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Modulation of the Bovine Trophoblastic Innate Immune Response by *Brucella abortus*[▽]

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Brucellosis is still a widespread zoonotic disease. Very little is known about the interaction between *Brucella abortus* and trophoblastic cells, which is essential for better understanding the pathogenesis of the *Brucella*-induced placentitis and abortion, a key event for transmission of the disease. The goal of this study was to evaluate the profile of gene expression by bovine trophoblastic cells during infection with *B. abortus*. Explants of chorioallantoic membranes were inoculated with *B. abortus* strain 2308. Microarray analysis was performed at 4 h after infection, and expression of cytokines and chemokines by trophoblastic cells was assessed by real-time reverse transcription-PCR at 6 and 12 h after inoculation. In addition, cytokine and chemokine expression in placentomes from experimentally infected cows was evaluated. Expression of proinflammatory genes by trophoblastic cells was suppressed at 4 h after inoculation, whereas a significant upregulation of CXC chemokines, namely, CXCL6 (GCP-2) and CXCL8 (interleukin 8), was observed at 12 but not at 6 h after inoculation. Placentomes of experimentally infected cows had a similar profile of chemokine expression, with upregulation of CXCL6 and CXCL8. Our data indicate that *B. abortus* modulates the innate immune response by trophoblastic cells, suppressing the expression of proinflammatory mediators during the early stages of infection that is followed by a delayed and mild expression of proinflammatory chemokines, which is similar to the profile of chemokine expression in the placentomes of experimentally infected cows. This trophoblastic response is likely to contribute to the pathogenesis of *B. abortus*-induced placentitis.

Brucella abortus is a facultative intracellular gram-negative bacterium that causes abortion and temporary infertility in cattle (13, 19, 29). It is also a zoonotic pathogen causing fever, weakness, endocarditis, arthritis, osteomyelitis, and meningitis in humans (51). In cattle, the infection tends to be chronic with tropism for the reproductive system of pregnant cows. Abortion is the most significant clinical sign, but an infected cow may be completely asymptomatic. Transmission of the disease occurs mainly after abortion or parturition of infected cows via contaminated fetus, fetal membranes, and uterine secretions (8, 37, 38). During the early stages of infection, *B. abortus* is found mostly in lymph nodes. The infection may progress to bacteremia and colonization of the uterus, where the organism replicates preferentially within trophoblasts in the rough endoplasmic reticulum (9, 10, 29). As a result, the cow develops placentitis, fetal death, and abortion, particularly during the last third of the gestation (2). *B. abortus* grows primarily in the extracotyledonary trophoblasts and then spreads to the cotyledonary (placental) trophoblasts (3). Therefore, proliferation of *B. abortus* within trophoblastic cells is a key event in the mechanism of abortion. Trophoblasts favor bacterial growth by producing erythritol and progesterone, which stimulate in vitro growth of *B. abortus* (15, 42). In acute infection of pregnant cows, more than 85% of the bacteria are located in the placenta and allantoic fluid (43). In spite of the importance of intracellular multiplication of *B. abortus* within trophoblastic cells and consequent placentitis, which are key events in the pathogenesis of *B. abortus*-induced abortion, information regarding the interaction between *B. abortus* and bovine trophoblast is extremely scarce.

Innate immunity against *B. abortus* involves a system of pattern recognition receptors which recognizes conserved sequences known as pathogen-associated molecular patterns such as *Brucella* lipopolysaccharide (LPS), which is recognized by Toll-like receptor 4 (TLR4). TLRs signal through an adapter molecule, MyD88, to activate the NF-κB pathway, resulting in cytokine induction and regulation of costimulatory molecules (30, 44). However, *Brucella* LPS is not a strong agonist of TLR4, which favors evasion of the host innate immunity (4, 11, 18, 26, 45). Conversely, *Brucella* lipoproteins, which are TLR2 ligands, have a proinflammatory effect (17). In spite of the knowledge accumulated over the last few years on the interaction of the host with *Brucella*, little is known about the interaction of this organism with trophoblastic cells, par-

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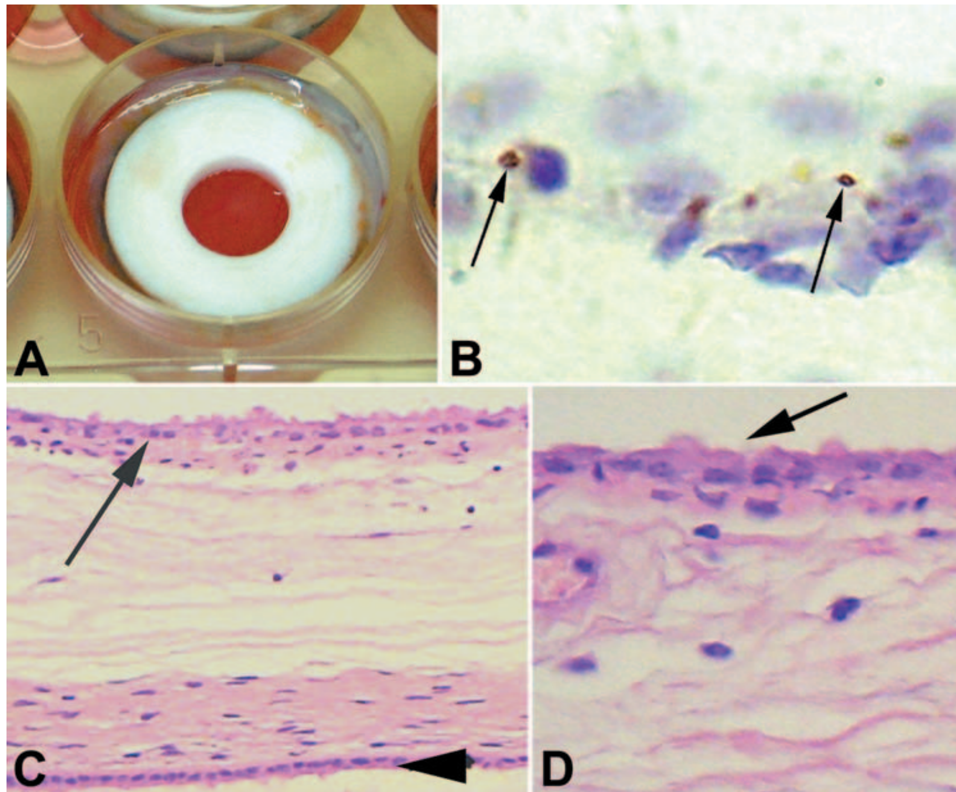

FIG. 1. Infection of cultured bovine CAM explants with *Brucella abortus*. (A) CAM explants in a six-well plate with the trophoblastic side toward the well in the center of the ring. (B) Immunohistochemical localization of *B. abortus* (arrows) within trophoblastic cells in a cultured CAM explant. (C and D) CAM cultured for 48 h. Trophoblastic cells are indicated by arrows, and the amnion is indicated by the arrowhead. H&E staining.

ticularly in regard to the role played by the trophoblastic cells in eliciting the placental inflammatory response. This is a biologically relevant theme, since in contrast to silent chronic infections, infection of placenta (primarily trophoblastic cells) with *Brucella* results in an acute inflammatory response (2, 3, 31). Due to the lack of bovine trophoblastic cell lines, phagocytic cells or poorly differentiated cell lines are often used for studying the pathogenesis of *B. abortus*. However, *Brucella*-elicited proinflammatory trophoblastic response may play a fundamental role in the pathogenesis of placentitis in bovine brucellosis.

Trophoblastic cells have been intensively studied in the context of implantation, maternal recognition of pregnancy, and placental development, including gene expression profiling, which resulted in identification of expression of several genes such as interferon tau (5), matrix metalloproteinases (50), proteoglycans, adhesion molecules, integrins, and growth factors (46–48). However, none of these studies have focused on the response of the trophoblast to intracellular pathogens, particularly *Brucella*. Considering the upregulation of proinflammatory genes in *Brucella*-infected mouse macrophage RAW 264.7 (14) as well as in vivo in the mouse (36), an evaluation of gene expression profile of bovine trophoblasts during infection can contribute significantly to our understanding of the pathogenesis of *B. abortus*-induced abortion.

In this study, we employed a previously developed model of infection of explants of the bovine chorioallantoic membranes (CAM) with *B. abortus* (37, 39) in association with microarray technology and quantitative real-time reverse transcription-PCR (RT-PCR). Therefore, considering our hypothesis that the interaction of *B. abortus* with trophoblasts results in a change in the profile of gene expression with upregulation of proinflammatory genes, the aim of this study was to identify differentially expressed genes in bovine trophoblastic cells during infection with *B. abortus*.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Brucella abortus* strain 2308 was used in all experimental inoculations in this study. Frozen stocks of *B. abortus* strain 2308 were prepared by growing the bacterium on tryptose agar (Difco) for 48 h at 37°C in 5% CO_2. Prior to experimental inoculation, *B. abortus* was grown in 5 ml of *Brucella* broth (Difco) for 24 h at 37°C under agitation (200 rpm).

CAM explant. CAM were obtained at local slaughterhouses from bovine pregnant uteruses with gestational ages ranging from 180 to 240 days as assessed by measuring the fetal crown-rump length as previously described (16). All tissues were obtained from cows serologically negative for brucellosis and were processed according to a previously described method (37, 39) with modifications as detailed below. CAM were aseptically removed from the uterus and immediately placed into RPMI 1640 (Invitrogen; Carlsbad, CA) with 50 U/ml of penicillin and 50 μg/ml of streptomycin (Invitrogen) for 20 min. The CAM were then washed two times with RPMI 1640 to remove antibiotic residues, mounted onto a support (Fig. 1), and placed into six-well cell culture plates with supplemented medium (RPMI 1640 with 10% fetal calf serum and 4 mM L-glutamine) in contact with the trophoblastic and allantoic or amniotic surfaces, which were completely separated from each other (Fig. 1).

CAM experimental infection. CAM were divided into two experimental groups (infected and control). CAM were inoculated on their trophoblastic surface with

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a suspension of 2.0×10^7 CFU of *B. abortus* in supplemented RPMI per well, corresponding to a multiplicity of infection of approximately 1:1,000, considering an average of 20,000 trophoblasts per explant, as determined morphometrically (described below). An equal volume of sterile medium was added to the trophoblastic surface of control CAM. After inoculation, CAM were cultured for 4, 6, or 12 h in RPMI 1640 supplemented with 10% fetal calf serum and 4 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2 followed by washing with the same medium and culture with medium containing 50 μg/ml of gentamicin for 1 h to inactivate extracellular bacteria. Additional CAM inoculated in parallel under the same conditions were processed for CFU counting as described below. At different time points after inoculation, the media were removed and RNA extraction was performed by adding Trizol (Invitrogen; Carlsbad, CA) directly onto the trophoblastic surface of the CAM explants.

In vivo experimental infection. The experimental protocol for in vivo infection used in this study has been previously described (31, 34). All heifers were serologically negative for brucellosis before challenge, as assessed by the Rose Bengal plate agglutination test. The heifers underwent estrous synchronization followed by artificial insemination. Pregnancy was confirmed by ultrasonography at 35 days after insemination. Between 6 and 7 months of pregnancy, the heifers were challenged by conjunctival administration of the virulent *B. abortus* strain 2308. Each heifer was inoculated with 50 μl in each eye (total of 100 μl per heifer) with a bacterial suspension containing a total of 3.0×10^7 CFU. The heifers were then kept under observation until abortion or calving. This experimental protocol was approved by the local Committee for Ethical Use of Experimental Animals (CETEA-UFMG, protocol 028/05). The heifers were divided in three groups: (i) heifers with negative bacteriology and term gestation ($n = 6$), (ii) heifers with positive bacteriology and term gestation ($n = 5$), and (iii) heifers with positive bacteriology and abortion ($n = 5$). Samples of placentomes were collected immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Additional samples were fixed and processed for histopathology.

Bacteriological analysis. Following treatment with gentamicin for inactivation of extracellular bacteria (as described above), trophoblastic cells from infected CAM explants were lysed with a solution of 0.1% sterile Triton X-100 (Roche; Mannheim, Germany). The lysate was serially diluted, plated on tryptose agar plates (Difco), and incubated for 48 h at 37°C with 5% CO_2 for CFU counting.

Histopathology and immunohistochemistry. Morphological integrity of CAM was assessed by histological analysis of CAM explants from each placenta. CAM explants were fixed for 24 h in 10% neutral-buffered formalin and further processed for paraffin embedding, sectioning, and staining with hematoxylin and eosin stain (H&E stain). Immunohistochemistry was performed as previously described (40) for confirmation of the intracellular localization of *B. abortus* in trophoblasts. Briefly, sections (4 μm) were deparaffinized, hydrated, and incubated in 4% hydrogen peroxide in phosphate-buffered saline (PBS) (0.01 M, pH 7.2) for 30 min, incubated with skim milk (1:10 dilution) as a blocking solution for 30 min, and then incubated with a polyclonal anti-*B. abortus* antibody (1:100 dilution) as the primary antibody for 30 min in a humidified chamber at room temperature. After being washed in PBS, the slides were incubated with biotinylated secondary antibody for 20 min at room temperature, washed in PBS again, and then incubated with streptavidin-peroxidase complex (LSAB1 kit; DAKO Corporation, Carpinteria, CA) for 20 min at room temperature. The reaction was developed with a 0.024% diaminobenzidine solution (Dako), and sections were counterstained with Mayer's hematoxylin. H&E-stained sections of CAM explants were morphometrically analyzed with a micrometric ocular to estimate the number of trophoblastic cells per explant.

Oligonucleotide microarray analysis. A high-density microarray containing 13,249 70-mer oligonucleotides (NCBI Gene Expression Omnibus number, GPL2853) was used in this study (28). Total RNA used for microarray analysis was obtained from six placentas with 12 infected and 12 control explants from each placenta. At 4 h after inoculation, total RNA was isolated from the trophoblastic surface of CAM by use of Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA was further purified with the RNeasy mini kit (Qiagen, Valencia, CA). RNA samples were analyzed by spectrophotometry and agarose gel electrophoresis. Three RNA pools from two placentas each were generated prior to cDNA synthesis. cDNA from infected and control CAM explants was synthesized and labeled using random hexamers and amino allyl dUTP. cDNAs from infected CAM explants were labeled with Alexa Fluor reactive dye 647 (Molecular Probes/Invitrogen) and cDNAs from control CAM explants were labeled with Alexa Fluor reactive dye 555 (Molecular Probes/Invitrogen). Labeled cDNAs were hybridized with the spotted oligonucleotides for 48 h in a hybridization station (Perkin Elmer HybArray). After being washed, the slides were scanned using the ProScanArray Microarray (Perkin Elmer). The images were analyzed using the GenePix Pro (Molecular Devices) and GeneSpring (Agilent Technologies) software.

Real-time RT-PCR analysis. Quantitative real-time RT-PCR was used for validation of the microarray results and to assess expression of cytokines and chemokines in cultured CAM explants and in placentomes of experimentally infected cows. The same pools of RNA used for the microarray analysis were also processed for validation of the microarray data by RT-PCR. For assessing cytokine and chemokine expression in CAM explants, another five placentas were collected and total RNA was extracted from infected and control CAM explants at 6 and 12 h after inoculation. Cytokine and chemokine expression in placentomes from experimentally infected cows was performed using total RNA isolated from placental tissues. Total RNA from CAM explants or tissue samples was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA from each sample was retrotranscribed in a total volume of 50 μl (TaqMan RT reagent; Applied Biosystems, Foster City, CA). Each real-time PCR was performed using 2 μl of cDNA, 600 nM of sense and antisense primers (Table 1), and 17 μl of SYBR green mix (Applied Biosystems). Cycling parameters for real-time PCR were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, using the 7900HT fast real-time PCR system (Applied Biosystems). The data were analyzed using the comparative threshold cycle (C_T) method (27). Severalfold changes in expression of target genes in infected CAM explants were calculated relative to the average level of the respective gene in control CAM explants from the corresponding time point after inoculation. C_T values were normalized based on GAPDH expression. The primers used in this study are listed in Table 1.

Statistical analysis. The microarray results were analyzed using the statistical tools of the GeneSpring software. The statistical significance of change in real-time RT-PCR assays was verified by the Student Newman Keuls test after logarithmic transformation of the raw data with Graphpad Instat software, version 3.05 (Graphpad Software, Inc., CA).

Microarray data accession number. The entire microarray data set has been submitted to the Gene Expression Omnibus database at NCBI (http://www.ncbi.nlm.nih.gov/geo/) and has been assigned accession number GSE10342.

RESULTS

Invasion of bovine trophoblastic cells by *Brucella abortus*. In order to evaluate the suitability of the CAM explant model for studying the early stages of infection, the invasion of trophoblastic cells by *B. abortus* in cultured CAM explants was evaluated by bacteriological and immunohistochemical methods. Morphometric analysis indicated an estimated number of trophoblasts ranging from 13,225 to 31,651 per well (Fig. 1). Based on an approximate average of 20,000 trophoblasts per CAM explant, the number of *B. abortus* per cell was estimated over the time course of infection between 2 and 12 h after inoculation (Fig. 2). A marked increase in the intracellular numbers of *B. abortus* during the first 4 h after inoculation reflects invasion rather than intracellular multiplication of the organism. Considering the number of intracellular organisms at 6 and 12 h after inoculation, the intracellular doubling time in trophoblasts was estimated to be approximately 8.7 h. Furthermore, immunohistochemistry confirmed that the organisms were indeed localized intracellularly in trophoblastic cells (Fig. 1). After 12 h in culture, marked losses of trophoblastic cells in CAM explants were observed. Morphologically, this loss was characterized by a reduction in the number and size of trophoblastic cells, which were flattened and vacuolated, with detachment of trophoblasts from the basal membrane. Therefore, all experiments with CAM explants in this study were limited to 12 h.

Profile of trophoblastic gene expression during the early stages of *Brucella abortus* infection. Changes in the profile of gene expression in trophoblastic cells of CAM explants during the early stages of infection with *B. abortus* were determined by microarray analysis at 4 h after inoculation. This time point was

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TABLE 1. List of genes and primers for real-time RT-PCR

Gene	Primers	Product size (nt)
CXCL1 (GROα)	5′-CTATTTTTGGGGAGAGGGTATTCC-3′ 5′-CGTGACCTATCTGTTTGCTTGAACC-3′	94
CXCL3 (GROγ)	5′-GGATGGCTGTTCCAGAAGTAGACC-3′ 5′-GGTGATTCCTCTTTTCCCTCTTTG-3′	64
CXCL6 (GCP-2)	5′-ATTCATCCCAAAACGGTCAGTG-3′ 5′-CAGACTTCCCTTCCATTCTTCAAG-3′	101
CXCL8 (IL-8)	5′-ACACATTCCACACCTTTCCACC-3′ 5′-GCAGACCTCGTTTCCATTGGTAAG-3′	117
IL-1 beta	5′-TCCTCCGACGAGTTTCTGTGTG-3′ 5′-GGGATTTTTGCTCTCTGTCCTGG-3′	76
IL-18	5′-GCTCTCCAATGCTTTCAGCG-3′ 5′-AGCCATCTTTATGCCTGTGCTC-3′	147
GAPDH	5′-ATGGTGAAGGTCGGAGTGAACG-3′ 5′-TGTAGTGAAGGTCAATGAAGGGGTC-3′	121
Complement component 9	5′-ACAGCAGGCTATGGGATCAA-3′ 5′-TGTCAAAAGGTGTGCTTAGGG-3′	58
IL-4	5′-TGTCACTGCAAATCGACACC-3′ 5′-ATGCCAGCAGGAAGAACAGA-3′	74
Lymphotoxin β	5′-CAGGAGCCACTTCTCTGGTG-3′ 5′-TTACCAGTCCTCCCTGATCCT-3′	96
Serum amyloid A4	5′-CTTCCTGCTCCTCTGCTCTC-3′ 5′-GTGACCCTGTGTCCCTGTCT-3′	77
TNF (ligand) superfamily member 8	5′-GAGGAGGTTGACACAGCACA-3′ 5′-GGATGAGAGGGACTTGAGAGAA-3′	84
CXCL11	5′-GTTCAAGGCTTCCCCATGTT-3′ 5′-TCTGCCACTTTCACTGCTTTT-3′	77

selected based on the curve of invasion of trophoblastic cells by *B. abortus* (Fig. 2) and a previous microarray study with *Brucella*-infected macrophages (14). RNAs from six independent experiments were pulled to generate three pools from two experiments each. Thus, the microarray analysis reflects the results of three independent hybridizations, which were analyzed by filtering based on severalfold change, considering up- or downregulated genes as those with at least a twofold increase or decrease from the level seen for the uninfected controls. The data were further filtered based on statistical significance ($P < 0.05$), thus eliminating genes whose increase or decrease in the amount of mRNA was not consistent in all of the three RNA pools. This approach resulted in the identification of 25 genes that had a statistically significant increase in expression (Table 2) and 37 downregulated genes (Table 3) at 4 h postinfection. Surprisingly, a significant downregulation of several proinflammatory genes was observed at 4 h after inoculation of CAM explants with *B. abortus* (Table 3). Considering that this study was focused on evaluating innate immunity and inflammatory response, reduced expression of genes associated with inflammation and immune response was confirmed by real-time RT-PCR analysis (Fig. 3), including complement component 9, interleukin 4 (IL-4), lymphotoxin beta, serum amyloid A4, member 8 of the tumor necrosis factor (TNF) superfamily, and chemokine CXC ligand 11 (CXCL11). Levels of expression of selected genes involved in inflammation were similar when analyzed by microarray and real-time RT-PCR, which also demonstrated a trend of decreased expression of these genes (Table 3 and Fig. 3), thus validating the microarray analysis.

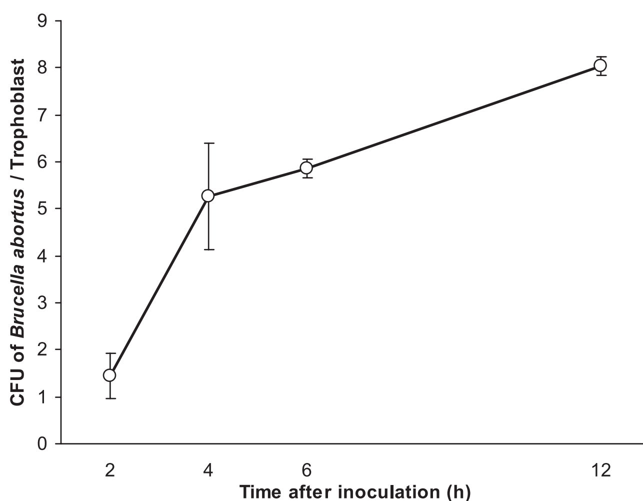

FIG. 2. Time course of invasion of bovine trophoblastic cells in CAM explants by *Brucella abortus*. Explants were inoculated with an MOI of 1,000 of *B. abortus* strain 2308, treated with gentamicin to kill extracellular bacteria, lysed, serially diluted, and plated for CFU counting. Data points represent means and standard errors of the mean for five independent experiments performed in triplicate.

Expression of proinflammatory chemokines and cytokines by bovine trophoblastic cells during infection with *Brucella abortus*. Considering the acute inflammatory response in the placentas of cattle infected with *B. abortus* and the lack of proinflammatory response by trophoblastic cells at 4 h after inoculation, as demonstrated by microarray analysis, we evaluated expression of CXC chemokines (CXCL8 [IL-8], CXCL1 [GROα], CXCL3 [GROγ], and CXCL6 [GCP-2]) as well as proinflammatory cytokines (IL-1β and IL-18) at later time points during infection (6 and 12 h). Epithelial cells are known to express CXC chemokines (12), and although the two proinflammatory cytokines selected for this study are primarily secreted by professional phagocytes, they were measured, since in addition to trophoblastic cells the tro-

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TABLE 2. Upregulated genes in cultured explants of bovine chorioallantoic membranes infected with *Brucella abortus* at 4 h after inoculation

Function and GenBank no.	Fold upregulation	*P* value	Gene or gene product/function
Inflammation and immune response			
BQ940635	2.95	0.00873	DnaJ (Hsp40) homolog, subfamily C, member 14
AL833759	10.0	0.00637	Hypothetical protein FLJ39827
Transcription			
BX537915	2.008	0.000012	Transcription elongation factor A (SII), 3
BQ575777	2.793	0.0126	TAF12 RNA polymerase II, TATA box binding protein-associated factor, 20 kDa
AF353674	2.849	0.0226	BTB (POZ) domain containing 6
Cell cycle			
AK090488	2.242	0.0239	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform
NM_057749	3.194	0.0464	Cyclin E2
AY376439	2.04	0.0381	Epithelial cell transforming sequence 2 oncogene
Transport			
NM_021614	10.0	0.00637	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
Signaling			
AK074259	4.184	0.00621	Pleckstrin homology domain containing family B (evectins) member 2
CN434563	2.439	0.028	HSPC054 protein
Regulation			
NM_024321	2.066	0.046	Hypothetical protein MGC10433
BM729023	2.77	0.0443	CUG triplet repeat, RNA binding protein 2
Adhesion			
BQ716387	2.061	0.00518	Cadherin 5, type 2, vascular epithelium cadherin
Metabolism			
BX647562	2.008	0.000808	Hypothetical protein MGC4767
CR627424	4.184	0.026	Transcribed locus, moderately similar to XP_526970.1 (*Pan troglodytes*)
NM_020773	2.415	0.0187	TBC1 domain family, member 14
Membrane			
AK091890	2.024	0.0482	CD34 antigen
Other			
BM563524	2.053	0.011	PWWP domain containing 1
BU729749	2.096	0.00666	Similar to CG14894-PA
NM_024067	2.169	0.024	Chromosome 7 open reading frame 26
NG010008A20C05	2.61	0.0485	Unknown
BM363643	2.688	0.00594	Unknown
W31247	3.69	0.0335	Phosphoprotein enriched in astrocytes 15
CN434037	2.564	0.0444	Unknown

phoblastic surface of the CAM explants contain other resident cells, which may include small but significant populations of lymphocytes and phagocytic cells. The limitation of using transcripts to measure IL-1β and IL-18 is noteworthy, since these cytokines require caspase 1 cleavage for activation (6, 25).

Infected CAM explants had a significant increase in expression of CXCL8 and CXCL6 at 12 h but not at 6 h postinfection, compared to uninfected controls (Fig. 4). Consistent with the microarray results at 4 h after inoculation, no significant increases in expression of the selected chemokines and proinflammatory cytokines were detected at 6 h after inoculation.

Placentomes from experimentally infected cows have a profile of proinflammatory chemokine expression that is similar to that of infected trophoblastic cells in CAM explants. To evaluate the correlation of proinflammatory gene expression in vivo and in vitro, expression of the same selected cytokines and chemokines that were analyzed for CAM explants was also assessed for placentomes from experimentally infected cows. Severalfold changes in expression of proinflammatory genes in placentomes from cows with positive bacteriology with or without abortion were calculated in comparison to what was seen for placentomes from cows with negative bacteriology and normal parturition. No significant differences were observed between the groups of bacteriologically positive cows with or without abortion (Fig. 5), in spite of differences in histopathology between these two groups (Fig. 6). Therefore, when severalfold changes in expression of these two groups with positive bacteriology were grouped together, there was a statistically significant increase in the expression of CXCL8 and CXCL6 (Fig. 5). Thus, the profile of chemokine expression in the placentomes of experimentally infected cows, with an approximately 10-fold increase in expres-

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TABLE 3. Downregulated genes in cultured explants of bovine chorioallantoic membranes infected with *Brucella abortus* at 4 h after inoculation

Function and GenBank no.	Fold downregulation	*P* value	Gene or gene product/function
Inflammation and immune response			
NM_001737	14.09	0.000306	Complement component 9
CD640452	7.379	0.0125	IL-4
BQ064345	3.321	0.00472	Lymphotoxin beta (TNF superfamily member 3)
BG618424	2.977	0.00241	Serum amyloid A4, constitutive
AW452023	2.831	0.0138	TNF (ligand) superfamily member 8
U66096	2.162	0.000687	CXCL11
Transcription			
NM_016276	3.451	0.0384	Serum/glucocorticoid-regulated kinase 2
AW266874	3.288	0.01	Unknown
Cell cycle			
D15049	2.124	0.00767	Protein tyrosine phosphatase, receptor type H
Stress			
AK098671	4.016	0.0199	Dimethylarginine dimethylaminohydrolase 2
CR455999	2.243	0.000287	Glutathione synthetase
Transport			
BC006404	2.753	0.00853	Suppressor of potassium transport defect 3
NM_170736	2.524	0.0103	Potassium inwardly rectifying channel, subfamily J, member 15
Signaling			
AK092516	2.657	0.0238	Hypothetical protein LOC150084
NM_145803	2.222	0.0169	TNF receptor-associated factor 6
Regulatory			
CN432381	24.54	0.0258	Unknown
NM_000035	5.368	0.0255	Aldolase B, fructose-bisphosphate
BG117301	3.564	0.0223	Pleiotropic regulator 1 (PRL1homolog, *Arabidopsis*)
BX648731	2.555	0.0426	Junction-mediating and regulatory protein
AI971356	2.482	0.0197	Transcribed locus, strongly similar to NP_000446.1 serine/threonine kinase 11
BE294317	2.142	0.0444	p53 and DNA damage regulated 1
Metabolism			
AA035275	2.886	0.00485	Transcribed locus, moderately similar to XP_126676.1 RIKEN cDNA 1810057P16 gene [Mus musculus]
NM_031438	2.884	0.045	Nudix (nucleoside diphosphate linked moiety X)-type motif 12
BC047129	2.519	0.0446	Flavin-containing monooxygenase 1
BM808152	2.255	0.0355	Adenine phosphoribosyltransferase
Y14385	2.235	0.0465	Inositol polyphosphate phosphatase-like 1
Membrane			
DN547823	3.502	0.0323	Unknown
Other			
CR456122	3.452	0.0454	Unknown
AK094654	3.094	0.00241	Chromosome 14 open reading frame 129
DN642227	3.082	0.00241	Unknown
NM_005382	2.912	0.0418	Neurofilament 3 (150-kDa medium)
CN438371	2.824	0.0337	Unknown
AK125351	2.805	0.0136	RNA binding motif protein 21
NM_182684	2.789	0.0297	Uroplakin IIIb
BC015335	2.721	0.00418	Immature colon carcinoma transcript 1
BF440457	2.649	0.0195	Transcribed locus

sion of CXCL8 and CXCL6, was similar to the profile observed for *B. abortus*-infected CAM explants (Fig. 4). Increased expression of these proinflammatory genes was associated with histopathological changes that were more severe than those seen for bacteriologically negative placentomes (Fig. 6).

DISCUSSION

Infection of pregnant cows with *B. abortus* results in placentitis and abortion (29). Although there is evidence to support the contention that intracellular multiplication of *B. abortus*

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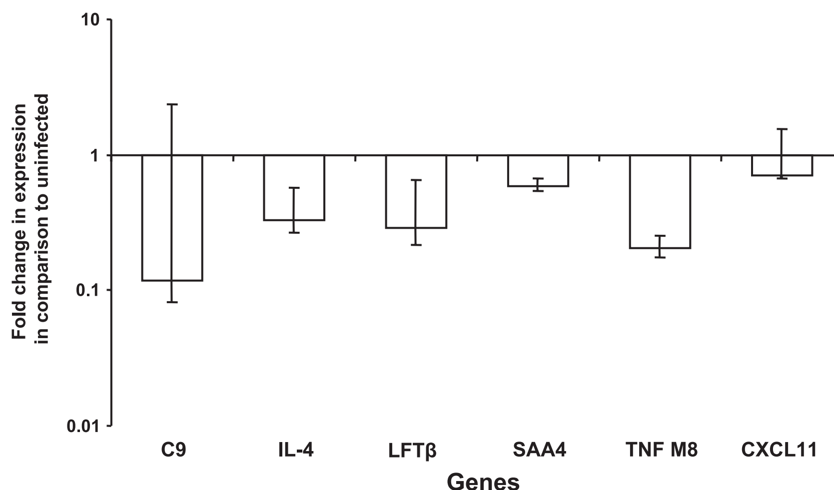

FIG. 3. Validation of the microarray results by real-time RT-PCR, indicating downregulated expression of proinflammatory genes in CAM explants infected with *Brucella abortus* in comparison to the uninfected group at 4 h postinoculation. Bars represent geometric means and standard errors of the mean for three pools of two placentas each, totaling six independent experiments. Abbreviations: C9, complement component 9; LFTβ, lymphotoxin beta; SAA4, serum amyloid A4; TNF M8, TNF (ligand) superfamily member 8.

within trophoblastic cells is important in the pathogenesis of *B. abortus*-induced abortion and placentitis (3), very little is known about the interaction between *B. abortus* and bovine trophoblastic cells, particularly about the role played by trophoblastic cells in the inflammatory response in placenta. Here we describe a thorough study aimed to identify changes in the profile of gene expression during the early stages of infection of trophoblastic cells with *B. abortus*, with emphasis on proinflammatory mediators. Our data can be summarized as follows. (i) There was a suppression of proinflammatory response by trophoblastic cells during the early stages of infection (i.e., 4 h after inoculation). (ii) Expression of proinflammatory chemokines was detected at later stages of infection (i.e., 12 h after inoculation). (iii) A pattern of proinflammatory response similar to that observed for infected CAM explants was also detected in vivo for placentomes from experimentally infected cows. Therefore, these data support the notion of a biphasic response of trophoblastic cells to *B. abortus* infection, with an initial suppression of proinflammatory genes that is followed

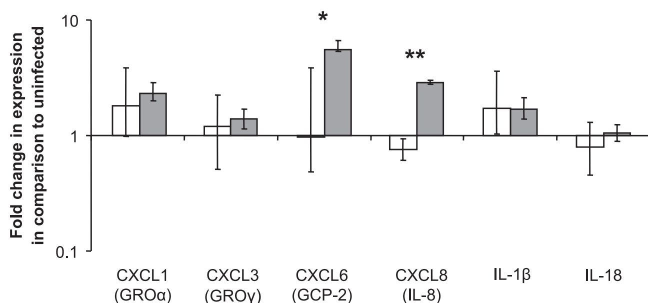

FIG. 4. Expression of proinflammatory chemokines and cytokines by CAM explants infected with *Brucella abortus* at 6 (white bars) and 12 (gray bars) h after inoculation. Normalized C_T values for CXCL6 (GCP-2) and CXCL8 (IL-8) are significantly different between 6 and 12 h after inoculation (*, $P = 0.0172$; **, $P = 0.0184$). Data points represent geometric means and standard errors for five independent experiments.

by a mild proinflammatory response at later stages of infection. Implications of these findings are discussed in detail below.

The model of infection based on explants of CAM proved suitable for studying the early events of the interaction between *B. abortus* and bovine trophoblastic cells. However, the explants are fully viable for only 12 h in culture (although some explants remain viable for up to 48 h in culture), so this model does not allow evaluation of later events such as the kinetics of intracellular bacterial multiplication and trophoblastic responses at later time points. *Brucella* sp. is able to infect phagocytes and nonphagocytic cells in vivo and in vitro (9, 10, 32, 33). However, *Brucella* sp. requires a long period of time to interact, invade, and replicate in nonphagocytic cells. Only 40 to 50% of Vero cells inoculated with *B. abortus* 2308 contain the organism at 8 h after inoculation (9, 10), which is pretty similar to the kinetics observed for trophoblastic cells in this study.

It has been shown that *Brucella* induces expression of proinflammatory cytokines such as TNF-α and IL-12 both in vivo and in vitro (7, 21, 49). Surprisingly, our microarray data demonstrated a suppression of proinflammatory response from trophoblastic cells in response to *B. abortus* infection during the first 4 h of infection. This response included downregulation of some inflammatory markers and CXCL11 (Table 3). Interestingly, our data are in good agreement with a recent study that demonstrated low levels of cytokine response during the early stages of *B. abortus* infection in mice (4). In the mouse model of *Brucella* infection, expression of CXC chemokines occurs in the spleen during the first days of infection, which correlates with an early neutrophilic response in the liver, which progresses toward a granulomatous inflammation by 1 week postinfection (36). These in vivo observations correlate well with previous microarray analysis of macrophages infected with *B. abortus*, which displayed a marked proinflammatory response at 4 h postinoculation, with upregulation of TNF and IL-1α and -β (14). Therefore, suppression of proinflammatory response in nonphagocytic cells may be an important mechanism for the evasion of innate immunity by *B. abor-*

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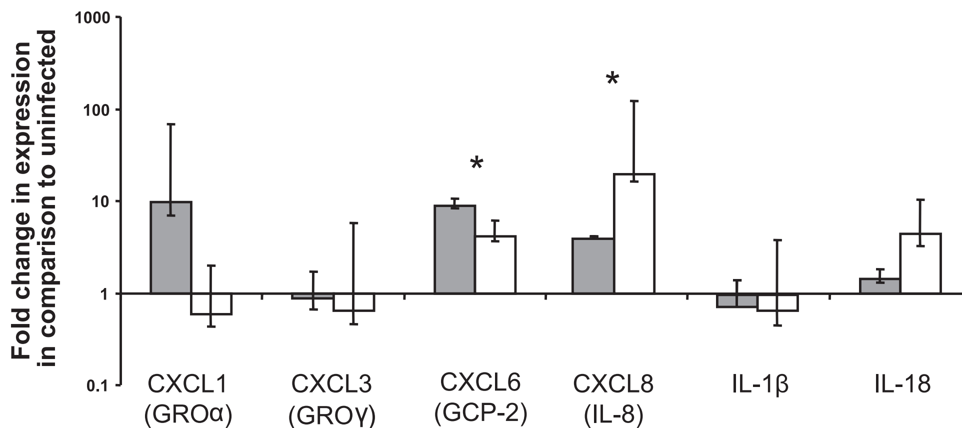

FIG. 5. Expression of proinflammatory chemokines and cytokines by placental tissue of cows experimentally infected with *Brucella abortus* divided in two groups: (i) positive bacteriology and term parturition (gray bars) and (ii) positive bacteriology with abortion (white bars). Severalfold change of expression was calculated in comparison to the control group level (bacteriologically negative and normal parturition). *, Normalized C_T values for CXCL6 (GCP-2) and CXCL8 (IL-8) are significantly different between controls and both positive bacteriology groups combined ($P < 0.05$; means were compared by Student Newman Keuls test after logarithmic transformation of the raw data). Columns represent geometric means and standard errors.

tus. This mechanism seems particularly appealing during the course of invasion of epithelial cells in the gastrointestinal tract during the initial steps of infection in vivo. It is noteworthy that the internalization of *B. abortus* by phagocytic cells occurs much faster than that by nonphagocytic cells (14, 24), which may account for some of the differences in the responses of these cells to infection with *B. abortus*.

Interestingly, the interaction of *B. abortus* with epithelial cells during the early stages of infection in vivo does not result in lesions or inflammatory response in the gastrointestinal tract, which is one of the primary sites of entry for *B. abortus*, the respiratory tract being the other. Experimental infections demonstrated that *B. abortus* is endocytosed by lymphoepithelial cells present in the intestinal tract. *Brucella* localizes within phagocytic cells in the lamina propria of domed villi from 2 to 4 h after infection. Invasion in this location does not trigger any significant inflammatory reaction (1). Conversely, intracellular localization of *B. abortus* in trophoblastic cells is associated with an intense inflammatory response in infected pregnant cows (29, 31). Importantly, the interaction of *Brucella* with intestinal epithelial cells is transient, whereas the interaction with trophoblasts occurs for prolonged periods of time. Therefore, the suppression of epithelial proinflammatory response is likely to be the default response triggered by *B. abortus*, which is eventually overcome by a proinflammatory response later on during infection, as demonstrated in this study.

Upregulation of neutrophil chemoattractants such as CXCL8 and CXCL6 in trophoblastic cells from CAM explants at 12 h after inoculation supports the hypothesis that trophoblastic cells may play an active role eliciting neutrophil influx to the placentome during infection with *B. abortus*. This trophoblastic response is likely to contribute to the pathogenesis of the neutrophilic and necrotizing placentitis observed in vivo as a result of *B. abortus* infection of pregnant cows. Importantly, *Brucella*-induced placentitis is thought to be a major factor causing abortion in bovine brucellosis, which is a key event for transmission of the disease. We also demonstrated that the profile of proinflammatory chemokine expression in placentomes infected by *B. abortus* in vivo was very similar to that observed for infected trophoblastic cells in vitro, which indicates that the CAM explant model reflects the response of the whole placental tissue during infection with *B. abortus*. Upregulation of these chemokines in vivo was associated with more-severe histopathological changes in vivo.

Our data indicate that following an initial suppression of proinflammatory genes, induction of CXC chemokines was observed at a later time point during infection of trophoblasts (i.e., at 12 h). However, this induction of proinflammatory mediators is delayed and mild compared to epithelial responses to other gram-negative organisms. Although a direct comparison between different tissues and animal models of infection has several limitations, *Salmonella enterica* induces a very strong and rapid proinflammatory response in intestinal tissues (35, 41). Supporting this notion, a recent study demonstrated that *Salmonella enterica* serotype Typhimurium induces higher levels of cytokines during the early stages of infection in mice; this is in contrast to *B. abortus* infection in the same model, which results in the induction of low levels of cytokines (4). This mild inflammatory response triggered by *B. abortus* in comparison to those seen for other gram-negative bacteria is at least in part due to the structure of its LPS, which is impaired for signaling through the TLR4 (11, 18, 26, 45).

Another interesting finding in this study was the suppression of expression of four genes belonging to the TNF superfamily in trophoblastic cells during the early stages of infection (i.e., 4 h after inoculation), namely, lymphotoxin beta, member 8 of TNF superfamily, CXCL11, and TNF receptor-associated factor 6. Although the majority of members of the TNF superfamily are expressed by immune cells, the expression of these genes has also been described for epithelial cells (23). These genes are thought to play a role favoring protective immunity in infectious diseases (20). There are several experimental evidences indicating that TNF-α plays a role in immunity to *B. abortus* in the mouse (7, 21, 49), although *Brucella suis* is able to suppress TNF-α production by human macrophages (22). Indeed, *Brucella* spp. have been recognized as stealthy invad-

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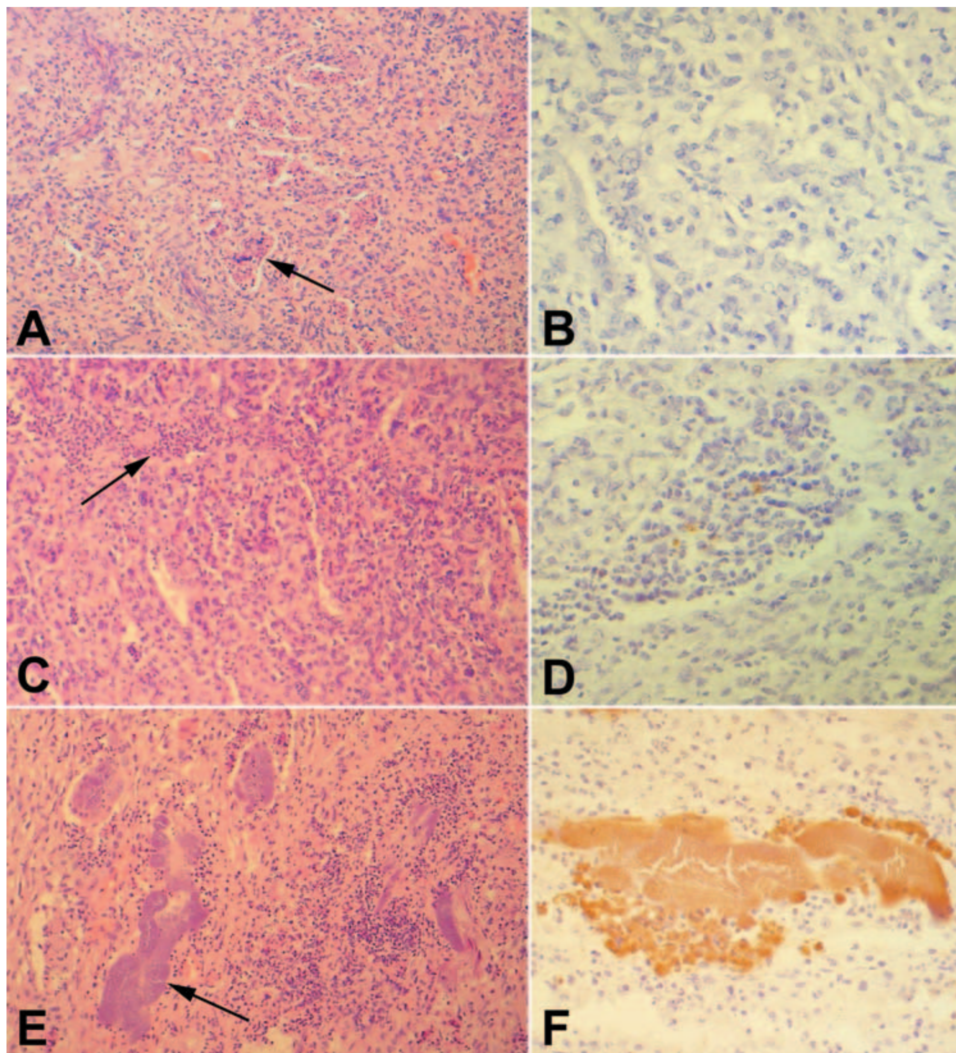

FIG. 6. Bovine placentomes from cows experimentally infected with *Brucella abortus*. (A) Histologically normal placentome from a bacteriologically negative cow. Chorionic villi within caruncular crypts are indicated by the arrow; H&E staining. (B) Same tissue sample as shown in panel A, with a negative result on immunohistochemistry; streptavidin-peroxidase staining. (C) Suppurative placentitis with a moderated and multifocal inflammatory infiltrate (arrow) in a cow with positive bacteriology and normal parturition. (D) Same tissue sample as shown in panel C, with immunohistochemical labeling of cell-associated *B. abortus* antigens; streptavidin-peroxidase staining. (E) Severe necrotizing placentitis with intense and multifocal inflammatory infiltrate associated with a myriad of intralesional bacterial colonies (arrow) in a cow with positive bacteriology and abortion; H&E staining. (F) Same tissue sample as shown in panel E, with a large colony of immunohistochemically labeled *B. abortus*; streptavidin-peroxidase staining.

ers, with very low impact on the immune cells (4). TNF acts as a cofactor for gamma interferon stimulation by lymphocytes, and it is also known to contribute to resistance through mechanisms independent of gamma interferon (52). The early activation of TNF after the infection of mice with *B. abortus* requires NF-κB activation through a TLR2 receptor MyD88-dependent pathway (26, 52). During the infection of mice, TNF depletion favors infection of macrophages by *B. abortus* in the spleen, resulting in the accumulation of cells and splenomegaly (52). Thus, the downregulation of TNF superfamily genes observed during the early stages of infection of trophoblasts with *B. abortus* further supports the hypothesis that the organism somehow suppresses a proinflammatory response by epithelial host cells.

In conclusion, taken together our data indicate that *B. abortus* modulates innate immune response by trophoblastic cells, suppressing the expression of proinflammatory mediators during the early stages of infection that is followed by a delayed and mild expression of proinflammatory chemokines by trophoblastic cells, which is similar to the profile of chemokine expression detected for the placentome of experimentally infected cows.

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