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Brucella abortus Inhibits Major Histocompatibility Complex Class II Expression and Antigen Processing through Interleukin-6 Secretion via Toll-Like Receptor 2[▽]

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The strategies that allow *Brucella abortus* to survive inside macrophages for prolonged periods and to avoid the immunological surveillance of major histocompatibility complex class II (MHC-II)-restricted gamma interferon (IFN-γ)-producing CD4$^+$ T lymphocytes are poorly understood. We report here that infection of THP-1 cells with *B. abortus* inhibited expression of MHC-II molecules and antigen (Ag) processing. Heat-killed *B. abortus* (HKBA) also induced both these phenomena, indicating the independence of bacterial viability and involvement of a structural component of the bacterium. Accordingly, outer membrane protein 19 (Omp19), a prototypical *B. abortus* lipoprotein, inhibited both MHC-II expression and Ag processing to the same extent as HKBA. Moreover, a synthetic lipohexapeptide that mimics the structure of the protein lipid moiety also inhibited MHC-II expression, indicating that any *Brucella* lipoprotein could down-modulate MHC-II expression and Ag processing. Inhibition of MHC-II expression and Ag processing by either HKBA or lipidated Omp19 (L-Omp19) depended on Toll-like receptor 2 and was mediated by interleukin-6. HKBA or L-Omp19 also inhibited MHC-II expression and Ag processing of human monocytes. In addition, exposure to the synthetic lipohexapeptide inhibited Ag-specific T-cell proliferation and IFN-γ production of peripheral blood mononuclear cells from *Brucella*-infected patients. Together, these results indicate that there is a mechanism by which *B. abortus* may prevent recognition by T cells to evade host immunity and establish a chronic infection.

Infection with *Brucella abortus* has been shown to potently activate both the innate and adaptive arms of the immune system, leading to a proinflammatory response that favors the differentiation of T-cell responses toward a T-helper 1 (Th1) profile (15, 55–57). Despite this immune response, *B. abortus* can persist for years inside macrophages, evading host immune responses.

Macrophages are an early barrier for defense against *Brucella*. They phagocytose and degrade invading microorganisms, participating actively in innate immunity. Additionally, by processing microorganisms within intracellular compartments, they present peptides in the context of the major histocompatibility complex (MHC) to T lymphocytes, promoting the adaptive immune response. Gamma interferon (IFN-γ) has a critical role in protective immunity against *Brucella*. This cytokine enhances both the microbicidal and antigen-presenting functions of macrophages (12, 18, 19, 48). Thus, the virulence of *B. abortus* relies on the ability of this organism to survive and replicate within vacuolar phagocytic compartments of macrophages (26, 30), and the macrophage-*Brucella* interaction is critical for the establishment of chronic *Brucella* infections.

Once inside macrophages, pathogens use a large array of strategies to evade or counteract host immune responses. For example, they can diminish or abrogate their antigen (Ag) presentation capacity, thus reducing T-cell-mediated immune responses (21, 37, 43). The mechanisms and pathogen factors involved in this process have been shown to differ from one pathogen to another, but globally the cause of the phenomenon remains unclear. It has been found that pathogens possess conserved molecular patterns termed pathogen-associated molecular patterns (PAMPs) (2), many of which signal through Toll-like receptors (TLRs). The PAMPs include CpG DNA (which signals via TLR9), *Escherichia coli* lipopolysaccharide (LPS) (which signals via TLR4), and bacterial lipoproteins (which signal via TLR2), among others. Recently, it has been demonstrated that prolonged exposure to *Mycobacterium tuberculosis* 19-kDa lipoprotein, as well as LPS and CpG DNA, inhibits MHC class II (MHC-II) expression and Ag processing and presentation by macrophages, which may allow certain pathogens to evade immune surveillance and promote chronic infection (9, 38, 51).

The strategies that allow *B. abortus* to survive for prolonged periods inside macrophages in the face of vigorous Th1-type responses are not completely understood. We have demonstrated that *Brucella* is able to dampen these Th1 responses during the chronic phase of the disease in humans (24). In addition, it has been demonstrated that *B. abortus* LPS, despite its low endotoxic activity compared with the activity of LPS from enterobacteria (25, 31), acts as a down-regulator of T-cell

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activation in murine peritoneal macrophages, impairing the MHC-II presentation pathway (20). This phenomenon is due to the formation of LPS macrodomains at the cell plasma membrane which interfere with the MHC-II presentation of peptides to specific T-cell hybridomas. However, little is known about other potential mechanisms or factors by which *B. abortus* may evade T-cell responses and promote chronic infection.

In this study we evaluated the effect of *B. abortus* on MHC-II expression and Ag presentation in human monocytes/macrophages. As model target cells we used the THP-1 human monocytic cell line. We first elucidated the ability of *B. abortus* to induce the down-modulation of MHC-II expression upon macrophage infection. Once the phenomenon was corroborated, we investigated the role of *Brucella* lipoproteins in the inhibition of MHC-II expression and Ag processing mediated by *B. abortus*. To do this, we used purified recombinant outer membrane protein 19 (Omp19) from *B. abortus* as the model stimulant. Here, we present the results of this study.

MATERIALS AND METHODS

Bacteria. *B. abortus* S2308 and *B. ovis* REO 198 were cultured in tryptose soy agar supplemented with yeast extract (Merck, Buenos Aires, Argentina). The numbers of bacteria in stationary-phase cultures were determined by comparing the optical densities at 600 nm with a standard curve. Where indicated below, *Brucella* cells were washed five times for 10 min each in sterile phosphate-buffered saline, heat killed at 70°C for 20 min, aliquoted, and stored at −70°C until they were used. The total absence of *B. abortus* viability after heat killing was verified by the absence of bacterial growth on tryptose soy agar.

Cloning, expression, and purification of recombinant lipidated Omp19 (L-Omp19) and unlipidated Omp19 (U-Omp19) from *B. abortus*. Lipoproteins were cloned and purified as previously described (25). To eliminate LPS contamination, recombinant Omps were adsorbed with Sepharose-polymyxin B (Sigma, St. Louis, MO). These proteins contained <0.25 endotoxin unit/μg protein, as assessed by the *Limulus* amebocyte assay (Associates of Cape Cod, Woods Hole, MA). The protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as the standard. The purified proteins were aliquoted and stored at −70°C until they were used.

LPS and antigens. *B. abortus* 2308 LPS and *E. coli* O111 strain K58H2 LPS were provided by I. Moriyón (University of Navarra, Pamplona, Spain). The purity and characteristics of these preparations have been described elsewhere (54). LPS was solubilized in water by sonication at the appropriate concentration and autoclaved before use. Recombinant Ag85B of *M. tuberculosis* was obtained from John T. Belisle (Colorado State University, Fort Collins). *B. abortus* cytoplasmic proteins (CP) were obtained as described previously (24). The lipohexapeptide tripalmitoyl-*S*-glyceryl-Cys-Ser-Lys_4-OH (Pam_3Cys) was obtained from Boehringer Mannheim (Indianapolis, IN).

Cells and media. Unless otherwise specified, all experiments were performed at 37°C in a 5% CO_2 atmosphere using standard medium composed of RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Gibco-BRL Life Technologies, Grand Island, NY), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured as previously described (25). To induce maturation, the cells were cultured in the presence of 0.05 μM 1,25-dihydroxyvitamin D_3 (Calbiochem-Nova Biochem International, La Jolla, CA) for 48 to 72 h. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation from human blood collected either from healthy adult individuals or from patients with active acute brucellosis (24). All volunteers gave informed consent prior to participation in the study. Monocytes were obtained after centrifugation of PBMCs on a Percoll (GE Healthcare Bio-Sciences) gradient and were resuspended in standard medium. The T-cell hybridoma DB1 (Ag85B specific) was kindly provided by W. H. Boom (Case Western Reserve University, Cleveland, OH) and was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented as indicated above. The viability of cells was more than 95% in all the experiments, as measured by a trypan blue exclusion test.

Flow cytometry. Vitamin D_3-treated THP-1 cells, monocytes, or PBMCs at a concentration of 0.5×10^6 cells/ml were incubated in round-bottom polypropylene tubes (Sarstedt, Nümbrecht, Germany) with 150 U/ml of recombinant human IFN-γ (Endogen, Rockford, IL) in the absence or presence of heat-killed *B. abortus* (HKBA), *B. abortus* LPS, L-Omp19, U-Omp19, Pam_3Cys, *E. coli* LPS, or *M. tuberculosis* lysate at the indicated concentration for 48 h. At the end of culture, cells were washed and incubated with fluorescein isothiocyanate-labeled anti-human HLA-DR monoclonal antibody (MAb) (clone L243; BD Pharmingen, San Diego, CA), anti-human-CD11b MAb (clone Bear 1; Beckman Coulter, Fullerton, CA) (only THP-1 cells), or isotype-matched control antibody (Ab) for 30 min on ice. The cells were then washed and analyzed with a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ), using CellQuest software (Becton-Dickinson). The results were expressed as mean fluorescence intensities (arithmetic means ± standard errors of the means).

In infection experiments, 0.5×10^6 THP-1 cells/ml were infected with *B. abortus* at different multiplicities of infection in the presence of IFN-γ (150 U/ml; Endogen) for 2 h in standard medium containing no antibiotics. Then the cells were extensively washed to remove uninternalized bacteria, and the infected cells were maintained in the presence of IFN-γ for an additional 48 h. After this, flow cytometry was conducted as indicated above. To monitor *Brucella* intracellular survival, concomitantly infected cells were lysed with 0.1% (vol/vol) Triton X-100 in H_2O after washing with phosphate-buffered saline, and serial dilutions of lysates were rapidly plated onto tryptose soy agar plates to enumerate the CFU.

To block TLRs, 0.5×10^6 THP-1 cells/ml were incubated with 20 μg/ml of anti-human TLR2 (clone TL2.1; eBioscience, San Diego, CA), anti-human TLR4 (clone HTA125; eBioscience), or an isotype-matched control for 30 min at 37°C. Following incubation with MAbs, cells were cultured with HKBA (1×10^8 bacteria/ml), L-Omp19 (100 ng/ml), LPS (50 ng/ml), or Pam_3Cys (10 ng/ml) in the presence of IFN-γ (150 ng/ml; Endogen) for 48 h as described above. After this, flow cytometry was conducted as indicated above.

Ag processing and presentation assays. THP-1 cells were incubated in 96-well flat-bottom plates (1.5×10^5 cells/well) with 10 ng/ml of phorbol myristate acetate (Sigma) in DMEM for 24 h to promote adherence to plates. The cells were washed once with DMEM and incubated with 150 U/ml of IFN-γ (Endogen) with or without HKBA, L-Omp19, U-Omp19, or *B. abortus* LPS at the indicated concentrations for 24 h. Following incubation, the medium was removed, and the cells were extensively washed prior to Ag exposure. The cells then were pulsed with Ag85B (0.1 to 30 μg/ml) for 6 h, followed by incubation with DB1 T hybridoma cells (10^5 cells/well). Supernatants were harvested after 20 to 24 h, and the amount of interleukin-2 (IL-2) produced by T hybridoma cells was determined by a standard enzyme-linked immunosorbent assay (ELISA).

Effect of IL-6 on HLA-DR expression and Ag processing. To study the effect of endogenous IL-6, 0.5×10^6 THP-1 cells/ml were incubated with 150 U/ml of IFN-γ (Endogen) and HKBA (1×10^8 bacteria/ml) or L-Omp19 (100 ng/ml) in the presence of neutralizing MAb to IL-6 (clone MQ2-13A5; eBioscience) or the respective isotype controls at a concentration of 20 μg/ml. After this, HLA-DR expression and Ag processing and presentation were evaluated as indicated above. To study the effect of exogenous IL-6 on HLA-DR expression, 0.5×10^6 THP-1 cells/ml were stimulated with 150 U/ml of IFN-γ (Endogen) in the presence or absence of different concentrations of human recombinant IL-6 (eBioscience) for 48 h. After culture, flow cytometry was conducted as indicated above.

Proliferation assay. A proliferation assay was performed as previously described (24), using mycobacterial purified protein derivative (PPD) (10 μg/ml) or CP (10 μg/ml) as the Ag. The results were expressed as the difference in the number of cpm (cpm of stimulated cultures − cpm of unstimulated cultures).

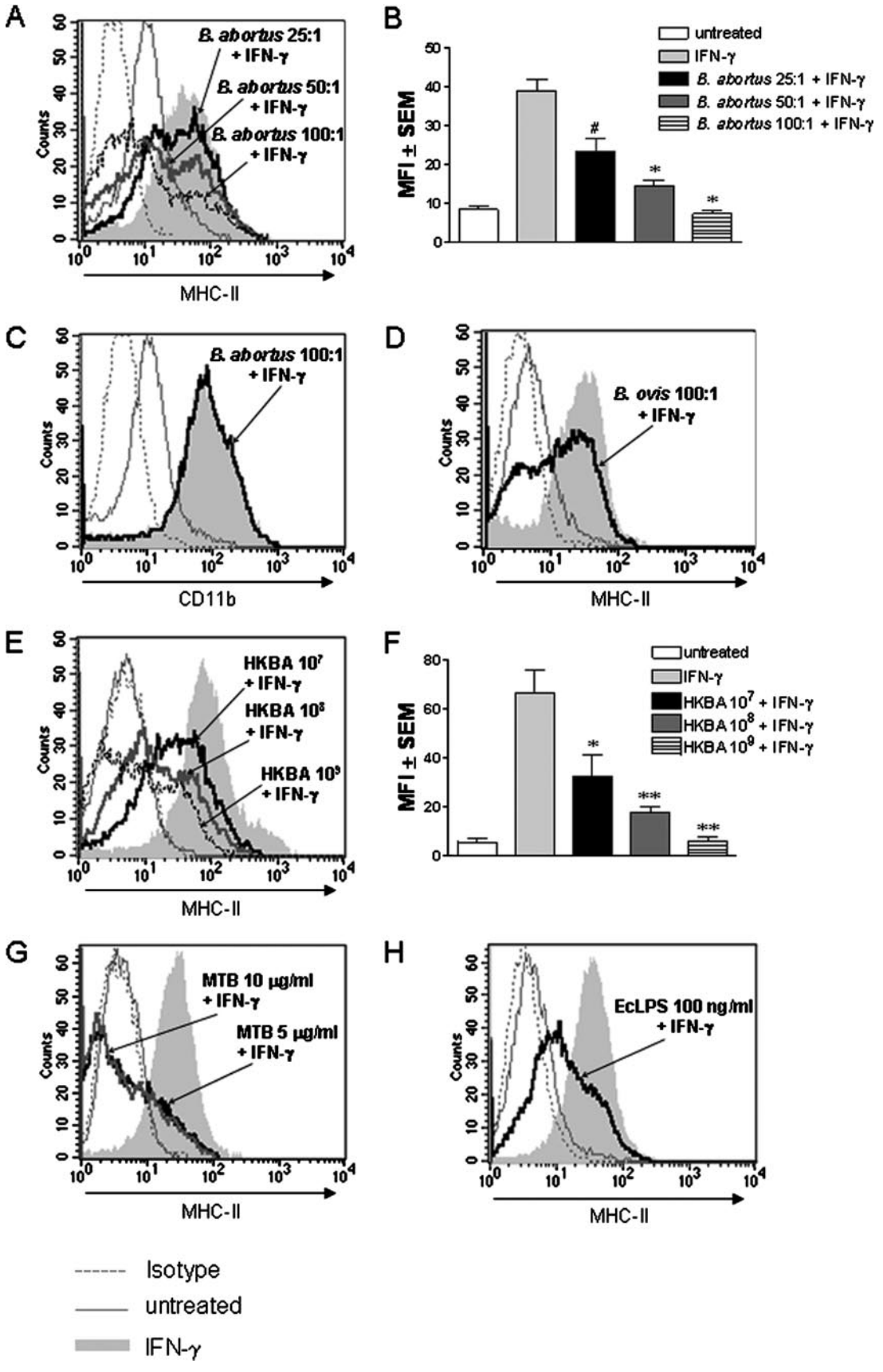
Stimulation of cytokine production. Cytokine production was stimulated as previously described (24), using PPD (10 μg/ml) or CP (10 μg/ml) as the Ag.

Measurement of cytokine concentrations. Concentrations of human IL-2 and IFN-γ and mouse IL-2 in culture supernatants were measured by a sandwich ELISA using paired cytokine-specific MAbs according to the manufacturer's instructions (BD PharMingen).

Statistical analysis. The statistical significance of results was calculated using the nonparametric Mann-Whitney U test with the InStat 2 software (GraphPad Software Inc., San Diego, CA).

RESULTS

***B. abortus* down-modulates the IFN-γ-induced expression of MHC-II molecules.** The ability of *B. abortus* to inhibit the IFN-γ-induced expression of MHC-II molecules on human THP-1 cells was determined. Cells were infected with *B. abortus* in the presence of IFN-γ for 2 h and washed to remove



uninternalized bacteria, and the infection was maintained in the presence of IFN-γ for an additional 48 h. The expression of MHC-II molecules (HLA-DR) was evaluated by flow cytometry. *B. abortus* infection inhibited the IFN-γ-induced surface expression of MHC-II molecules in a dose-dependent manner (Fig. 1A and B). Significant ($P < 0.05$) MHC-II down-modulation was detected in cultures infected with 25 or more bacteria per cell. In contrast, the surface expression of CD11b in control cells did not differ from the surface expression of CD11b in infected cells (Fig. 1C), indicating that *B. abortus* did not down-modulate all surface molecules globally. Inhibition was not due to a loss of cell viability in infected cultures. Two independent measures, trypan blue exclusion and the Annexin V assay, revealed no differences in the percentages of viable cells in infected and uninfected cultures, even at the highest infection level (data not shown). Infection of THP-1 cells with *B. ovis* also inhibited the expression MHC-II molecules (Fig. 1D). To test whether viable bacteria were necessary to induce inhibition of MHC-II expression, the ability of HKBA to down-modulate IFN-γ-induced MHC-II expression was examined. HKBA also inhibited the IFN-γ-induced expression of MHC-II. Inhibition was a function of the amount of bacteria present in the culture (Fig. 1E and F). Significant ($P < 0.01$) MHC-II down-modulation was detected at bacterium/cell ratios similar to the ratios used for infection, and the level of inhibition was similar to that obtained with *M. tuberculosis* lysate or *E. coli* LPS (Fig. 1G and H), two well-known down-modulators of MHC-II expression (51) used as controls. These results suggest that MHC-II inhibition is caused by a constitutive component of *B. abortus*. This component might also be present in other *Brucella* species, such as *B. ovis*.

***B. abortus* LPS is unable to down-modulate the expression of MHC-II molecules induced by IFN-γ.** As mentioned above, despite the low endotoxic activity of *B. abortus* LPS compared with the activity of LPS of enterobacteria (25, 31), it has been demonstrated that *B. abortus* LPS impairs the MHC-II presentation pathway (20). Thus, experiments were conducted to evaluate the contribution of *B. abortus* LPS to the down-modulation of MHC-II expression mediated by HKBA. To do this, THP-1 cells were incubated with HKBA as described above but in the presence or absence of polymyxin B, a specific inhibitor of the activity of LPS (34), and after 48 h of incubation the expression of MHC-II molecules was evaluated by flow cytometry. Again, HKBA significantly ($P < 0.01$) inhibited the IFN-γ-induced surface expression of MHC-II molecules in a dose-dependent fashion (Fig. 2A and C). MHC-II down-modulation was not due to *B. abortus* LPS, as addition of polymyxin B had no effect on HKBA-induced MHC-II down-modulation (Fig. 2B and C) under conditions in which it completely blocked MHC-II down-modulation in response to 100 ng/ml of *E. coli* LPS (Fig. 2D and E). Moreover, a high concentration (5,000 ng/ml) of highly purified *B. abortus* LPS was unable to reduce the IFN-γ-induced expression of MHC-II (Fig. 2F). Taken together, our results indicate that *B. abortus* LPS does not contribute to the down-modulation of MHC-II induced by HKBA, corroborating and extending previous results obtained with murine macrophages (20).

***B. abortus* lipoprotein Omp19 down-modulates the MHC-II expression induced by IFN-γ.** As *B. abortus* LPS is not involved in the down-regulation of MHC-II expression and taking into account the finding that other bacterial lipoproteins inhibit IFN-γ-induced MHC-II expression (22, 23, 38, 41), we hypothesized that *Brucella* lipoproteins could be the constitutive components involved in MHC-II down-modulation. To test this hypothesis, we used recombinant L-Omp19 as a *Brucella* lipoprotein model. THP-1 cells were incubated with IFN-γ in the presence or absence of L-Omp19, and after 48 h of incubation the IFN-γ-induced expression of MHC-II was evaluated by flow cytometry. L-Omp19 down-modulated the expression of MHC-II in a dose-dependent fashion. Significant inhibition ($P < 0.01$) was seen with as little as 10 ng/ml of L-Omp19, and maximum inhibition was observed with 1,000 ng/ml (Fig. 3A). To ensure that MHC-II down-modulation was not due to *E. coli* LPS that might have been copurified with the recombinant L-Omp19, cultures were incubated with or without polymyxin B. Inhibition was not due to LPS contamination, as addition of polymyxin B had no effect on L-Omp19-induced MHC-II down-modulation (Fig. 3A). Inhibition of MHC-II expression was dependent on the lipidation of L-Omp19, as U-Omp19 failed to down-modulate MHC-II expression even at a concentration of 5,000 ng/ml (Fig. 3B). To ascertain whether the effects elicited by L-Omp19 could be extended to all *B. abortus* lipoproteins, THP-1 cells were incubated with various concentrations of a synthetic lipohexapeptide (Pam_3Cys) that mimics the structure of the lipoprotein lipid moiety, and the expression of MHC-II was evaluated by flow cytometry after 48 h of stimulation. The range of Pam_3Cys concentrations used encompassed the molar concentration of Omp19 used (1,000 ng/ml). Pam_3Cys inhibited MHC-II expression to a degree that was commensurate with the degree of inhibition induced by L-Omp19 (Fig. 3C). These results indicate that the Pam_3-modified cysteine is the molecular structure that down-modulates the IFN-γ-induced expression of MHC-II; thus, this down-modulation could be brought about by any *B. abortus* lipoprotein.

Inhibition of MHC-II expression mediated by HKBA and L-Omp19 is TLR2 dependent. TLR2 has been shown previously to mediate responses to bacterial lipoproteins in cells of

FIG. 1. *B. abortus* down-modulates the IFN-γ-induced expression of MHC-II molecules. (A and B) THP-1 cells were infected with *B. abortus* at different multiplicities of infection in the presence of IFN-γ (150 U/ml) for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. (C) THP-1 cells were infected with *B. abortus* as described above. (D) THP-1 cells were infected as described above but with *B. ovis*. (E and F) THP-1 cells were incubated with medium (untreated), IFN-γ (150 U/ml), or HKBA plus IFN-γ for 48 h. (G and H) THP-1 cells were incubated with medium (untreated), IFN-γ (150 U/ml), or *M. tuberculosis* lysate (MTB) plus IFN-γ (G) or with *E. coli* LPS (EcLPS) plus IFN-γ (H) for 48 h. MHC-II and CD11b (C) expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. The bars in panels B and F indicate the arithmetic means of five experiments, and the error bars indicate the standard errors of the means. MFI, mean fluorescence intensity. Nonspecific binding was determined using a control isotype Ab. Number sign, $P < 0.05$ for a comparison with IFN-γ; one asterisk, $P < 0.01$ for a comparison with IFN-γ; two asterisks, $P < 0.001$ for a comparison with IFN-γ.

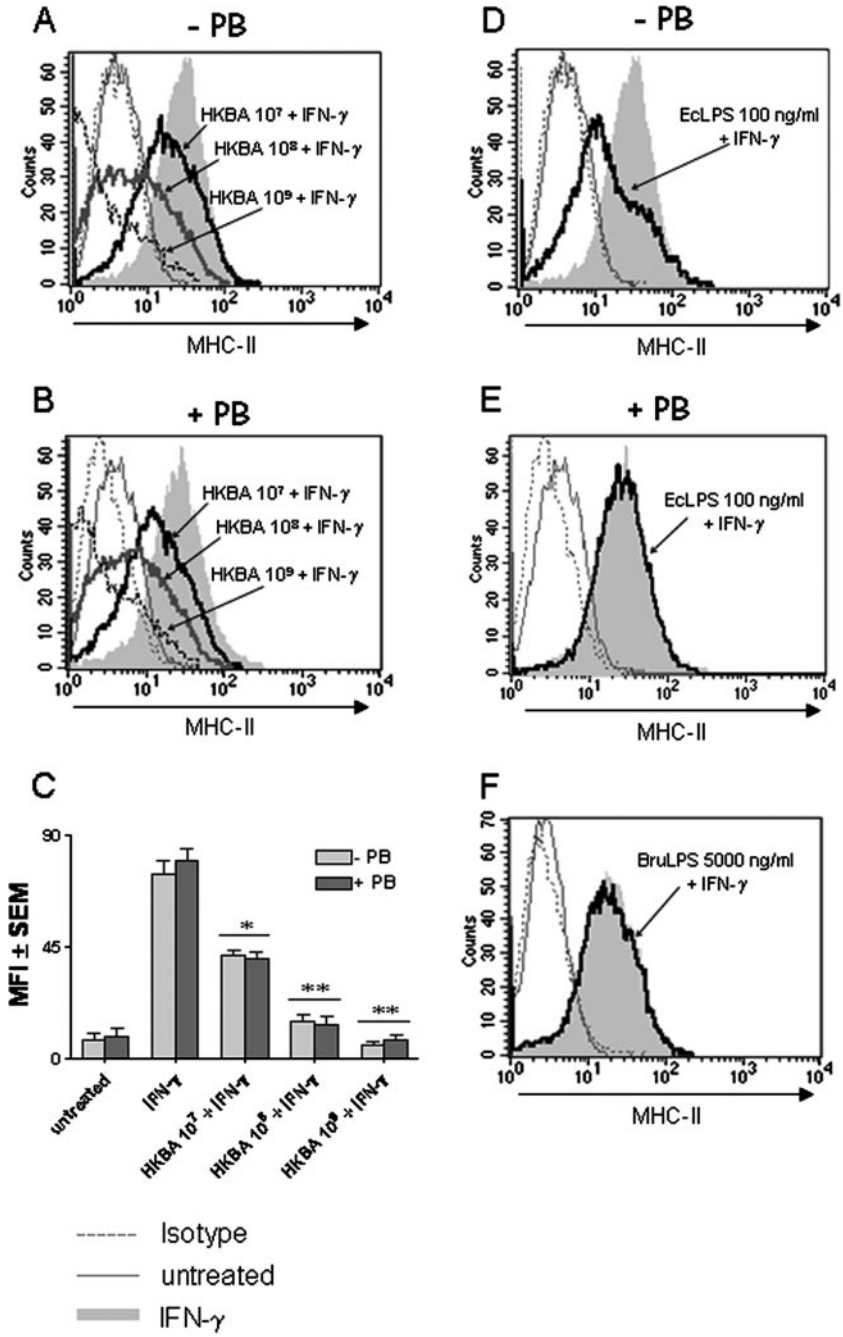


FIG. 2. *B. abortus* LPS is unable to down-modulate the IFN-γ-induced expression of MHC-II molecules. (A to C) THP-1 cells were incubated in the conditions described in the legend to Fig. 1E but in the absence (A) or presence (B) of polymyxin B (PB). (D and E) THP-1 cells were incubated in the conditions described in the legend to Fig. 1H but in the absence (D) or presence (E) of PB. (F) THP-1 cells were incubated with medium (untreated), IFN-γ (150 U/ml), or *B. abortus* LPS (BruLPS) plus IFN-γ for 48 h. MHC-II expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. The bars in panel C indicate the arithmetic means of five experiments, and the error bars indicate the standard errors of the means. MFI, mean fluorescence intensity. Nonspecific binding was determined using a control isotype Ab. One asterisk, $P < 0.01$ for a comparison with IFN-γ; two asterisks, $P < 0.001$ for a comparison with IFN-γ.

the monocytic lineage (28). Consequently, the role of TLR2 in mediating *B. abortus* lipoprotein-induced MHC-II down-regulation was tested. THP-1 cells were preincubated with anti-TLR2 Ab or anti-TLR4 Ab or the respective isotype controls and then cultured with L-Omp19 or HKBA. The expression of MHC-II was evaluated by flow cytometry after incubation. *E. coli* LPS, a TLR4 ligand, and Pam_3Cys, a TLR2 ligand, were used as controls. Preincubation of THP-1 cells with anti-TLR2 significantly blocked ($P < 0.01$) the L-Omp19-mediated inhibition of MHC-II expression induced by IFN-γ. Anti-TLR2 also significantly inhibited ($P < 0.01$) the HKBA-mediated inhibition of MHC-II expression. Anti-TLR4 Ab or isotype

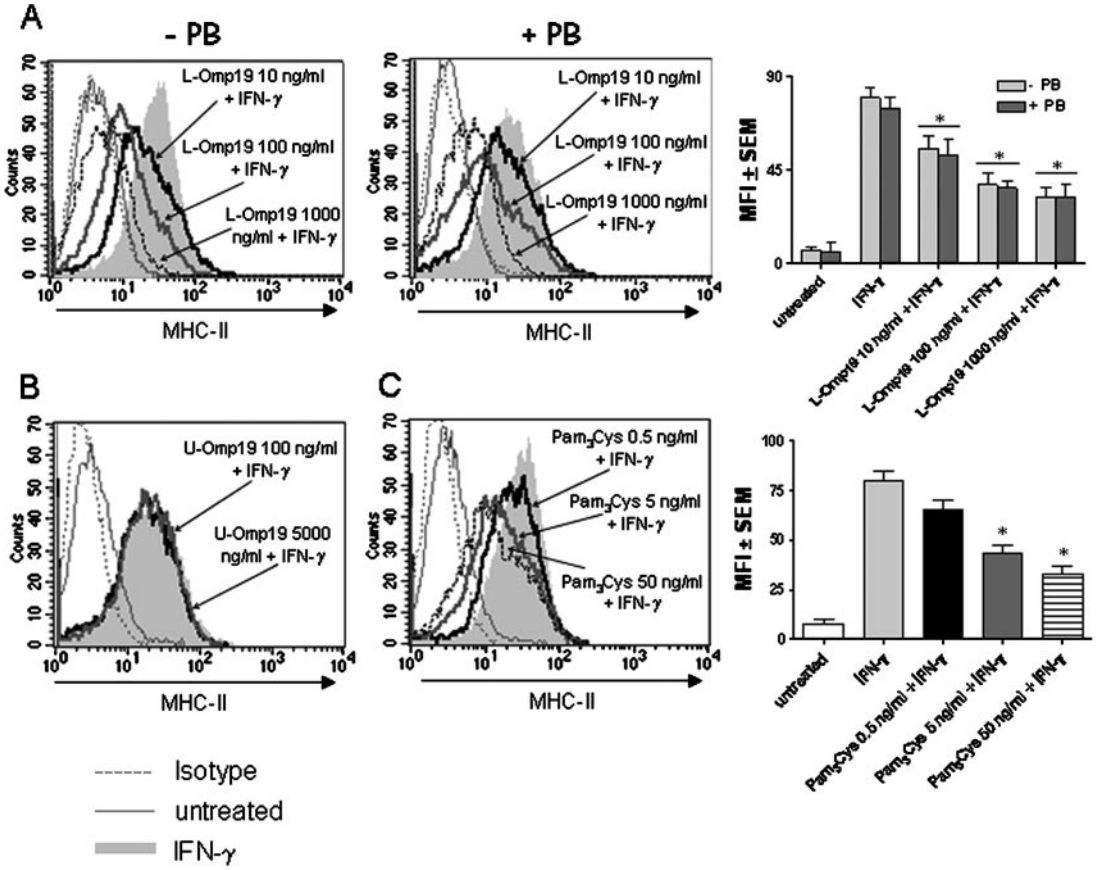


FIG. 3. *B. abortus* lipoprotein Omp19 down-modulates the IFN-γ-induced expression of MHC-II. (A) THP-1 cells were incubated with medium (untreated), IFN-γ (150 U/ml), or L-Omp19 plus IFN-γ for 48 h in the absence or presence of polymyxin B (PB). (B) THP-1 cells were incubated with medium (untreated), IFN-γ (150 U/ml), or U-Omp19 plus IFN-γ for 48 h. (C) THP-1 cells were incubated with medium (untreated), IFN-γ (150 U/ml), or Pam_3Cys plus IFN-γ for 48 h. MHC-II expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. The bars indicate the arithmetic means of five experiments, and the error bars indicate the standard errors of the means. MFI, mean fluorescence intensity. Nonspecific binding was determined using a control isotype Ab. Asterisk, $P < 0.01$ for a comparison with IFN-γ.

control Ab had no effect on the response investigated (Fig. 4A and B). As expected, preincubation of THP-1 cells with anti-TLR4 significantly blocked the *E. coli* LPS-mediated MHC-II down-regulation, whereas anti-TLR2 significantly inhibited the MHC-II down-regulation induced by Pam_3Cys (Fig. 4C and D). These results indicate that the inhibition of MHC-II expression induced by HKBA and L-Omp19 depends on TLR2.

HKBA and L-Omp19 inhibit MHC-II-restricted Ag processing and presentation by THP-1 cells. To determine if inhibition of IFN-γ-induced MHC-II expression by HKBA and L-Omp19 was associated with changes in Ag processing and presentation of soluble Ags for MHC-II-restricted T cells, THP-1 cells were pretreated with IFN-γ in the presence of HKBA or L-Omp19 for 24 h, followed by incubation with Ag85B from *M. tuberculosis* and the DB1 T-cell hybridoma, which recognize soluble Ag85B processed and presented by THP-1 cells (HLA-DR1/DR2) (23). THP-1 cells treated with IFN-γ alone processed and presented epitopes recognized by DB1 cells efficiently at Ag85B concentrations of 5 μg/ml or higher (Fig. 5). Treatment with HKBA (10^8 and 10^9 bacteria/ml) for 24 h significantly inhibited ($P < 0.05$) HLA-DR Ag processing of Ag85B by IFN-γ-treated THP-1 cells at all Ag concentrations (Fig. 5A). Likewise, treatment with L-Omp19 (100 and 1,000 ng/ml) significantly inhibited ($P < 0.05$) HLA-DR Ag processing at an Ag concentration of 10 μg/ml or higher (Fig. 5B). U-Omp19 had no effect on HLA-DR Ag processing and presentation (Fig. 5C), indicating that acylation of lipoproteins is critical in the phenomenon observed. As described by others (20), *B. abortus* LPS was also able to significantly ($P < 0.05$) down-modulate Ag presentation (Fig. 5D), in spite of being unable to down-regulate MHC-II expression (Fig. 2F). Yet, this effect was obtained with 5,000 ng/ml of *B. abortus* LPS but not with 1,000 ng/ml (Fig. 5D). Thus, HKBA and L-Omp19 inhibited IFN-γ-regulated processing and presentation of soluble Ag by THP-1 cells.

IL-6 contributes to inhibition of responses to IFN-γ mediated by HKBA and L-Omp19. *B. abortus* and L-Omp19 induce the production of multiple cytokines, including IL-6 and IL-10, in macrophages/monocytes (25). Taking into account the finding that IL-6 and IL-10 are cytokines that are able to down-modulate MHC-II expression (14, 35), we evaluated whether these cytokines were responsible for the inhibition of IFN-γ-induced responses. THP-1 cells were

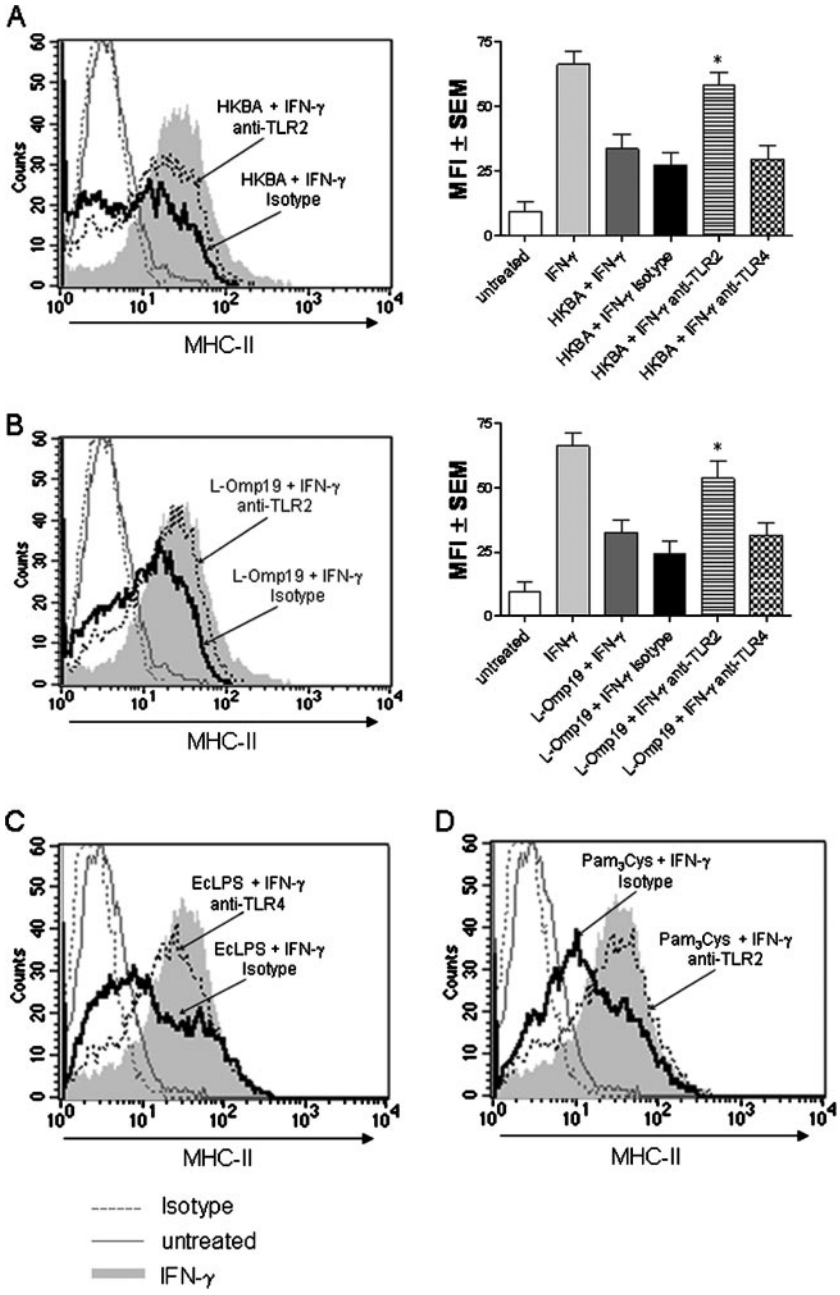


FIG. 4. Inhibition of MHC-II expression mediated by HKBA and L-Omp19 is TLR2 dependent. THP-1 cells were incubated with 20 μg/ml of anti-TLR2, anti-TLR4, or an isotype-matched control for 30 min at 37°C before the addition of (A) HKBA (1×10^8 bacteria/ml), (B) L-Omp19 (100 ng/ml), (C) *E. coli* LPS (EcLPS) (100 ng/ml), or (D) Pam_3Cys (10 ng/ml) in the presence of IFN-γ (150 U/ml). After 48 h of culture, MHC-II expression was assessed by flow cytometry. The histograms indicate the results of one representative of three independent experiments. The bars indicate the arithmetic means of three experiments, and the error bars indicate the standard errors of the means. MFI, mean fluorescence intensity. Nonspecific binding was determined using a control isotype Ab. Asterisk, $P < 0.01$ for a comparison with HKBA plus IFN-γ (A) or L-Omp19 plus IFN-γ (B).

cultured with HKBA or L-Omp19 plus IFN-γ for 48 h in the presence or absence of neutralizing Abs to IL-6 or IL-10 or the respective isotype controls. The expression of MHC-II was then evaluated by flow cytometry. Neutralization of IL-6 resulted in significant ($P < 0.01$) recovery of the inhibition of IFN-γ-induced MHC-II expression mediated by HKBA and L-Omp19 (Fig. 6A and B), while neutralization of IL-10 had no effect (Fig. 6E). Exposure of THP-1 to recombinant human IL-6 also inhibited the expression of MHC-II. Incubation with increasing concentrations of the cytokine markedly inhibited the up-regulation of MHC-II induced by IFN-γ (Fig. 6C). Moreover, neutralization of IL-6 also resulted in partial recovery of the inhibition of IFN-γ-induced MHC-II-restricted Ag presentation mediated by L-Omp19

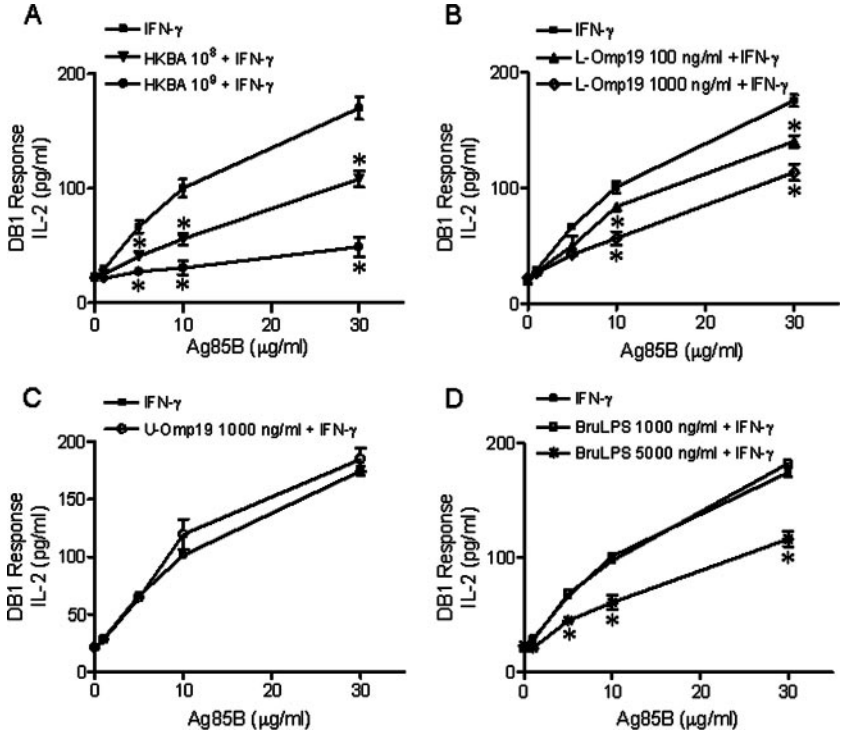


FIG. 5. HKBA and L-Omp19 inhibit antigen processing and presentation. THP-1 cells were incubated with IFN-γ (150 U/ml) with or without HKBA, L-Omp19, or *B. abortus* LPS (BruLPS) for 24 h. Cells were then pulsed with Ag85B for 6 h, followed by incubation with DB1 cells for 24 h. Supernatants were harvested, and the amount of IL-2 was determined by an ELISA. The results are expressed as the means ± standard deviations. The experiments were performed three times in duplicate. Asterisk, $P < 0.05$ for a comparison with IFN-γ.

(Fig. 6D). Therefore, the inhibition of responses to IFN-γ induced by either HKBA or L-Omp19 is mediated, at least in part, by IL-6.

HKBA and L-Omp19 down-modulate MHC-II expression and Ag processing of human monocytes. The next experiments were designed to determine whether the effect of HKBA and L-Omp19 on MHC-II expression induced by IFN-γ could be extended to human monocytes. For this purpose, either human PBMCs or purified monocytes were treated with IFN-γ, HKBA plus IFN-γ, L-Omp19 plus IFN-γ, or U-Omp-19 plus IFN-γ for 48 h. After this, the expression of MHC-II was evaluated by flow cytometry. HKBA and L-Omp19, but not U-Omp19, down-regulated the IFN-γ-induced expression of MHC-II in purified human monocytes or in the monocytes present in the PBMC population (Fig. 7). We also asked whether HKBA or L-Omp19 had the capacity to inhibit Ag processing and presentation by human monocytes. For this, PBMCs from PPD responder healthy individuals were treated with IFN-γ in the presence or absence of HKBA, L-Omp19, or Pam_3Cys for 48 h. Then the cells were washed and used in proliferation or IFN-γ production assays with PPD as the antigen. PBMCs proliferated and secreted IFN-γ when they were cultured with PPD (Fig. 8). Treatment with HKBA significantly ($P < 0.05$) reduced proliferation (Fig. 8A) and production of IFN-γ (Fig. 8B) to PPD in a dose-dependent fashion. Both L-Omp19 and Pam_3Cys were also able to reduce T-cell proliferation and IFN-γ production (Fig. 8C and D, respectively). Altogether, these results indicate that *B. abortus* lipoproteins are able to down-modulate MHC-II expression and Ag processing of soluble Ags by human monocytes, and the Pam_3-modified cysteine was the molecular structure that induced this phenomenon.

Exposure to Pam_3Cys inhibits Ag-specific T-cell proliferation and IFN-γ production of PBMCs from *Brucella*-infected patients. We observed that PBMCs from patients with acute brucellosis display a Th1-type response with cell proliferation and production of IFN-γ and IL-2, while patients with the chronic form of the disease do not do this, and we hypothesized that prolonged exposure to *B. abortus* components might allow the bacterium to dampen this response, allowing its intracellular survival during the chronic phase of the disease (24). This led us to investigate whether prolonged exposure (48 h) of PBMCs from patients with acute brucellosis to Pam_3Cys, the molecular component of brucellar lipoproteins involved in down-modulation of MHC-II expression and Ag processing, inhibited T-cell proliferation and IFN-γ production upon antigenic stimulation. Hence, PBMCs from acute brucellosis patients were treated with IFN-γ and Pam_3Cys for 48 h, washed, and used in proliferation or IFN-γ production assays with *B. abortus* CP (24) as the antigen. PBMCs from patients with acute brucellosis proliferated (as measured by IL-2 secretion) and secreted IFN-γ when they were cultured with CP. Treatment with Pam_3Cys significantly ($P < 0.05$) reduced secretion of both IL-2 and IFN-γ to CP in a dose-dependent fashion (Fig. 9). These results indicate that prolonged exposure to Pam_3Cys affects Ag presentation in PBMCs from *Brucella*-infected patients, leading to a diminished Th1 response.

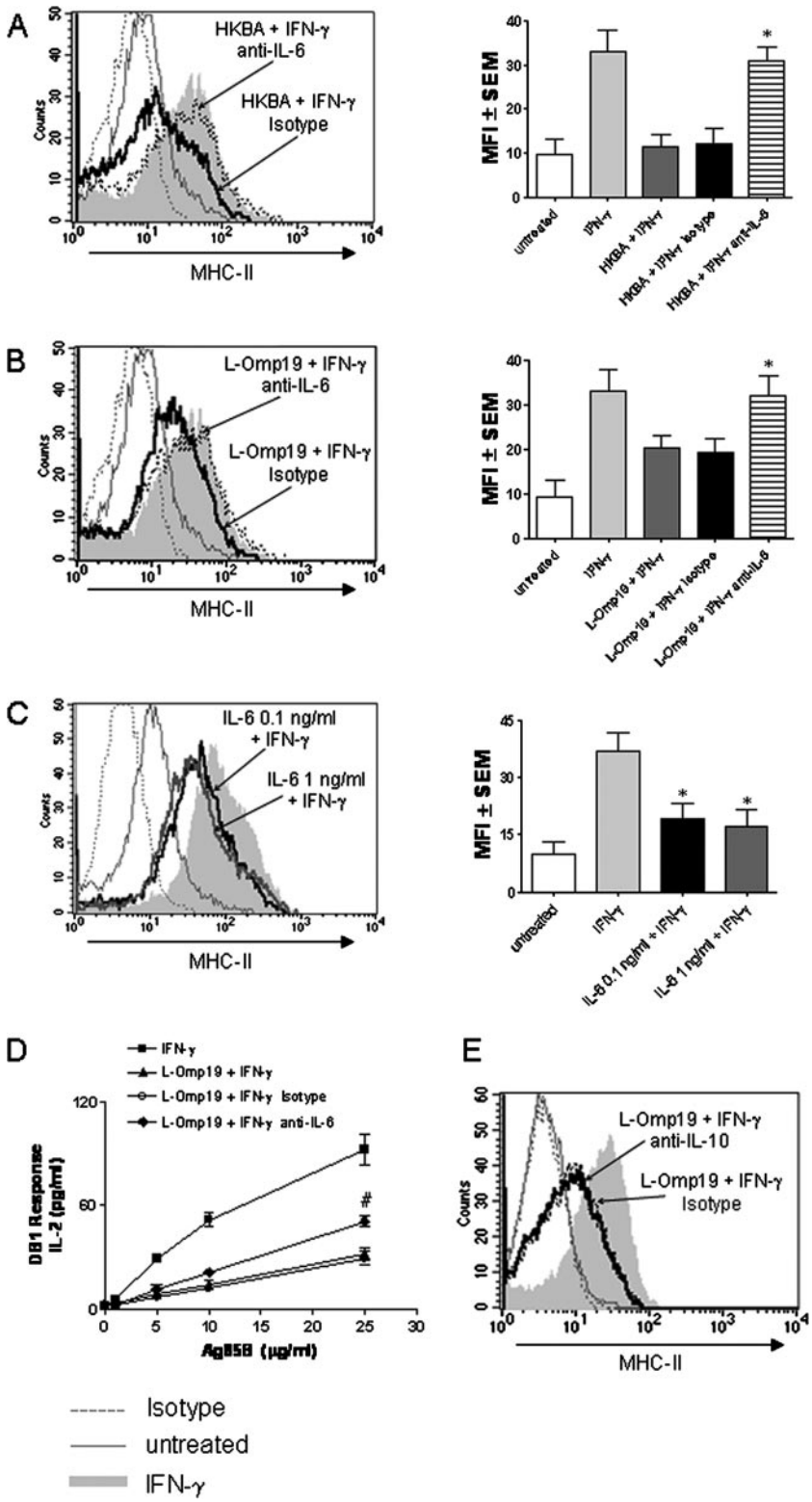


FIG. 6. IL-6 inhibits responses to IFN-γ mediated by HKBA and L-Omp19. (A and B) THP-1 cells were cultured with (A) HKBA (1×10^8 bacteria/ml) or (B) L-Omp19 (100 ng/ml) plus IFN-γ (150 U/ml) for 48 h in the presence or absence of 20 μg/ml of anti-IL-6 or an isotype-matched control. (C) THP-1 cells were cultured with IL-6 plus IFN-γ (150 U/ml) for 48 h. MHC-II expression was assessed by flow cytometry. The histograms indicate the results of one representative of three independent experiments. The bars indicate the arithmetic means of three experiments, and the error bars indicate the standard errors of the means. MFI, mean fluorescence intensity. Nonspecific binding was determined using a control isotype Ab. Asterisk, $P < 0.01$ for a comparison with HKBA plus IFN-γ (A), L-Omp19 plus IFN-γ (B), or IFN-γ (C). (D) An antigen processing and presentation experiment was conducted as described in the legend to Fig. 5 in the presence or absence of 20 μg/ml of anti-IL-6 or an isotype-matched control. Number sign, $P < 0.05$ for a comparison with IFN-γ. (E) THP-1 cells were incubated in the conditions described above for panel B but in the presence or absence of 20 μg/ml of anti-IL-10 or an isotype-matched control.

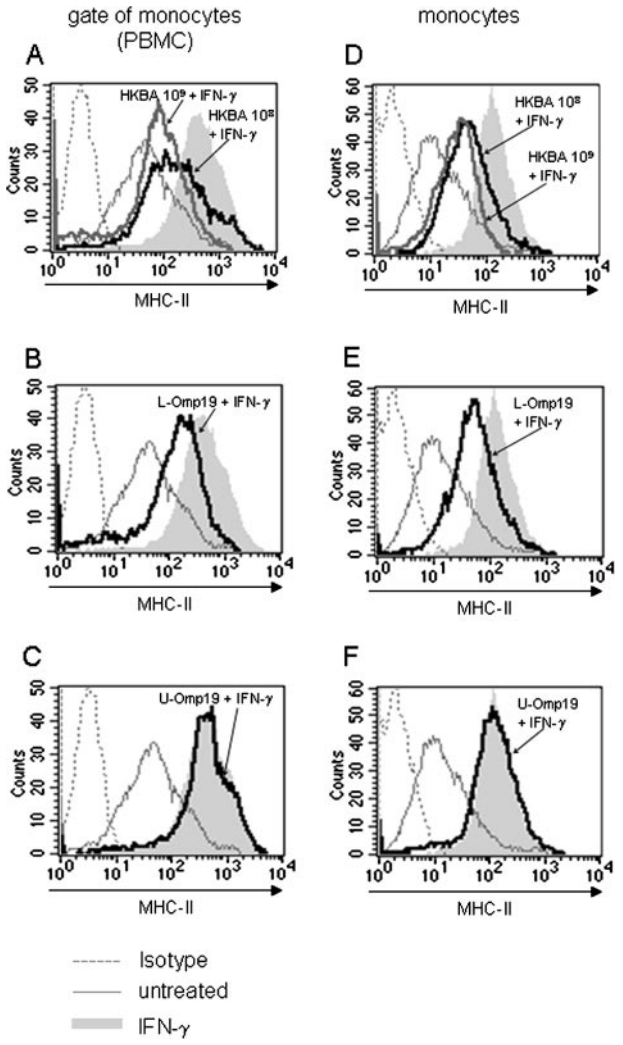


FIG. 7. HKBA and L-Omp19 down-modulate MHC-II expression of human monocytes. Human PBMCs or monocytes were incubated with medium (untreated), IFN-γ (150 U/ml), or HKBA plus IFN-γ (A and D), with L-Omp19 (1,000 ng/ml) plus IFN-γ (B and E), or with U-Omp19 (1,000 ng/ml) plus IFN-γ (C and F) for 48 h. MHC-II expression was assessed by flow cytometry. The monocyte population in PBMCs was analyzed using the monocyte-specific forward and side light scatter gates. The histograms indicate the results of one representative of three independent experiments. Nonspecific binding was determined using a control isotype Ab.

DISCUSSION

To fight, flee, or hide is imperative for long-term survival of a persistent infectious microbe. Thus, *B. abortus* has a panoply of defensive resources, including limited exposure of antigenic targets (5, 10), seclusion in immune privileged sites (6), and the capacity to circumvent innate (16, 44, 54) and/or adaptive immune responses (24).

Early in infection, resistance to complement (29) and cationic peptide-mediated attacks (32), as well as protection against host recognition of PAMPs (31), enhance *Brucella* survival before the bacterium reaches its intracellular niche, the macrophage. Immediately after entry into macrophages, *Brucella* resides in an acidified compartment that fuses with components of the early endosomal pathway (7). There, the vast majority of the ingested bacteria are rapidly killed by macrophages, initiating, through the processing and presentation of bacterial Ags in the context of the MHC, the adaptive immune

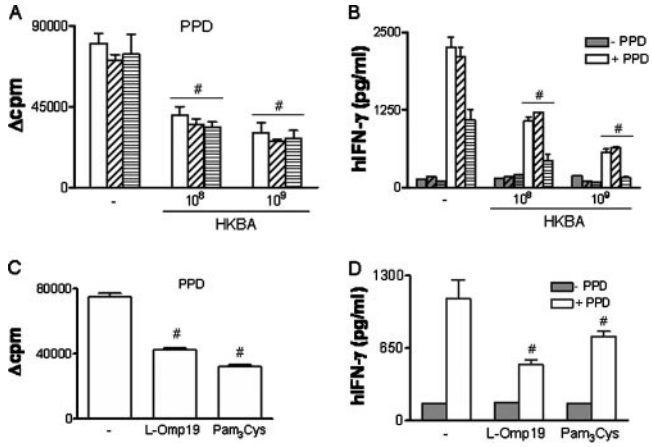


FIG. 8. HKBA and L-Omp19 down-modulate proliferation and IFN-γ secretion of human monocytes. PBMCs from PPD responder individuals (1×10^6 cells/ml) were treated with IFN-γ (150 U/ml) with or without HKBA (A and B), L-Omp19 (100 ng/ml), or Pam_3Cys (10 ng/ml) (C and D) for 48 h. Cells were then washed and cultured with or without PPD for 48 h. At the end of culture blastogenesis (A and C) or IFN-γ production (B and D) was determined. Each bar indicates the mean difference in cpm or the mean IFN-γ concentration of PBMCs from a different donor based on triplicate determinations, and the error bars indicate the standard deviations. Number sign, $P < 0.05$ for a comparison with the control.

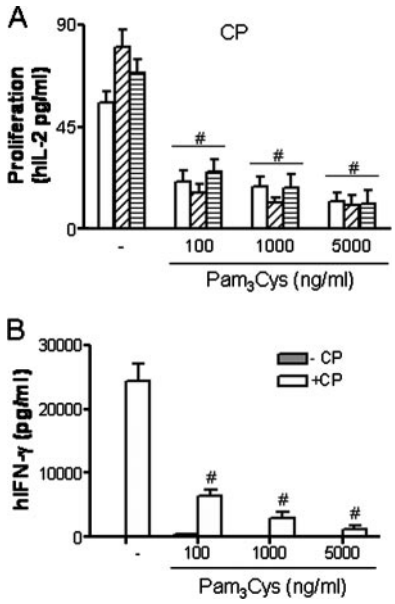


FIG. 9. Exposure to Pam_3Cys inhibits T-cell proliferation and IFN-γ production of PBMCs from *Brucella*-infected patients. PBMCs from acute brucellosis patients (1×10^6 cells/ml) were treated with IFN-γ (150 U/ml) with or without Pam_3Cys for 48 h. Cells were then washed and cultured with or without *B. abortus* CP for 48 h. Supernatants were harvested, and the amount of human IL-2 (hIL-2) (A) or human IFN-γ (hIFN-γ) (B) was determined by an ELISA. Each bar indicates the mean cytokine concentration of PBMCs from a different donor based on duplicate determinations, and the error bars indicate the standard deviations. Number sign, $P < 0.05$ for a comparison with the control.

response. However, some of the surviving bacteria are instead trafficked to an intracellular compartment known as the replicative phagosome or the "brucellosome" through continual interactions between the *Brucella*-containing vacuoles and the endoplasmic reticulum of the host macrophages. Once inside the replicative phagosome, bacteria are resistant to further attack and begin to multiply dramatically (7, 47). *Brucella* also inhibits macrophage apoptosis, which in turn favors pathogen survival and replication (17, 27).

Recent advances in assigning functions to *Brucella* virulence factors in the various stages of its intracellular cycle have demonstrated that the VirB type IV secretion system is involved in controlling the maturation of the *Brucella*-containing vacuole into a replication permissive organelle (11, 13), that cyclic 1-2-glucans help prevent phagosome-lysosome fusion, allowing bacterial intracellular replication (3), and that the O polysaccharide inhibits phagocytosis, protecting the bacteria from the phagolysosome and inhibiting host cell apoptosis (17, 42). Despite these refined escape mechanisms *Brucella* has to face a second challenge if it is to persist in the host for a long time. It should be able to inhibit Ag processing and presentation by *Brucella*-containing macrophages to avoid the immunological surveillance of MHC-II-restricted IFN-γ-producing $CD4^+$ T lymphocytes, thus promoting chronic infection.

In this paper we present evidence indicating that infection with *B. abortus* down-modulates expression of MHC-II on THP-1 cells. This effect can be extended to other *Brucella* species since *B. ovis* was also able to inhibit MHC-II expression. Down-modulation of MHC-II expression correlates with inhibition of Ag processing and presentation of soluble Ags to MHC-II-restricted T cells. Both inhibition of MHC-II expression and Ag processing were not dependent on bacterial viability, since they were also induced by exposure to HKBA, suggesting that they were elicited by a constitutive bacterial component. Among the factors possibly implicated, *B. abortus* LPS was a probable candidate for this role, since LPS from other bacteria were shown to diminish MHC-II expression (51). Yet, our results indicate that the inhibition of the MHC-II expression induced by *B. abortus* is independent of its LPS. Polymyxin B, a specific inhibitor of the activity of LPS (34), was unable to inhibit MHC-II expression induced by HKBA in THP-1 cells. Moreover, highly purified *B. abortus* LPS was also unable to reduce MHC-II expression.

B. abortus possesses lipoproteins (50). Studies conducted in our laboratory have demonstrated that *B. abortus* lipoproteins can elicit not only inflammatory but also immunomodulatory mediators, e.g., IL-10 and IL-6 from monocytes (25). These findings support the contention that lipoproteins, together with LPS, are important virulence factors for *Brucella* survival and replication in the host. As other bacterial lipoproteins have the ability to inhibit IFN-γ-induced MHC-II expression and Ag presentation by murine and human macrophages (22, 23, 38, 41), we hypothesized that *B. abortus* lipoproteins could be the constitutive components involved in the phenomena observed. L-Omp19, a prototypical *B. abortus* lipoprotein, inhibited both MHC-II expression and Ag presentation in a dose-dependent fashion. U-Omp19 had no inhibitory activity, demonstrating that acylation of Omp19 is required for its biological activity. Not only L-Omp19 but also Pam_3Cys was able to inhibit MHC-II expression. Since all brucellar lipoproteins likely share the Pam_3Cys modification, this indicates that any lipoprotein should be able to down-modulate MHC-II expression and Ag presentation. As the *B. abortus* genome contains no less than 80 genes encoding putative lipoproteins (8), it follows that lipoprotein-induced inhibition of MHC-II expression and Ag presentation could be effective enough to explain why *B. abortus* can persist in the host for a long time in the face of a vigorous T-cell response.

Despite the inability of *B. abortus* LPS to down-modulate MHC-II expression, our results indicate that this molecule was able to inhibit Ag presentation of soluble Ags for a MHC-II-restricted T-cell hybridoma. This apparent discrepancy can be explained by the results of Forestier et al. (20). In these investigators' hands the deficient Ag presentation elicited by *B. abortus* LPS was not due to reduced MHC-II surface expression; rather, *B. abortus* LPS formed macrodomains at the cell plasma membrane which interfered with the MHC-II presentation of peptides to specific T-cell hybridomas. Thus, *B. abortus* possesses at least two possible mechanisms for interfering with MHC-II-restricted Ag presentation: (i) sequestration of MHC-II molecules inside LPS macrodomains (20) and (ii) lipoprotein-mediated down-modulation of the expression of MHC-II molecules.

The relative roles of TLR2 and TLR4 in mediating *B. abortus*-induced MHC-II down-modulation merit discussion. Our results indicate that HKBA inhibits MHC-II expression via TLR2 and not via TLR4. As *B. abortus* LPS utilizes TLR4 (25), these results strengthen the contention that LPS is not the molecule employed by *B. abortus* to down-modulate MHC-II expression; in addition, they provide proof of concept that *B. abortus* lipoproteins are the TLR2 ligands employed by the bacterium to down-modulate MHC-II. As TLR2 can sample material present in phagosomal compartments (39, 52), phagosomal *Brucella* may chronically activate TLR2 in infected cells, leading to decreased MHC-II Ag processing.

We have shown that *B. abortus* and L-Omp19 stimulate macrophages via TLR2 to produce IL-6 (25). Under some circumstances, this cytokine has been involved, in mediating the inhibition of MHC-II expression induced by other bacteria (35, 53). The present study provides evidence that one mechanism exploited by *B. abortus* to inhibit IFN-γ-induced MHC-II expression and Ag presentation is stimulation of macrophages to secrete IL-6. Neutralization of IL-6 resulted in significant recovery of the inhibition of IFN-γ-induced MHC-II expression and Ag presentation mediated by HKBA and L-Omp19. Our findings thus concur with the observations mentioned above and add new evidence to the compelling information indicating that IL-6 has inhibitory effects on macrophage functions (1, 4).

The down-modulation of MHC-II expression and Ag processing was not a phenomenon unique to THP-1 cells. *B. abortus*, as well as L-Omp19 and Pam_3Cys, also diminished MHC-II expression and inhibited the ability of human monocytes to process and present soluble Ags (PPD) to polyclonal T cells from human donors. The finding that *Brucella* lipoproteins inhibited the ability of human monocytes to activate Ag-specific T cells suggests that these molecules could be implicated in the down-modulation of T-cell responses reported in human brucellosis (24, 33, 45, 46). Indeed, our results indicate that prolonged exposure to Pam_3Cys of PBMCs from acute

patients, which display a Th1-type response with cell proliferation and production of IFN-γ and IL-2, transforms this response towards the nonresponder phenotype of chronically infected patients (24). This strongly supports our contention that the diminished Th1-type responses observed during the chronic phase of the disease are due to prolonged exposure to *B. abortus* components (e.g., lipoproteins).

Although the mechanisms by which *B. abortus* inhibits IFN-γ-induced gene expression may be induced by other bacteria (9, 38, 40), *B. abortus* may be particularly successful in inducing and exploiting these responses. First, *B. abortus* resides mainly inside macrophages, a cell type that expresses TLRs and exhibits IFN-γ-dependent modulation of many genes involved in host defense (including genes involved in MHC-II Ag processing and presentation). Second, *B. abortus* possesses means to resist acute innate microbicidal mechanisms, allowing it to persist inside macrophages for a sufficient period to provide chronic exposure to PAMPs. Third, lipoproteins are shed from live intracellular bacteria (36), making them available to stimulate TLR2 in association with TLR1, both of which are recruited to phagosomal compartments (39, 52). Prolonged TLR2 signaling by lipoproteins may then reduce MHC-II expression and the antigen-presenting function of infected macrophages, which would then serve as a niche in which *B. abortus* could persist without detection by $CD4^+$ T cells. This supports a model in which at least three factors contribute to the establishment of a successful chronic infection by *Brucella*. These factors are bacterial macrophage invasion and persistence in the replicative phagosome, interaction of lipoproteins with TLRs, and inhibition of MHC-II expression and Ag presentation by modulatory cytokines (e.g., IL-6) produced in the microenvironment of the invaded cell.

Finally, our results that demonstrate that *Brucella* lipoproteins inhibit Ag presentation, together with the evidence that indicates that *Brucella* LPS also interfere with the MHC-II presentation pathway (20) and the recent identification of a *Brucella* proline racemase directly involved in the immune modulation of the host (49), indicate that this bacterium possesses multiple mechanisms to restrain immunity. Thus, it easy to envision that a microorganism such as *B. abortus* equipped with several redundant or complementary survival strategies is more likely to succeed in a "tug of war" with the immune system.

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