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Proinflammatory Response of Human Trophoblastic Cells to *Brucella abortus* Infection and upon Interactions with Infected Phagocytes[1]

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ABSTRACT

Trophoblasts are targets of infection by *Brucella* spp. but their role in the pathophysiology of pregnancy complications of brucellosis is unknown. Here we show that *Brucella abortus* invades and replicates in the human trophoblastic cell line Swan-71 and that the intracellular survival of the bacterium depends on a functional *virB* operon. The infection elicited significant increments of interleukin 8 (IL8), monocyte chemotactic protein 1 (MCP-1), and IL6 secretion, but levels of IL1beta and tumor necrosis factor-alpha (TNF-alpha) did not vary significantly. Such proinflammatory response was not modified by the absence of the *Brucella* TIR domain-containing proteins BtpA and BtpB. The stimulation of Swan-71 cells with conditioned medium (CM) from *B. abortus*-infected human monocytes (THP-1 cells) or macrophages induced a significant increase of IL8, MCP-1 and IL6 as compared to stimulation with CM from non-infected cells. Similar results were obtained when stimulation was performed with CM from infected neutrophils. Neutralization studies showed that IL1beta and/or TNF-alpha mediated the stimulating effects of CM from infected phagocytes. Reciprocally, stimulation of monocytes and neutrophils with CM from *Brucella*-infected trophoblasts increased IL8 and/or IL6 secretion. These results suggest that human trophoblasts may provide a local inflammatory environment during *B. abortus* infections either through a direct response to the pathogen or through interactions with monocytes/macrophages or neutrophils, potentially contributing to the pregnancy complications of brucellosis.

Brucella, *cellular interactions*, *inflammatory response*, *trophoblast*

INTRODUCTION

Brucellosis is a zoonotic disease caused by infection with *Brucella* species, which affects over 500 000 people annually worldwide. *Brucella melitensis*, *B. suis* and *B. abortus* are the most pathogenic species for humans and are responsible for most human cases [1]. The disease affects several domestic animals, including cattle, sheep, goats, pigs, and dogs, and can be also found in wild species (foxes, deer, feral swine, seals, dolphins, and others). Infection is usually transmitted to humans by direct contact with infected animal tissues and consumption of dairy products.

In humans, brucellosis manifestations are mainly due to inflammatory phenomena, which may be found both in the acute and chronic phases of the disease and in virtually all the affected organs. The most common clinical features of human brucellosis are undulant fever, sweating, arthralgias, myalgias, lymphadenopathy, and hepatosplenomegaly [2]. Among animals, involvement of the reproductive organs is common, and reproductive problems, including abortion and perinatal death, are among the most frequent findings.

Brucellosis may be also a cause of abortion in humans, although the incidence of this complication varies widely among studies, ranging from 7% to 40% [3–6]. According to an extensive survey in the Middle West, the incidence of brucellosis during pregnancy was 1.3 cases per 1000 deliveries. Among infected women, the incidence of spontaneous abortion was 43% during the first and second trimesters and 2% in the third trimester [7]. The pathophysiology of brucellar abortion in humans is completely unknown, but studies performed in animals may provide some clues to the mechanisms involved. In cattle, *Brucella* infection occurs through the trophoblastic cells in the base of chorionic villi, from which the bacteria disseminate to other trophoblasts. Bacterial replication within these cells induces the infiltration of inflammatory cells, trophoblast necrosis, vasculitis, and ulceration of the chorioallantoid membrane, finally leading to abortion [8, 9]. Therefore, these studies point to a central role of infected trophoblasts in the pathogenesis of brucellar abortion.

Several studies have assessed the innate immune response of trophoblasts to infection or microbial antigens. The stimulation of primary human villous trophoblasts from term placentas with pathogenic strains of *Escherichia coli*, *Bacteroides fragilis*, *Mycoplasma hominis*, *Staphylococcus aureus*, or *Streptococcus agalactiae* induced increased secretion of interleukin 1β (IL1β), IL6, IL8, and IL10 [10]. Exposure of the HTR-8/SVneo human first-trimester trophoblast cell line to *E. coli* LPS resulted in increased secretion of tumor necrosis factor-α (TNF-α) and IL8 [11], and its exposure to viral single stranded RNA led to increases in IL6 and IL8 secretion [12]. The responses of this same cell line to infection with *Porphyromonas gingivalis* included an increased production of IL1β and IL8 [13]. Primary human syncytiotrophoblast cells secreted chemokines (CXCL8, CCL3, CCL4) and TNF-α in response to *Plasmodium falciparum* hemozoin [14]. The human first trimester trophoblast cell lines HTR8 and Swan-

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71 exhibited a strong induction of IL1β secretion upon infection by *Chlamydia trachomatis*, and HTR8 also exhibited strong induction of IL8 [15]. In primary third-trimester cytotrophoblasts, the stimulation with a bacterial agonist of the Nod1 intracellular receptor (γ-D-glutamyl-meso-diaminopimelic acid) induced an increased secretion of IL6, GRO-α, and MCP-1 [16].

The different proinflammatory mediators produced by trophoblasts may induce several pathological mechanisms that affect pregnancy. TNF-α alone induces apoptosis in cultured placental trophoblast cells, the activity of which is enhanced by the presence of interferon-γ (IFN-γ) [17]. IL1β and TNF-α induce IL8 production by cultured chorionic cells, which may stimulate neutrophilic infiltration [18]. IL1β and TNF-α increase the protein levels of MMP-9 in isolated human amniochorion tissues, resulting in actual physical weakening of fetal membranes [19].

While histological studies indicate that trophoblasts are a main target of *Brucella* infection during pregnancy, the cytokine response of human trophoblasts to such infection has not been explored. In the present study we employed the human trophoblastic cell line Swan-71 to address this issue.

MATERIALS AND METHODS

Culture of Cell Lines

The telomerase-immortalized first trimester trophoblast cell line Swan-71 was kindly provided by Dr. Gil Mor (Yale University School of Medicine). These cells exhibit most of the phenotypical characteristics and biological responses of primary trophoblasts [20]. Cells were grown in Dulbecco modified Eagle medium (DMEM)/Ham F12 medium (1:1) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. THP-1 cells were obtained from American Type Culture Collection and were grown and maintained in RPMI 1640 medium supplemented as above. To induce maturation, THP-1 cells were cultured in the presence of 0.05 μM 1,25-dihydroxyvitamin D_3 (Calbiochem-Nova Biochem International) for 48 to 72 h. For infection assays, cells were seeded at 5×10^4 cells/well (trophoblasts) or 5×10^5 cells/well (THP-1 cells) in 24-well plates and cultured in a 5% CO_2 atmosphere at 37°C for 24 h in antibiotic-free culture medium.

Neutrophil and Monocyte Isolation and Macrophage Differentiation

Peripheral blood samples were obtained from healthy volunteers after approval by the Institutional Review Board of Instituto de Estudios de la Inmunidad Humoral (IDEHU). Written informed consent was obtained from all volunteers. Human neutrophils were isolated following standard procedures [21]. Briefly, whole blood diluted with sterile buffered saline was carefully layered on Ficoll-Paque (density 1.077 g/ml) (GE Healthcare) and centrifuged at $400 \times g$ for 30 min. The pellet containing red cells and granulocytes was harvested, resuspended in buffered saline, and mixed with 6% dextran. After incubation at room temperature to allow erythrocyte sedimentation, the leukocyte-rich supernatant was aspirated and centrifuged at $500 \times g$ for 10 min at 4°C. The remaining red cells were eliminated by quick hypotonic lysis. Harvested neutrophils were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium supplemented with 5% serum from the same donor and 1 mM glutamine (Gibco-BRL Life Technologies). Cell viability was >98%, as determined by trypan blue exclusion. The purity of the final neutrophil preparation was >95% as determined by morphological examination with Giemsa staining and flow cytometry light scatter patterns.

Human monocytes were isolated by standard procedures. Briefly, whole blood diluted with sterile buffered saline was carefully layered on Ficoll-Paque (density: 1.077 g/ml) and centrifuged at $400 \times g$ for 30 min. The buffy coat was carefully harvested, resuspended in RPMI 1640 medium supplemented with 1 mM glutamine and left to adhere in 24-well plates for 4 h. Cells were washed with sterile PBS to eliminate nonadherent cells, and RPMI medium supplemented with 5% sera from the same donors, 100 U/ml penicillin, and 100 μg/ml streptomycin were added. Cell viability was >98%, as determined by trypan blue exclusion. This monocyte-enriched population was used for in vitro macrophagic differentiation following standard procedures [22]. Monocytes were cultured in medium supplemented with autologous serum at 37°C in a 5% CO_2 atmosphere for 7 days, when morphological changes were observed. The purity of the final macrophagic preparation was >95% as determined by flow cytometry light scatter patterns (macrophages have increased size and specially increased granularity compared to monocytes). Antibiotics were removed 24 h prior to infection.

Bacterial Strains and Growth Conditions

B. abortus 2308 (wild type strain) was obtained from our collection. *B. abortus virB10*$^-$ mutant and *B. abortus btpAbtpB*$^-$ double mutant (both on *B. abortus* 2308 background) were kindly provided by Dr. Diego Comerci (Universidad de San Martín, Argentina). All strains were grown in tryptic soy broth at 37°C with agitation. Bacteria were washed twice with sterile PBS, and inocula were prepared in sterile PBS on the basis of optical density readings, but the actual concentration was later checked by plating on agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities.

Cellular Infections

Swan-71 cells were infected with *B. abortus* 2308 or with *virB10*$^-$ or *btpAbtpB*$^-$ mutants at multiplicity of infection (MOI) of 250 bacteria/cell. Neutrophils, monocytes (primary and THP-1 cells) and macrophages were infected at MOI 100. After the bacterial suspension was dispensed, the plates were centrifuged for 10 min at $400 \times g$ and then incubated for 2 h at 37°C in a 5% CO_2 atmosphere. Cells were extensively washed with medium to remove extracellular bacteria and incubated in medium supplemented with 100 μg/ml gentamicin and 50 μg/ml streptomycin to kill extracellular bacteria (complete medium). Conditioned medium (CM) from infected cells was harvested at 24 and/or 48 h postinfection (p.i.) for measuring cytokines and chemokines, and for stimulation experiments.

Evaluation of Cytotoxicity

To analyze the effect of infection on cell integrity, the release of lactate dehydrogenase (LDH) from infected trophoblasts was determined. Cells were infected with *B. abortus* 2308 at different MOIs as described above. Culture supernatants were harvested at 24 and 48 h p.i. and LDH levels were measured using CytotTox 96 nonradiactive cytotoxicity assay (Promega). Results were expressed as the ratio between LDH levels measured in the samples (infected or noninfected cultures) and those corresponding to a 100% cell lysis (obtained by hypotonic lysis of the same number of cells).

Stimulation of Trophoblasts with CM from Brucella-infected Monocytes, Macrophages or Neutrophils

CM from THP-1 monocytes, macrophages, or neutrophils infected with *B. abortus* 2308 (MOI 100) were harvested at 24 h p.i., sterilized by filtration through a 0.22-μm nitrocellulose filter and used to stimulate noninfected Swan-71 cells. CM was used diluted 1:10 in complete medium. After 24 and 48 h, supernatants from stimulated cultures were harvested to measure cytokines and chemokines.

To determine which factors might be involved in the stimulating effects of CM, in some experiments, CM samples from either monocytes or neutrophils infected with *B. abortus* were preincubated for 1 h at 37°C with a neutralizing monoclonal antibody against TNF-α or its isotype control before being transferred to trophoblasts cultures. Alternatively, trophoblasts were preincubated with the IL1β receptor antagonist IL1Ra (R&D Systems) for 1 h at 37°C before stimulation with CM from infected monocytes or neutrophils.

Stimulation of Monocytes or Neutrophils with CM from Brucella-infected Trophoblasts

Swan-71 cells were plated at 3×10^5 cells/well in 6-well plates and infected with *B. abortus* 2308 at MOI 100. CM was harvested at 48 h p.i., sterilized by filtration through a 0.22- μm nitrocellulose filter, and used to stimulate noninfected THP-1 cells or freshly isolated peripheral blood neutrophils. After 24 and 48 h, supernatants from stimulated cultures were harvested to measure cytokines and chemokines.

Measurement of Cytokines and Chemokines

Levels of human IL1β, IL6, IL8, MCP-1, and TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured in culture supernatants by sandwich ELISA, using paired cytokine-specific monoclonal antibodies according to the manufacturer's instructions (BD Pharmingen

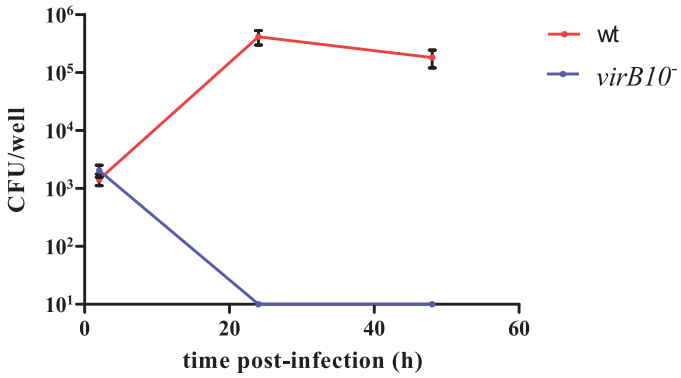

FIG. 1. *Brucella abortus* invades and replicates in Swan-71 trophoblastic cells. Trophoblasts were infected for 2 h at MOI 250 with *B. abortus* 2308 (wild type [wt]) or the *virB10*⁻mutant and were later treated with antibiotics to kill extracellular bacteria. Cell lysates obtained at different post-infection times were plated on agar to determine the number of live intracellular bacteria (colony-forming units [CFU]). Data are means ± SEM from three independent experiments performed in triplicate.

and R&D Systems). For determinations in culture supernatants from Swan-71 cells stimulated with CM from *Brucella*-infected monocytes, macrophages or neutrophils, levels of cytokines and chemokines already present in the CM were subtracted. Similarly, for determinations in culture supernatants from THP-1 cells or neutrophils stimulated with CM from *Brucella*-infected Swan-71 cells, levels already present in this later medium were subtracted.

Statistical Analysis

Statistical analysis was performed with one-way ANOVA, followed by Post Hoc a Tukey Test or Dunnett's Test using GraphPad Prism 4.0 software. Data are represented as mean ± SD.

RESULTS

Brucella abortus *Invades and Replicates in Swan-71 Trophoblastic Cells*

Swan-71 cells were infected with *B. abortus* for 2 h and were later treated with antibiotics to kill extracellular bacteria. Cell lysates were obtained at different p.i. times to determine the number of live intracellular bacteria. As shown in Figure 1, the number of viable bacteria increased by approximately 3 logs during the first 24 h p.i. and stayed at similar levels at 48 h p.i. To rule out a cytotoxic effect of the infection, culture supernatants obtained at each time point were assayed for LDH activity. No difference of LDH activity was found between infected and non-infected cells for any time point (not shown).

Brucella virB genes, which encode several proteins that take part in a bacterial type IV secretion system (T4SS), have been shown to be critical determinants of the ability of *Brucella* to survive and replicate in different cell types, including human primary trophoblasts [23, 24]. In contrast, the replication of *B. abortus* 2308 in the trophoblastic cell line JEG-3 seems to be *virB*-independent [24]. To evaluate the role of the T4SS in *B. abortus* survival in Swan-71 cells, these trophoblasts were also infected with a *virB10* insertional polar mutant. As shown in Figure 1, the mutant invaded Swan-71 cells with the same efficiency as its wild-type counterpart, but it did not survive intracellularly as no CFU were recovered at 24 or 48 h p.i.

Brucella abortus *infection elicits a proinflammatory response in Swan-71 cells*

Conditioned medium from *B. abortus*-infected trophoblasts was harvested at 24 and 48 h p.i. and analyzed by ELISA for the presence of proinflammatory cytokines and chemokines. As shown in Figure 2, the infection elicited significant IL8, IL6,

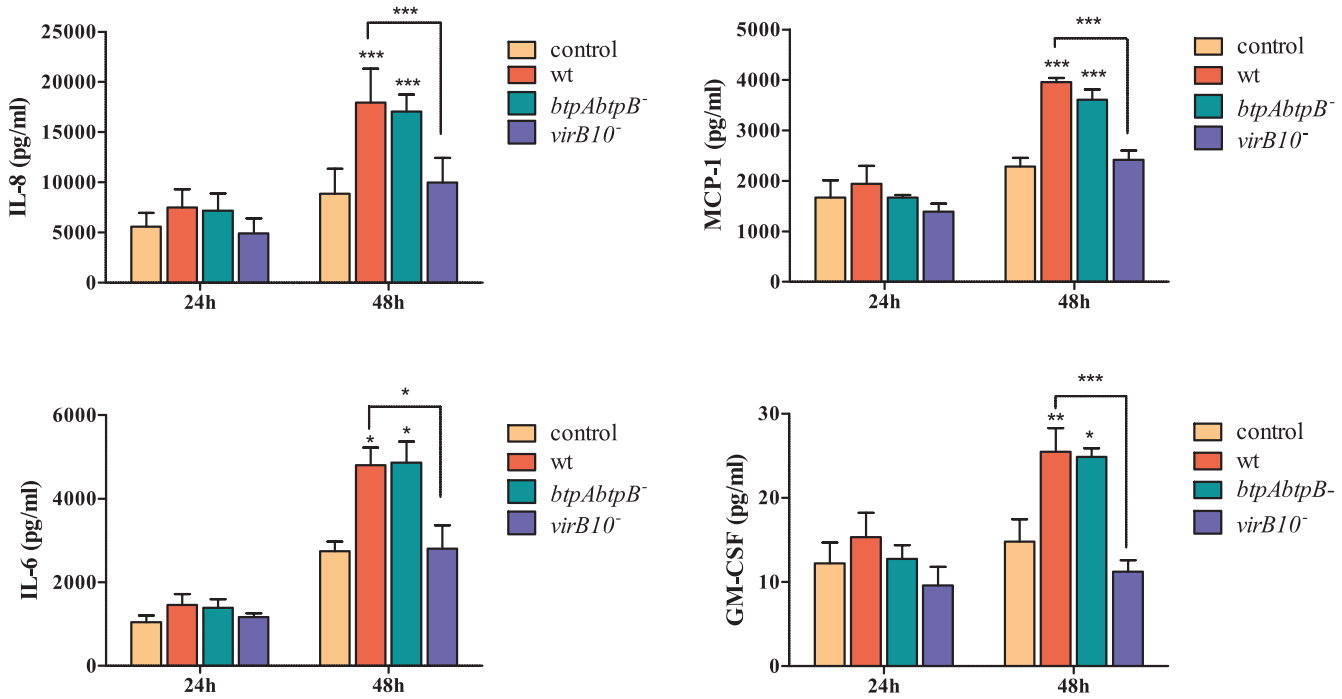

FIG. 2. *Brucella abortus* generates a proinflammatory immune response in Swan-71 trophoblastic cells upon infection. Trophoblasts were infected with *B. abortus* 2308 (wild type [wt]), the *btpAbtpB*⁻ double mutant, and the *virB10*⁻ mutant at MOI 250 and culture supernatants were harvested at 24 and 48 h post infection for measuring IL8, MCP-1, IL6, and GM-CSF, using ELISA. Data are means ± SEM from three independent experiments performed in triplicate. Asterisks over bars indicate significant differences versus uninfected (control) cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ANOVA followed by Tukey test).

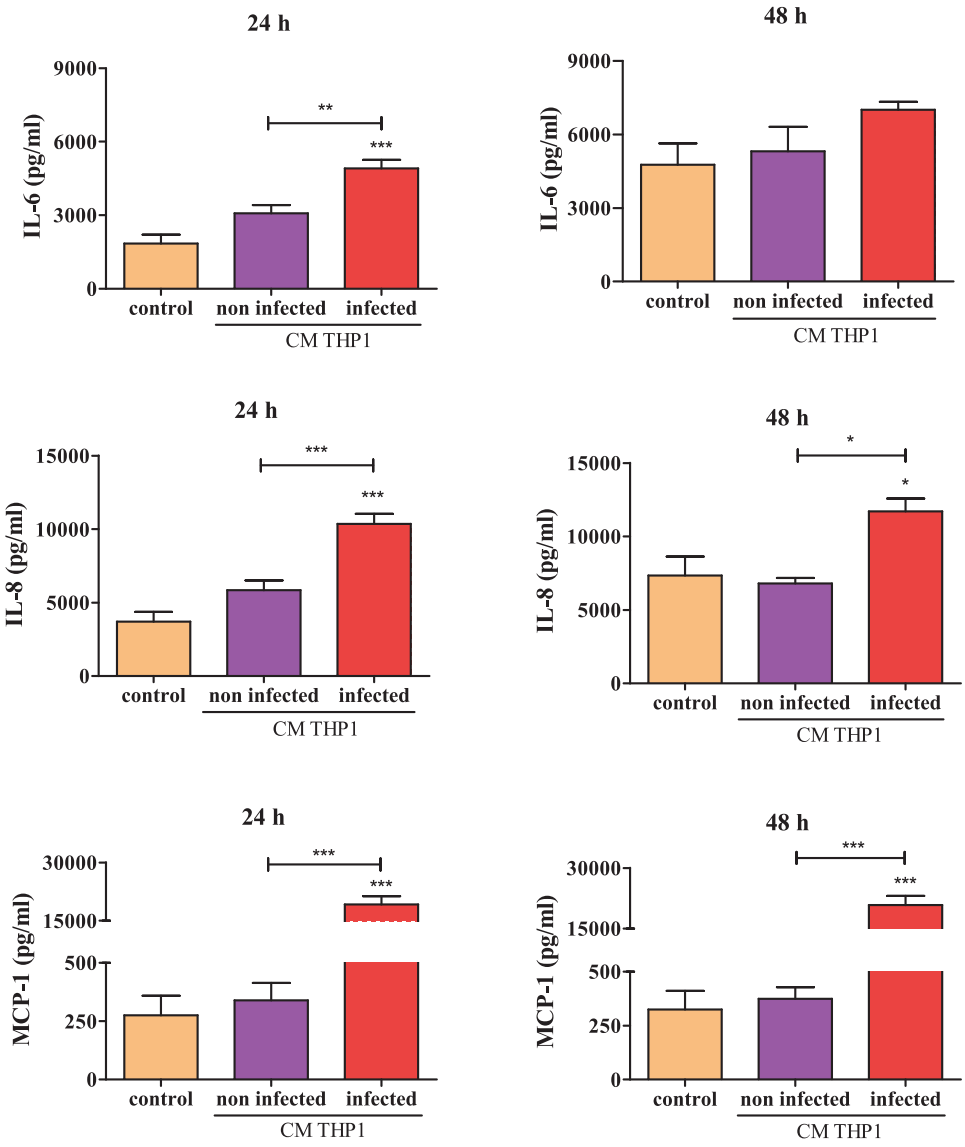

FIG. 3. Factors produced by *B. abortus*-infected monocytes stimulate the production of chemokines and cytokines by Swan-71 trophoblastic cells. THP-1 cells (human monocytes) were infected for 24 h with *B. abortus*, and conditioned medium (CM) was used to stimulate Swan-71 cells. As a control, other cultures were stimulated with CM from noninfected THP-1 cells. Culture supernatants were harvested at 24 and 48 h after stimulation for measuring IL6, IL8, and MCP-1 by ELISA. Data are means ± SEM from three independent experiments performed in triplicate. Asterisks over bars indicate significant differences versus unstimulated (control) cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ANOVA followed by a Tukey test).

and MCP-1 responses at 48 h p.i. as compared to non-infected controls. Infection also elicited a low but significant GM-CSF response. In contrast, no significant increase in the level of any of these factors was observed for cells infected with the *virB10*$^{-}$ mutant. Levels of IL1β or TNF-α did not vary significantly during the post-infection period as compared to uninfected cells (not shown).

Previous studies have shown that *B. abortus* expresses two TIR domain-containing proteins, BtpA and BtpB, which inhibit TLR signaling and modulate the innate immune response of dendritic cells to *Brucella* infection [25]. In order to test the influence of the Btp proteins on the response of human trophoblasts to *B. abortus*, Swan-71 cells were infected with a double mutant lacking these modulators (*btpAbtpB*$^{-}$). The mutant strain invaded trophoblasts as efficiently as the wild-type strain as no significant differences in intracellular CFU were detected at 2 h p.i. (1458 ± 380 vs. 1125 ± 165 CFU/well for the mutant and wild-type strains, respectively, not shown). As shown in Figure 2, absence of the Btp proteins did not modify significantly the production of the cytokines and chemokines measured in this study, at least during the first 48 h p.i.

Swan-71 Cells Secrete Cytokines and Chemokines in Response to Factors Produced by Brucella*-Infected Monocytes and Macrophages*

During in vivo infections, cells may secrete proinflammatory mediators not only in response to the pathogen itself but also in response to soluble factors produced by other infected cell types. In the natural context trophoblasts are in contact with immune cells present in the decidua, mainly macrophages [26]. As cells of the monocytic/macrophagic lineage have been consistently shown to be the preferred replication niche of *Brucella* spp. [27], we wondered whether factors produced by infected monocytes and macrophages could influence the

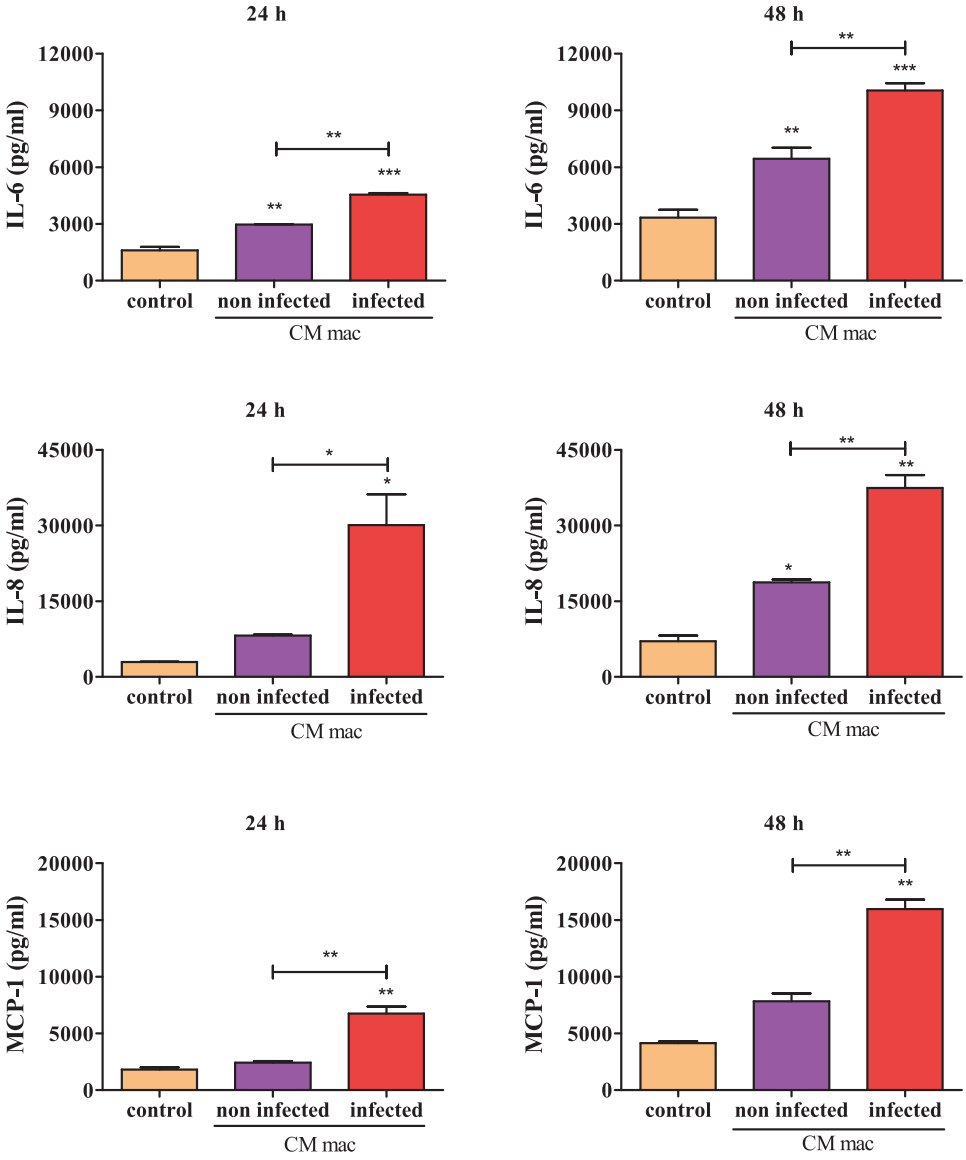

FIG. 4. Factors produced by *B. abortus*-infected macrophages stimulate the production of chemokines and cytokines by Swan-71 trophoblastic cells. Macrophages differentiated in vitro from monocytes isolated from human venous blood were infected for 24 h with *B. abortus* and conditioned medium (CM) was used to stimulate Swan-71 cells. As a control, other cultures were stimulated with CM from non-infected macrophages. Culture supernatants were harvested at 24 and 48 h after stimulation for measuring IL6, IL8, and MCP-1 by ELISA. Data are means ± SEM from three independent experiments performed in triplicate. Asterisks over bars indicate significant differences versus unstimulated (control) cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ANOVA followed by a Tukey test).

production of cytokines and chemokines by trophoblasts. To test this hypothesis, Swan-71 cells were stimulated with CM obtained from THP-1 cells (human monocytic cell line) and primary macrophages that had been infected with *B. abortus* 24 h before. Culture supernatants from these stimulated trophoblasts were harvested at 24 and 48 h post-stimulation to analyze the presence of cytokines and chemokines. Prior to their use for stimulation, CM from infected monocytes and macrophages were analyzed for their cytokine and chemokine levels. As shown in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org; in the public domain), levels of TNF-α, IL1β, IL6, MCP-1, and IL8 were similarly increased by *B. abortus* infection in all these cell types. Levels of these factors already present in the transferred CM were subtracted from those measured in culture supernatants from CM-stimulated Swan-71 cells in order to determine the secretion specifically elicited by the stimulation. As shown in Figure 3, CM from infected THP-1 cells elicited a specific stimulation of IL8, MCP-1, and IL6 secretion by Swan-71 cells. At 48 h post stimulation, cytokine levels specifically stimulated by the infection (i.e., the difference between mean levels found in trophoblasts stimulated with CM from infected monocytes and those in trophoblasts stimulated with CM from uninfected monocytes) were 4916 pg/ml for IL8, 1695 pg/ml for IL6, and 20452 pg/ml for MCP-1. The specific increase was particularly remarkable for MCP-1, as the chemokine level attained after stimulation with CM from infected monocytes was 52-fold higher than that attained with CM from non-infected controls.

Similar results were obtained when Swan-71 cells were stimulated with CM from *Brucella*-infected human macrophages. As shown in Figure 4, levels of IL8, IL6 and MCP-1 were significantly higher in trophoblasts stimulated with CM from infected macrophages than in cells stimulated with CM

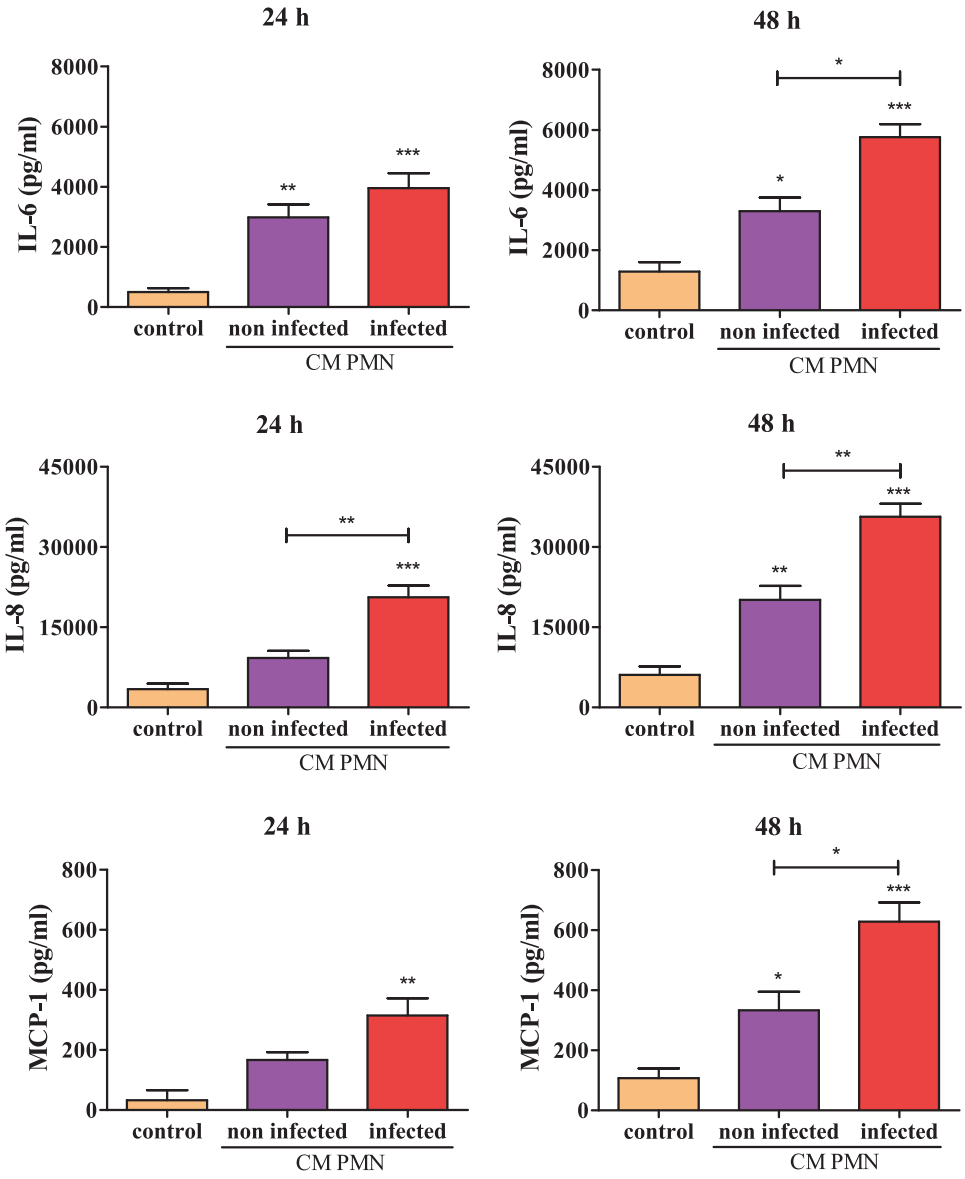

FIG. 5. Factors produced by *B. abortus*-infected neutrophils stimulate the production of chemokines and cytokines by Swan-71 trophoblastic cells. Neutrophils isolated from human venous blood were infected for 24 h with *B. abortus*, and the conditioned medium (CM) was used to stimulate Swan-71 cells. As a control, other cultures were stimulated with CM from non-infected neutrophils. Culture supernatants were harvested at 24 and 48 h after stimulation for measuring IL6, IL8, and MCP-1 by ELISA. Data are means ± SEM from three independent experiments performed in triplicate. Asterisks over bars indicate significant differences versus unstimulated (control) cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ANOVA followed by a Tukey test).

from uninfected controls. At 48 h post-stimulation cytokine levels specifically stimulated by the infection were 18667 pg/ml for IL8, 3621 pg/ml for IL6, and 8112 pg/ml for MCP-1.

In contrast to these stimulations, CM from *Brucella*-infected THP-1 cells or macrophages did not induce a significant specific TNF-α or IL1β secretion by Swan-71 cells (not shown). Overall, CM from *Brucella*-infected monocytes (THP-1 cells) or macrophages elicited a similar cytokine response in Swan-71 cells.

Swan-71 Cells Secrete Cytokines and Chemokines in Response to Factors Produced by Brucella*-Infected Neutrophils*

Histological studies have consistently shown a neutrophilic infiltrate in placentomes and endometrium in *Brucella*-infected bovines [9]. This may in turn increase the probability of interactions between trophoblasts and neutrophils, with potential immunological consequences. Therefore, experiments similar to those described above were carried out to test whether factors produced by *Brucella*-infected neutrophils may stimulate cytokine and chemokine production in trophoblastic cells. As shown in Figure 5, levels of IL8, MCP-1 and IL6 produced by Swan-71 increased significantly upon stimulation with CM from *Brucella*-infected neutrophils, and such increase was significantly higher than that induced by uninfected cells. At 48 h post-stimulation cytokine levels specifically stimulated by the infection were 15528 pg/ml for IL8, 2464 pg/ml for IL6, and 295 pg/ml for MCP-1. In contrast, CM from infected neutrophils did not induce a significant specific TNF-α or IL1β secretion by Swan-71 cells (not shown).

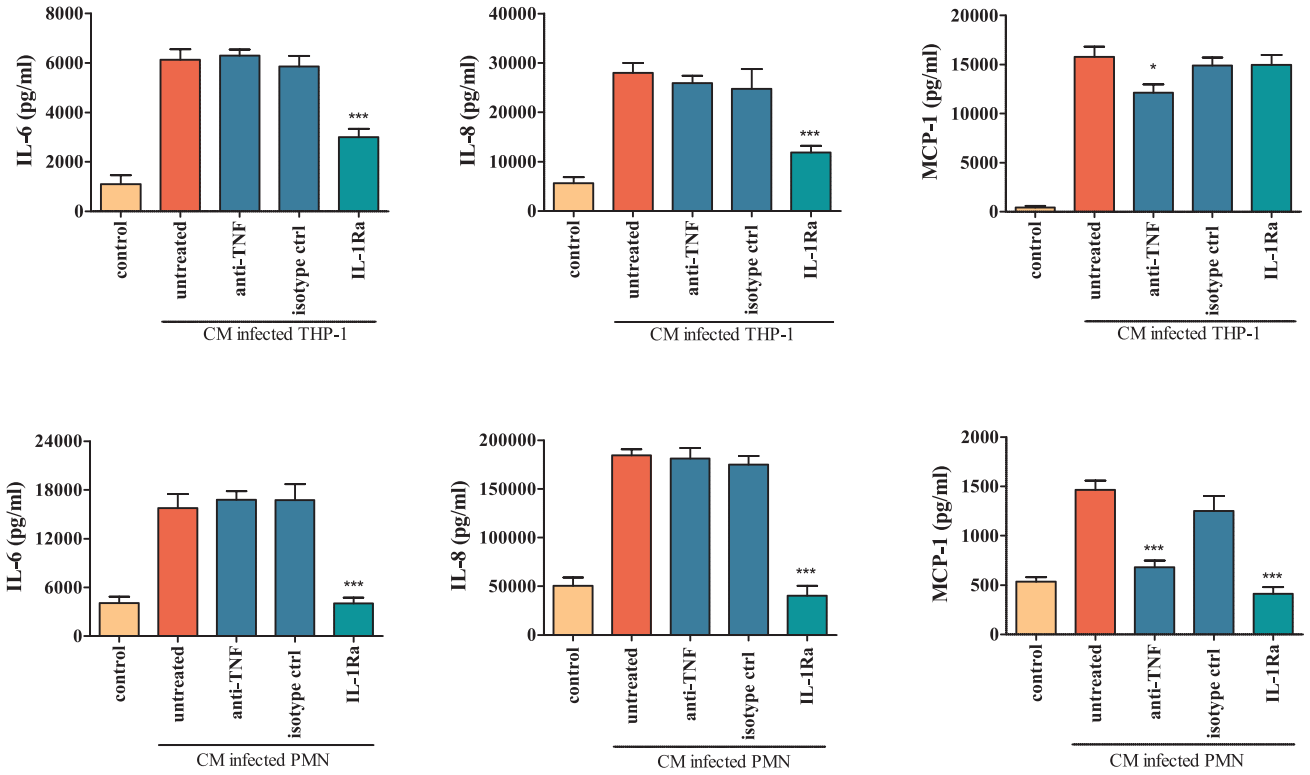

FIG. 6. IL1β and TNF-α partially mediated the stimulating effects of conditioned media (CM) from *Brucella*-infected monocytes and neutrophils on Swan-71 cells. THP-1 cells and neutrophils were infected with *B. abortus* for 24 h, and CM was used to stimulate Swan-71 cells after 1-h preincubation of CM with a neutralizing anti-TNF-α antibody (or its isotype control) or after 1-h preincubation of trophoblasts with IL1Ra. Data are means ± SEM from three independent experiments performed in triplicate.*$P < 0.05$; ***$P < 0.001$ versus untreated cells (ANOVA followed by a Tukey test).

IL1β and TNF-α Mediate the Stimulating Effect of Monocytes and Neutrophils on Cytokine and Chemokine Secretion by Swan-71 Cells

Previous studies have shown that primary human trophoblasts and some trophoblastic cell lines produce IL8 or MCP-1 in response to IL1β and TNF-α [28, 29]. We speculated that these proinflammatory cytokines, which were detected in CM from *Brucella*-infected phagocytes (Supplemental Table S1), may be also involved in the induction of IL8, MCP-1 and IL6 secretion in Swan-71 cells stimulated with CM from *Brucella*-infected monocytes and neutrophils. Given that, to our best knowledge, the chemokine response of the Swan-71 cell line to IL1β and TNF-α has not been determined, we first tested such response in vitro. The secretion of IL8, IL6, and MCP-1 by Swan-71 increased significantly upon stimulation with TNF-α and IL1β doses ranging from 1 to 10 ng/ml (Supplemental Figure S1). Next, to determine whether these proinflammatory cytokines may mediate the induction of IL8, MCP-1, and IL6 production in Swan-71 cells by CM from *Brucella*-infected monocytes and neutrophils, blocking experiments were performed using an anti-TNF-α antibody or IL1Ra. As shown in Figure 6, preincubation with IL1Ra produced a significant reduction of IL8 and IL6 secretion in trophoblasts stimulated with CM from either THP-1 cells or neutrophils infected with *B. abortus*. The induction of MCP-1 by CM from infected neutrophils was significantly abrogated by IL1Ra, and was also significantly abrogated by the anti-TNF-α antibody (but not by the isotype control). This antibody also produced a small but significant reduction of the MCP-1 increase induced by CM from infected monocytes. Collectively, these results indicate that IL1β is involved in the stimulating effect of CM from *Brucella*-infected monocytes and neutrophils on the production of IL8, IL6, and MCP-1 by Swan-71 cells. Additionally, TNF-α is also involved in the induction of MCP-1 exerted by CM from infected neutrophils and monocytes.

Human Neutrophils and Monocytes Increase Their Cytokine Secretion in Response to Factors Produced by Brucella*-Infected Trophoblasts*

Different in vitro infection models, including some performed with *Brucella* spp. [30, 31], have shown that the induction of immune mediators during the interaction of professional phagocytes with nonphagocytic cells may be bidirectional, so that factors produced by non-phagocytic cells can also induce the secretion of immune mediators by phagocytes. To test this possibility in our model, CM from *Brucella*-infected Swan-71 cells was used to stimulate THP-1 cells or human neutrophils in vitro, and proinflammatory cytokines and chemokines were measured in culture supernatants at 24 and 48 h post stimulation. As shown in Figure 7, IL6 secretion by THP-1 cells was significantly induced (compared to untreated cells) by CM from infected trophoblasts, and this effect was significantly higher than that elicited by CM from uninfected cells. No specific induction was observed on MCP-1 levels as these increased similarly upon stimulation with CM from either infected or uninfected trophoblasts at both time points. At 24 h post-stimulation IL8 levels were significantly higher in monocytes stimulated with CM from infected trophoblasts than in those stimulated with the uninfected controls, but this difference disappeared at 48 h. Figure 8 shows the results obtained when neutrophils were stimulated with CM from *Brucella*-infected Swan-71 cells. Although the

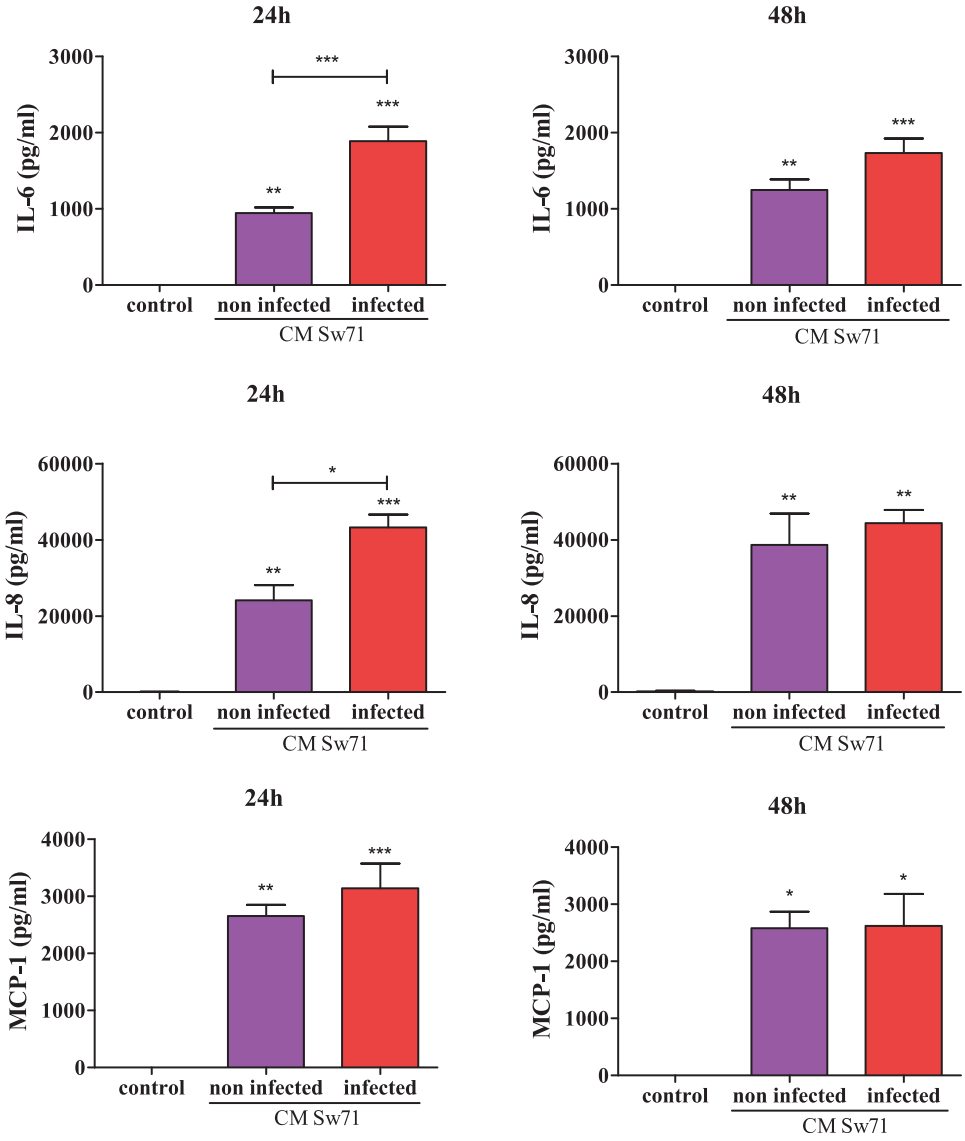

FIG. 7. Factors produced by *B. abortus*-infected Swan-71 trophoblastic cells stimulate the production of chemokines and cytokines by monocytes. Swan-71 cells were infected for 48 h with *B. abortus* and the conditioned medium (CM) was used to stimulate cultures of THP-1 cells. As a control, other cultures were stimulated with CM from non-infected Swan-71 cells. Culture supernatants were harvested at 24 and 48 h after stimulation for measuring IL6, IL8, and MCP-1 by using ELISA. Data are means ± SEM from three independent experiments performed in triplicate. Asterisks over bars indicate significant differences versus unstimulated (control) cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ANOVA followed by a Tukey test).

levels of all the cytokines tended to be higher in neutrophils stimulated with CM from infected trophoblasts than in neutrophils stimulated with CM from non-infected cells, differences only reached statistical significance for IL6. No induction of IL1β or TNF-α was detected in either monocytes or neutrophils upon stimulation with CM from either infected trophoblasts or uninfected controls (not shown).

DISCUSSION

Brucella infections have been associated with pregnancy complications, including abortion, in animals [32] and humans [3–6]. Studies performed in animals have shown that *Brucella* infects and replicates in trophoblasts, and that such infection is followed by the infiltration of inflammatory cells [8, 9]. Inflammatory responses to pathogens in placental tissues have been related to infection-triggered abortion, and in many cases trophoblasts have been shown to contribute to such inflammation [33–35]. As trophoblasts have been shown to respond to some infections with the production of proinflammatory cytokines and chemokines, the aim of the present study was to evaluate whether *Brucella* infection induces a proinflammatory response in human trophoblasts.

We found that *B. abortus* infects and replicates in the human trophoblastic cell line Swan-71. These findings agree with those reported recently by Salcedo et al. [24] who evaluated *Brucella* infection in choriocarcinoma cell lines (JEG-3, BeWo, JAR), a SV40-transformed cell line (HTR8), and primary trophoblasts isolated from term placentas. In that study, the defective expression of *virB* genes affected the replication of *B. abortus* in most cell lines and in primary trophoblasts but not in the JEG-3 cell line. Our results with Swan-71 cells are in agreement with the findings reported for primary trophoblasts as the *virB10*$^{-}$ mutant could not replicate in this cell line in spite of displaying an invasion level similar to the wild type strain at the initial time point.

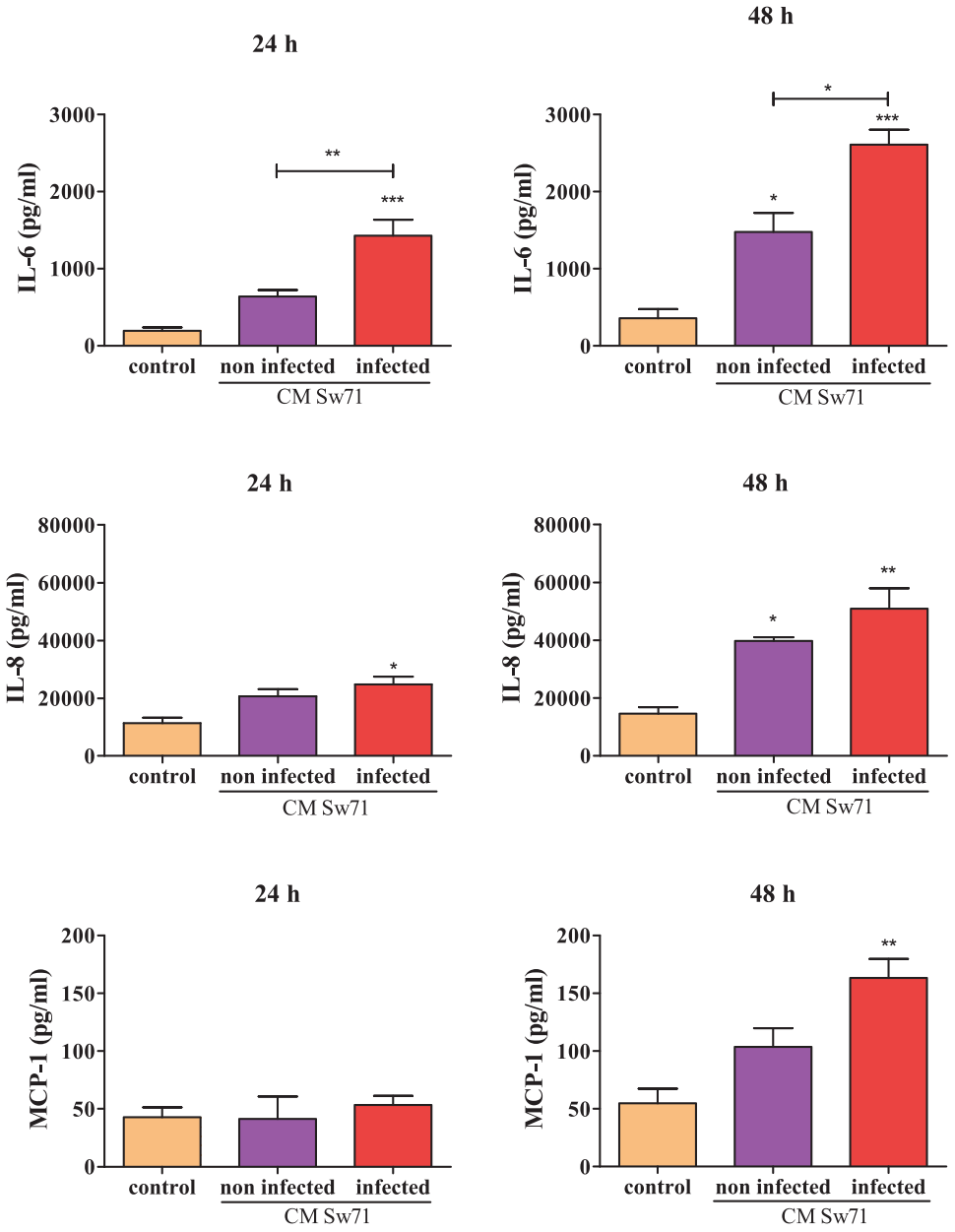

FIG. 8. Factors produced by *B. abortus*-infected Swan-71 cells stimulate the production of cytokines by neutrophils. Swan-71 cells were infected for 48 h with *B. abortus* and the conditioned medium (CM) was used to stimulate cultures of human neutrophils (5×10^5 cells/well). As a control, other cultures were stimulated with CM from non-infected Swan-71 cells. Culture supernatants were harvested at 24 and 48 h after stimulation for measuring IL6, IL8 and MCP-1 by ELISA. Data are means ± SEM from three independent experiments performed in triplicate. Asterisks over bars indicate significant differences versus unstimulated (control) cells (*$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$, ANOVA followed by a Tukey test).

Despite the known role of trophoblasts as producers of proinflammatory mediators during infections, the cytokine and chemokine response of human trophoblasts to *Brucella* spp. has not been examined except for a study that compared the changes in IL6 and TNF-α in cells infected with *B. melitensis* M5-90 or a phosphoglucomutase mutant strain [36]. The present study shows that human trophoblastic cells (Swan-71) respond to *B. abortus* infection with a significantly increased production of IL6, IL8, MCP-1, and GM-CSF. These results suggest that *Brucella*-infected human trophoblasts may mediate inflammatory responses through the recruitment of phagocytes to the infection site. The findings are in line with those reported by Carvalho Neta et al. for explants of bovine chorioallantoic membranes infected with *B. abortus* on their trophoblastic surface [9]. These authors found increased expression of IL8 mRNA at 12 h p.i. but no significant change in IL1β expression.

At variance with studies showing that the expression of the *Brucella* TIR domain-containing proteins (BtpA and BtpB) modulates the TNF-α and/or IL-12 response to this pathogen in dendritic cells [25], we did not find a significant difference in the secretion of cytokines and chemokines between cells infected with a mutant lacking these proteins and those infected with the wild type strain. Of note, in a previous study on the early transcriptional response of bovine chorioallantoic membranes to *B. abortus* only a handful of inflammation-related genes had upregulated transcription in response to infection with a Δ*btpB* mutant and the list did not include IL8, IL6 or MCP-1 [37]. Possibly, Btp proteins have a more pronounced

influence on the immune responses of professional phagocytes than on those of non-phagocytic cells.

In the natural context, trophoblasts are in contact with immune cells present in the decidua, among which macrophages represent a significant proportion [26]. The fact that macrophages are the preferred replication niche of *Brucella* spp. [27] makes likely a scenario in which *Brucella*-infected macrophages interact with adjacent trophoblasts. Thus, we wondered whether factors produced by infected monocytes or macrophages could influence the production of cytokines and chemokines by trophoblasts. Notably, CM from infected human monocytes (THP-1 cells), and macrophages stimulated the secretion of IL8, MCP-1, and IL6 by Swan-71 cells, always at levels significantly higher than those elicited by CM from noninfected phagocytes. These results suggest that, whereas trophoblasts mount a significant proinflammatory response to *Brucella* infection, they could also do so in the natural context as a result of their interaction with adjacent *Brucella*-infected cells of the monocytic/macrophagic lineage. Moreover, the levels of IL6 and MCP-1 attained during monocyte-trophoblast interactions were higher than those produced by infected trophoblasts alone, and this was especially noteworthy for MCP-1. Similarly, cytokine levels produced during macrophage-trophoblast interactions were higher than those produced by trophoblast infection. A similar scenario has been found in previous studies in which non-phagocytic cells produce a modest proinflammatory response to direct *Brucella* infection but mount a robust response upon stimulation with factors produced by *Brucella*-infected monocytes/macrophages [30, 31].

An interaction analogous to that described above can be postulated between trophoblasts and infected neutrophils, especially because the number of neutrophils in the decidua usually increases markedly during gestational infections [38, 39]. In the case of *Brucella* infection, one possible contributor to the influx of neutrophils could be the IL8 secreted by trophoblasts upon *B. abortus* infection or upon interaction with *Brucella*-infected monocytes, as commented on above. In a fashion similar to that described for monocyte-trophoblast interaction, factors produced by *Brucella*-infected neutrophils stimulated the production of IL8, MCP-1, and IL6 by trophoblastic cells. Of note, levels of IL8 produced by stimulated trophoblasts were markedly higher than those produced by *Brucella*-infected trophoblasts.

On the basis of previous studies that showed that IL8 and MCP-1 production by human trophoblasts was stimulated by IL1β and TNF-α [28, 29], we tested whether these cytokines might mediate the stimulating effects of CM from *Brucella*-infected monocytes and neutrophils. In line with those previous studies, neutralization experiments revealed that IL1β and TNF-α are involved in the stimulation of MCP-1 and IL8 secretion exerted by CM from infected phagocytes and are also involved in the stimulation of IL6 secretion.

It is interesting to note that CM from monocytes, macrophages, and neutrophils stimulated trophoblasts to secrete IL8 and MCP-1, which are chemoattractants for neutrophils and monocytes, respectively. This suggests a potential amplification mechanism by which factors produced by *Brucella*-infected phagocytes may stimulate trophoblasts to produce chemokines that recruit more phagocytes to infected tissue. Whereas neutrophils have a limited ability to control infections by smooth *Brucella* species due to resistance mechanisms of these bacteria [40], they nevertheless respond with oxidative burst and secretion of matrix metalloproteinases (MMPs) [41, 42]. Notably, both oxidative stress and an increased MMP-9 activity have been shown to induce pregnancy complications in experimental models of gestational infection [43, 44]. Thus, an increased recruitment of neutrophils to placental tissues during *Brucella* infection may have detrimental consequences for gestation.

In the context of the interaction of trophoblasts with resident or recruited phagocytes, factors produced by infected trophoblasts may also modulate the immune responses of phagocytes. In line with this hypothesis, we found that IL6 secretion by monocytes and neutrophils was significantly induced by CM from infected trophoblasts, and the same was true for IL8 secretion by monocytes. Therefore, trophoblasts may contribute to the inflammatory process of *Brucella* placental infections not only by recruiting phagocytes to the site of infection but also by stimulating these cells to secrete proinflammatory chemokines and cytokines.

Globally, a scenario can be postulated in which trophoblasts produce chemoattractants for monocytes and neutrophils both in response to *B. abortus* infection and in response to IL1β and TNF-α produced by *Brucella*-infected resident phagocytes in the placenta. Whereas the recruited monocytes/macrophages probably serve as a replication niche for *Brucella*, the recruited neutrophils may induce local tissue damage through several mechanisms. These phenomena may be amplified by the reciprocal stimulation between the three cell types through secreted cytokines, and may be long-lasting due to the ability of *Brucella* to survive and replicate within macrophages and trophoblasts. Altogether, these proinflammatory processes may contribute to the gestational complications of brucellosis.

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