 2 microglobulin
97538_at	110006	6.8×10^3	−0.6 ± 0.2	0.999	b-gus	β glucuronidase
160107_at	15452	6.5×10^3	0.07 ± 0.1	0.999	Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1
103957_at 103958_g_at	22042	3.4×10^3	−0.5 ± 1.2	0.991	Tfrc	Transferrin receptor
99950_at	21374	6.7×10^2	−0.09 ± 0.2	0.996	Tbp	TATA box binding protein

[a] Signal log ratio = LOG_2 (experimental *Brucella* spp. infected signal/control uninfected signal) ± SE.

Table 2

Genes with Increased Transcription in *Brucella* spp. Infected as Compared to Uninfected RAW 264.7 Macrophages

H_1: Mφ ≠ Mφ + *B. melitensis* = Mφ + *B. neotomae* = Mφ + *B. ovis*

		SLR[a]							
Probe set	**Entrez gene**	***M*[b]**	***N*[b]**	***O*[b]**	**Average ± SEM**	**EBarrays probability**	**Gene symbol**	**Gene product**	**Additional statistics[c]**
Chemotaxis									
102736_at	20296	6.4	5.8	6.5	6.2 ± 0.5	0.996	Ccl2	Chemokine (C-C motif) ligand 2 (MCP-1)	*a, b, c*
103486_at	16176	7.5	6.1	6.7	6.8 ± 0.2	0.869	Il1b	Interleukin 1 beta	*a, b, c*
94761_at	20306	5.6	4.6	6.0	5.4 ± 0.3	0.764	Ccl7	Chemokine (C-C motif) ligand 7 (MCP-3)	*a, b, c*
98406_at	20304	2.7	2.2	3.8	2.9 ± 0.6	0.658	Ccl5	Chemokine (C-C motif) ligand 5 (RANTES)	
101160_at	20310	4.8	4.4	4.6	4.6 ± 0.1	0.997	Cxcl2	Chemokine (C-X-C motif) ligand 2 (MIP-2α)	*a, b, c*
94146_at	20303	3.6	3.3	3.6	3.5 ± 0.2	0.999	Ccl4	Chemokine (C-C motif) ligand 4 (MIP-1β)	*a, b, c*
98822_at	53606	2.3	2.0	3.0	2.4 ± 0.3	0.891	Isg15	ISG15 ubiquitin-like modifier	
102424_at	20302	2.6	2.4	2.9	2.6 ± 0.2	0.905	Ccl3	Chemokine (C-C motif) ligand 3 (MIP-1α)	*a, b*
104388_at	20308	2.8	2.4	2.7	2.6 ± 0.2	0.932	Ccl9	Chemokine (C-C motif) ligand 9 (MIP-1γ)	*a, b*
93858_at	15945	1.7	2.1	2.2	2.0 ± 0.2	0.522	Cxcl10	Chemokine (C-X-C motif) ligand 10	*c*
Defense response									
92948_at	12981	6.4	5.0	5.1	5.5 ± 0.4	0.723	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	*a, b*
100981_at	15957	4.6	4.2	5.6	4.8 ± 0.3	0.720	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	*a, b, c*
102629_at	21926	4.3	4.1	4.2	4.2 ± 0.2	0.999	Tnf	Tumor necrosis factor	*a, b, c*
94142_at	12985	4.7	3.7	4.6	4.3 ± 0.2	0.987	Csf3	Colony stimulating factor 3 (granulocyte)	*a, b, c*
103639_at	15958	2.8	3.2	3.7	3.2 ± 0.4	0.907	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	
93871_at	16181	4.0	3.4	3.9	3.7 ± 0.2	0.996	Il1rn	Interleukin 1 receptor antagonist	*a, b, c*
102921_s_at	14102	2.5	3.5	2.7	2.9 ± 0.4	0.914	Fas	Fas (TNF receptor superfamily member)	*c*
102712_at	20210	3.4	2.3	2.9	2.9 ± 0.2	0.948	Saa3	Serum amyloid A 3	*c*

H_1: Mφ ≠ Mφ + *B. melitensis* = Mφ + *B. neotomae* = Mφ + *B. ovis*

Probe set	Entrez gene	SLR[a] *M*[b]	SLR[a] *N*[b]	SLR[a] *O*[b]	SLR[a] Average ± SEM	EBarrays probability	Gene symbol	Gene product	Additional statistics[c]
94928_at	21938	2.5	2.0	2.5	2.4 ± 0.2	0.885	Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	*a, b, c*
92534_at	14579	2.6	2.0	2.9	2.5 ± 0.2	0.839	Gem	GTP binding protein (gene overexpressed in skeletal muscle)	
98988_at	80859	3.1	2.7	2.1	2.6 ± 0.2	0.94304859	Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	
Protein-nucleus import									
97238_at	21335	2.8	3.2	2.9	3.0 ± 0.6	0.940	Tacc3	Transforming, acidic coiled-coil containing protein 3	
104149_at	18035	2.6	2.1	1.9	2.2 ± 0.2	0.704	Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	*a, b*
161135_f_at	66406	2.3	1.2	2.6	2.0 ± 0.3	0.43736716	Sac3d1	SAC3 domain containing 1	
Apoptosis									
94186_at	22029	5.0	4.4	4.6	4.7 ± 0.2	0.943	Traf1	Tnf receptor-associated factor 1	*a, b, c*
104712_at	17869	3.7	4.0	3.0	3.6 ± 0.4	0.890	Myc	Myelocytomatosis oncogene	*c*
99392_at	21929	2.6	2.3	2.0	2.3 ± 0.5	0.814	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	*c*
161666_f_at	17873	3.8	3.4	3.6	3.6 ± 0.2	0.970	Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	*a, b*
102779_at	17873	2.7	2.4	2.7	2.6 ± 0.3	0.947	Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	*c*
Fatty acid biosynthesis									
104647_at	19225	5.6	4.7	4.4	4.9 ± 0.3	0.929	Ptgs2	Prostaglandin-endoperoxide synthase 2	*a, b, c*
94057_g_at	20249	2.2	2.4	2.2	2.3 ± 0.2	0.849	Scd1	Stearoyl-coenzyme A desaturase 1	
Cell surface receptor linked signal transduction									
95344_at	16165	3.0	3.3	3.0	3.1 ± 0.3	0.941	Il13ra2	Interleukin 13 receptor, alpha 2	*c*
97733_at	11541	2.8	2.6	2.5	2.7 ± 0.2	0.931	Adora2b	Adenosine A2b receptor	*a, b*
104498_at	26556	2.4	3.2	1.3	2.3 ± 0.3	0.421	Homer1	Homer homolog 1 (Drosophila)	*b*
102663_at	18793	2.7	2.2	2.7	2.5 ± 0.2	0.953	Plaur	Urokinase plasminogen activator receptor	*a, b, c*
Regulation of biological process									
92232_at	12702	5.6	4.4	5.2	5.1 ± 0.2	0.912	Socs3	Suppressor of cytokine signaling 3	*a, b, c*

H_1: Mφ ≠ Mφ + *B. melitensis* = Mφ + *B. neotomae* = Mφ + *B. ovis*

Probe set	Entrez gene	SLR[a]			Average ± SEM	EBarrays probability	Gene symbol	Gene product	Additional statistics[c]
		M[b]	*N*[b]	*O*[b]					
162206_f_at	12702	3.9	3.1	3.4	3.5 ± 0.1	0.967	Socs3	Suppressor of cytokine signaling 3	*a*, *b*
92471_i_at	20556	2.4	1.6	2.6	2.2 ± 0.3	0.595	Slfn2	Schlafen 2	
Regulation of transcription, DNA-dependent									
101415_i_at	81845	2.5	2.9	1.9	2.4 ± 0.3	0.777	Bat4	HLA-B associated transcript 4	
102709_at	15260	2.4	2.5	2.7	2.5 ± 0.1	0.915	Hira	Histone cell cycle regulation defective homolog A (S. cerevisiae)	*a*, *b*
103651_r_at	68705	2.4	2.2	2.0	2.2 ± 0.2	0.808	Gtf2f2	General transcription factor IIF, polypeptide 2	
102882_at	22704	2.4	2.1	2.5	2.3 ± 0.3	0.579	Zfp46	Zinc finger protein 46	
Protein metabolism									
160829_at	21664	5.1	4.7	4.5	4.8 ± 0.4	0.958	Phlda1	Pleckstrin homology-like domain, family A, member 1	*a*, *b*
93352_at	21817	2.2	1.8	2.0	2.0 ± 0.5	0.530	Tgm2	Transglutaminase 2, C polypeptide	
102782_at	71340	3.3	3.5	2.8	3.2 ± 0.2	0.926	Riok1	RIO kinase 1	*a*, *b*
97548_at	328110	3.1	2.7	2.9	2.9 ± 0.2	0.950	Prpf39	PRP39 pre-mRNA processing factor 39 homolog (yeast)	
Cell growth and/or maintenance									
104451_at	18174	2.4	1.8	2.4	2.2 ± 0.1	0.805	Slc11a2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (Nramp2)	*a*, *b*
94379_at	16561	3.2	2.8	3.2	3.1 ± 0.1	0.945	Kif1b	Kinesin family member 1B	*a*, *b*
94384_at	15937	2.8	2.6	2.2	2.5 ± 0.3	0.890	Ier3	Immediate early response 3	*b*, *c*
160729_f_at	21884	3.3	2.1	3.4	2.9 ± 0.2	0.736	Fabp9	Fatty acid binding protein 9, testis	*a*, *b*
161281_f_at	15937	3.6	3.0	1.8	2.8 ± 0.3	0.44599685	—	Immediate early response 3	
Physiological process									
160084_at	546355	3.2	2.7	3.1	3.0 ± 0.3	0.995	Odc	Similar to ornithine decarboxylase	*b*
AFFX-GapdhMur/M 32599_5_st	14433	2.9	2.3	1.7	2.3 ± 0.4	0.761	Gapdh	Similar to glyceraldehyde-3-phosphate dehydrogenase	
102749_at	12865	1.9	2.4	2.5	2.3 ± 0.4	0.672	Cox7a1	Cytochrome c oxidase, subunit VIIa 1	
94147_at	18787	2.6	2.3	1.9	2.3 ± 0.3	0.813	Serpine1	Serine (or cysteine) proteinase inhibitor, clade E, member 1	*c*
102694_at	26436	2.5	1.5	3.4	2.5 ± 0.2	0.498	Psg16	Pregnancy specific glycoprotein 16	*a*, *b*

H_1: Mφ ≠ Mφ + *B. melitensis* = **Mφ + *B. neotomae*** = **Mφ + *B. ovis***

Probe set	Entrez gene	SLR[a] *M*[b]	SLR[a] *N*[b]	SLR[a] *O*[b]	SLR[a] Average ± SEM	EBarrays probability	Gene symbol	Gene product	Additional statistics[c]
Miscellaneous classification									
96515_at	14204	1.9	1.9	2.5	2.1 ± 0.4	0.550	Il4i1	Interleukin 4 induced 1	
98774_at	16365	4.0	4.2	4.1	4.1 ± 0.3	0.973	Irg1	Immunoresponsive gene 1	*a, b, c*
98773_s_at	16365	3.4	3.1	3.3	3.3 ± 0.3	0.998	Irg1	Immunoresponsive gene 1	*a, b*
94971_at	72391	2.7	1.9	2.6	2.4 ± 0.4	0.851	Cdkn3	Cyclin-dependent kinase inhibitor 3	
162384_f_at	12457	1.9	3.4	2.8	2.7 ± 0.4	0.748	Ccrn4l	CCR4 carbon catabolite repression 4-like	
94389_at	66373	1.9	2.4	1.9	2.1 ± 0.4	0.503	Lsm5	LSM5 homolog, U6 small nuclear RNA associated (S. cerevi-siae)	
97714_r_at	54130	2.7	1.7	3.2	2.5 ± 0.3	0.753	Actr1a	ARP1 actin-related protein 1 homolog A (yeast)	*b*
96162_at	50764	1.6	3.4	2.3	2.5 ± 0.2	0.492	Fbxo15	F-box only protein 15	*b*
93869_s_at	12044	2.3	2.2	2.3	2.3 ± 0.2	0.627	Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	*b, c*
94505_at	67245	1.9	1.8	2.2	2.0 ± 0.1	0.512	Peli1	Pellino 1	*a, b*
104177_at	58185	2.8	1.9	3.0	2.5 ± 0.3	0.827	Rsad2	Radical S-adenosyl methionine domain containing 2	
100669_at	25465	2.1	2.0	2.5	2.2 ± 0.6	0.583	—	Interleukin 17	
161511_f_at	53606	2.1	2.1	3.1	2.4 ± 0.5	0.825	Isg15	Interferon stimulated gene, ubiquitin-like modifier	
97693_at	30865	5.6	5.0	5.4	5.3 ± 0.2	0.965	C78513	EST C78513	
104477_at		3.0	3.3	3.8	3.4 ± 0.3	0.919	—	Transcribed locus	
99849_at	319202	2.7	2.0	2.7	2.5 ± 0.2	0.908	1200016 E24Rik	RIKEN cDNA 1200016E24 gene	

[a] Signal log ratio = LOG_2 (experimental *Brucella* spp. infected signal/control uninfected signal) ± SEM.

[b] *M, N, O* indicate *Brucella* spp. used to infect macrophages *B. melitensis*, *B. neotomae* and *B. ovis*, respectively.

[c] Identified by additional statistical analyses: *a*, ANOVA $P \geq 0.01$; *b*, SAM delta = 0.05; *c*, *B. abortus* microarrays (14).

Table 3

Genes with Decreased Level of Transcript in *Brucella* spp. Infected as Compared to Uninfected RAW 264.7 Macrophages

H_1: Mφ ≠ Mφ + *B. melitensis* = Mφ + *B. neotomae* = Mφ + *B. ovis*

Probe set	Entrez gene	SLR[a]			Average ± SEM	EBarrays probability	Gene symbol	Gene product	Additional statistics[c]
		M[b]	*N*[b]	*O*[b]					
Small GTPase mediated signal transduction									
97415_at	19340	−2.6	−2.0	−3.2	−2.6 ± 0.3	0.866	Rab3d	RAB3D, member RAS oncogene family	*b, c*
97227_at	14673	−2.2	−2.5	−1.9	−2.2 ± 0.3	0.807	Gna12	Guanine nucleotide binding protein, alpha 12	*a*
97549_at	12632	−2.1	−2.2	−1.9	−2.1 ± 0.3	0.662	Cfl2	Cofilin 2, muscle	
93850_at	29875	−2.7	−2.0	−1.3	−2.0 ± 0.4	0.494	Iqgap1	IQ motif containing GTPase activating protein 1	
Cell proliferation									
161417_r_at	18109	−2.5	−2.8	−2.5	−2.6 ± 0.5	0.968	Mycn	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	
93943_f_at	12193	−2.5	−2.4	−2.4	−2.4 ± 0.5	0.925	Zfp36l2	Zinc finger protein 36, C3H type-like 2	
93713_at	20416	−2.5	−2.7	−2.2	−2.5 ± 0.3	0.914	Shc1	Src homology 2 domain-containing transforming protein C1	
92300_at	17428	−2.4	−2.0	−2.9	−2.4 ± 0.3	0.889	Mnt	Max binding protein	*c*
99076_at	353187	−2.2	−1.7	−2.8	−2.3 ± 0.3	0.786	Nr1d2	Nuclear receptor subfamily 1 group D member 2	*c*
101571_g_at	16010	−2.0	−1.7	−3.0	−2.3 ± 0.2	0.685	Igfbp4	Insulin-like growth factor binding protein 4	
99024_at	17122	−2.0	−1.8	−2.2	−2.0 ± 0.3	0.557	Mad4	Max dimerization protein 4	
100444_at	12568	−2.4	−1.9	−1.6	−2.0 ± 0.4	0.520	Cdk5	Cyclin-dependent kinase 5	
Carboxylic acid metabolism									
93320_at	12894	−3.4	−1.9	−3.1	−2.8 ± 0.4	0.663	Cpt1a	Carnitine palmitoyltransferase 1a, liver	*c*
96799_at	30839	−2.7	−2.2	−2.3	−2.4 ± 0.3	0.897	Fbxw5	F-box and WD-40 domain protein 5	
96126_at	20397	−2.3	−2.4	−2.0	−2.2 ± 0.2	0.704	Sgpl1	Sphingosine phosphate lyase 1	
94405_at	21366	−2.7	−1.5	−1.7	−2.0 ± 0.2	0.444	Slc6a6	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	
Response to external stimulus									
101433_at	15064	−2.4	−2.4	−2.4	−2.4 ± 0.3	0.918	Mr1	Major histocompatibility complex, class I-related	
104228_at	668701	−2.2	−2.2	−2.6	−2.3 ± 0.4	0.865	EG668701	Similar to Rap guanine nucleotide exchange factor 2	
103250_at	54722	−2.7	−2.1	−2.0	−2.3 ± 0.2	0.838	Dfna5h	Deafness, autosomal dominant 5 homolog	
104372_at	64296	−2.6	−2.2	−1.9	−2.2 ± 0.2	0.793	Abhd8	Abhydrolase domain containing 8	

H_1: Mφ ≠ Mφ + *B. melitensis* = **Mφ + *B. neotomae*** = **Mφ + *B. ovis***

Probe set	Entrez gene	SLR[a] *M*[b]	SLR[a] *N*[b]	SLR[a] *O*[b]	Average ± SEM	EBarrays probability	Gene symbol	Gene product	Additional statistics[c]
99349_at	16171	−1.7	−2.5	−2.1	−2.1 ± 0.3	0.681	Il17a	Interleukin 17A	
93218_at	20947	−2.3	−2.0	−1.7	−2.0 ± 0.4	0.573	Swap70	SWAP complex protein	
Protein modification									
99643_f_at	12876	−3.7	−3.8	−2.7	−3.4 ± 0.4	0.949	Cpe	Carboxypeptidase E	*b*
161848_r_at	19260	−2.5	−2.2	−2.4	−2.4 ± 0.4	0.870	Ptpn22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	
92427_at	21812	−2.4	−1.9	−2.3	−2.2 ± 0.4	0.729	Tgfbr1	Transforming growth factor, beta receptor I	
99642_i_at	12876	−3.0	−2.1	−1.3	−2.2 ± 0.7	0.618	Cpe	Carboxypeptidase E	
100427_at	19277	−2.0	−1.4	−2.7	−2.0 ± 0.3	0.552	Ptpro	Protein tyrosine phosphatase, receptor type, O	*b, c*
Transcription from Pol II promoter									
104591_g_at	17260	−2.4	−2.9	−2.5	−2.6 ± 0.2	0.932	Mef2c	Myocyte enhancer factor 2C	*b, c*
104590_at	17260	−2.8	−2.0	−2.2	−2.3 ± 0.2	0.821	Mef2c	Myocyte enhancer factor 2C	*a, b*
96171_at	54006	−2.1	−1.6	−2.4	−2.0 ± 0.2	0.580	Deaf1	Deformed epidermal autoregulatory factor 1 (Drosophila)	*b*
Cytoskeleton organization and biogenesis									
95705_s_at	11461	−2.2	−3.1	−2.4	−2.6 ± 0.5	0.887	Actb	Actin, beta, cytoplasmic	
161981_r_at	14246	−2.6	−1.6	−2.4	−2.2 ± 0.4	0.693	Flg	Filaggrin	
Metabolism									
94872_at	57319	−2.8	−2.5	−2.8	−2.7 ± 0.4	0.934	Smpdl3a	Sphingomyelin phosphodiesterase, acid-like 3A	
161733_at	59010	−2.4	−2.9	−2.1	−2.5 ± 0.3	0.919	Sqrdl	Sulfide quinone reductase-like	
101972_at	16541	−2.8	−2.4	−2.5	−2.6 ± 0.3	0.915	Napsa	Napsin A aspartic peptidase	
99667_at	12862	−2.3	−2.2	−2.5	−2.3 ± 0.5	0.878	Cox6a2	Cytochrome c oxidase, subunit VI a, polypeptide 2	
103538_at	21386	−1.8	−2.1	−2.5	−2.1 ± 0.3	0.680	Tbx3	T-box 3	
96035_at	12039	−1.7	−2.0	−2.4	−2.0 ± 0.4	0.606	Bckdha	Branched chain ketoacid dehydrogenase E1, alpha polypeptide	
97560_at	19156	−1.4	−3.0	−1.7	−2.0 ± 0.4	0.381	Psap	Prosaposin	
Cell communication									
160932_at	17973	−2.6	−2.6	−2.6	−2.6 ± 0.1	0.964	Nck1	Non-catalytic region of tyrosine kinase adaptor protein 1	*a, b*
97768_at	13506	−2.2	−2.1	−2.6	−2.3 ± 0.3	0.803	Dsc2	Desmocollin 2	
Cell growth and/or maintenance									

H_1: Mφ ≠ Mφ + *B. melitensis* = **Mφ + *B. neotomae*** = **Mφ + *B. ovis***

		SLR[a]							
Probe set	**Entrez gene**	***M*[b]**	***N*[b]**	***O*[b]**	**Average ± SEM**	**EBarrays probability**	**Gene symbol**	**Gene product**	**Additional statistics[c]**
92695_at	14296	−2.0	−1.6	−2.7	−2.1 ± 0.3	0.658	Frat1	Frequently rearranged in advanced T-cell lymphomas	
103534_at	15130	−2.8	−2.0	−1.5	−2.1 ± 0.2	0.626	Hbb-b2	Hemoglobin, beta adult minor chain	*b*
93736_at	21452	−2.0	−2.0	−2.3	−2.1 ± 0.3	0.606	Tcn2	Transcobalamin 2	
Miscellaneous classification									
96494_at	75785	−2.9	−2.7	−2.5	−2.7 ± 0.2	0.959	Klhl24	Kelch-like 24 (Drosophila)	
103933_at	22619	−2.9	−1.9	−2.8	−2.5 ± 0.2	0.895	Siae	Sialic acid acetylesterase	*b*
104299_at	224454	−3.0	−1.9	−2.5	−2.5 ± 0.3	0.852	Zdhhc14	Zinc finger, DHHC domain containing 14	
94299_at	69654	−2.5	−2.0	−2.2	−2.2 ± 0.2	0.788	Dctn2	Dynactin 2	
162116_r_at	116891	−3.0	−2.7	−1.7	−2.5 ± 0.9	0.741	Derl2	Der1-like domain family, member 2	
160934_s_at	73094	−3.0	−2.6	−1.6	−2.4 ± 0.5	0.692	Sgip1	SH3-domain GRB2-like (endophilin) interacting protein 1	
104714_at	105445	−2.2	−1.7	−2.2	−2.0 ± 0.2	0.611	Dock9	Dedicator of cytokinesis 9	
162075_r_at	17476	−2.7	−1.5	−3.8	−2.7 ± 0.4	0.551	Mpeg1	Macrophage expressed gene 1	
96464_at	140570	−2.1	−2.2	−1.9	−2.1 ± 0.2	0.518	Plxnb2	Plexin B2	
160905_s_at	80515	−2.2	−2.2	−2.1	−2.1 ± 0.3	0.735	A030009 H04Rik	RIKEN cDNA A030009H04 gene	
97119_at	99029	−2.6	−2.5	−2.2	−2.4 ± 0.3	0.911	AI596198	Expressed sequence AI596198	
97752_at	99358	−2.0	−2.1	−2.8	−2.3 ± 0.3	0.858	E13001 3N09Rik	RIKEN cDNA E130013N09 gene	
103748_at	74440	−2.9	−1.6	−2.1	−2.2 ± 0.2	0.645	4933407 C03Rik	RIKEN cDNA 4933407C03 gene	
94069_r_at	67163	−3.4	−2.3	−2.2	−2.6 ± 0.3	0.946	2610204 L23Rik	RIKEN cDNA 2610204L23 gene	

[a] Signal log ratio = LOG_2 (experimental *Brucella* spp. infected signal/control uninfected signal) ± SEM.

[b] *M, N, O* indicate *Brucella* spp. used to infect macrophages *B. melitensis, B. neotomae* and *B. ovis*, respectively.

[c] Identified by additional statistical analyses: *a*, ANOVA $P \geq 0.01$; *b*, SAM delta = 0.05; *c*, *B. abortus* microarrays (14).

Table 4A

Differences in Macrophage Transcript Expression when Infected by Different *Brucella* Species

A. *B. ovis* and *B. neotomae* Infected Macrophages Express Transcripts Different from *B. melitensis* or Uninfected Macrophages

H2: Mφ ≠ Mφ + *B. melitensis* ≠ Mφ + *B. neotomae*= Mφ + *B. ovis*

		SLR[a]						
Probe set	**Entrez gene**	***B. melitensis***	***B. neotomae***	***B. ovis***	**EBarrays probability**	**Gene symbol**	**Gene product**	**Additional statistics[b]**
94337_at	14453	−4.2 ± 0.7	−1.1 ± 0.4	−1.3 ± 0.5	1.000	Gas2	Growth arrest specific 2	
162240_r_at	13010	0.8 ± 0.3	3.4 ± 0.2	3.9 ± 0.1	0.999	Cst3	Cystatin C	*a, b*
101793_at	14129	−3.1 ± 0.1	−1.4 ± 0.3	−0.5 ± 0.3	0.903	Fcgr1	Fc receptor, IgG, high affinity I (FcγRI)	*a, b, c*
102794_at	12767	−3.6 ± 0.5	−1.4 ± 0.7	−1.7 ± 0.3	0.898	Cxcr4	Chemokine (C-X-C motif) receptor 4	*c*
101070_at	54484	−3.6 ± 0.5	−1.6 ± 0.6	−1.7 ± 0.3	0.885	Mkrn1	Makorin, ring finger protein, 1	
161878_r_at	52250	−2.2 ± 0.8	0.5 ± 0.1	0.3 ± 0.2	0.852	Reep1	Receptor accessory protein 1	
93569_f_at	545005	0.6 ± 0.3	2.6 ± 0.4	3.3 ± 0.7	0.829	LOC544988	Similar to Spetex-2F protein	
99377_at	18249	2.7 ± 0.6	0.5 ± 0.9	−0.6 ± 0.6	0.781	Obp1a	Odorant binding protein Ia	
94240_i_at	19944	1.7 ± 0.9	−0.8 ± 1.3	−0.8 ± 1.0	0.734	Rpl29	Ribosomal protein L29	
104697_at	80837	−3.0 ± 0.7	−1.2 ± 0.9	−0.8 ± 0.4	0.622	Rhoj	ras homolog gene family, member J	
92857_at	19934	−3.3 ± 0.7	−1.2 ± 0.7	−2.0 ± 0.9	0.571	Rpl22	Ribosomal protein L22	
102706_i_at	16625	−2.7 ± 0.9	−0.6 ± 0.4	−0.1 ± 0.3	0.566	Serpina3c	Serine (or cysteine) proteinase inhibitor, clade A, member 3C	

Table 4B

B. *B. melitensis* and *B. neotomae* Infected Macrophages Express Transcripts Different from *B. ovis* or Uninfected Macrophages

H_3: Mφ ≠ Mφ + *B. melitensis* = Mφ + *B. neotomae* ≠ Mφ + *B. ovis*

		SLR[a]					
Probe set	**Entrez gene**	***B. melitensis***	***B. neotomae***	***B. ovis***	**EBarrays probability**	**Gene symbol**	**Gene product**
161420_r_at	13478	−0.3 ± 0.7	−0.2 ± 1.3	−3.2 ± 0.5	0.993	Dpagt1	Dolichyl-phosphate acetylgluco-saminephosphotransferase 1 (GlcNAc-1-P transferase)
160797_r_at	12412	−2.1 ± 0.4	−2.0 ± 0.4	0.6 ± 0.2	0.975	Cbx1	Chromobox homolog 1 (Drosophila HP1 beta)
92600_f_at	13117	−2.4 ± 0.6	−2.4 ± 0.7	0.0 ± 0.1	0.953	Cyp4a10	Cytochrome P450, family 4, subfamily a, polypeptide 10
161128_r_at	544834	0.5 ± 1.3	1.8 ± 0.9	3.7 ± 0.8	0.9361	6030426L16Rik	Similar to Hippocalcin-like protein 1, Visinin-like protein 3
99885_at	16019	0.6 ± 0.9	1.0 ± 0.3	3.1 ± 0.6	0.738	Igh-6	Immunoglobulin heavy chain 6 (heavy chain of IgM)
93566_at	74246	−1.5 ± 0.2	−2.6 ± 0.6	0.1 ± 0.3	0.711	Gale	Galactose-4-epimerase, UDP
162076_r_at	14800	−2.2 ± 0.7	−3.0 ± 0.5	−0.5 ± 0.4	0.681	Gria2	Glutamate receptor, ionotropic, AMPA2 (alpha 2)
160728_r_at	73945	−2.1 ± 1.1	−0.4 ± 0.6	−4.4 ± 0.6	0.6579	Otud4	OTU domain containing 4
AFFX-BioDn-3_st	1037747	−1.6 ± 0.3	−0.4 ± 0.1	1.4 ± 0.1	0.6294	—	Biotin synthesis protein bioC
161131_r_at	228889	−0.6 ± 0.4	−0.7 ± 0.1	−2.7 ± 0.1	0.5167	—	Dead (asp-glu-ala-asp) box polypeptide 27
102299_at	18750	−1.8 ± 0.7	−1.2 ± 0.5	−3.3 ± 0.4	0.508	Prkca	Protein kinase C, alpha

Table 4C

C. *B. melitensis* and *B. ovis* Infected Macrophages Express Transcripts Different from *B. neotomae* or Uninfected Macrophages

H_4: Mφ ≠ Mφ + *B. melitensis* = Mφ + *B. ovis* ≠ Mφ + *B. neotomae*

		SLR[a]						
Probe set	**Entrez gene**	***B. melitensis***	***B. neotomae***	***B. ovis***	**EBarrays probability**	**Gene symbol**	**Gene product**	**Additional statistics[b]**
94051_at	13612	3.0 ± 0.4	0.5 ± 0.4	3.2 ± 0.3	0.998	Edil3	EGF-like repeats and discordin I-like domains 3	*a*, *b*
96785_at	80880	−3.2 ± 0.6	−0.5 ± 1.1	−2.7 ± 0.7	0.994	Ankrd47	Ankyrin repeat domain 47	
92913_at	26874	−4.1 ± 0.3	−2.0 ± 0.4	−3.9 ± 0.3	0.920	Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	*a*, *b*
103467_g_at	54151	−2.3 ± 0.3	−0.6 ± 0.3	−2.9 ± 0.3	0.897	Cyhr1	Cysteine and histidine rich 1	
94755_at	16175	3.9 ± 0.4	1.7 ± 0.7	3.1 ± 0.4	0.693	Il1a	Interleukin 1 alpha	*b*, *c*
93568_i_at		1.4 ± 0.0	−1.1 ± 0.7	1.2 ± 0.5	0.556	LOC544988	Similar to Spetex-2F protein	
92504_at	15574	2.1 ± 0.6	−0.1 ± 0.4	1.8 ± 0.7	0.501	Hus1	Hus1 homolog (S. pombe)	
104592_i_at	17260	−3.7 ± 0.2	−1.9 ± 0.3	−3.3 ± 0.2	0.473	Mef2c	Myocyte enhancer factor 2C	*a*, *b*
102798_at	11535	0.9 ± 0.6	2.4 ± 0.6	−0.3 ± 0.7	0.444	Adm	Adrenomedullin	
102374_at	53902	−2.9 ± 0.3	−1.0 ± 0.4	−2.2 ± 0.5	0.425	Dscr1l2	Down syndrome critical region gene 1-like 2	*a*, *b*

[a] Signal log ratio = LOG_2 (experimental *Brucella* spp. infected signal/control uninfected signal) ± SEM.

[b] Identified by additional statistical analyses: *a*, ANOVA $P \geq 0.01$; *b*, = SAM delta = 0.05; *c*, *B. abortus* microarrays (14).