Brucella melitensis Triggers Time-Dependent Modulation of Apoptosis and Down-Regulation of Mitochondrion-Associated Gene Expression in Mouse Macrophages

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Brucella spp. are facultative intracellular bacteria that cause brucellosis in humans and other animals. *Brucella* spp. are taken up by macrophages, and the outcome of the macrophage-*Brucella* interaction is a basis for establishment of a chronic *Brucella* infection. Microarrays were used to analyze the transcriptional response of the murine macrophage-like J774.A1 cell line to infection with virulent *Brucella melitensis* strain 16M. It was found that most significant changes in macrophage gene transcription happened early following infection, and global macrophage gene expression profiles returned to normal between 24 and 48 h postinfection. These findings support the observation that macrophages kill the majority of *Brucella* cells at the early infection stage, but the surviving *Brucella* cells are able to avoid macrophage brucellacidal activity inside replicative phagosomes at the later infection stage. At 4 h postinfection, macrophage genes involved in cell growth, metabolism, and responses to endogenous stimuli were down-regulated, while the inflammatory response (e.g., tumor necrosis factor alpha and Toll-like receptor 2), the complement system, the responses to external stimuli, and other immune responses were up-regulated. It is likely that the most active brucellacidal activity happened between 0 and 4 h postinfection. Mitochondrion-associated gene expression, which is involved in protein synthesis and transport, electron transfer, and small-molecule transfer, and many other mitochondrial functions were significantly down-regulated at 4 h postinfection. Although there were both pro- and antiapoptosis effects, *B. melitensis* 16M appears to inhibit apoptosis of macrophages by blocking release of cytochrome *c* and production of reactive oxygen species in the mitochondria, thus preventing activation of caspase cascades.

Brucellosis in humans and other animal species is caused by facultative intracellular bacteria belonging to the genus *Brucella*. Unlike many pathogenic bacteria, the brucellae lack classical virulence factors, such as invasive proteases, exotoxins, endotoxic lipopolysaccharide (LPS), capsules, fimbriae, pili, virulence plasmids, and lysogenic phages (29, 48). *Brucella* virulence relies on the ability of the organism to survive and replicate within vacuolar phagocytic compartments of macrophages (37). The host macrophage-*Brucella* interaction is critical for establishment of chronic *Brucella* infections. For example, both the type IV secretion system encoded by the *virB* operon (7) and the two-component regulatory system encoded by the *bvrRS* operon (44) are necessary for successful replication of *Brucella* inside macrophages. Smooth *Brucella* strains with intact LPS O side chains are virulent and invade macrophages through lipid rafts (56). Immediately after entry into

macrophages, *Brucella* strains reside in an acidified compartment that fuses with components of the early endosomal pathway (56). The majority of *Brucella* strains are killed at the early infection stage (14, 56). However, a subpopulation of virulent *Brucella* strains is instead trafficked to an intracellular compartment known as the replicative phagosome or the brucellosome through continual interactions between the *Brucella*-containing vacuoles and the endoplasmic reticulum of the host macrophages. Once inside the replicative phagosome, *Brucella* cells are resistant to further attack and begin to multiply dramatically (37, 56). Virulent *Brucella* strains also inhibit macrophage apoptosis, which in turn favors pathogen survival and replication (25, 28). However, the genetic basis for the inhibition of apoptosis is not known.

Previous studies (24, 32, 35, 64) have indicated that smooth virulent *Brucella* strains undergo rapid clearance (>90% of *Brucella* cells are killed) within the first 24 h, which is followed by growth of the surviving bacteria after infection of murine J774.A1 or peritoneal macrophages. Macrophage responses against virulent *Brucella* strains have been characterized mostly using technologies that reveal intracellular processes involved in killing and trafficking of *Brucella* spp. during different infec-

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tion stages (14, 38). Eskra et al. analyzed the RAW267.4 macrophage transcriptional responses against infection by *Brucella abortus* strain 2308 at a single time, 4 h postinfection (23). A time course study of host gene transcription profiles that covers different infection stages would be useful for clarifying the quality and quantity of responses that occur. In our investigation we used the Affymetrix mouse GeneChip 430 2.0 array to analyze mouse macrophage gene expression profiles during the course of virulent *B. melitensis* strain 16M infection. Our goal was to analyze apoptosis-related genes and cellular components in order to determine the mechanisms of apoptosis inhibition. Our results suggest that a *B. melitensis* infection triggers most transcriptional changes in mouse macrophages early following infection that are consistent with modulation of apoptotic genes and down-regulation of genes in the mitochondrion- and cytochrome *c*-mediated stress pathways.

MATERIALS AND METHODS

***Brucella* and macrophage cell line.** *B. melitensis* strain 16M was originally obtained from Alexander J. Winter's laboratory at Cornell University and was grown either in tryptic soy broth or on tryptic soy agar plates. The characteristics of this strain were confirmed by using AMOS (*B. abortus*-*B. melitensis*-*B. ovis*-*B. suis*) PCRs (10), crystal violet staining, antibiotic resistance analysis, the urease test, and thionin and fuchsin susceptibility analysis (61). The J774.A1 macrophage cell line was purchased from the American Type Culture Collection (Manassas, Va.). The cells were cultured at 37°C with 5% CO₂ in complete tissue culture medium (c-DMEM) consisting of Dulbecco's modified Eagle's medium (American Type Culture Collection) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.). The J774.A1 cells continued to be able to generate the dose-dependent oxidative burst response, as detected by the nitro blue tetrazolium reduction assay after exposure to phorbol myristate acetate (8).

Macrophage infection and survival assay. J774.A1 macrophage cells were plated in 24-well plates in c-DMEM without antibiotics at a concentration of 2.5×10^5 cells per well and incubated overnight at 37°C with 5% (vol/vol) CO₂. The J774.A1 cells were then infected with *B. melitensis* strain 16M in triplicate wells of a 24-well plate at a multiplicity of infection (MOI) of 200:1. Following 4 h of incubation at 37°C in an atmosphere containing 5% CO₂, the cells were washed three times with phosphate-buffered saline and treated with 50 µg/ml gentamicin to kill extracellular *Brucella*. Infected cells were lysed with 0.1% Triton X-100 in phosphate-buffered saline at 4 h, 8 h, 24 h, 48 h, and 72 h postinfection (12). The number of viable CFU was determined by plating a series of 1:10 dilutions on tryptic soy agar plates (20).

Quantitation of cytopathic cell death. Cells cultured in 24-well plates were infected with *B. melitensis* strain 16M in triplicate wells as described above. The culture supernatants were collected at 0, 4, 8, 24, 48, and 72 h, and the lactate dehydrogenase (LDH) released from the cell cytoplasm was tested by using a CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) according to the manufacturer's instructions. Cytopathic cell death was expressed as a percentage of the LDH released, as follows: $100 \times (\text{optical density at } 490 \text{ nm } [OD_{490}] \text{ of infected cells} - OD_{490} \text{ of uninfected cells}) / (OD_{490} \text{ of lysed uninfected cells} - OD_{490} \text{ of uninfected cells})$ (51).

Total RNA isolation. J774.A1 macrophage cells were plated in 12 75-cm² flasks in c-DMEM without antibiotics 1 day prior to infection at a concentration of 8×10^6 cells per flask. The 12 flasks were divided into four groups of three flasks each. Nine flasks (three groups) of cells were infected with *B. melitensis* strain 16M at an MOI of 200:1. *Brucella* cultures derived from different *Brucella* colonies were used to infect different groups of macrophage cells to obtain independent infections. Following 4 h of incubation at 37°C in 5% CO₂, the cells were washed three times with phosphate-buffered saline and treated with 50-µg/ml gentamicin to kill extracellular *Brucella* bacteria. At zero time (no *Brucella* infection) and at 4 h, 24 h, and 48 h postinfection, 7.5 ml of TRIzol reagent (Invitrogen Corp., Carlsbad, CA) was added to each flask in a group. Uninfected J774.A1 cells were counted to determine the value for zero time postinfection. The homogenized samples were incubated for 5 min at room temperature. The lysed cells were aliquoted into 10 tubes, centrifuged, and stored at -80°C. Total RNA of J774.A1 cells was isolated by the TRIzol method and was further cleaned with a QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA) used ac-

cording to the manufacturers' protocols. An Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) was used to assess the concentrations and quality of RNA samples. The same set of RNA samples was used for both real-time reverse transcription (RT)-PCR and Affymetrix microarray hybridization.

Affymetrix microarray experiment and data analysis. Twenty micrograms of total RNA per sample was processed for microarray analysis, and the Affymetrix mouse GeneChip 430 2.0 array was used for hybridization. Preparation of cRNA, hybridization, and scanning of the GeneChip 430 2.0 arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). The Affymetrix GCOS expression analysis software was used to process image data, and all the raw signals were first globally scaled to a target intensity of 250. Quality controls, including scaling factors, average intensities, presence calls, background intensities, noise, and raw Q values, were all within acceptable limits as defined by the Affymetrix protocol (1). Hybridization controls, including BioB, BioC, BioD, and CreX, were called present on all chips and yielded the expected intensity increases (1). GeneSpring (version 7.0; Silicon Genetics, Redwood, CA) and the microarray data analysis suite of the ToolBus/PathPort (<http://pathport.vbi.vt.edu>) web service federation (22) were used for further microarray data analysis. The probe sets of the microarray data were called either present, absent, or marginal based on the default *P* values set up in the GCOS system. Briefly, a probe set is called present when the detection *P* value is less than 0.04, absent when the detection *P* value is greater than 0.06, and marginal when the detection *P* value is between 0.04 and 0.06 (1). A list of probe sets labeled absent in any one chip was first filtered out. After the variability across these biological replicative samples was evaluated, the signal intensity cutoff value was determined to be 50. The probe sets with signal intensities less than 50 for any one chip were also filtered out in the final list of target probe sets. The Welch *t* test (*P* < 0.05) with the multiple testing correction of the Benjamini and Hochberg false discovery rate was used for statistical analysis of the uninfected control group and treatment groups at different infection times (59). The fold change cutoff values (<0.75 or > 1.3333) were also used to filter out genes with small fold changes between two groups (45).

Gene ontology (GO) annotation of genes identified as differentially expressed was carried out with the Onto-Express software package (available at <http://vortex.cs.wayne.edu/Projects.html>). Probabilities of overrepresentation were estimated with a chi-square-based probability model with the false discovery rate multiple testing correction.

Real-time RT-PCR. Probes sets of 15 genes were designed using the Primer3 software (57) and were purchased from Sigma-Genosys (The Woodlands, TX). To confirm microarray data, the same triplicate biological RNA samples used for microarrays were also used for the real-time RT-PCR experiment. To determine if the changes in cell growth and cell density influenced the macrophage gene expression profiles, only c-DMEM was added to uninfected J774.A1 cell cultures at zero time, 4 h, 24 h, and 48 h, with zero time defined like it was for the microarray experiment. The cells were collected at these different times, and RNA was prepared as described above. Fifty nanograms of RNA was used per reverse transcription reaction (Superscript reverse transcription kit; Invitrogen, California). The primer pair spanning the 5' end of glyceraldehyde-3-phosphate dehydrogenase was used for internal housekeeping gene control; 12.5 µl of real-time PCR master mixture (Platinum qPCR super mix UDG; Invitrogen, California), 0.5 µl of SYBR (Cambrex, New Jersey), and 2 µl of cDNA were used for each reaction. A melting curve analysis was also carried out. The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, and 54.5°C for 15 s, and 72°C for 30 s. Data analysis was carried out using the $2^{-\Delta\Delta C_T}$ method (43) for computing fold changes between samples for differential gene expression analysis.

RESULTS

Two-phase *B. melitensis* survival inside J774.A1 cells and inhibition of macrophage cell death. To exclude the possibility that changes in gene expression were not observed because of an inadequate oxidative burst response in the J774.A1 macrophage cell line, the nitro blue tetrazolium reduction assay (8) was used. The J774.A1 cells used in these experiments remained able to generate a dose-dependent oxidative burst response (data not shown). J774.A1 cells were infected with virulent *B. melitensis* strain 16M, and the numbers of viable CFU were determined at 4, 8, 24, 48, 72 h postinfection as described in Materials and Methods (Fig. 1). The majority of

FIG. 1. Intracellular growth of *B. melitensis* strain 16M in J774.A1 macrophages. J774.A1 cells were infected with strain 16M at an MOI of 200:1, and the number of live intracellular bacteria was determined by determining the number of CFU/well, as described in Materials and Methods. The results are the results of one representative experiment of three similar experiments, and the values are means $\pm$ standard errors of the means for triplicate infections performed concomitantly. Different stages of infection are indicated.

Brucella cells were killed during the first 24 h (approximately 1.0 log difference), while the surviving bacteria began to replicate after 24 h postinfection. This two-phase survival pattern is consistent with previous reports (24, 32, 35, 64). Based on these results, the time series microarray analysis was performed using times of 0, 4, 24, and 48 h postinfection, representing an uninfected control, the early infection stage, the

transition from the early stage to the late infection stage, and the late infection stage, respectively.

The supernatants of uninfected and *B. melitensis* strain 16M-infected J774.A1 macrophages were collected at different times, and the amounts of LDH released from dead macrophages were determined (data not shown). No significant LDH was detected for these macrophages at any of the times, and this suggested that apoptosis or macrophage cell death did not occur in uninfected and *B. melitensis*-infected J774.A1 cells.

More genes were suppressed than transcribed at any time. A total of 12 microarray chips were used, with three chips for each time. Of 45,101 probe sets in the mouse array, the percentages of present probe sets (see Materials and Methods) at 0, 4, 24, 28 h postinfection were $39.3\% \pm 0.6\%$, $36.9\% \pm 1.0\%$, $38.5\% \pm 0.9\%$, and $37.2\% \pm 1.1\%$, respectively; the percentages of absent probe sets were $59.0\% \pm 0.6\%$, $61.4\% \pm 1.1\%$, $59.5\% \pm 1.0\%$, and $61.2\% \pm 1.1\%$, respectively; and the percentages of marginally present probe sets were $1.6\% \pm 0.06\%$, $1.7\% \pm 0.1\%$, $1.6\% \pm 0.1\%$, and $1.63\% \pm 0.06\%$, respectively. The results indicate that the percentages of genes with present, marginally present, and absent expression at different times were similar. Only a small portion of all the genes was marginally transcribed ($0.04 < P < 0.06$) at any time. All the other genes were either transcribed (present) or suppressed (absent), and fewer transcribed genes were found at each time.

Gene expression profiles at 4 h postinfection. For microarray data analysis, 22,831 probe sets were first selected for further analysis by filtering out 21,463 probe sets that did not exhibit transcriptional changes in one chip and the 807 probe sets that exhibited very low signals (see Materials and Methods). More than one probe set in a mouse array can represent one gene (4). Transcription of 1,426 probe sets (1,296 genes) was significantly up- or down-regulated between 0 and 4 h (Fig. 2). For the 1,426 probe sets, expression of 1,138 probe sets (1,053 genes) was down-regulated (Fig. 2A) and expression of 288 probe sets (243 genes) was up-regulated (Fig. 2B) at 4 h

FIG. 2. Time course for 1,426 significantly regulated probe sets (1,296 genes) at 4 h postinfection. The 1,426 probe sets included 1,138 down-regulated probe sets (1,053 genes) (A) and 288 up-regulated probe sets (243 genes) (B). More than one probe set may represent one gene. The general gene expression patterns at 24 h and 48 h postinfection are similar to those at zero time. The results suggest that most up- or down-regulated gene expression at 4 h postinfection returned to the normal level at 24 h and 48 h postinfection. The y axis represents log transformation of the normalized gene expression signal intensity. Standard errors of means are indicated for each probe set. The figure was created by GeneSpring. Supplemental Tables 1 and 2 at <http://sitemaker.umich.edu/helab> provide signal data and gene information for these up- and down-regulated probe sets.

TABLE 1. Functional families overrepresented in the macrophage transcriptional response to *B. melitensis* infection at 4 h postinoculation

Biological process	<i>P</i> value (no. of genes) ^a
Up-regulated processes	
Macromolecule biosynthesis	0.0 (18)
Lipid metabolism.....	0.0 (16)
Sterol metabolism.....	0.0 (9)
Immune cell activation	0.0 (7)
Humoral immune response.....	0.0 (6)
Inflammatory response	3.0E-5 (5)
Cellular defense response	1.3E-4 (3)
Response to external stimulus.....	1.9E-4 (12)
Cell adhesion	0.0021 (12)
Cell proliferation.....	0.0077 (15)
Nucleic acid metabolism	0.047 (8)
Intracellular transport.....	0.047 (5)
Down-regulated processes	
Cell growth and/or maintenance	0.0 (217)
Macromolecule metabolism.....	0.0 (214)
Nucleic acid metabolism.....	0.0 (161)
Regulation of biological process	0.0 (131)
Biosynthesis.....	0.0 (87)
Amino acid metabolism.....	0.0 (33)
Apoptosis.....	0.0 (29)
Electron transfer	0.0 (25)
Behavioral fear response	0.0 (3)
Response to endogenous stimulus	2.0E-5 (18)
Lipid metabolism.....	0.001 (27)
Cell adhesion	0.0038 (6)
Intracellular signaling cascade	0.026 (39)
Response to oxidative stress.....	0.045 (3)

^a 0.0 represents a *P* value of less than 1.0E-6.

postinfection. The levels of most up- or down-regulated gene expression observed at 4 h postinfection returned to normal levels between 24 h and 48 h postinfection.

The Onto-Express software package was used to sort these up- and down-regulated genes based on the GO annotation system. This allowed identification of biological processes with statistically significant overrepresentation ($P < 0.05$) and at least three members present in the data sets, as shown in Table 1. Macrophage gene expression during early *Brucella* infection was marked by several distinct trends. The general cellular activities were suppressed substantially, as shown by down-regulated genes involved in cell growth and/or maintenance, macromolecule and nucleic acid metabolism, biosynthesis and biological process regulation, and the intracellular signaling cascade. The apoptosis and electron transfer systems were also suppressed, which is described in detail below. Both up- and down-regulated genes involved in lipid metabolism were observed at 4 h postinfection, which might have been related to sensitive macrophage responses to bacterial lipid components (for example, the lipid A in the LPS). One noteworthy phenomenon is that macrophages displayed up-regulated responses to external stimuli but down-regulated responses to endogenous stimuli (Table 1), suggesting that at this time infecting *Brucella* had started to escape active macrophage responses to intracellular stress (due to *Brucella* infection) although inflammatory and other defense responses described below were still observed. More down-regulated gene expression profiles than up-regulated gene expression profiles also

suggests that the most active brucellacidal activity might have happened in the first 4 h postinfection.

It was important to determine if gene products necessary for killing *Brucella* during the first 24 h were activated. *Brucella* infection significantly induced gene expression in the immune response category, such as immune cell activation and humoral and innate immune responses (Table 1). An up-regulated inflammatory immune response was observed and correlated with clearance of *Brucella*. For example, the gene encoding Toll-like receptor 2 (TLR2) was significantly up-regulated (4.5-fold; $P = 0.044$) by 4 h postinfection. Transcription of the gene encoding tumor necrosis factor alpha (TNF- α) was also stimulated so that there was a 9.9-fold ($P = 0.039$) increase by 4 h postinfection, and then the level of transcription returned to normal levels at 24 h and 48 h postinfection. In addition to the genes encoding TLR2 and TNF- α , other up-regulated genes directly or indirectly related to inflammatory responses included Ncf1, Fcgr2b, Il1b, Ccl9, Tnfrsf1b, Tlr7, Il3ra1, Il2rg, and Il1rn. Up-regulated complement expression might help phagocytosis and favor killing of *Brucella* inside macrophages (25). Our data also indicated that genes encoding complement component factor h (Cfh), complement component 1 q subcomponent receptor 1 (C1qr1), complement component 5 receptor 1 (C5r1), and complement component 3a receptor 1 (C3ar1) were up-regulated at 4 h postinfection. The C1qbp gene encoding complement component 1 q subcomponent binding protein was also down-regulated at 4 h postinfection.

B. melitensis 16M prevents phagosome-lysosome fusion inside macrophages (17). Our study showed that five lysosomal genes (Idua, Dpp7, Cttns, Ap3m1, and Gla) encoding five different enzymes were down-regulated and that none of the lysosomal genes listed in the GO biological component category were up-regulated (data not shown). Ap3m1 encodes the mu 1 subunit of adaptor-related protein complex 3, which mediates trans-Golgi network-to-lysosome protein transport and also endosome-to-lysosome protein transport (55). Decreased Ap3m1 transcription might affect regular protein transport to lysosomes (40, 55) and contribute to prevention of phagosome-lysosome fusion.

Smooth *Brucella* cells are phagocytosed via lipid rafts on the macrophage cell surface, and the acidified *Brucella*-containing vacuoles immediately fuse with the early endosomes (56). The eps15 homology domain-containing protein EHD1 is localized in transferrin-containing endosomes and is involved in regulated endocytosis (47). The Ehd1 gene encoding EHD1 was also up-regulated at 4 h postinfection. Transcription of all five probe sets for transferrin receptor was significantly increased (approximately two- to fivefold; *P* value range, 0.027 to 0.034) at 4 h, which is consistent with the observation that iron bound to transferrin mediates killing or prevents *Brucella* replication inside macrophages (33). Transferrin receptors are synthesized in the host cell endoplasmic reticulum and transported to the host cell surface, where they bind to transferrin and the receptor-ligand complexes are internalized in early endosomes (15). Acidification of the early endosomes to pH 6.5 to 6.0 causes release of iron from transferrin. Our study also showed that there was fourfold up-regulation of natural resistance-associated macrophage protein 2 (Nramp2) ($P = 0.0122 < 0.05$), which functionally couples with transferrin receptors to mediate pH-dependent iron uptake across the endosomal mem-

TABLE 2. Significantly regulated apoptosis-related genes at 4 h postinfection^a

Gene	Fold change	P value	Description
Apoptosis regulators			
Irak2	2.07	0.0381	<i>Mus musculus</i> adult male olfactory brain cDNA, B cell
Bcl10	2.67	0.0394	Leukemia/lymphoma 10
AW260063	3.82	0.0389	<i>Mus musculus</i> transcribed sequences
Bcl2	0.30	0.0231	B-cell leukemia/lymphoma 2
Bat3	0.42	0.0426	HLA-B-associated transcript 3
Bak1	0.42	0.0424	BCL2 antagonist/killer 1
Timm50	0.50	0.027	Translocase of inner mitochondrial membrane 50 homolog (yeast)
Hip1	0.53	0.0284	Huntingtin interacting protein 1
Bad	0.55	0.0301	Bcl-associated death promoter
Bcl2l2	0.56	0.0427	Bcl2-like 2
Taf10	0.58	0.0476	TAF10 RNA polymerase II, TATA box binding protein-associated factor
Bag1	0.60	0.00533	Bcl2-associated athanogene 1
Bcl2l	0.65	0.0416	Bcl2-like
Pmaip1	0.67	0.0479	Phorbol-12-myristate-13-acetate-induced protein 1
Casp2	0.67	0.0279	Caspase 2
Apoptosis inhibitors			
Mkl1	0.59	0.0285	RIKEN cDNA 1810012I01 gene
Birc1b	0.70	0.0474	Baculoviral IAP repeat-containing 1b
Apoptosis related			
TNF	9.90	0.0392	Tumor necrosis factor alpha
Tnfrsf5	2.88	0.0267	Tumor necrosis factor receptor superfamily, member 5
Myc	3.84	0.0271	Myelocytomatosis oncogene
Tnfrsf6	6.79	0.0267	Tumor necrosis factor receptor superfamily, member 6
Ddit3	0.18	0.0267	DNA damage-inducible transcript 3
Ifld2	0.21	0.042	Induced in fatty liver dystrophy 2
Ifld2	0.29	0.0352	Induced in fatty liver dystrophy 2
Siva ^b	0.29	0.0394	Cd27 binding protein
Siva	0.40	0.0408	Cd27 binding protein
Ngfrap1	0.53	0.031	Nerve growth factor receptor (TNFRSF16)-associated protein 1
Casp8ap2	0.53	0.0309	Caspase 8-associated protein 2
Trp53inp1	0.55	0.0433	Transformation-related protein 53-inducible nuclear protein 1
Tia1	0.58	0.031	Cytotoxic granule-associated RNA binding protein 1
Gsk3b	0.60	0.0394	Glycogen synthase kinase 3 beta
Pdcd8	0.62	0.0283	Programmed cell death 8
Bbc3	0.62	0.0231	Bcl-2 binding component 3
Elmo2	0.65	0.0271	Engulfment and cell motility 2, ced-12 homolog (<i>Caenorhabditis elegans</i>)
Cfdp	0.68	0.0469	Craniofacial development protein 1
Pdcd5	0.74	0.0393	Programmed cell death 5

^a The Ifld2 and Siva genes contain two probe sets each. The 36 probe sets (34 genes) are grouped into apoptosis regulators, apoptosis inhibitors, and apoptosis-related genes according to GO assignment. Both pro- and antiapoptosis effects are found. Supplemental Table 3 at <http://sitemaker.umich.edu/helab> contains signal data and gene information for all the 36 probe sets at different times. No apoptosis is explained in the text.

^b Siva is the Hindu God of destruction.

brane (62). Iron augments macrophage-mediated killing of virulent *Brucella* alone and in conjunction with gamma interferon (33). Since lysosomal activity is not enhanced and virulent *Brucella* prevents phagosome-lysosome fusion, the transferrin-containing endosomes and early phagosomes might have played an important role in the initial killing of *Brucella*, which is promoted by the iron secreted from transferrin in the acid endosome environment.

(i) Time-dependent apoptosis modulation. Thirty-six apoptosis-related probe sets (34 genes) were significantly up- or down-regulated at the early infection stage (Table 2). Both antiapoptosis and proapoptosis effects correlated with these gene expression profiles. The proapoptosis gene expression profiles included the profiles of two down-regulated apoptosis inhibitors (Mkl1 and Birc1b) and seven up-regulated apoptosis regulators or apoptosis-related genes (Bcl10, Irak2, AW260063, TNF- α , Tnfrsf5, Tnfrsf6, and Myc) (Table 2). The antiapoptosis effect was exhibited by 25 down-regulated proapoptosis or apop-

osis-related genes (for example, Bad, Bat3, Bak1, Hip1, and Pmaip1) (Table 2). Tnfrsf5 (Cd40) and Tnfrsf6 (Fas) are two members of the TNF receptor superfamily. The up-regulated TNF- α transcription might lead to apoptosis by the TNF signaling pathway (5). This pathway was not transcriptionally active, probably due to the down-regulated caspase 2 expression that is required for the apoptosis cascade (Table 2). Caspase 8-associated protein 2 expression was also down-regulated. Activation of the caspase cascades is required for apoptosis, but none of the caspase genes were up-regulated. Activation of NF- κ B protects cells from TNF-induced apoptosis (5). The Nfkb1 gene encoding NF- κ B subunit 1 was up-regulated 2.1-fold ($P = 0.049$) at 4 h postinfection. The Fas-mediated apoptosis signal pathway is another important death receptor of the apoptosis pathway (5). Although the Fas gene (Tnfrsf6) was up-regulated at 4 h postinfection, the Fas ligand gene (FasL or Tnfrsf6) was not expressed at that time. Ligation of Fas by FasL promotes the recruitment of the cytosolic adap-

tor protein Fas-associated death domain protein. The gene encoding the Fas-associated death domain protein was also down-regulated at 4 h. Caspase 8 and caspase 3 are also required for this pathway, but their corresponding genes were not up-regulated either. Although both pro- and antiapoptosis effects were observed, virulent *Brucella* strain 16M appeared to inhibit apoptosis by down-regulating or preventing activation of caspase apoptosis cascades.

(ii) Down-regulation of mitochondrion-associated gene expression at 4 h postinfection. Our data showed that 106 of 1,525 (7.0%) mitochondrion-associated probe sets (90 genes) found in the GO system were down-regulated and only 4 (0.26%) mitochondrion-associated probe sets (4 genes) were up-regulated at the early infection stage (Table 3). Approximately 22-fold more down-regulated mitochondrion-associated genes than up-regulated mitochondrion-associated genes were found. This change is much larger than the approximately fivefold difference between the total numbers of down-regulated genes (1,053 genes) and up-regulated genes (243 genes) at 4 h postinfection. Further Onto-Express analysis also indicated that there was significantly down-regulated mitochondrion-associated gene expression ($P < 1E-6$) (see Fig. 2 in the supplemental material at <http://sitemaker.umich.edu/helab>).

Many mitochondrion-associated genes important in different stages of protein synthesis and import were down-regulated (Table 3). For example, 10 mitochondrial ribosomal proteins (e.g., Lactb, Mrpl1, and Mrps24) were down-regulated, and none were up-regulated. Down-regulation of three heat shock proteins (Hspe1, Hspa9a, and Hsp75) suggested that there was inhibition of mitochondrial protein import in response to *Brucella* infection. Translocator of inner mitochondrial membrane 44 (Timm44) recruits mitochondrial Hsp70 to drive protein translocation into the mitochondrion (63). Down-regulation of Timm44 at the early infection stage might slow protein import from the cytoplasm to the mitochondrion. Three mitochondrial translation factors (Tsfm, Tufm, and Mto1) were also down-regulated. Down-regulation of mitochondrial protein synthesis and import suggested that there was suppressed mitochondrial activity in response to *Brucella* at the early infection stage. Three genes involved in the tricarboxylic acid cycle (Aco2, Idh3b, and Cs) were also down-regulated, suggesting that there was suppressed energy production.

Mitochondria have been viewed as the central executor of programmed cell death (46). The down-regulation of specific mitochondrion-associated genes might be another reason for inhibition of apoptosis. Mitochondria also control the activation of the cell death machinery by sequestering caspase activators, such as cytochrome *c* or apoptosis-inducing factor, in the intermembrane space (46). Mitochondria undergoing permeability transitions liberate the apoptosis-inducing factor and induce nuclear apoptosis (46). This important proapoptosis factor was slightly down-regulated at 4 h and was unchanged at 24 h and 48 h postinfection (data not shown). Mitochondria are actively involved in the cytochrome *c*-mediated caspase activation apoptosis pathway (5, 34, 52). When cells are damaged or stressed, mitochondria release the electron carrier protein cytochrome *c* into the cytosol. Released cytochrome *c* induces the key mediator Apaf-1 to form the apoptosome that activates caspase 9 and the downstream executioner caspases. Several cytochrome *c*-related macrophage genes were down-

regulated, including the Uqcrc1, Uqcrc2, Uqcr11, and Cyp27a1 genes (Table 3). Expression of the cytochrome *c* gene was not significantly altered during the whole infection process. These data suggest that additional cytochrome *c* protein is not produced to trigger apoptosis in *Brucella*-infected macrophages. Several genes involved in release of cytochrome *c* from mitochondria were down-regulated at 4 h postinfection, including Pmaip1 (or Noxa) (1.5-fold decrease; $P = 0.048$), Bcl2 (3.3-fold decrease; $P = 0.023$), and Timm50 (2.0-fold decrease; $P = 0.027$). Bak1 induces caspase activation via released cytochrome *c* (16). However, our analysis showed that there was a 2.4-fold ($P = 0.043$) decrease in Bak1 transcription at 4 h postinfection. These observations are consistent with the prevention of release of cytochrome *c* from mitochondria. At 24 and 48 h postinfection, the transcriptional activity of the macrophage cell returned to normal, in spite of a significant increase in the number of *Brucella* cells (Fig. 1).

Reactive oxygen species (ROS), such as superoxide anions, hydrogen, organic peroxides, and free radicals, are generated by all aerobic cells as by-products of metabolic reactions and in response to various stimuli (46). The mitochondrial ROS also serve as the signaling messengers during the early induction phase of apoptosis. Mitochondria are a major site of ROS production; for example, superoxide radical is produced by transfer of a single electron to molecular oxygen at the level of the respiratory chain (46). The electron-transferring flavoprotein transfers the electrons to the main mitochondrial respiratory chain via electron-transferring flavoprotein-ubiquinone oxidoreductase (9). Down-regulation of Etfb suppressed the electron transfer to start the process. Electron transfer chain complexes I and III generate the majority of ROS inside mitochondria (13). While NADH is the starting point of the electron transfer chain, 10 subunits (e.g., Ndufa2, Ndufs7, and Ndufv2) of the NADH dehydrogenase of complex I were down-regulated (Table 3). Three of 11 complex III genes (Uqcrc1, Uqcrc2, and Uqcr11) were also down-regulated. Uqcrc2 encodes a core protein required for complex III assembly. The ROS production was likely to be decreased due to the many down-regulated complex I and III genes. One complex V gene, the Atp5o gene, was also down-regulated (Table 3). It is possible that *Brucella* infection triggers the down-regulation of the mitochondrial stress pathway that leads to decreased ROS production and inhibition of the early signal transduction of apoptosis. Ferredoxin reductase transfers electrons from NADPH to cytochrome P450 in mitochondria and sensitizes cells to ROS-mediated apoptosis (42). Down-regulation of the Fdxr gene is consistent with the decreased ROS production and sensitivity to apoptosis. The down-regulation of the antioxidants Gpx4 and Prdx3 observed at the early infection stage might have been due to insufficient ROS production.

It was also found that transport of small molecules between the mitochondrion and the cytoplasm was strongly affected, as shown by the results for nine down-regulated small-molecule transport genes (Table 3). One example is the voltage-dependent anion channel protein, which is the primary transporter of ions and metabolites across the outer mitochondrial membranes (58). The increase in the outer mitochondrial membrane permeability is a central event in apoptotic cell death because it releases several apoptogenic factors, such as cyto-

TABLE 3. Selected mitochondrion-associated genes significantly regulated at 4 h postinfection^a

Gene	Fold change	P value	Description
Down-regulated mitochondrion genes			
Protein synthesis and import			
Mrpl1	0.58	0.0321	Mitochondrial ribosomal protein L1
Mrpl4	0.57	0.0267	Mitochondrial ribosomal protein L4
Mrpl19	0.61	0.031	Mitochondrial ribosomal protein L19
Mrpl19	0.52	0.048	Mitochondrial ribosomal protein L19
Mrpl22	0.74	0.048	Mitochondrial ribosomal protein L22
Mrpl41	0.48	0.0321	Mitochondrial ribosomal protein L41
Mrps9	0.48	0.043	Mitochondrial ribosomal protein S9
Mrps15	0.5	0.0428	Mitochondrial ribosomal protein S15
Mrps24	0.6	0.0469	Mitochondrial ribosomal protein S24
Mrpl53	0.59	0.0275	Mitochondrial ribosomal protein L53
Lactb	0.7	0.031	Beta-lactamase, mitochondrial ribosomal protein L56
Mto1	0.45	0.0128	Mitochondrial translation optimization 1 homolog (<i>Saccharomyces cerevisiae</i>)
Tsfm	0.52	0.0464	Ts translation elongation factor, mitochondrial
Tufm	0.62	0.42	Tu translation elongation factor, mitochondrial
Timm44	0.74	0.0488	Translocator of inner mitochondrial membrane 44
Gfm	0.61	0.0386	G elongation factor
Hspe1	0.53	0.0483	Heat shock protein 1 (chaperonin 10)
Hspa9a	0.65	0.0438	Heat shock protein A
Hsp75	0.57	0.027	Heat shock protein 75, chaperone
Electron transfer chain			
Ndufa2	0.73	0.415	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2
Ndufa4	0.72	0.0292	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4
Ndufb5	0.71	0.0423	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5
Ndufb8	0.66	0.0354	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8
Ndufc1	0.60	0.048	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1
Ndufc1	0.55	0.0384	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1
Ndufs5	0.64	0.0426	NADH dehydrogenase (ubiquinone) Fe-S protein 5
Ndufs7	0.65	0.031	NADH dehydrogenase (ubiquinone) Fe-S protein 7
Ndufs7	0.55	0.0391	NADH dehydrogenase (ubiquinone) Fe-S protein 7
Ndufv2	0.57	0.0383	NADH dehydrogenase (ubiquinone) flavoprotein 2
Uqerc1	0.72	0.0393	Ubiquinol-cytochrome <i>c</i> reductase core protein 1
Uqerc2	0.67	0.0479	Ubiquinol-cytochrome <i>c</i> reductase core protein 2
Uqcr11	0.63	0.0394	Ubiquinol-cytochrome <i>c</i> reductase (6.4-kDa) subunit 11
Atp5o	0.57	0.042	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O unit
Fdxr	0.61	0.0496	Ferredoxin reductase
Etfb	0.72	0.0487	Electron transferring flavoprotein, beta polypeptide
Txn2	0.62	0.0267	Thioredoxin 2
TCA cycle			
Aco2	0.72	0.031	Aconitase 2, mitochondrial
Idh3b	0.58	0.0394	Isocitrate dehydrogenase 3 (NAD ⁺) beta
Cs	0.63	0.0338	Citrate synthase
Cs	0.54	0.027	Citrate synthase
Pdhb	0.68	0.0321	Pyruvate dehydrogenase (lipoamide) beta
Antioxidant			
Gpx4	0.5	0.0384	Glutathione peroxidase 4
Prdx3	0.65	0.0476	Peroxiredoxin 3
Small-molecule transport			
C330005L02Rik	0.67	0.0267	Belongs to the mitochondrial carrier family
3010027G13Rik	0.5	0.0423	Rap2 interacting protein
3010027G13Rik	0.55	0.0371	Rap2 interacting protein
3010027G13Rik	0.44	0.31	Rap2 interacting protein
Clic4	0.74	0.43	Chloride intracellular channel 4 (mitochondrial)
Sfxn1	0.54	0.0271	Sideroflexin 1
Mscp	0.57	0.0284	Mitochondrial solute carrier protein
Slc25a15	0.67	0.0442	Solute carrier family 25 (mitochondrial ornithine transporter)
Ucp2	0.43	0.0319	Uncoupling protein 2, mitochondrial
Vdac1	0.49	0.0492	Voltage-dependent anion channel 1
Vdac1	0.5	0.0271	Voltage-dependent anion channel 1
Vdac1	0.62	0.0267	Voltage-dependent anion channel 1
Mtch1	0.62	0.0314	Mitochondrial carrier homolog 1 (<i>Caenorhabditis elegans</i>)
Apoptosis			
Bad	0.55	0.0301	Bcl-associated death promoter
Bcl2l	0.65	0.0416	Bcl2-like
Bbc3	0.62	0.0231	Bcl-2 binding component 3
Pdcd8	0.62	0.0283	Programmed cell death 8
Pmaip1	0.67	0.0479	Phorbol-12-myristate-13-acetate-induced protein 1
Up-regulated mitochondrion genes			
Immt	2.4	0.0479	Inner membrane protein, mitochondrial
Cbr2	7.26	0.0321	Carbonyl reductase 2
Aqp9	2.28	0.0285	Aquaporin 9
Acat2	2.1	0.0285	Acetyl-coenzyme A acetyltransferase 2

^a Altogether, 90 genes (106 probe sets) were down-regulated and only 4 genes were up-regulated. Supplemental Table 4 at <http://sitemaker.umich.edu/helab> contains signal data and gene information for all the 110 probe sets at different times. Many genes, including Cs, 3010027G13Rik, Vdac1, Mrpl9, Ndufb1, and Ndufs7, contain more than one probe set.

FIG. 3. Expression profiles of 12 significantly regulated genes between 0 and 24 h postinfection. The 12 probe sets (12 genes) include 5 down-regulated genes and 7 up-regulated genes. There were also three genes in the 1,426 probe sets found at 4 h postinfection: Cd28, BC031407, and Gsn. The color bar indicates the level of expression, which was determined by log transformation of the normalized gene expression signal intensity. Different colors at different times for a gene indicate the time course of the gene expression pattern for that gene. The figure was created by using the microarray data analysis package of the ToolBus/PathPort web service federation, which is comparable and in agreement with the figure made by GeneSpring (see supplemental Fig. 1 at <http://sitemaker.umich.edu/helab>).

chrome *c*, into the cytoplasm, which activate the downstream apoptosis pathway. Voltage-dependent anion channel protein is an important component of the mitochondrial permeability transition pore complex and plays an essential role in the increase in mitochondrial membrane permeability (58). Down-regulation of Vdac1 is consistent with inhibition of apoptosis by virulent *Brucella* infection.

Gene expression profiles at 24 h and 48 h postinfection. Only 12 probe sets for 12 genes were found to have significant transcriptional differences at 24 h postinfection (Fig. 3). No macrophage gene transcription was found to be significantly different at 0 and 48 h postinfection. Of the 12 genes differentially regulated at 24 h postinfection, 7 (H2-D1, Cd28, Cd72, Ly6a, Rbm22, Tapbp, and Gsn) were up-regulated and 5 (BC031407, Tuba4, Gpcr25, Jag1, and 5730592L21Rik) were down-regulated. Cd28 and Gsn were also up-regulated and BC031407 was also down-regulated at 4 h postinfection (Fig. 3). It is noteworthy that the Gsn gene encoding gelsolin was induced; gelsolin is an actin-severing protein that modulates actin assembly and disassembly and regulates cell motility in vivo through modulation of the actin network. Regulation of the cellular machinery that controls actin cytoskeleton assembly is considered an important virulence mechanism in many intracellular bacteria, such as *Listeria*, *Shigella*, and *Salmonella* (41). Gelsolin was also reported to strongly inhibit apoptosis induced by anti-Fas antibody, C2-ceramide, or dexamethasone (50). CD28 is considered a costimulatory signal on T cells that binds to the B7 ligand on antigen-presenting cells. Transcription of the B7 gene (Cd52; Affymetrix no. 1460218_at) in macrophages was increased 1.53-fold ($P > 0.05$) at 4 h postin-

fection. The CD5 ligand CD72 is another costimulatory molecule on macrophages (2). The costimulatory signals are necessary to activate naïve T cells. The H2-D antigen is an important major histocompatibility complex class I molecule and is associated with a potent cytotoxic T-lymphocyte response (6). Ly6a encodes lymphocyte activation protein 6A, also known as stem cell antigen 1 (Sca-1). Lymphocyte activation protein 6A regulates the developmental program of hematopoietic stem cells and specific progenitor populations (31). Rbm22 encodes RNA binding motif protein 22. Among the five down-regulated genes, Tuba4 encodes α -tubulin 4, which is important for microtubule polymerization and microtubule-based movement. Gpcr25 (or Gpr65) encodes G-protein-coupled receptor 65 that binds sphingosine-1-phosphate (21). Tapbp encodes tapasin, an immunoglobulin superfamily molecule that links major histocompatibility complex class I molecules to a transporter associated with antigen processing (TAP) in the endoplasmic reticulum (60). The Jagged1 protein encoded by Jag1 is a receptor of the Notch proteins that are indispensable for T-cell development (65).

The microarray data analysis package of the ToolBus/PathPort web service federation (22) was used with the same algorithms and parameter settings as GeneSpring for our microarray data analysis. Under these conditions, both programs generated the same lists of up- and down-regulated genes in our study, as expected. Our microarray data are available from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE5202. Supplemental material, including the data files for the Affymetrix arrays analyzed, can be accessed at the website <http://sitemaker.umich.edu/helab>.

TABLE 4. Verification of microarray data by real-time RT-PCR^a

Gene (Affymetrix no.)	Microarray data			Real-time RT-PCR	
	0 h	4 h	4 h/0 h ratio	Left primer/right primer	4 h/0 h ratio
Jak3 (1425750_a_at)	160.9	135.6	0.75	CCGGAGTCCCTATCTGACAA/CTGCAGCTCTTGTGCGCAGT	0.64
Pdcd8 (1418127_a_at)	1,199	738	0.64	TCGGAAATCACAATTCCTCC/ACGTTCCATAGCACAAATCCC	0.79
Ccl4 (1421578_at)	2,108	2,711	1.40	CCTGACCAAAAGAGGCAGAC/GAGGAGGCCTCTCCTGAAGT	6.50
Biklk (1437148_at)	11,112	11,728	1.07	CCGCTAACTCTTGGAAGT/ACCCAAATGGAGAATCCCTC	1.05
Clk (1426124_a_at)	1,348	1,168	0.88	AGAAAAACCAGGAAACGCAGA/CTTCAGAGGTTTACAGCGCC	1.07
B2m (1449289_a_at)	10,481	13,574	1.34	GAGCCCAAGACCGTCTACTG/TGCTATTTCTTCTGCGTGC	1.02
Cpd (1418018_at)	402.2	1,208	2.25	TGCATCTGCTCCATCAAGTC/TCCGTTTCATCCTGGAAGTC	2.05
Lcn2 (1427747_a_at)	262	770	0.34	AGCTACAATGTGCAAGTGGC/ACAGCTCCTTGTTCTTCCA	0.52
Lsp1 (1417756_a_at)	4,972	5,416	1.12	AAATTGCTTCCTGTTGCCAG/TTGGTCAAAGGATGAGAGGG	0.85
Saa3 (1450826_a_at)	108.4	205.8	1.26	ACATGTGGCGAGCCTACTCT/GCTGATGACTTTAGCAGCCC	1.26
Mt2 (1428942_at)	2,883	2,013	0.81	CCGATCTCTCGTCGATCTTC/AGGAGCAGCAGCTTTTCTTG	1.02
Tes (1460378_a_at)	1,430	2,018	1.42	AAAACCGAGAATTGGCACCTG/GACACACCAACGATGGTTC	1.35
Anxa2 (1419091_a_at)	4,743	8,101	1.58	GACATCTGGAGAGCATCAA/ACCTTGCTCTCGAGTCCCCTT	1.18
β-Actin 5 (M12481_5_at)	14,999	17,272	1.22	TGTTACCAACTGGGACGACA/CTGGGTCATCTTTTCACGGT	1.01

^a The same RNA samples used for microarray experiments were used for real-time RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase 5' was used as the internal control for real-time RT-PCR ratio calculations. The transcriptional signal ratios for 4 h and 0 h (uninfected) after infection are generally in agreement with each other.

Confirmation of microarray data. Real-time RT-PCR, which has been found to be useful for validation of microarray data (53), was performed to confirm changes in expression of selected genes identified from our microarray data in a manner similar to manner used by Livak et al. (43). The glyceraldehyde-3-phosphate dehydrogenase 5' RT-PCR product was used as the internal control, and its expression remained unchanged in all the samples tested. Table 4 shows the comparative expression results for 14 genes for 0 and 4 h postinfection. In general, the genes that were differentially expressed in the microarray were found to be in good agreement with the real-time RT-PCR results. Similar comparative results were observed when other times were studied. An independent real-time RT-PCR experiment using RNA samples from uninfected J774.A1 macrophages at different times indicated that these 14 genes generally maintained their gene expression profiles (data not shown), suggesting that cell growth and density did not significantly influence the gene expression profiles in uninfected J774.A1 macrophages during the 48 h examined.

DISCUSSION

Our microarray study was the first study to show that most macrophage gene expression changes occur at the early infection stage and that the global gene expression profiles return to normal between 24 and 48 h postinfection. These findings can be used to support the observation that macrophages kill the majority of *Brucella* cells at the early infection stage, but the surviving *Brucella* cells are able to avoid macrophage brucellacidal activity at the later infection stage. Our findings can be explained by the fact that the surviving *Brucella* cells mask themselves from their host by replicating in membrane-bound replicative phagosomes (37, 56). Immediately after entry into the macrophages *Brucella* cells reside in an acidified membrane-bound compartment. Later, *Brucella* cells gain access to their growth niche, known as the replicative phagosome, after continual interactions between the *Brucella*-containing compartment and the endoplasmic reticulum of macrophages (56). Celli et al. (14) indicated that the majority of infected *Brucella*

cells were in the early phagosome at 4 h postinfection and then most of the *Brucella*-containing vacuoles matured into either degradative or replicative compartments at 24 h postinfection. The replicative phagosomes do not fuse with lysosomes and provide a more hospitable environment for intracellular survival than the early stage acidified compartment provides. Gorvel and Moreno (27) reported that crucial macrophage cell cycle processes, such as chromosome condensation of DNA synthesis, mitosis, karyokinesis, and cytokinesis, were not inhibited while a large number of *Brucella* cells lived in the replicating niche. On the other hand, intracellular *Brucella* replication does not require de novo host protein synthesis as treatment of Vero cells with cycloheximide does not inhibit *Brucella* replication (18). This suggests that the significant *Brucella* growth (24 to 48 h) inside the replicative phagosomes does not interfere with the important host physiological pathways.

Interestingly, the replicative phagosome is nutrient poor and microaerobic, and *Brucella* undergoes significant nutrient deprivation (37, 56). Genetic screens using transposon mutants have identified numerous *Brucella* genes required for survival and replication in macrophages and for successful chronic infection. Many of the mutated *Brucella* genes are the genes required for cellular metabolism in the replicative phagosome, including genes encoding enzymes involved in purine, pyrimidine, and amino acid biosynthesis pathways (36). It is likely that the replicative phagosomes take up minimal carbohydrates and other metabolites necessary for *Brucella* to further synthesize precursors needed for growth (e.g., amino acids). However, the brucellae inside replicative phagosomes probably use their own enzymes for necessary cell growth and metabolism. The self-supply mechanism inside replicative phagosomes gives *Brucella* an advantage that minimizes the interaction with the host environment, and therefore, a minimal host response is stimulated. It is likely that regulated gene expression in *Brucella* is responsible for this adaptation to the replicative niche and that *Brucella* replication is dependent on the generation of signal molecules expressed in vivo by the pathogen. Our next step is to use a *Brucella* microarray to analyze the time course of *Brucella* gene expression profiles inside macrophages.

Both TLR2 and TNF- α were significantly up-regulated. TLR2 recognizes pathogen-associated immunostimulants, such as bacterial lipoproteins, peptidoglycan, and certain types of LPS, and triggers innate immunity against *Brucella* infection (11). Activation of TLR2 also triggers an inflammatory response and TNF- α production when the cells are stimulated with heat-killed *B. abortus* (26). TNF- α plays a critical role in activation of first-line defenses of a host against foreign organisms. TNF- α is involved in controlling the number of *Brucella* cells in BALB/c mice, as shown by neutralization of TNF- α with monoclonal antibodies, which results in a significant increase in the number of splenic CFU recovered at 3 weeks postinfection (49). Campos et al. (11) reported that TLR2 knockout mice controlled *Brucella* infection as efficiently as wild-type mice controlled *Brucella* infection at weeks 1, 3, and 6 after infection. On the other hand, Huang et al. (30) found that the TNF- α response in TLR2-deficient mice was significantly delayed and reduced, and the early TNF- α response to *Brucella* was dependent on TLR2, while the delayed response was TLR2 independent but MyD88 dependent. These observations suggest that *Brucella* may use several pathways triggering proinflammatory responses. It is likely that activation of TLR2 at the early infection stage can trigger TNF- α production and might contribute to the clearance mechanism responsible for the early dramatic drop in viability. While the gene expression profiles at 4 h postinfection cannot provide conclusive evidence about why the majority of infecting *Brucella* cells were killed during the early infection stage, our results suggested that the most active brucellacidal activity of macrophages might have occurred in the first 4 h after infection. Analysis of the gene expression profiles at the earlier infection stage may provide more insight into an effective brucellacidal mechanism in macrophages.

It is possible that the rate of infection of macrophages with *B. melitensis* strain 16M at the late infection stage is low. Our analysis with Giemsa staining (32) showed that all the macrophages contained *Brucella* at 4 and 24 h postinfection and 84% of macrophages contained *Brucella* at 48 h after infection with *B. melitensis* strain 16M (see supplemental Table 5 at <http://sitemaker.umich.edu/helab>). The data suggested that all the macrophages were infected with *B. melitensis* at the early infection stage and that the majority of macrophages still contained live or undigested dead *Brucella* at the late infection stage. Therefore, the gene expression profiles observed in this study mostly reflected the reality of *B. melitensis*-infected macrophages at these different times.

Our microarray results indicate for the first time that there is a close relationship between mitochondrial activity, apoptosis, and *Brucella* survival inside macrophages. Previous studies indicated that virulent *B. abortus* (51), *B. suis* (19, 28), and *B. melitensis* (25) inhibit apoptosis in macrophages. Our LDH release assay demonstrated that *B. melitensis* strain 16M inhibits apoptosis in the macrophage-like J774.A1 cell line (Fig. 1). In our study we systematically analyzed the gene expression profiles of all the apoptosis-related murine genes in the course of *Brucella* infection. Our data showed that there was a mixture of proapoptosis and antiapoptosis effects. Both extrinsic and intrinsic apoptosis pathways are executed by caspases (5). Although several up-regulated genes favor certain apoptosis pathways (e.g., FasL signaling and TNF-mediated cell signaling

pathways), none of the genes in the caspase cascades was up-regulated, and transcription of Casp2 and Casp8ap2 was indeed down-regulated at the early infection stage. This observation suggested that some step upstream of caspase activation (for example, cytochrome *c* release from the mitochondria) was blocked. Since mitochondria are critical for execution of the intrinsic cell death pathway, we systemically analyzed mitochondrion-associated gene expression profiles. Our results showed that at the early infection stage virulent *B. melitensis* significantly suppressed mitochondrial activity, as reflected by the down-regulated genes involved in mitochondrial protein synthesis and import, the tricarboxylic acid cycle, the electron transfer chain, and many other important mitochondrial functions (Table 3). The down-regulation of Bcl2, Pmaip1, Timm50, Bak1, and other genes suggests that cytochrome *c* was not released, consistent with our observation that the caspase activation pathway was not initiated during the course of *Brucella* infection. Our data also suggested that ROS production was suppressed and was not available to send signals to initiate apoptosis pathways. The hypothesis that ROS production was suppressed also supports the report that virulent *Brucella* avoids inducing an oxidative burst when it invades its host cell (38). The down-regulation of Vdac1 suggests that the mitochondrial permeability transition pore is not formed, consistent with blocked cytochrome *c* release. In summary, although there were both pro- and antiapoptosis effects, *B. melitensis* 16M appears to prevent apoptosis in macrophages by suppressing mitochondrial gene expression involved in cytochrome *c* release, ROS production, and mitochondrial membrane permeability and thereby preventing activation of caspase cascades. Prevention of apoptosis in macrophages by *B. melitensis* strain 16M ensures extensive *Brucella* replication after the initial killing stage.

The theory of the endosymbiotic origin of mitochondria is generally accepted (39, 46, 54). Phylogenetic analyses indicate that mitochondria originated from the α -proteobacteria (3). *Brucella* also belongs to this class, and the similarities between *Brucella* and mitochondria have been documented (54). For example, in contrast to most gram-negative bacteria, in which phosphatidylethanolamine is the major phospholipid component of the membrane, in *Brucella* phosphatidylcholine and cardiolipin are major components (54). In this respect, *Brucella* possess the lipid pattern of *Agrobacterium* and mitochondria. An interesting possibility is that similar to mitochondria, *Brucella* contains homologous genes for endosymbiosis to facilitate intracellular survival. *Brucella* may also mimic mitochondrial features inside macrophages and thus avoid extensive attack from the host. Indeed, α -proteobacteria encode abundant and diverse homologs of several key enzymes of the apoptotic machinery, including paracaspases, metacaspases, apoptotic ATPases, and mitochondrial HtrA-like proteases (39).

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