# Lipoproteins, Not Lipopolysaccharide, Are the Key Mediators of the Proinflammatory Response Elicited by Heat-Killed *Brucella abortus*[1]

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**Inflammation is a hallmark of brucellosis. Although *Brucella abortus*, one of the disease's etiologic agents, possesses cytokine-stimulatory properties, the mechanism by which this bacterium triggers a proinflammatory response is not known. We examined the mechanism whereby heat-killed *B. abortus* (HKBA), as well as its LPS, induces production of inflammatory cytokines in monocytes/macrophages. Polymyxin B, a specific inhibitor of LPS activity, did not inhibit the production of TNF-α- and IL-6-induced HKBA in the human monocytic cell line THP-1. HKBA induced the production of these cytokines in peritoneal macrophages of both C3H/HeJ and C3H/HeN mice, whereas *B. abortus* LPS only stimulated cells from C3H/HeN mice. Anti-TLR2 Ab, but not anti-TLR4 Ab, blocked HKBA-mediated TNF-α and IL-6 production in THP-1 cells. Because bacterial lipoproteins, a TLR2 ligand, have potent inherent stimulatory properties, we investigated the capacity of two *B. abortus* lipoproteins, outer membrane protein 19 (Omp19) and Omp16, to elicit a proinflammatory response. Lipidated (L)-Omp16 and L-Omp19, but not their unlipidated forms, induced the secretion of TNF-α, IL-6, IL-10, and IL-12 in a time- and dose-dependent fashion. Preincubation of THP-1 cells with anti-TLR2 Ab blocked L-Omp19-mediated TNF-α and IL-6 production. Together, these results entail a mechanism whereby *B. abortus* can stimulate cells from the innate immune system and induce cytokine-mediated inflammation in brucellosis. We submit that LPS is not the cause of inflammation in brucellosis; rather, lipoproteins of this organism trigger the production of proinflammatory cytokines, and TLR2 is involved in this process.** *The Journal of Immunology,* 2004, 173: 4635–4642.

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acteria of the genus *Brucella* are Gram-negative, facultative intracellular organisms that cause severe disease in both animals and humans. Brucellosis remains endemic in many developing countries, where it undermines animal health and productivity, causing important economic losses (1). It also takes a toll in human health (2). *Brucella* organisms invade cells of the reticuloendothelial system, and can be sequestered in macrophages at specific locations within the body, such as spleen, brain, joints, heart, liver, and bone marrow (3). After infection, most patients show an acute disease phase, which can progress either to recovery or to chronicity. Inflammation is present both in the acute and chronic phases of human brucellosis and in virtually all of the organs affected by this disease. Clinical signs of such inflammation are undulant fever, endocarditis, arthritis, osteomyelitis, meningitis, pleocytosis, lymphocytic and monocytic infiltration of the joints, orchitis, nephritis, hepatic granuloma, etc. (4).

With the growing realization that cytokines play a role in inflammation, research on cytokine-mediated inflammatory reactions in brucellosis is receiving great attention. Studies conducted to date have revealed that *Brucella abortus* can induce in a variety of cell types the release of proinflammatory cytokines such as IL-1β (5–7), IL-6 (6, 7), IL-12 (8–11), and TNF-α (6, 7, 9–11). These proinflammatory cytokines generally have potent effector functions that overlap extensively with each other to bring about the many components of inflammation, e.g., tissue necrosis (12), chemotaxis of cellular infiltrates, induction of collagenase and PG secretion by sinovial fibroblast and chondrocytes (13, 14), bone resorption, and cartilage destruction (15), as well as a plethora of microbicidal mechanisms (16). Thus, potent cytokine-stimulatory properties possessed by *B. abortus* may explain the correlation between tissue invasion and localized inflammation. *B. abortus* also has been shown to induce the production of IL-10 (9, 17), a cytokine that may eventually down-modulate the proinflammatory response elicited by the bacterium (9).

The mechanism by which these bacteria trigger a proinflammatory response is not known. Most features related to pathogenicity seem to be concentrated or to act at the *Brucella* surface (18). Among the factors possibly implicated, the unusual LPS molecule located in the outer membrane of *Brucella* species has received considerable attention (19). It has been demonstrated that most of the biological activities induced by *Brucella* LPS are quantitatively and qualitatively different from the classical enterobacterial LPS (20, 21). Unlike many endotoxins, *Brucella* LPS is nonpyrogenic, it does not induce a localized Shwartzman reaction, it fails to increase the host's susceptibility to histamine or to activate complement, and is a very weak mitogen for murine B cells. At least

100 times more *Brucella* LPS than enterobacterial LPS is required to kill a host. As for induction of proinflammatory mediators, it has been demonstrated that the biological activity of *Brucella* LPS is at least 3 orders of magnitude lower than enterobacterial LPS (8, 9, 22). The weak biological activities induced by *Brucella* LPS seem to be related to the unique structure of its lipid A (21, 23).

Alternatively, *B. abortus* could use its DNA to elicit a proinflammatory response. Bacterial DNA is enriched in unmethylated CpG motifs that have been shown to activate the innate immune system (24). However, it has been demonstrated that DNA from *B. abortus* is relatively inefficient in eliciting cytokine production (9).

Recently, it has been shown that heat-killed *B. abortus* (HKBA)[3] induces the production of TNF-$\alpha$ through a TLR2-dependent pathway, independently of TLR4 and TLR9 (10). As LPS and bacterial DNA use, respectively, TLR4 and TLR9 to stimulate cytokine production, this finding supports the notion that *B. abortus* LPS and DNA are not involved in eliciting proinflammatory responses in brucellosis.

Studies conducted in our laboratory, as well as research done by others, have demonstrated that bacterial lipoproteins, a TLR2 ligand (25), are able to stimulate cellular proliferation as well as inflammatory cytokine production in normal cells. This phenomenon is caused by the lipid moiety of the protein (26–28). In addition, we have demonstrated that bacterial lipoproteins also can elicit the production of IL-10, an anti-inflammatory cytokine, in both human and monkey monocytes (29). Both autocrine and exocrine inhibition of IL-10 production resulted in an increase in the production of the proinflammatory cytokines IL-6 and IL-12 (30). The findings that lipoproteins can elicit not only inflammatory, but also anti-inflammatory mediators, e.g., IL-10 from monocytes, have added support to the contention that lipoproteins are crucial factors in the pathogenesis of bacterial infections.

It has been demonstrated by molecular cloning and sequencing that three genes encoding the outer membrane proteins (Omp) of *B. abortus*, Omp10, Omp16, and Omp19, exhibit structural features of bacterial lipoprotein precursors, namely, an amino-terminal signal peptide ending with a tetrapeptide sequence that conforms to the consensus sequence required for lipoprotein modification and processing (31, 32). The apparently correct processing of Omp10, Omp16, and Omp19 in *Escherichia coli* suggests that the pathway for lipoprotein maturation is functionally shared between *Brucella* spp. and *E. coli* (32). Physicochemical and functional analyses have recently confirmed that Omp10, Omp16, and Omp19 are lipoproteins and that they are surface exposed (33). It has also been demonstrated that these lipoproteins are present in several *Brucella* strains. These strains represent all six *Brucella* species and all their biovars.

As *B. abortus* LPS and DNA seem not to be involved in eliciting an inflammatory response, it is conceivable that *Brucella* lipoproteins might be the molecules that stimulate the cells from the innate immune system and induce cytokine-mediated inflammation in brucellosis. To test this hypothesis, we cloned, expressed in *E. coli*, and purified recombinant lipidated Omp16 (L-Omp16) and Omp19 (L-Omp19) and used them as model stimulants. The use of these (or any other) lipoproteins as a model is justified in so far as their immunological effects are elicited by the lipid, not the protein moiety. The lipid moiety is likely shared by all bacterial lipoproteins. As the model target cell we used the THP-1 human monocytic cell line. These cells were used to ensure consistent results

(26, 30). We also stimulated the THP-1 cells with HKBA. *B. abortis* cells were used to allow for inferences on whether lipoproteins or LPS were the principal stimulants of monocytes/macrophages in this bacterium. We present the results of this study in this article.

## Materials and Methods

### Mice

Four- to 6-wk-old female C3H/HeJ mice (obtained from The Jackson Laboratory, Bar Harbor, ME) and C3H/HeN (obtained from Charles River Laboratories, Wilmington, MA) were acclimated and randomly distributed into experimental groups. Mice were kept in conventional animal facilities and received water and food ad libitum.

### Bacterial strains

*E. coli* strain JM109 (Promega, Madison, WI) was used as host during the cloning experiments and for propagation of plasmids. BL21 (DE3) (Stratagene, La Jolla, CA) was used for expression of the recombinant proteins. Bacterial strains were routinely grown at 37°C in Luria-Bertani broth or agar, supplemented when required with 100 $\mu$g/ml ampicilin. *B. abortus* S2308 was cultured in Argentina in tryptose-soy agar supplemented with yeast extract (Merck, Buenos Aires, Argentina). Bacterial numbers on stationary phase cultures were determined by comparing the OD at 600 nm with a standard curve. *Brucella* organisms were washed five times for 10 min each in sterile PBS, heat killed by boiling for 20 min, aliquoted, and stored at $-70°C$ until used. Total absence of *B. abortus* viability subsequent to heat killing was verified by the absence of bacterial growth in tryptose-soy agar.

### Cloning, expression, and purification of recombinant lipidated and unlipidated Omp16 and Omp19 from B. abortus

The open reading frames of Omp16 and Omp19 were cloned in the pET 22b$^+$ vector (Novagen, Madison, WI). Briefly, the sequence information previously reported by Tibor et al. (31, 32) was used to design specific primers for the entire sequence with *Nde*I and *Xho*I restriction sites at the 5′ ends. The primers were as follows: lipidated *B. abortus* Omp16 (L-Omp16), forward, p1–5′-CTCTGGCATATGCGCCGTATC-3′; L-Omp16, reverse, p2–5′-TTGCCGCTCGAGCCGTCCGGCCCC-3′; L-Omp19, forward, p1–5′-CTGGCCCATATGCAGAGCTCCCG-3′; and L-Omp19, reverse, p2–5′-AAACTCGAGGCGCGACAGCGTCAC-3′.

The unlipidated version of the Omps devoid of the putative signal peptide and the N-terminal cysteine was cloned using different forward primers: unlipidated *B. abortus* Omp16 (U-Omp16), forward, p3–5′-CGTTGCCATATGTGCGTCAAAGAA-3′; U-Omp19, forward, p3–5′-CCTGGCCATATGCCAGAGCTCCCG-3′.

*B. abortus* 544 genomic DNA was used as template for PCR with PFU DNA polymerase (Stratagene). The ligation mix was used to transform JM109 competent cells. Miniprep plasmid DNA was purified from overnight cultures of 10 colonies using the Wizard Miniprep kit (Promega). The resulting plasmids (pET-L-Omp16, pET-U-Omp16, pET-L-Omp19, pET-U-Omp19) contained the genes with a COOH-terminal 6× histidine tag. Competent *E. coli* BL21(DE3) (Stratagene) were transformed with the corresponding constructs, and rOmps were successfully expressed after induction with isopropyl $\beta$-D-thiogalactoside (1 mM). Recombinant L-Omp16 and L-Omp19 were isolated from bacterial membranes by sonication and selective extraction by phase partitioning with 2% Triton X-114 (34). This preparation was further purified by affinity chromatography with a Ni-NTA resin (Qiagen, Dorking, U.K.). U-Omp16 and U-Omp19 were isolated from bacterial cytoplasm by sonication and were further purified by affinity chromatography with a Ni-NTA resin (Qiagen). To eliminate LPS contamination, rOmps were adsorbed with Sepharose-polymyxin B. They contained <0.25 endotoxin U/$\mu$g protein, as assessed by *Limulus* amebocyte assay (Associates of Cape Cod, Woods Hole, MA). Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL) using BSA as standard. The purified proteins were aliquoted and stored at $-70°C$ until used. Expression and purification of the recombinant proteins were monitored by SDS-PAGE, followed by silver staining. Identity of Omps was confirmed by Western blot developed with anti-Omp16 or anti-Omp19 mAbs.

### LPS and lipoproteins

*B. abortus* 2308 LPS and *E. coli* O111 K58H2 LPS were provided by I. Moriyon (University of Navarra, Pamplona, Spain). The purity and the characteristics of these preparations have been published elsewhere (35). LPS was solubilized in water by sonication at the appropriate concentration

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[3] Abbreviations used in this paper: HKBA, heat-killed *B. abortus*; Omp, outer membrane protein; L-Omp, lipidated *B. abortus* Omp; L-OspA, lipidated *B. burgdorferi* outer surface protein A; PS, poststimulation; U-Omp, unlipidated *B. abortus* Omp; U-OspA, unlipidated *B. burgdorferi* outer surface protein A.

and autoclaved before use. Recombinant lipidated *Borrelia burgdorferi* outer surface protein A (L-OspA) and unlipidated outer surface protein A (U-OspA) were obtained from J. Dunn, Brookhaven National Laboratories (Brookhaven, NY).

*Cells*

THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured, as previously described (26, 30). To induce CD14 expression, the cells were cultured in 0.05 $\mu$M 1,25-dihydroxyvitamin $D_3$ (Calbiochem-Novabiochem International, La Jolla, CA) for 48–72 h. Thioglycolate-elicited peritoneal macrophages were isolated, as previously described (36), from C3H/HeJ and C3H/HeN mice.

*Stimulation of cytokine production*

Vitamin $D_3$-treated THP-1 cells or thioglycolate-elicited peritoneal macrophages ($1 \times 10^6$/ml) were cultured in flat-bottom polypropylene 48-well plates (Corning, Corning, NY) in RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 10% heat-inactivated FBS (HyClone, Logan, UT), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.25 $\mu$g/ml fungizone per ml (RPMI 1640). For dose-response studies, cells were stimulated with HKBA, L-Omp16, U-Omp16, L-Omp19, U-Omp19, *B. abortus* LPS, *E. coli* LPS, L-OspA, or U-OspA at the indicated concentration. Cultures were incubated at 37°C in a humidified atmosphere (5% $CO_2$ and 95% air) for 24 h, except where indicated in the text. Where indicated, cultures also contained 10 $\mu$g/ml polymyxin B sulfate (Sigma-Aldrich, St. Louis, MO). At the end of the culture, cells were centrifuged at 400 × g at 4°C for 10 min, and the supernatants were aliquoted and stored at −70°C until they were used.

*Blocking of TLRs*

THP-1 cells ($1 \times 10^6$/ml) were incubated with 20 $\mu$g/ml anti-human TLR2 (clone TL2.1; eBioscience, San Diego, CA), anti-human TLR4 (clone HTA125; eBioscience), or IgG2a isotype control for 30 min at 37°C. This preparation was subsequently incubated with *E. coli* LPS, *B. abortus* LPS, L-OspA, HKBA, or L-Omp19 to reach a final concentration of 10 ng/ml *E. coli* LPS, 1000 ng/ml *B. abortus* LPS, 250 ng/ml L-OspA, $1 \times 10^8$ bacteria/ml HKBA, or 500 ng/ml L-Omp19 in a final volume of 0.5 ml. Cultures were incubated for 2 or 24 h, as in the above paragraph, and supernatants were assayed for TNF-$\alpha$ or IL-6, respectively.

*Kinetics of cytokine production*

For the study of kinetics of cytokine production, cell-free supernatants were collected after THP-1 cells had been stimulated for 2, 4, 8, 12, 24, 48, and 72 h. The concentrations of stimulants used were 500 ng/ml L-OspA, U-OspA, L-Omp16, U-Omp16, L-Omp19, U-Omp19, or 100 ng/ml *E. coli* LPS.

*Measurement of cytokine concentrations*

Human IL-6, IL-12 (p40), IL-10, and TNF-$\alpha$, and mouse IL-6 and TNF-$\alpha$ were measured in culture supernatants by sandwich ELISA using paired cytokine-specific mAbs, according to the manufacturer's instructions (BD Pharmingen, San Diego, CA).

*Statistical analysis*

Comparisons of cytokine production were made by the nonparametric Mann-Whitney *U* test with the InStat 2 Software (GraphPad, San Diego, CA).

## Results

*B. abortus is a more potent inducer of pro- and anti-inflammatory cytokines than its LPS*

The ability of HKBA and *B. abortus* LPS to induce the secretion of pro- and anti-inflammatory cytokines in THP-1 cells was determined. Cells were incubated with HKBA, *B. abortus* LPS, or *E. coli* LPS as a control, and, after 24 h of culture, the production of IL-6, IL-10, IL-12, and TNF-$\alpha$ was quantified in the culture supernatants by ELISA. The production of all cytokines was markedly enhanced in culture supernatants of THP-1 cells that were stimulated with HKBA when compared with the unstimulated cells. Cytokine production was a function of the amount of bacteria present in the culture. A significant ($p < 0.0001$) IL up-regulation was detected in cultures containing between $1 \times 10^7$ and $1 \times 10^9$
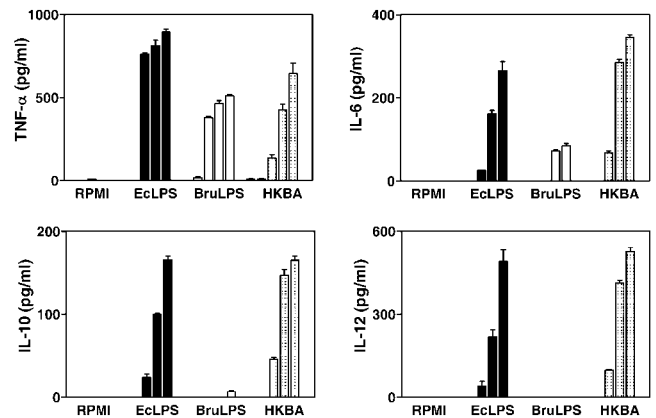


**FIGURE 1.** HKBA is a more potent inducer of pro- and anti-inflammatory cytokines than *B. abortus* LPS. THP-1 cells ($1 \times 10^6$/ml) were incubated with supplemented medium (RPMI 1640), various concentrations of HKBA (from *left* to *right*, $10^5$ to $10^9$ bacteria/ml, in multiples of 10), various concentrations of *B. abortus* LPS (BruLPS) (from *left* to *right*, 10, 100, 500, and 1000 ng/ml), or *E. coli* LPS (EcLPS) (1, 10, and 100 ng/ml). After 24 h, TNF-$\alpha$, IL-6, IL-10, and IL-12 in the cell supernatants were quantified by Ab capture ELISA. Results are expressed as the mean (pg/ml) ± SD. These experiments were performed twice in duplicate.

bacteria/ml. Cytokine production in cultures that contained $1 \times 10^6$ bacteria/ml or less dropped dramatically to the levels of unstimulated cells (Fig. 1). Conversely, *B. abortus* LPS was a weak inducer of cytokine production. At concentrations comparable to the concentrations estimated to be present in $1 \times 10^7$ to $1 \times 10^9$ bacteria/ml, *B. abortus* LPS induced the secretion of marginal
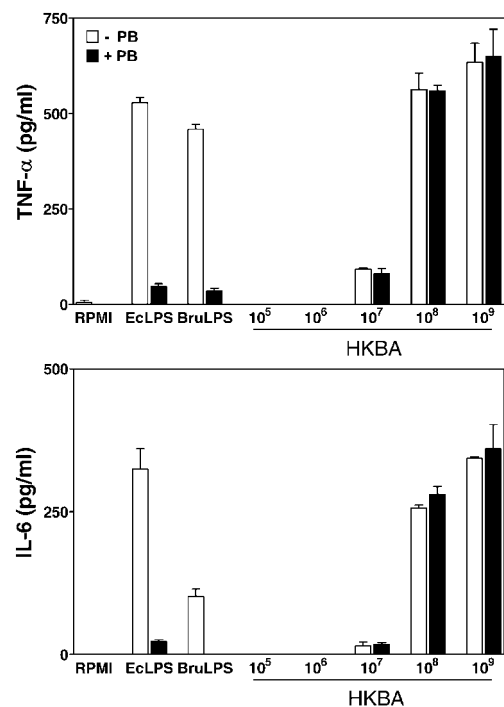


**FIGURE 2.** *B. abortus* LPS is not involved in the production of cytokines mediated by HKBA. THP-1 cells ($1 \times 10^6$/ml) were incubated for 24 h with RPMI 1640, various concentrations of HKBA, *B. abortus* LPS (BruLPS) (1000 ng/ml), or *E. coli* LPS (EcLPS) (100 ng/ml) in the presence (■) or absence (□) of polymyxin B (PB). TNF-$\alpha$ and IL-6 in the cell supernatants were quantified by Ab capture ELISA. Results are expressed as the mean (pg/ml) ± SD. These experiments were performed twice in duplicate.

amounts of IL-10 and failed to induce IL-12 production ($10^7$ *B. abortus* 2308 cells contain an estimate amount of 10 ng of LPS; similar amounts of LPS have been informed to be present in *B. abortus* RB51 cells (37)). *B. abortus* LPS was only able to induce IL-6 and TNF-$\alpha$ secretion in THP-1 cells, although significant ($p < 0.0001$) cytokine production was achieved at concentrations of 100 ng/ml or higher (Fig. 1). These results indicate that HKBA is able to induce the production of pro- and anti-inflammatory cytokines in THP-1. They also suggest that due to its low biological activity, *B. abortus* LPS seems not to be involved in eliciting such response.

### B. abortus *LPS is not involved in the production of cytokines mediated by HKBA*

Experiments were then conducted to evaluate the contribution of *B. abortus* LPS to the production of cytokines elicited by HKBA. For that purpose, THP-1 cells were incubated with HKBA, *B. abortus* LPS, or *E. coli* LPS in the presence or absence of polymyxin B, a specific inhibitor of the activity of LPS (38), and, after 24 h of culture, the production of IL-6 and TNF-$\alpha$ was evaluated in the culture supernatants by ELISA. Again, HKBA induced significant production ($p < 0.0001$) of IL-6 and TNF-$\alpha$ in a dose-response fashion. IL secretion was not due to *B. abortus* LPS, as the addition of polymyxin B had no effect on HKBA-induced cytokine production under conditions in which it completely blocked cytokine production in response to 100 ng/ml *E. coli* LPS and 1000 ng/ml *B. abortus* LPS (Fig. 2).

To further determine the contribution of *B. abortus* LPS to the production of cytokines elicited by *B. abortus*, the production of
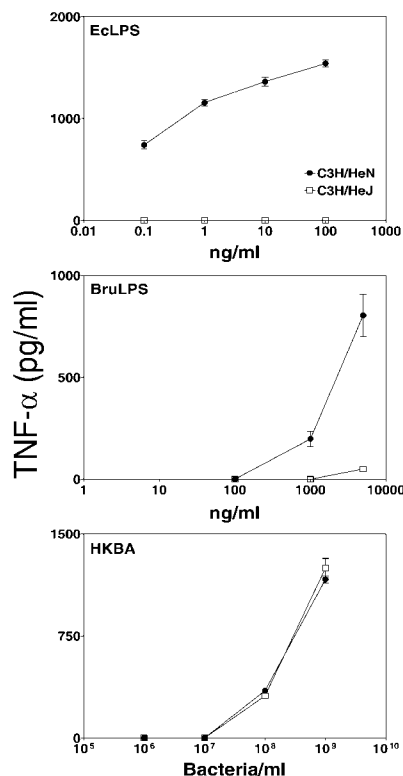


**FIGURE 3.** HKBA induction of TNF-$\alpha$ secretion in vitro is TLR4 independent. Peritoneal macrophages from C3H/HeN and C3H/HeJ mice were cultured with various concentrations of *E. coli* LPS (EcLPS), *B. abortus* LPS (BruLPS), or HKBA. After 24 h, TNF-$\alpha$ in the cell supernatants was quantified by Ab capture ELISA. Each bar represents the mean ± SD of duplicate cultures. Data are representative of two separate experiments.
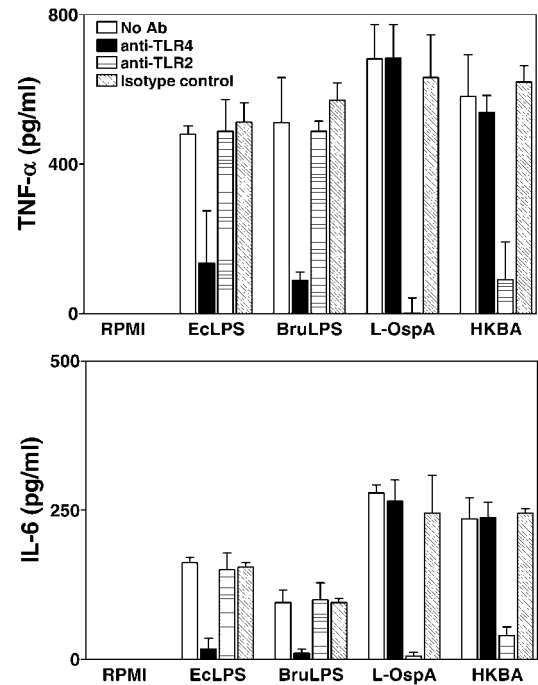


**FIGURE 4.** The production of cytokines induced by HKBA is TLR2 dependent. THP-1 cells ($1 \times 10^6$/ml) were left untreated (□), preincubated with anti-TLR4 (■), anti-TLR2 (▤), or IgG2a isotype control (▧) for 30 min before the addition of *E. coli* LPS (EcLPS) (10 ng/ml), *B. abortus* LPS (BruLPS) (1000 ng/ml), L-OspA (250 ng/ml), or HKBA ($10^8$ bacteria/ml). After 2 or 24 h, TNF-$\alpha$ or IL-6 in the cell supernatants was quantified by Ab capture ELISA. Results are expressed as the mean (pg/ml) ± SD. These experiments were performed twice in duplicate.

TNF-$\alpha$ induced by HKBA was evaluated in peritoneal macrophages from LPS-nonresponder (TLR4-mutant) C3H/HeJ mice stimulated with HKBA, *B. abortus* LPS, or *E. coli* LPS, as compared with wild-type C3H/HeN mice. C3H/HeN cells treated with *B. abortus* LPS and *E. coli* LPS produced TNF-$\alpha$ in a dose-dependent fashion. Both LPS preparations failed to induce TNF-$\alpha$ in cells from C3H/HeJ mice (Fig. 3). In contrast, HKBA was able to elicit the same amounts of TNF-$\alpha$ ($p > 0.05$) from peritoneal macrophages of either mouse strain, indicating that HKBA activation is LPS independent (Fig. 3). Similar results were obtained when IL-6 production was evaluated (data not shown).

Taken together, our results indicate that *B. abortus* LPS does not contribute to the production of cytokines induced by HKBA. The results also indicate that *B. abortus* LPS, but not HKBA, activates macrophages through TLR4.

### *The production of cytokines induced by HKBA is TLR2 dependent*

To further examine the role of the TLR4 and TLR2 in the cytokine production induced by HKBA in human monocytes, THP-1 cells were preincubated with anti-TLR2, anti-TLR4, or their respective isotype controls, and then cultured with HKBA or *B. abortus* LPS. The production of TNF-$\alpha$ and IL-6 was evaluated in culture supernatants by ELISA after culture. *E. coli* LPS and L-OspA were used as controls. As expected, preincubation of THP-1 cells with anti-TLR4 significantly blocked ($p < 0.01$) the *E. coli* LPS- and *B. abortus* LPS-mediated production of both TNF-$\alpha$ and IL-6, whereas anti-TLR2 inhibited significantly ($p < 0.01$) the cytokine production induced by L-OspA (Fig. 4). Anti-TLR2 also blocked significantly ($p < 0.01$) the HKBA-mediated production of TNF-$\alpha$ and IL-6. In contrast, anti-TLR4 had no effect on this response
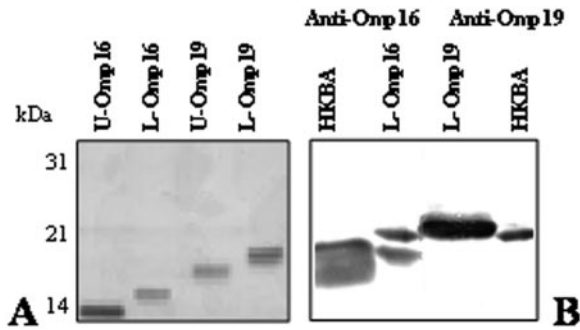
**FIGURE 5.** Purification and identification of *B. abortus* Omp16 and Omp19 lipoproteins. Recombinant U-Omp16, L-Omp16, U-Omp19, and L-Omp19 were purified by affinity chromatography, run on a 12% SDS-PAGE gel, and silver stained (*A*). L-Omp16, L-Omp19, and HKBA were run on a 15% SDS-PAGE gel, transferred to a nitrocellulose membrane, and revealed with anti-Omp16 or anti-Omp19 mAbs (*B*).

(Fig. 4). Isotype control mAbs had no effect on any of the responses studied. These results indicate that activation of monocytes and cytokine production induced by *B. abortus* depends on TLR2.

*Cloning, expression, and purification of* B. abortus *Omp16 and Omp19 lipoproteins*

As cytokine production induced by HKBA proceeds via TLR2 and taking into account that bacterial lipoproteins are potent inducers of cytokines in a variety of cells (26–28) through a TLR2-mediated pathway (25), we hypothesized that *Brucella* lipoproteins could be responsible for the cytokine stimulation seen in HKBA-stimulated THP-1 cells. To test this hypothesis, *B. abortus* lipoproteins Omp16 and Omp19 were cloned, expressed in *E. coli*, and purified to use them as model lipoprotein stimulants. Additionally, to investigate whether the active moiety of Omp16 and Omp19 was the N terminus tripalmitoyl-Cys moiety, the unlipidated version of the Omps devoid of the putative signal peptide and the N-terminal cysteine were cloned and purified. The recombinant lipoproteins were expressed in *E. coli* strain BL21(DE3). The ex-

pressed proteins were purified using nickel affinity chromatography. Each resulting preparation was of >95% purity (Fig. 5*A*). The identity of the Omp16 and Omp19 was assessed using specific mAbs. L-Omp16 and L-Omp19 have a similar m.w. than that of the native proteins (Fig. 5*B*). The mAb against Omp16 detected an additional band of slightly higher molecular mass in the L-Omp16 preparation (Fig. 5*B*). As protein acylation is a posttranslational modification, this band may correspond to a precursor form of Omp16 (33).

B. abortus *lipoproteins Omp16 and Omp19 induce the production of pro- and anti-inflammatory cytokines in the THP-1 cell line*

The ability of Omp16 and Omp19 to induce the secretion of pro- and anti-inflammatory cytokines in THP-1 cells was determined. Cells were incubated with L-Omp16, U-Omp16, L-Omp19, or U-Omp19, and, after 24 h of culture, the production of IL-6, IL-10, IL-12, and TNF-α was evaluated in the culture supernatants by ELISA. L-OspA and *E. coli* LPS were used as control. L-Omp16 and L-Omp19 induced IL-6, IL-10, IL-12, and TNF-α in a dose-dependent fashion. Significant cytokine production ($p < 0.001$) was seen with as little as 1 ng/ml L-Omp19, and maximum production was achieved with 1000 ng/ml (Fig. 6). Both Omps were similarly effective in the induction of TNF-α. L-Omp16 induced significant amounts ($p < 0.001$) of IL-6, IL-10, and IL-12 at 10–100 ng/ml. Cytokine production by lipoprotein-stimulated THP-1 cells was dependent on the lipidation of the molecules, as U-Omp16 and U-Omp19 failed to induce IL-6, IL-10, IL-12, and TNF-α at all the concentrations tested (Fig. 6). To ensure that the cytokine production was not due to *E. coli* LPS that might have been copurified with the rOmps, cultures were incubated with or without polymyxin B. Cytokine secretion was not due to LPS contamination, as the addition of polymyxin B had no effect on L-Omp16- or L-Omp19-induced cytokine production under conditions in which it completely blocked cytokine production in response to 100 ng/ml *E. coli* LPS (Fig. 6). As expected, L-OspA induced high levels of all cytokines tested, and these levels were not affected by the addition of polymyxin B. These results demonstrate that *B. abortus* lipoproteins induced pro- and anti-



**FIGURE 6.** *B. abortus* lipoproteins induce pro- and anti-inflammatory cytokines in THP-1 cells. THP-1 cells ($1 \times 10^6$/ml) were incubated for 24 h with RPMI 1640, *E. coli* LPS (EcLPS) (100 ng/ml), L-Ospa (500 ng/ml), U-OspA (500 ng/ml), or various concentrations of L-Omp19, U-Omp19, L-Omp16, and U-Omp16 (from *left* to *right*, 1, 10, 100, 500, and 1000 ng/ml) in the presence (■) or absence (□) of polymyxin B (PB). TNF-α, IL-6, IL-10, and IL-12 in the cell supernatants were quantified by Ab capture ELISA. Results are expressed as the mean (pg/ml) ± SD. These experiments were performed twice in duplicate.
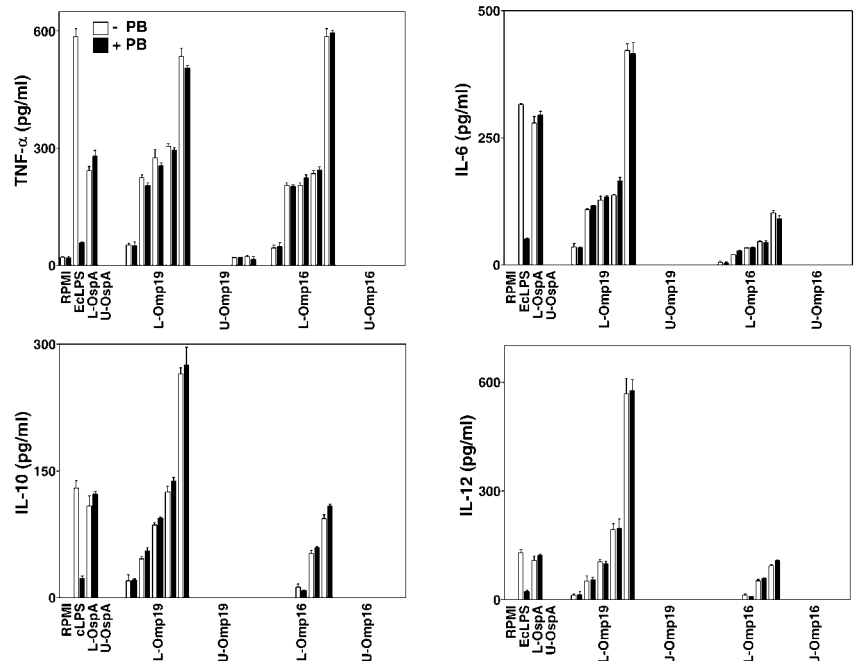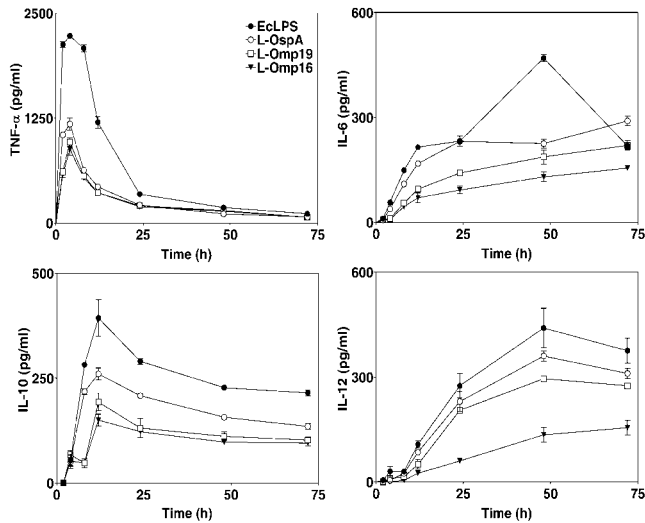
**FIGURE 7.** Kinetics of cytokine production by THP-1 cells in response to *B. abortus* lipoproteins. THP-1 cells ($1 \times 10^6$/ml) were incubated with *E. coli* LPS (EcLPS) (100 ng/ml), L-OspA (500 ng/ml), L-Omp19 (500 ng/ml), or L-Omp16 (500 ng/ml), and cell-free supernatants were collected at 2, 4, 8, 12, 24, 48, and 72 h after addition of stimulants. TNF-$\alpha$, IL-6, IL-10, and IL-12 in the cell supernatants were quantified by Ab capture ELISA. Results are expressed as the mean (pg/ml) $\pm$ SD. These experiments were performed twice in duplicate.

inflammatory cytokines in THP-1 cells. The results also indicate that the lipid modification of Omp16 and Omp19 is essential for the induction of cytokines in monocytes.

The kinetics of cytokine production by THP-1 cells also was determined using L-Omp16, U-Omp16, L-Omp19, U-Omp19, L-OspA, and *E. coli* LPS as stimulants. Cytokine concentrations



**FIGURE 8.** The production of cytokines induced by *B. abortus* lipoproteins is TLR2 dependent. THP-1 cells ($1 \times 10^6$/ml) were left untreated (□), preincubated with anti-TLR4 (■), anti-TLR2 (▤), or IgG2a isotype control (▨) for 30 min before the addition of L-Omp19 (500 ng/ml) or HKBA ($10^8$ bacteria/ml). After 2 or 24 h, TNF-$\alpha$ or IL-6 in the cell supernatants was quantified by Ab capture ELISA. Results are expressed as the mean (pg/ml) $\pm$ SD. These experiments were performed twice in duplicate.

were determined at 2, 4, 8, 16, 24, 48, and 72 h after adding the stimulants to the cultures. Neither U-Omp16 nor U-Omp19 alone induced the production of any cytokine at any time (data not shown). The concentrations of all of the cytokines studied increased rapidly within the first 20 h of stimulation with L-Omp16, L-Omp19, L-OspA, and *E. coli* LPS. After this time, cytokine concentrations either continued to increase at a lesser rate (IL-6 and IL-12) or declined gradually (IL-10 and TNF-$\alpha$) (Fig. 7). The concentration of TNF-$\alpha$ reached its peak value at 2 h poststimulation (PS), and declined sharply thereafter. The IL-10 concentration peaked between 8 and 16 h PS and declined thereafter. The concentrations of both IL-6 and IL-12 increased more slowly than those of the previous two cytokines and reached a plateau by 48 h PS.

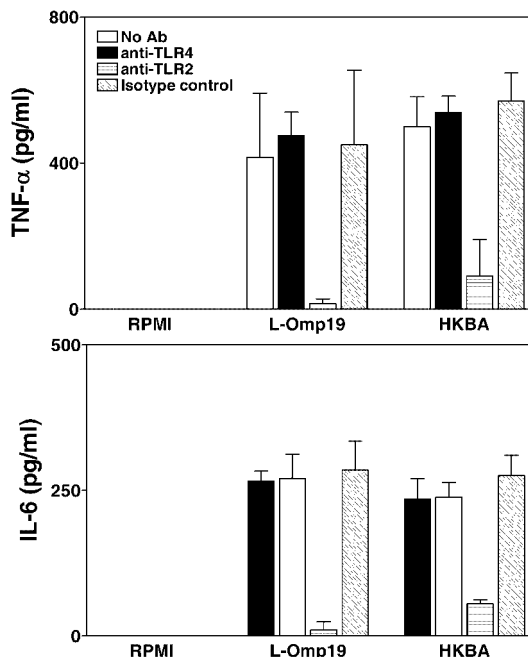### TLR2 mediates the production of cytokines induced by B. abortus lipoproteins

TLR2 has been shown previously to mediate responses to bacterial lipoproteins in cells of the monocytic lineage (39). Consequently, the role of TLR2 in mediating *B. abortus* lipoprotein-induced cytokine production was tested. THP-1 cells were preincubated with anti-TLR2 Ab, anti-TLR4 Ab, or their respective isotype controls, and then cultured with HKBA or L-Omp19. The production of TNF-$\alpha$ and IL-6 was evaluated in culture supernatants by ELISA after culture. Preincubation of THP-1 cells with anti-TLR2 significantly blocked ($p < 0.01$) L-Omp19-mediated production of both TNF-$\alpha$ and IL-6. Anti-TLR2 also inhibited significantly ($p < 0.01$) the HKBA-mediated production of TNF-$\alpha$ and IL-6 (Fig. 8). Anti-TLR4 Ab or isotype control Ab had no effect on any of the responses investigated. These results indicate that production of cytokines induced by *B. abortus* lipoproteins is TLR2 dependent.

## Discussion

The clinical manifestations of human brucellosis are protean. Yet, despite the diversity of clinical signs and symptoms, inflammation is a hallmark of this disease. Intermittent inflammatory arthritis is present in the early phases of brucellosis in humans, and some patients may go on to develop chronic inflammatory arthritis that resembles other forms of human inflammatory arthritis (4). Synovial tissue characteristically reveals a lymphocytic infiltrate and hyperplasia of synovial lining cells (40). Joint damage is often associated with presence of *Brucella* organisms in these joints, as was shown by in vitro culture of synovial tissue and fluid (41). In the liver, *Brucella* infections induce hepatic lesions that can be both granulomatous and nongranulomatous (42, 43). Histologically, granulomas show central necrosis, a polymorphic cellular infiltrate, few giant cells, and peripheral fibrosis (44). The most characteristic sign of inflammation in neurobrucellosis is meningitis, which is caused by invasion of the CNS by *Brucella* (45). Examination of the cerebrospinal fluid in *Brucella* meningitis generally reveals an elevated protein content and lymphocytic pleocytosis (45). Surgical biopsies of brain or meninges and postmortem examinations of nerve tissue yield evidence of perivascular lymphocytic infiltrates and of granuloma formation (45).

In domestic ruminants, the signs of inflammation are orchitis in males and placentitis in females (46). *Brucella* organisms can infect the bovine placenta and induce abortion. Macrophages, immunoreactive for *Brucella* Ags, neutrophils, and lymphocytic cells, can be present in the placental cellular infiltrates (47).

Although enterobacterial LPS has been clearly documented to play a central role in the pathogenesis of Gram-negative bacterial infections (48), the capacity of *Brucella* LPS to elicit an inflammatory response has been questioned (9, 10, 22). In this study, we present evidence indicating that the production of inflammatory

cytokines induced by *B. abortus* is independent of its LPS. HKBA induced the production of TNF-$\alpha$ and IL-6 in peritoneal macrophages of both C3H/HeJ and C3H/HeN mice, whereas *B. abortus* LPS only stimulated cells from C3H/HeN mice. Moreover, polymyxin B, a specific inhibitor of the activity of LPS (38), was unable to inhibit the production of TNF-$\alpha$ and IL-6 induced by HKBA in THP-1 cells.

Because bacterial lipoproteins have potent inherent stimulatory properties (26–28), we hypothesized that *B. abortus* lipoproteins were responsible for the inflammation associated with infection (4, 45). L-Omp16 and L-Omp19 induced the secretion of TNF-$\alpha$, IL-6, IL-10, and IL-12 in a time- and dose-dependent fashion. Neither U-Omp16 nor U-Omp19 induced cytokine secretion in THP-1 cells, demonstrating that acylation of the lipoprotein molecule is required for the production of cytokines in cells of the monocyte/macrophage lineage. L-Omp16 and L-Omp19 were equally potent in inducing TNF-$\alpha$ production. L-Omp16 was less efficient in inducing IL-6, IL-10, and IL-12. The reasons for this difference are unknown, but may relate to heterogeneity in the lipidation of the Omp16 preparation (see Fig. 5*B*). As different TLR are involved in the signaling of fully and partially acylated lipoproteins (49, 50), it is possible that the array of TLRs (and other) receptors involved in transducing the Omp16 signal toward production of IL-6, IL-10, and IL-12 is less efficient with partly acylated Omp16 than with Omp19, which is uniformly acylated. In that respect, experiments conducted with TLR knockout mice indicated that different TLR-mediated signaling pathways may result in the selective induction of anti-inflammatory or proinflammatory cytokines (49).

The lipid modification of *B. abortus* lipoproteins is likely to be identical in all of the lipoprotein molecules of this organism. This entails that other lipoproteins also would have the ability to induce cytokine production. Because the *B. abortus* genome contains no less than 80 genes encoding putative lipoproteins (D. Comerci, unpublished observations), it follows that lipoprotein-induced production of proinflammatory mediators could be potent enough to explain why it is that inflammation in brucellosis is manifestly overt even when the bacteria are hard to find in lesions or bodily fluids. These results also indicate that innate immune responses, together with bacterial persistence in the tissues, might lead to the inflammatory pathology of brucellosis. Indeed, splenocytes from mice with the *scid* mutation (*scid* mice), which are devoid of T and B cells, but have monocytes, produced similar levels of IL-6 than did splenocytes from immunocompetent mice when stimulated with HKBA in vitro (51).

Purified *B. abortus* LPS dispensed at high concentrations (1 $\mu$g/ml) was able to induce the production of TNF-$\alpha$ and IL-6 in monocytes. This result, obtained in vitro, is probably not relevant physiologically. As many as $10^8$ *Brucella* cells/ml, a bacillary concentration not likely to occur in vivo, elicited comparable cytokine levels, but by a pathway that could not be inhibited with polymyxin B. Hence, not even this high bacterial concentration has enough available LPS for the latter to be functionally detectable.

Our findings demonstrate that *Brucella* lipoproteins can induce not only inflammatory cytokines, but also IL-10, a potent anti-inflammatory cytokine, in the same cell type, the monocyte. That these cytokines could thus act in concert within the same microenvironment of *B. abortus*-infected tissues underscores the contention that IL-10 may play a role in the control of inflammation in brucellosis (9). Moreover, the ability of *B. abortus* lipoproteins to induce anti-inflammatory cytokines such as IL-10 might explain the focal and transient nature of inflammatory episodes during *Brucella* infections (4).

The relative involvement of TLR2 and TLR4 in mediating *B. abortus*-induced cytokine production merits discussion. Our results indicate that HKBA induces production of TNF-$\alpha$ and IL-6 via TLR2, and agree in this regard with the results obtained by Huang et al. (10). In these investigators' hands, HKBA also induced TNF-$\alpha$ production in mouse cells via TLR2. In contrast, Campos et al. (11) obtained evidence to suggest that production of TNF-$\alpha$, as induced by HKBA, was TLR4 dependent. Both investigations demonstrated, as ours did too, that *B. abortus* LPS uses TLR4. We showed, moreover, in agreement with the results of Huang et al., that LPS is not a mediator of the proinflammatory activity of HKBA, and provided, in addition, proof of concept that *B. abortus* lipoproteins would be the TLR2 ligands used by the bacterium to trigger the release of pro- and anti-inflammatory mediators.

HKBA has been proposed as a powerful adjuvant and vaccine carrier, due to its ability to induce a vigorous Th1 response, by promoting IL-12 production in cells of the innate immune system (8, 10). Our results indicate that this IL-12-mediated Th1-promoting activity may be due to a single type of molecule: *Brucella* lipoproteins. If the nature of the adjuvant activity of *B. abortus* lipoproteins is the same as that of the whole bacterium, the development of more effective Th1-inducing adjuvants based on *B. abortus*-derived components should be greatly simplified.

Finally, the finding that purified L-Omp16 and L-Omp19 stimulate cytokine production by cells from the innate immunity may be important in disease pathogenesis. The invasion of spleen, brain, joints, heart, liver, and bone marrow by *Brucella* organisms would allow the introduction of these potent molecules into the tissues. The interaction of Omp16 and Omp19, or other *B. abortus* lipoproteins, with resident cells such as macrophages could cause cytokine production, proliferation, and recruitment of inflammatory cells, leading to tissue inflammation. This supports a model in which at least three factors contribute to the development of cytokine-mediated inflammation in brucellosis. These factors are bacterial tissue invasion and persistence, interaction of lipoproteins with effector cells, and regulation of this effect by modulatory cytokines produced in the microenvironment of the invaded tissues. In view of our results, it may be that the pathologic fate of brucellosis, in particular the transient and recurrent nature of the disease's inflammatory episodes, depends on a delicate balance between pro- and anti-inflammatory responses that are elicited by brucellar lipoproteins.

## Acknowledgments

## References

1. Zavala, I., A. Nava, J. Guerra, and C. Quiros. 1994. Brucellosis. *Infect. Dis. Clin. N. Am. 8:225.*

2. López-Merino, A. 1989. Brucellosis in Latin America. In *Brucellosis: Clinical and Laboratory Aspects.* E. J. Young and M. J. Corbel, eds. CRC Press, Boca Raton, p. 151.

3. Young, E. J. 1983. Human brucellosis. *Rev. Infect. Dis. 5:821.*

4. Young, E. J. 1989. Clinical manifestations of human brucellosis. In *Brucellosis: Clinical and Laboratory Aspects.* E. J. Young and M. J. Corbel, eds. CRC Press, Boca Raton, p. 97.

5. Stevens, M. G., and S. C. Olsen. 1994. In vitro effects of live and killed *Brucella abortus* on bovine cytokine and prostaglandin $E_2$ production. *Vet. Immunol. Immunopathol. 40:149.*

6. Zhan, Y., A. Kelso, and C. Cheers. 1993. Cytokine production in the murine response to *Brucella* infection or immunization with antigenic extracts. *Immunology 80:458.*

7. Zhan, Y., and C. Cheers. 1995. Differential induction of macrophage-derived cytokines by live and dead intracellular bacteria in vitro. *Infect. Immun. 63:720.*

8. Zaitseva, M., H. Golding, J. Manischewitz, D. Webb, and B. Golding. 1996. *Brucella abortus* as a potential vaccine candidate: induction of interleukin-12 secretion and enhanced B7.1 and B7.2 and intercellular adhesion molecule 1 surface expression in elutriated human monocytes stimulated by heat-inactivated *B. abortus. Infect. Immun.* 64:3109.

9. Huang, L. Y., A. M. Krieg, N. Eller, and D. E. Scott. 1999. Induction and regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharide, and heat-inactivated bacteria. *Infect. Immun.* 67:6257.

10. Huang, L. Y., J. Aliberti, C. A. Leifer, D. M. Segal, A. Sher, D. T. Golenbock, and B. Golding. 2003. Heat-killed *Brucella abortus* induces TNF and IL-12p40 by distinct MyD88-dependent pathways: TNF, unlike IL-12p40 secretion, is Toll-like receptor 2 dependent. *J. Immunol.* 171:1441.

11. Campos, M. A., G. M. Rosinha, I. C. Almeida, X. S. Salgueiro, B. W. Jarvis, G. A. Splitter, N. Qureshi, O. Bruna-Romero, R. T. Gazzinelli, and S. C. Oliveira. 2004. Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. *Infect. Immun.* 72:176.

12. Waage, A., A. Halstensen, and T. Aspevik. 1987. Association between tumor necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet* 1:355.

13. Dayer, J. M., B. De Rochemonteix, B. Burrus, S. Demczuk, and C. A. Dinarello. 1986. Human recombinant interleukin 1 stimulates collagenase and prostaglandin E$_2$ production by human synovial cells. *J. Clin. Invest.* 77:645.

14. Evequoz, V., F. Bettens, F. Kristensen, U. Trechsel, B. M. Stadler, J. M. Dayer, A. L. De Weck, and H. Fleisch. 1984. Interleukin 2-independent stimulation of rabbit chondrocyte collagenase and prostaglandin E$_2$ production by an interleukin 1-like factor. *Eur. J. Immunol.* 14:490.

15. Vassalli, P. 1992. The pathophysiology of tumor necrosis factor. *Annu. Rev. Immunol.* 10:411.

16. Takemura, R., and Z. Werb. 1984. Secretory products of macrophages and their physiological functions. *Am. J. Physiol.* 246:C1.

17. Svetic, A., Y. C. Jian, P. Lu, F. D. Finkelman, and W. C. Gause. 1993. *Brucella abortus* induces a novel cytokine gene expression pattern characterized by elevated IL-10 and IFN-γ in CD4$^+$ T cells. *Int. Immunol.* 5:877.

18. Moreno, E., and I. Moriyon. 2002. *Brucella melitensis*: a nasty bug with hidden credentials for virulence. *Proc. Natl. Acad. Sci. USA* 99:1.

19. Cherwonogrodzsky, J. W., G. Dubray, E. Moreno, and H. Meyer. 1990. Antigens of *Brucella*. In *Animal Brucellosis*. K. Nielsen and B. Duncan, eds. CRC Press, Boca Raton, p. 19.

20. Moreno, E., D. T. Berman, and L. A. Boettcher. 1981. Biological activities of *Brucella abortus* lipopolysaccharides. *Infect. Immun.* 31:362.

21. Berman, D. T., and R. S. Kurtz. 1987. Relationship of biological activities to structure of *Brucella abortus* endotoxin and lipopolysaccharide. *Ann. Inst. Pasteur Microbiol.* 138:98.

22. Goldstein, J., T. Hoffman, C. Frasch, E. F. Lizzio, P. R. Beining, D. Hochstein, Y. L. Lee, R. D. Angus, and B. Golding. 1992. Lipopolysaccharide (LPS) from *Brucella abortus* is less toxic than that from *Escherichia coli*, suggesting the possible use of *B. abortus* or LPS from *B. abortus* as a carrier in vaccines. *Infect. Immun.* 60:1385.

23. Qureshi, N., K. Takayama, U. Seydel, R. Wang, J. Cotter, P. K. Agrawall, C. A. Bush, R. Kurtz, and D. T. Berman. 1994. Structural analysis of lipid A derived from the lipopolysaccharide of *Brucella abortus. J. Endotoxin Res.* 1:137.

24. Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20:709.

25. Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science* 285:736.

26. Giambartolomei, G. H., V. A. Dennis, B. L. Lasater, and M. T. Philipp. 1999. Induction of pro- and anti-inflammatory cytokines by *Borrelia burgdorferi* lipoproteins in monocytes is mediated by CD14. *Infect. Immun.* 67:140.

27. Ma, Y., and J. J. Weis. 1993. *Borrelia burgdorferi* outer surface lipoproteins OspA and OspB possess B-cell mitogenic and cytokine-stimulatory properties. *Infect. Immun.* 61:3843.

28. Radolf, J. D., L. L. Arndt, D. R. Akins, L. L. Curetty, M. E. Levi, Y. Shen, L. S. Davis, and M. V. Norgard. 1995. *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytes/macrophages. *J. Immunol.* 154:2866.

29. Giambartolomei, G. H., V. A. Dennis, and M. T. Philipp. 1998. *Borrelia burgdorferi* stimulates the production of IL-10 in human and rhesus monkey normal cells. *Infect. Immun.* 66:2691.

30. Giambartolomei, G. H., V. A. Dennis, B. L. Lasater, P. K. Murthy, and M. T. Philipp. 2002. Autocrine and exocrine regulation of IL-10 production in THP-1 cells stimulated with *Borrelia burgdorferi* lipoproteins. *Infect. Immun.* 70:1881.

31. Tibor, A., V. Weynants, P. Denoel, B. Lichtfouse, X. De Bolle, E. Saman, J. N. Limet, and J. J. Letesson. 1994. Molecular cloning, nucleotide sequence, and occurrence of a 16.5-kilodalton outer membrane protein of *Brucella abortus* with similarity to pal lipoproteins. *Infect. Immun.* 62:3633.

32. Tibor, A., E. Saman, P. de Wergifosse, A. Cloeckaert, J. N. Limet, and J. J. Letesson. 1996. Molecular characterization, occurrence, and immunogenicity in infected sheep and cattle of two minor outer membrane proteins of *Brucella abortus. Infect. Immun.* 64:100.

33. Tibor, A., B. Decelle, and J. J. Letesson. 1999. Outer membrane proteins Omp10, Omp16, and Omp19 of *Brucella* spp. are lipoproteins. *Infect. Immun.* 67:4960.

34. Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard. 1998. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. pallidum by phase partitioning with the nonionic detergent Triton X-114. *Infect. Immun.* 56:490.

35. Velasco, J., J. A. Bengoechea, K. Brandenburg, B. Lindner, U. Seydel, D. González, U. Zähringer, E. Moreno, and I. Moriyón. 2000. *Brucella abortus* and its closest phylogenetic relative, *Ochrobactrum* spp., differ in outer membrane permeability and cationic peptide resistance. *Infect. Immun.* 68: 3210.

36. Munford, R. S., and C. L. Hall. 1985. Uptake and deacylation of bacterial lipopolysaccharides by macrophages from normal and endotoxin-hyporesponsive mice. *Infect. Immun.* 48:464.

37. Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. *Physiology of the Bacterial Cell*. Sinauer Associates, Sunderland, p. 4.

38. Morrison, D. C., and D. M. Jacobs. 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* 13:813.

39. Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. *J. Immunol.* 163:2382.

40. Gotuzzo, E., G. S. Alarcon, T. S. Bocanegra, C. Carrillo, J. C. Guerra, I. Rolando, and L. R. Espinoza. 1982. Articular involvement in human brucellosis: a retrospective analysis of 304 cases. *Semin. Arthritis Rheum.* 12:245.

41. Kelly, P. J., W. J. Martin, A. Schirger, and L. A. Weed. 1960. Brucellosis of the bones and joints: experience with sixty-six patients. *J. Am. Med. Assoc.* 174:347.

42. Young, E. J. 1979. *Brucella melitensis* hepatitis: the absence of granulomas. *Ann. Intern. Med.* 91:414.

43. Jordans, H. G., and K. D. DeBruin. 1980. Granulomas in *Brucella melitensis* infection. *Ann. Intern. Med.* 92:264.

44. Colmenero, J. D., M. I. Queipo-Ortuno, M. J. Reguera, A. M. Suarez-Munoz, S. Martin-Carballino, and P. Morata. 2002. Chronic hepatosplenic abscesses in brucellosis: clinico-therapeutic features and molecular diagnostic approach. *Diagn. Microbiol. Infect. Dis.* 42:159.

45. Bouza, E., M. Garcia de la Torre, F. Parras, A. Guerrero, M. Rodriguez-Creixems, and J. Gobernado. 1987. Brucellar meningitis. *Rev. Infect. Dis.* 9:810.

46. Enright, F. M. 1990. The pathogenesis and pathobiology of *Brucella* infection in domestic animals. In *Animal Brucellosis*. K. Nielsen and J. R. Duncan, eds. CRC Press, Boca Raton, p. 301.

47. Palmer, M. V., N. F. Cheville, and A. E. Jensen. 1996. Experimental infection of pregnant cattle with the vaccine candidate *Brucella abortus* strain RB51: pathologic, bacteriologic, and serologic findings. *Vet. Pathol.* 33:682.

48. Beutler, B. 2002. LPS in microbial pathogenesis: promise and fulfillment. *J. Endotoxin Res.* 8:329.

49. Alexopoulou, L., V. Thomas, M. Schnare, Y. Lobet, J. Anguita, R. T. Schoen, R. Medzhitov, E. Fikrig, and R. A. Flavell. 2002. Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice. *Nat. Med.* 8:878.

50. Takeuchi, O., T. Kawai, P. F. Muhlradt, M. Morr, J. D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13:933.

51. Liu, Z., and C. Cheers. 1993. The cellular source of interleukin-6 during *Listeria* infection. *Infect. Immun.* 61:2626.