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# *Brucella abortus* Lipopolysaccharide in Murine Peritoneal Macrophages Acts as a Down-Regulator of T Cell Activation<sup>1</sup>

Claire Forestier,<sup>2,\*</sup> Fabienne Deleuil,<sup>2,3,\*</sup> Nicolas Lapaque,<sup>\*</sup> Edgardo Moreno,<sup>†</sup> and Jean-Pierre Gorvel<sup>4,\*</sup>

Macrophages play a central role in host immune responses against pathogens by acting as both professional phagocytic cells and as fully competent APCs. We report here that the LPS from the facultative intracellular Gram-negative bacteria *Brucella abortus* interferes with the MHC class II Ag presentation pathway. LPS inhibits the capacity of macrophages to present hen egg lysozyme (HEL) antigenic peptides to specific CD4<sup>+</sup> T cells but not those of OVA to specific CD8<sup>+</sup> T cells. This defect was neither related to a decrease of MHC class II surface expression nor to a deficient uptake or processing of HEL. In addition, *B. abortus* LPS did not prevent the formation of SDS-resistant MHC class II complexes induced by HEL peptides. At the cell surface of macrophages, we observed the presence of LPS macromolecules highly enriched in MHC class II molecules, which may be responsible for the significant down-regulation of CD4<sup>+</sup> T cell activation. This phenomenon may account for the avoidance of the immune system by certain bacterial pathogens and may explain the immunosuppression observed in individuals with chronic brucellosis. *The Journal of Immunology*, 2000, 165: 5202–5210.

Macrophages constitute an early barrier of defense against pathogens. 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 MHC to T lymphocytes, promoting in this manner the adaptive immune response. Once inside macrophages, pathogens display a large array of strategies to evade or counteract host immune responses. For instance, they can diminish or abrogate their Ag presentation capacity, thus reducing the T cell-mediated immune responses. The mechanisms and the pathogen factors involved in this effect have been shown to differ from one pathogen to another but globally the cause of the phenomena remains unclear. It has been suggested that *Leishmania amazonensis* and *Leishmania major* down-regulate the activation of T cells by lowering the surface expression of Ia-peptide complexes on APCs (1, 2). *Trypanosoma cruzi* seems to interfere with Ag presentation by decreasing the adhesion of T cells to infected macrophages (3). *Mycobacterium tuberculosis*-infected monocytes exhibit a defective transport of class II molecules through the endosomal/lysosomal pathway (4), which results in an impaired capacity of MHC class II-restricted Ag presentation (5). In contrast, the inhibition of

T cell activation observed with *Mycobacterium bovis*-infected macrophages seems to be related to the secretion by infected APCs of inhibitory factors such as IL-6 (6).

Even though activated macrophages succeed in eliminating virulent microorganisms, they still have to degrade pathogen-derived fragments including proteins, glycolipids, and polysaccharides. In contrast to proteins, cell wall-derived polysaccharides and glycolipids are not readily digested by lysosomal enzymes and are efficiently retained for long periods of time inside macrophages (7). Consequently, APCs exposed to particulate Ags failed to activate T lymphocytes in response to proteins (7–10). LPS, the major component of the Gram-negative bacteria cell wall, has been shown to modulate the immune response. It favors the Ag presentation function of certain APCs by stimulating B lymphocytes (11) and dendritic cells (12), whereas it triggers an opposite effect in sinusoidal endothelial APCs (13) and induces an immunosuppression by inhibiting the Ab response to T-dependent Ags in mice (14).

In this study, we analyzed the effect of the nonenterobacteria *Brucella abortus* LPS on the ability of macrophages to present OVA- and hen egg lysozyme (HEL)<sup>5</sup>-derived peptides to specific T cell hybridomas in the context of MHC class I and II molecules, respectively. In contrast to enterobacteriaceae, *B. abortus* LPS displays a very low toxic activity, a property that makes this molecule suitable for immunological studies (15, 16). We have previously demonstrated that *B. abortus* LPS specifically associates with MHC class II molecules in APC (17). We also found that LPS accumulates inside lysosomal compartments of peritoneal macrophages for long periods of time, without detectable degradation. Then, the LPS is exported to the cell surface where it forms stable macromolecules (18). Here, we demonstrate that, in macrophages, *B. abortus* LPS significantly impairs the MHC class II presentation pathway but not that of MHC class I. The deficient Ag presentation is not due to a deficient uptake and catabolism of the native Ag and to a reduced MHC class II surface expression. In addition, the

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<sup>5</sup> Abbreviations used in this paper: HEL, hen egg lysozyme; KDO, 2-keto-3-deoxyoctonate; Err, experimental radioactive release; SAG, superantigen; Tot, total count release; Srr, spontaneous radioactive release; NP40, Nonidet P-40.

induction of SDS-resistant MHC class II molecules by HEL is not impaired by the presence of LPS. Interestingly, the LPS macrodomains we previously described at the macrophage plasma membrane (18) are highly enriched in MHC class II molecules. We propose that the LPS-MHC class II macrodomains may impair the appropriate recognition of HEL peptide-MHC class II complexes by CD4<sup>+</sup> T cell hybridomas.

## Materials and Methods

### Macrophage preparation

Eight-week-old female C3H/HeN (H-2<sup>b</sup>) and C57BL/6 (H-2<sup>b</sup>) mice were purchased from Jackson ImmunoResearch (West Grove, PA). Peritoneal fluids were harvested as described in (18), centrifuged at 1200 rpm for 10 min at 4°C, and the pellets were resuspended in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 10 mM HEPES (Life Technologies), 10 mM sodium pyruvate (Life Technologies), 10 mM nonessential amino acids (Life Technologies), 2 mM glutamine (Life Technologies), 2 × 10<sup>-5</sup> M 2-ME (Sigma, St. Louis, MO), and 1 mM penicillin/streptomycin (Life Technologies). Peritoneal cells were plated and incubated for 2 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Nonadherent cells were removed from the wells by aspiration, and the adherent macrophages were rinsed and incubated in fresh medium.

### Hybridomas and Ags

HEL and OVA were purchased from Sigma. The HEL<sub>34-45</sub> and HEL<sub>46-61</sub> peptides were synthesized using an Applied Biosystems peptide synthesizer (Perkin-Elmer Biosystems, Courtaboeuf, France) and the OVA<sub>257-264</sub> peptides were provided by Dr. Patrick Machy (Centre d'Immunologie de Marseille Luminy, Marseille, France). OVA was passively adsorbed to latex beads (Polysciences, Warrington, PA) in accordance with the manufacturer's protocol, followed by extensive washing in medium. The HEL-specific CD4<sup>+</sup> T cell hybridomas 3A9 and 3B11 are specific for HEL<sub>46-61</sub>-IK<sup>k</sup> and HEL<sub>34-45</sub>-IA<sup>k</sup>, respectively (19). The OVA-specific CD8<sup>+</sup> T cell hybridoma 4B10 is specific for OVA<sub>257-264</sub>-K<sup>b</sup> (20). The IL-2-dependent CTLL-2 cell line was used for detecting the presence of secreted IL-2 in the culture medium. All of these cell lines were cultured in the supplemented RPMI medium described above.

### Lipopolysaccharide

*Brucella abortus* 2308 and *Shigella flexneri* 5a LPSs were provided by Dr. I. Moriyon (University of Navarra, Pamplona, Spain) and Dr. P. Sansonetti (Pasteur Institute, Paris, France), respectively. The purity and the characteristics of these preparations have been published elsewhere (21, 22). LPSs were solubilized in water by sonication at the appropriate concentration and autoclaved before use. Determination of 2-keto-3-deoxyoctonate (KDO) was performed as described by Karkhanis et al. (23). The *Limulus* lysate gelation activity (24), which estimates the state of aggregation of endotoxins, was 2 ng for *B. abortus* LPS and 1 ng for *S. flexneri* LPS.

### Antibodies

The mAb (Baps C/Y) directed against *B. abortus* LPS C/Y epitope directly coupled to peroxidase and antisera from infected cows (25) were used to detect LPS from *B. abortus* in immunoblotting and immunofluorescence experiments, respectively. The mouse H1005/28 anti-H-2<sup>k</sup> mAb provided by Dr. Michel Pierres (Centre d'Immunologie de Marseille Luminy) and the mouse 10.2.16 anti-I-A<sup>k</sup> mAb (IgG2b) (17) were used to detect MHC class I and II molecules, respectively. The rat anti-mouse Fc receptor Ab (24G2) provided by Dr. L. Leserman (Centre d'Immunologie de Marseille Luminy) was used to saturate Fc receptors in immunofluorescence experiments. Secondary Abs, the goat Texas Red-conjugated anti-cow and the donkey FITC-conjugated anti-mouse were purchased from Immunotech (Marseille, France).

### Toxicity assay

Resting peritoneal macrophages (5 × 10<sup>5</sup> cells/ml) were incubated at 37°C for 40 min with 25 μCi of <sup>51</sup>Cr (specific activity 300 mCi/mg <sup>51</sup>Cr, Amersham, Little Chalfont, U.K.). Cells were washed twice with cell culture medium and incubated at 4°C for 30 min and then washed three times with cell culture medium. Macrophages were incubated with different concentrations of *B. abortus* and *S. flexneri* LPSs diluted in 0.5 ml of cell culture medium and incubated under a 5% CO<sub>2</sub> atmosphere at 37°C. After 12 h, supernatants were collected and the experimental radioactive release (Err)

counted. Total counts (Tot) were estimated from supernatants of frozen and thawed macrophages, whereas spontaneous radioactive release (Srr) was estimated from nontreated cells. The percentage of specific radioactive release of <sup>51</sup>Cr was estimated as 100(Err - Srr)/(Tot - Srr). Measurements of cell viability by the trypan blue exclusion method gave similar results.

### Ag presentation assay

Peritoneal macrophages were used as APCs. Two strategies were used to ensure high and uniform expression of MHC class II molecules. In the former, MHC class II expression was induced in vitro by treatment of macrophages plated in 96-well plates (10<sup>5</sup> cells/well; Costar, Cambridge, MA) with 10 ng/ml of IFN-γ for 48 h (R&D Systems, Abingdon, U.K.). In the latter, MHC class II expression was induced in vivo by the injection in the peritoneal cavity of *B. abortus* LPS (300 μg/mouse) or 10 ng/ml of IFN-γ. Macrophages were harvested from injected mice and plated in 96-well plates (10<sup>5</sup> cells/well). Then macrophages treated in vitro with IFN-γ and those from LPS-injected mice were incubated with various concentrations of LPS for 8 h at 37°C. After washing, cells were further incubated with HEL, synthetic HEL peptides, OVA, synthetic OVA peptides, and the corresponding specific T cell hybridomas (10<sup>5</sup> cells/well). After a 24-h incubation, culture supernatants were assayed for the presence of the T cell growth factor IL-2 by measuring the incorporation of [<sup>3</sup>H]thymidine in the IL-2-dependent CTLL cell line (10<sup>4</sup> cells/well).

### Flow cytometric analysis of Ia molecules

Macrophages activated in vivo by LPS or activated in vitro and in vivo by IFN-γ were plated at 10<sup>6</sup> cells/35-mm dish (Nunc, Naperville, IL) and incubated for 30 min at 4°C in PBS/10% mouse serum and then with the FITC-conjugated 10.2.16 Ab for 1 h at 4°C. Samples were washed and resuspended in 10 mM PBS/EDTA for analysis by FACScan flow cytometry (Becton Dickinson, Mountain View, CA).

### Ag uptake and degradation

HEL was labeled with <sup>125</sup>I by the Iodogen method (Pierce, Rockford, IL) to a specific activity of ~0.5 mCi/mg (10<sup>6</sup> cpm/μg). Macrophages (10<sup>6</sup> cells/well) in 24-well culture plates (Costar) were incubated with the radiolabeled HEL (2 μg/well) in cell culture medium for 1 h at 37°C. Cells were extensively washed in PBS containing 0.5% BSA until no radioactivity could be detected in the washing medium and then reincubated in radioiodinated HEL-free medium for various times at 37°C. At each time point, macrophages were solubilized in PBS/1% Triton X-100 (Sigma) and centrifuged for 20 min at 14,000 rpm at 4°C. Lysate supernatants were incubated for 30 min at 4°C with 20% TCA to separate soluble from insoluble fractions, and the cell-associated radioactivity was counted in each fraction using a Cobra 5005 counter (Packard, Meriden, CT).

### <sup>35</sup>S metabolic labeling and immunoprecipitations

10<sup>6</sup> macrophages from LPS-injected mice were cultured in cysteine/methionine-free RPMI 1640 medium containing 5% dialyzed FCS supplemented or not with 2 mg/ml of HEL. After 1 h at 37°C, cells were pulsed in medium containing 0.25 mCi/ml of 80% [<sup>35</sup>S]methionine and 20% [<sup>35</sup>S]cysteine mix (DuPont-NEN, Harrisburg, PA). After 2 h at 37°C, cells were washed and lysed in solubilization buffer (1% Nonidet P-40 (NP40); 10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2 mM EDTA; and 1 mM PMSF) for 30 min at 4°C. Samples were precleared by an incubation with protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) before incubation with the 10.2.16 anti-class II Ab preadsorbed on protein A-Sepharose beads. Immunoabsorbents were collected by centrifugation, washed three times with 1% NP40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.1% sodium deoxycholate, and 0.5% SDS, then twice with the same buffer without SDS and sodium deoxycholate, twice with 0.5% NP40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM EDTA, then twice with 10 mM Tris-HCl, pH 7.5. The washed beads were resuspended in 40 μl SDS-PAGE sample buffer containing 5 mM DTT. In the presence of HEL (Sigma), half of each sample was heated at 95°C for 5 min and the other half was left at room temperature for 1 h. The eluted proteins were then analyzed by SDS-PAGE on 12% acrylamide gels. Gels were treated with Enhance (DuPont-NEN, Boston, MA), dried, and submitted to autoradiography. Quantification of the band signal intensities were performed using a MacBAS v2.2 program.

### Confocal microscopy

Immunofluorescence experiments were performed on macrophages from LPS-injected mice without cell permeabilization to detect cell surface-associated molecules, as previously described (18). Macrophages (5 × 10<sup>5</sup> cells/well) were plated on 12-mm glass coverslips in 24-well tissue culture



plates (Costar) and were fixed at room temperature with 3.7% paraformaldehyde in PBS, pH 7.4, for 20 min followed by a 10-min incubation with 0.1 M glycine. Then, cells were incubated with a PBS solution containing 5% mouse serum/5% horse serum for 20 min to block unspecific binding. Primary Abs diluted in the same buffer were added to the cells for 30 min. After extensive washings with PBS, macrophages were incubated for 30 min with fluorescent secondary Abs. LPS was revealed using an antiserum from *B. abortus*-infected cows followed by anti-cow IgG Ab conjugated to Texas Red. MHC class II molecules were revealed by the FITC-conjugated 10.2.16 anti-I-A<sup>k</sup> murine IgGs. For MHC class I detection, cells were fixed with paraformaldehyde, incubated with the rat anti-mouse Fc receptor Ab (24G2), and incubated with the mouse H1005/28 anti-H-2<sup>k</sup> Ab, which was revealed by goat anti-mouse IgG Abs conjugated to fluorescein. Coverslips with adherent macrophages were washed, mounted in Mowiol (Hoechst, Frankfurt, Germany), and viewed under a Leica TCS 4D confocal microscope (Leica Lasertechnik, Heidelberg, Germany).

## Results

### *Brucella abortus* LPS affects the Ag presentation of two distinct HEL epitopes

In APCs, association of antigenic peptides with MHC class II molecules can occur in different compartments of the endocytic pathway (26). For instance, the HEL<sub>46–61</sub> epitope requires newly synthesized MHC class II molecules and the invariant chain expression to be presented to 3A9 hybridoma cells and has been shown to associate with class II molecules inside late endocytic compartments. In contrast, the HEL<sub>34–45</sub> epitope, which is more peripheral, can associate with mature or immature class II molecules inside early endocytic compartments and can be presented to 3B11 hybridoma cells independently of protein synthesis and of invariant chain (27). We have analyzed the effect of *B. abortus* LPS on these different Ag presentation pathways. We observed that LPS inhibits the presentation by macrophages of both HEL<sub>46–61</sub> (Fig. 1A) and HEL<sub>34–45</sub> epitopes (Fig. 1D) generated from exogenously added HEL. In parallel, we analyzed the availability of surface MHC class II molecules by testing the capacity of macrophages to present exogenously added purified peptides to T cell hybridomas (Fig. 1, B and C). The presentation of class II-restricted epitopes such as the HEL<sub>46–61</sub> (Fig. 1B) and the HEL<sub>34–45</sub> (Fig. 1D) peptides was also reduced in the presence of LPS. The inhibitory effect of LPS was Ag dose-dependent (Fig. 1, A and B), and increasing Ag concentrations could only partially restore the ability of macrophages to present HEL. Altogether, these results show that *B. abortus* LPS inhibits the presentation of different HEL epitopes generated in distinct endocytic compartments, affecting in this way the presentation by neosynthesized MHC class II, recycled MHC class II, and vacant surface MHC class II molecules.

### *LPS does not affect the MHC class I-mediated Ag presentation*

Because MHC class II presentation pathway is inhibited by the presence of LPS in macrophages, we tested whether *B. abortus* LPS could affect the MHC class I presentation pathway by using the OVA Ag and its specific CD8<sup>+</sup> T cell hybridoma. Fig. 2 shows that LPS does not significantly impair the presentation by MHC class I molecules of both OVA (Fig. 2A) and OVA<sub>257–264</sub> peptide (Fig. 2B) to CD8<sup>+</sup> T cells. These results indicate that the inhibitory effect of LPS is specific to the MHC class II pathway. This can be correlated with our previous results showing a specific association of *B. abortus* LPS with MHC class II molecules in B lymphocytes (17).

### *B. abortus* LPS inhibits MHC class II-mediated Ag presentation in a dose-dependent manner

The effect of *Brucella* LPS on Ag presentation by macrophages was compared with that of a classical endotoxin, the *Shigella flexneri* LPS, which displays distinct structural and functional prop-

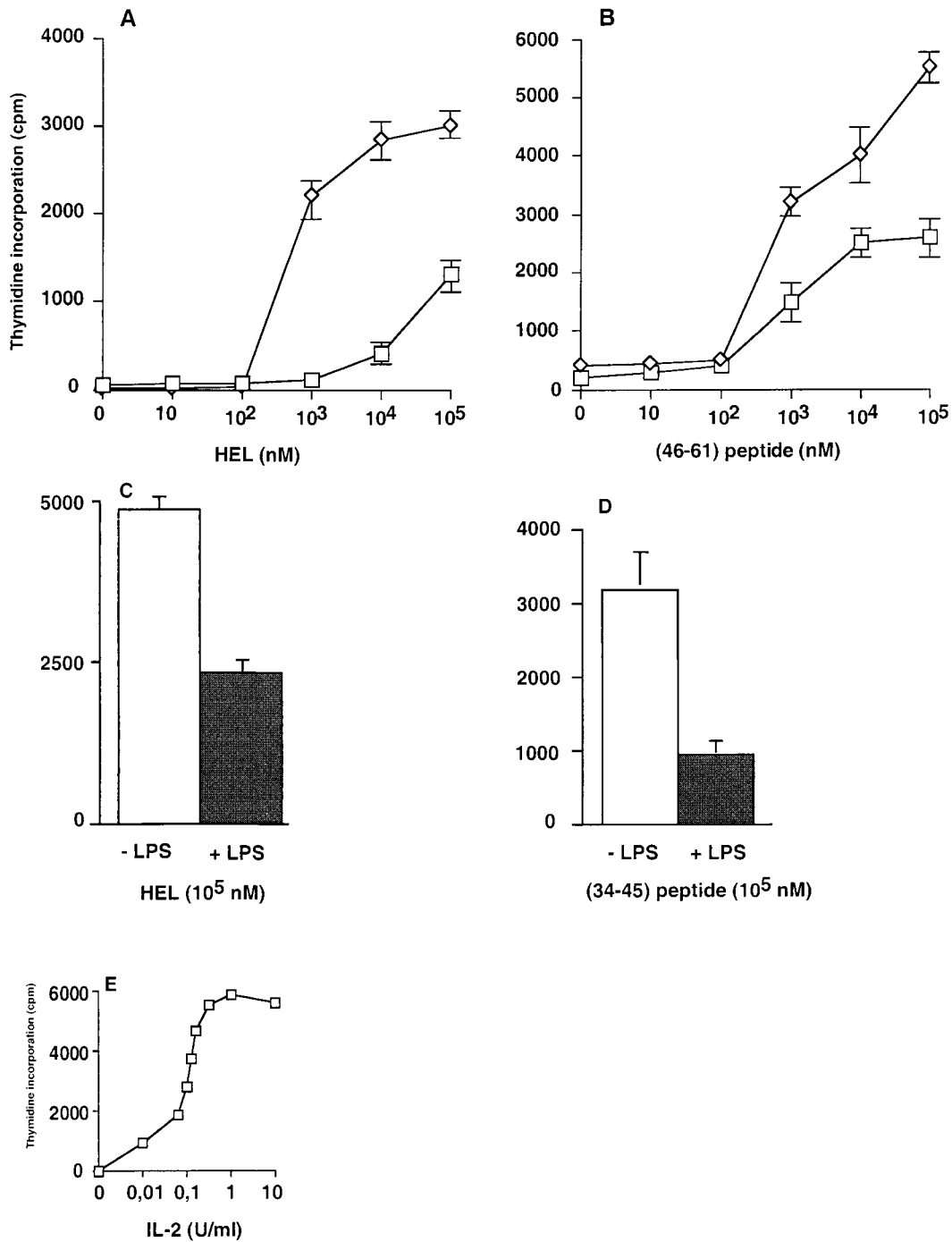
erties from *B. abortus* LPS (28). At low doses (0.1 µg/ml), *Brucella* LPS already inhibited 48% of HEL presentation, whereas *Shigella* LPS had a negligible effect (Fig. 3). The inhibition rate increased with the LPS dose, reaching 90% for *B. abortus* LPS and 70% for *S. flexneri* at 20 µg/ml. Similar results were observed when we used purified HEL<sub>46–61</sub> and HEL<sub>34–45</sub> peptides (data not shown), which do not require either internalization or processing in APCs. These results indicate that both LPSs were able to prevent HEL presentation to T cells in a dose-dependent manner; however, *B. abortus* LPS appears as the most active LPS. One parameter that could be responsible for the decreased ability of macrophages to present Ags is the cell toxicity displayed by LPSs. In agreement with previous reports, both LPSs exhibit very similar *Limulus* lysate gelation activity (1 ng for *Shigella* and 2 ng for *Brucella*). However, the classical *S. flexneri* endotoxin displayed very high toxicity when compared with that of *B. abortus* LPS (Fig. 4). For instance, at 12 nM of KDO, *Shigella* LPS was already 7 times more toxic than *Brucella* LPS. At 27 nM of KDO, *Shigella* was 18 times more toxic as compared with *Brucella* LPS. Moreover, at higher concentrations (200 nM of KDO), *Brucella* LPS did not display a significant toxic effect on cells, in agreement with previous reports (15, 29). Because LPS toxicity is dependent on time, it is very difficult to dissociate the toxic effect induced by *Shigella* LPS from the inhibition of HEL presentation. In contrast, the Ag presentation impairment observed in *B. abortus* LPS-treated macrophages was not related to a toxic effect on APCs, but rather to an inhibitory phenomenon. We then investigated further the mechanisms by which *B. abortus* LPS down-regulates T cell activation.

### *B. abortus* LPS does not affect the capacity of macrophages to internalize and catabolize native HEL

We previously showed that *Brucella* LPS concentrates in lysosomes between 6 and 24 h after its internalization (18). This retention could alter the intracellular trafficking of internalized proteins and the ability of these APCs to process Ags. To examine this possibility, HEL was radioiodinated, incubated with macrophages, and the cell-associated radioactivity of both normal and LPS-pulsed macrophages was determined after various times of chase. The efficiency of uptake was determined by incubating macrophages with <sup>125</sup>I-HEL and by measuring the radioactivity present in Triton X-100 lysates just after the pulse. Table I shows that the same amounts of native Ag (TCA-insoluble fraction) were associated with macrophages loaded or not with LPS, indicating that uptake of HEL was not affected by the presence of LPS. To analyze the catabolism of native HEL, the radioactivity was measured after different times of chase. Cell-associated TCA-precipitable radioactivity progressively diminished with incubation time, reflecting the degradation of native HEL by macrophages. No increase of radioactivity was detected in the cell-associated TCA-soluble fraction due to the release into the cell culture supernatants of peptidic fragments resulting from HEL processing. The time course of degradation and the amount of HEL degraded appeared very similar in both cell preparations, demonstrating that macrophages conserved their capacity to process native HEL even in the presence of LPS. So, the inhibition of presentation of HEL by LPS-loaded macrophages is not due to either a decrease in HEL uptake or in its intracellular processing.

### *The level of surface class II molecule expression is not impaired by the presence of LPS in vitro*

The responsiveness of CD4<sup>+</sup> T lymphocyte hybridomas to protein Ags depends on the expression of MHC class II molecules bound to a given Ag. Because LPS may modify the level of MHC class II molecule expression, we analyzed by flow cytometry the total



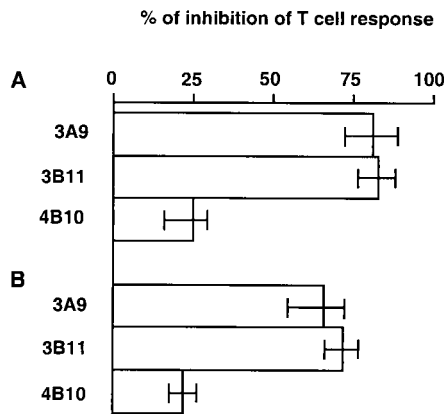
**FIGURE 1.** Inhibition of HEL presentation by LPS. Macrophages were cultured ( $10^6$  cells/ml) in 96-well plates with 10 ng/ml IFN- $\gamma$  for 48 h and treated ( $\square$ ) or not ( $\diamond$ ) with 10  $\mu$ g/ml *B. abortus* LPS during the last 8 h. Macrophages were incubated with various doses of HEL (A)- or HEL<sub>46-61</sub>-purified peptide (B) and 3A9 T cell hybridomas (A and B). In a second set of experiments, macrophages were incubated with native HEL (C) or HEL<sub>34-45</sub> peptide (D) and 3B11 hybridoma (C and D). After 24 h, culture supernatants were recovered and their IL-2 content was measured using CTLL cells, as described in *Materials and Methods*. Data in (E) corresponds to the proliferation of CTLL cells in the presence of various doses of recombinant IL-2 and correlates the thymidine incorporation values with the amount of IL-2 (U/ml) produced by T cells. Experiments were performed in triplicate.

amount of surface class II molecules present on IFN- $\gamma$ -activated macrophages loaded or not with LPS. Fig. 5 clearly indicates that IFN- $\gamma$ , in contrast to LPS, up-regulates MHC class II molecule expression in vitro, and that subsequent addition of *B. abortus* LPS on IFN- $\gamma$ -activated macrophages does not affect the surface expression of MHC class II molecules. Therefore, the inhibition of Ag presentation by macrophages in the presence of LPS cannot be

attributed to a reduced amount of MHC class II molecules at the macrophage surface.

*B. abortus* LPS induces the expression of MHC class II molecules in vivo

We also measured the level of MHC class II expression at the cell surface of macrophages obtained after injection of LPS into the

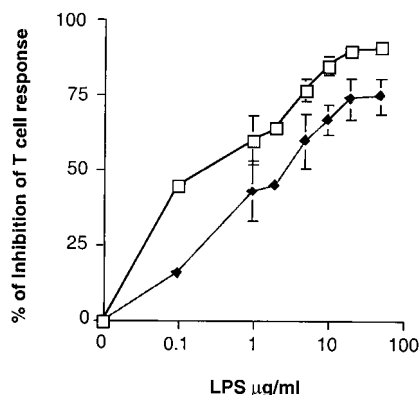


**FIGURE 2.** LPS inhibits MHC class II but not MHC class I Ag presentation. Macrophages were incubated with 10 ng/ml IFN- $\gamma$  for 48 h and treated with 10  $\mu$ g/ml *B. abortus* LPS during the last 8 h. Macrophages were then incubated with HEL or OVA (A) or with their corresponding peptides (B). 3A9, 3B11, and 4B10 T cell hybridomas recognize HEL<sub>46–61</sub>, HEL<sub>34–45</sub>, and OVA<sub>257–264</sub> peptides, respectively. Results are expressed as a percentage of inhibition of T cell responses and represent an average of four different experiments.

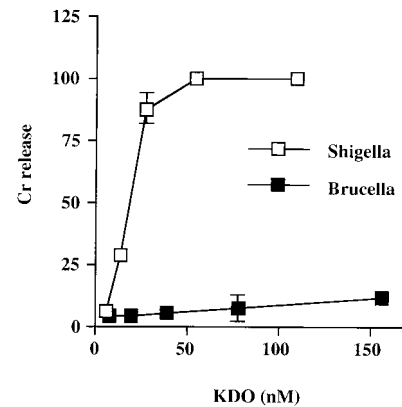
peritoneal cavity of mice. The injection of LPS resulted in a strong up-regulation of MHC class II molecules on the macrophage cell surface (Fig. 6), probably due to an indirect mechanism, the level of which was similar to that of macrophages from IFN- $\gamma$ -injected mice (data not shown).

#### MHC class II presentation pathway is inhibited in vivo by LPS

We then compared the antigenic presentation abilities between macrophages from LPS- and IFN- $\gamma$ -injected mice. Fig. 7 clearly shows that LPS-treated macrophages are less efficient in presenting HEL peptides than macrophages treated with IFN- $\gamma$ . These results corroborate those presented in Fig. 1 showing an inhibitory effect of LPS on processed HEL presentation in macrophages treated in vitro. In addition, as in vitro, the MHC class I presentation pathway was not affected (data not shown). We further investigated



**FIGURE 3.** Inhibition of MHC class II Ag presentation by *Brucella* and *Shigella* LPSs. Macrophages treated with IFN- $\gamma$  for 48 h were incubated with various concentrations of *B. abortus* ( $\square$ ) and *S. flexneri* ( $\blacklozenge$ ) LPSs for 8 h at 37°C. After washing, they were incubated with 10  $\mu$ M HEL and with 3A9 T cell hybridoma for 24 h. Supernatants were tested for their IL-2 content using CTLL-2 as described in *Materials and Methods*. Results are expressed as a percentage of inhibition of T cell responses. 0% of inhibition corresponds to the value of [ $^3$ H]thymidine incorporation obtained in the absence of LPS and 100% of inhibition correspond to 0 cpm.

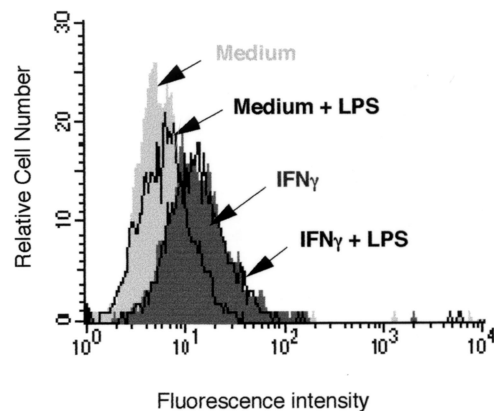


**FIGURE 4.** Macrophage cell death measured by  $^{51}$ Cr release. Macrophage death was measured as the percentage of  $^{51}$ Cr release of cells treated with different doses of LPS. Experiments were run in triplicate and the SD of each group was calculated. Specific radioactive  $^{51}$ Cr release =  $100(\text{Err} - \text{Srr})/(\text{Tot} - \text{Srr})$ .

what could be the defect explaining the interference with the Ag presentation process using the in vivo model.

#### LPS forms macrodomains, which are enriched in class II molecules

We recently demonstrated that after internalization in mouse peritoneal macrophages, *Brucella* LPS is recycled to the cell surface where it forms macrodomains (18). These membrane domains were observed in macrophages treated either in vitro or in vivo with LPS or after infection with an attenuated strain of *B. abortus* (18). In the absence of LPS, MHC class I and II molecules analyzed by confocal microscopy on macrophages from IFN- $\gamma$ -treated mice displayed an homogenous random distribution at their cell surface (Fig. 8, A and B). In contrast, the surface distribution of MHC class II molecules, but not that of class I, was modified after LPS treatment (Fig. 8, E and F). MHC class II molecules were concentrated in cell surface macrodomains, most of which colocalized with LPS (Fig. 8C). In contrast, MHC class I (Fig. 8F) did



**FIGURE 5.** *Brucella* LPS does not affect the level of expression of surface MHC class II molecules in vitro. Macrophages were incubated (medium + LPS) or not (medium) with 20  $\mu$ g/ml of *B. abortus* LPS for 8 h or were stimulated with IFN- $\gamma$  for 48 h and then treated (IFN- $\gamma$  LPS) or not (IFN- $\gamma$ ) with 20  $\mu$ g/ml of *Brucella* LPS in cell culture medium for 8 h. After washing, cells were immunolabeled with FITC-conjugated anti-IA<sup>k</sup> Ab for 1 h. The relative fluorescence of all samples was analyzed on a FACScan flow cytometer (Becton Dickinson). The figure shows one representative experiment among four.

Table I. Uptake and catabolism of  $^{125}\text{I}$ -HEL in the presence or absence of LPS<sup>a</sup>

Chase Time (min)	TCA-Precipitable (cpm)		TCA-Soluble (cpm)	
	-LPS		+LPS	
0	3143 ± 528	291 ± 13	2912 ± 497	234 ± 27
30	1742 ± 341	160 ± 41	1479 ± 187	152 ± 18
60	1543 ± 330	136 ± 23	1184 ± 156	213 ± 30
120	897 ± 65	89 ± 1	618 ± 20	65 ± 3

<sup>a</sup> Macrophages were incubated with IFN- $\gamma$  for 48 h and treated (+LPS) or not (-LPS) with LPS for 8 h. Then,  $^{125}\text{I}$ -HEL (2  $\mu\text{g}$ ) was added to the cells for 1 h at 37°C and, after extensive washing, the radioactivity was chased for various periods of time. Cell-associated TCA-soluble and -insoluble radioactivity was measured after each time period.

not colocalize with LPS (Fig. 8D). These results led us to hypothesize that the LPS-MHC class II molecule macrodomains may correspond to specialized plasma membrane units, which could be involved in the modulation of Ag presentation by macrophages.

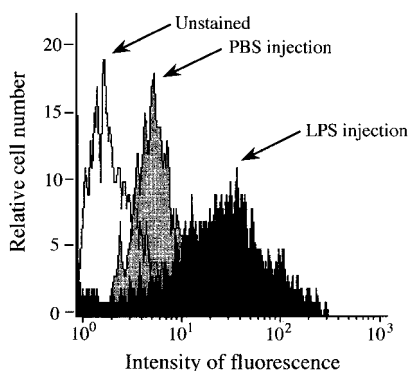
#### LPS does not modulate the formation of SDS-resistant MHC class II dimers induced by processed HEL

Because MHC class II molecules and LPS were clustered at the cell surface of macrophages, we hypothesized that the intrinsic capacity of Ia molecules to bind antigenic peptides could be modified in the presence of LPS. Macrophages were first treated with IFN- $\gamma$  to allow MHC class II expression followed by an incubation or not with LPS in the presence or the absence of HEL. We analyzed the influence of LPS on the formation of SDS-resistant MHC class II dimers (compact forms) induced upon HEL internalization, a hallmark of HEL peptide binding to MHC class II molecules. In the absence of HEL and LPS, macrophages from IFN- $\gamma$ -primed mice expressed compact forms (Fig. 9, -HEL, -LPS), the addition of LPS did not significantly change their amount (Fig. 9, -HEL, +LPS). Indeed, the value of the compact forms/free  $\alpha$  and  $\beta$  forms ratio was 0.65 in the absence and 0.5 in the presence of LPS. As expected, in the presence of HEL, a strong induction of compact forms was observed (Fig. 9, +HEL, -LPS), the amount of which remained constant in the presence of LPS (Fig. 9, +HEL, +LPS). The compact forms/free  $\alpha$  and  $\beta$  forms ratio reached 0.9 and 0.87 after HEL addition in the absence or the presence of LPS, respectively. These results show that the generation of HEL-induced compact forms correctly occurred even in the presence of LPS and suggest that LPS does not prevent the access of processed peptides to the MHC class II binding groove.

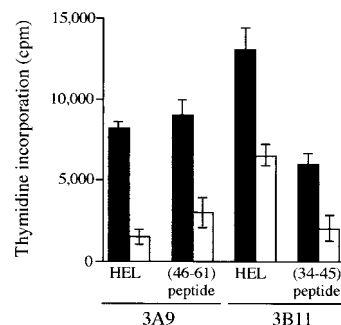
## Discussion

*Brucella* LPS, like those from other intracellular pathogens such as *Legionella*, *Campylobacter*, *Coxiella*, and *Chlamydia*, presents major differences when compared with enterobacterial LPSs (15–18, 24, 29–32). For instance, the lipid A moieties and the core oligosaccharides of these LPSs are structurally different from the classical enterobacterial LPSs, being, in general, more hydrophobic and less substituted, and possessing longer aliphatic chains. The LPSs from these intracellular bacteria display attenuated biological activities and exert very low endotoxicity for animals. In this report, we provide new insights into the mechanisms of cellular immune response modulation by the low endotoxic LPS, purified from *B. abortus*, a facultative intracellular parasite capable of infecting both animals and humans.

Here we demonstrate that the low endotoxic *B. abortus* LPS dramatically reduces the protein Ag-dependent stimulation of T lymphocytes. This event can be related to the intracellular trafficking of *Brucella* LPS and its capacity to form macrodomains with MHC class II molecules. The inhibitory effect on Ag presentation cannot be due to a direct suppressive action of LPS on T cells without any macrophage requirement, because LPS was never found free in the culture medium. Indeed, we have previously shown that intracellular LPS is not exocytosed or recycled by macrophages in the external milieu (18). It has been generally established that endotoxins enhance the accessory function of APCs by inducing the expression of molecules implied in T cell activation. For instance, *Escherichia coli* and *Salmonella* LPSs have been shown to up-regulate B7 costimulatory molecules in B lymphocytes (11) and in human monocytes (33). Endotoxins also trigger the maturation of both human and murine dendritic cells

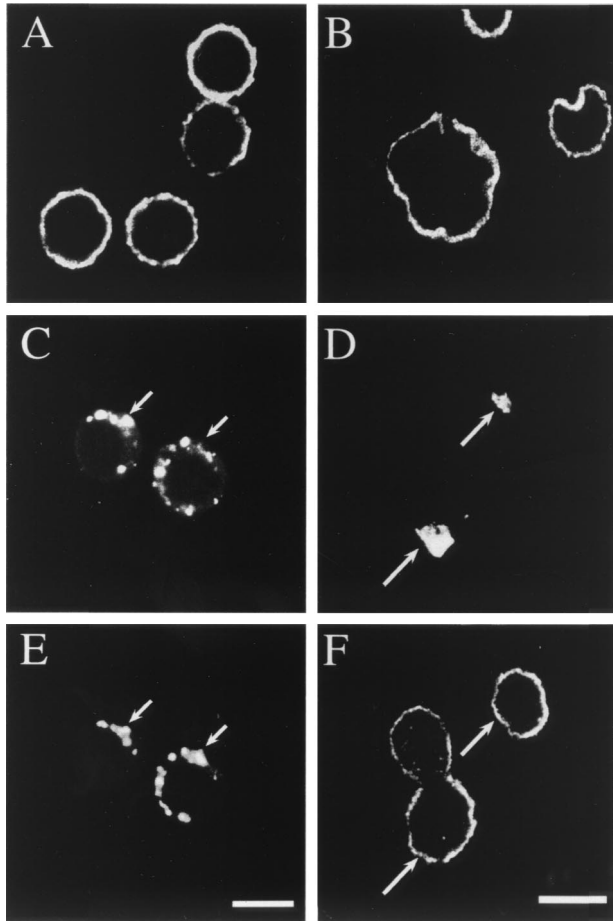


**FIGURE 6.** Macrophages for LPS-injected mice express MHC class II molecules. Macrophages were harvested from mice injected (or not) with LPS and directly submitted to flow cytometry analysis to detect the amount of MHC class II expression on macrophages cell surfaces (as described in Fig. 4), control macrophages that were not labeled with the fluorescent AB



**FIGURE 7.** LPS inhibits HEL Ag presentation in vivo. Macrophages were recovered from LPS (open columns)- or IFN- $\gamma$  (filled columns)-injected mice and were tested for their Ag presentation ability (see *Materials and Methods*) using HEL or HEL<sub>46–61</sub> and HEL<sub>34–45</sub> peptides recognized in the context of MHC class II molecules by their specific T cell hybridomas.

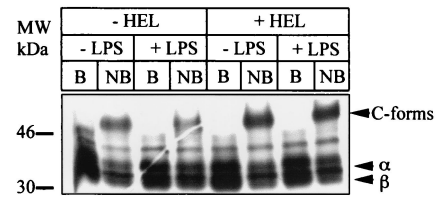




**FIGURE 8.** Detection of LPS-MHC class II macrodomains at the macrophage cell surface. Macrophages were analyzed by immunofluorescence without permeabilizing the cells to detect surface MHC class II (A and E), MHC class I (B and F), and LPS (C and D). In A and B, macrophages were harvested from IFN- $\gamma$ -injected mice, whereas in C, D, E, and F they were harvested from LPS-injected mice. Double immunofluorescence labeling (C and E) shows the expression of LPS (C) and MHC class II molecules (E), whereas in D and F labeling shows the expression of LPS (D) and MHC class I molecules (F). Arrows point out the position of LPS macrodomains.

from Ag-capturing cells to efficient presenting cells. This differentiation event is characterized by an up-regulation of B7 costimulatory molecules, the adhesion molecule ICAM-1, and MHC class II molecules at the cell surface (12, 34, 35). However, in sinusoidal endothelial cells pretreated with *E. coli* LPS, T cell activation was almost completely abrogated, in part, by a reduced expression of costimulatory molecules (13). We found that *B. abortus* LPS did not modulate the expression levels of either B7 membranous costimulatory molecules in peritoneal macrophages (data not shown). In *E. coli* LPS-treated liver endothelial cells, the major cause of the defect in Ag presentation was due to the accumulation of LPS inside the APC, leading to a modification of the pH of endocytic compartments and to a defect in the loading of MHC class II molecules with immunogenic peptides (13). We were able to exclude such an effect because the uptake of HEL, its processing, and the loading of peptides on MHC class II were not directly affected by *Brucella* LPS.

To be presented to T cells, protein Ags have to be internalized and processed by APCs into peptides, which associate with MHC class II molecules within MHC class II-enriched compartments. Then, the peptide-MHC class II complexes recycle to the cell sur-



**FIGURE 9.** Induction of SDS-resistant MHC class II dimers by HEL in LPS-treated or -untreated macrophages. Macrophages from LPS-injected mice were plated and treated (or not) with 20  $\mu\text{g/ml}$  of LPS for 8 h before a 4-h metabolic labeling at 37°C with [ $^{35}\text{S}$ ]cysteine/methionine mix in the presence or the absence of 2 mg/ml of HEL. Macrophages were lysed and supernatants submitted to immunoprecipitation with the 10.2.16 anti-class II Ab. Samples were boiled (B) or not (NB), allowing the detection of compact forms (C-forms) in nonboiling conditions after SDS-PAGE and autoradiography.

face (36). Several studies have revealed that phagocytosis of some microbial particulate materials, like soluble polysaccharides or polyanions, affected the presentation of proteins by macrophages to specific T cells (7). Although the underlying mechanisms have not been clearly identified, it was suggested that the encounter between Ia molecules and processed peptides could be impaired after ingestion of these poorly degradable molecules. Similarly, we have observed that *B. abortus* LPS is retained in macrophages without undergoing degradation, it accumulates for several hours within the lysosomes before recycling to the macrophage plasma membrane where it concentrates in some cell surface areas (18). In this study, we report that plasma membrane LPS macrodomains were specifically enriched in MHC class II molecules, confirming the tight association between these two molecules found by co-precipitation experiments in murine macrophages (data not shown) and human and murine B lymphocytes (17). Despite this fact, the presence of MHC class II-LPS macrodomains seemed to not prevent the binding of HEL peptides into the groove of MHC class II molecules because the formation of HEL-induced compact forms was similar in both LPS-treated and -untreated macrophages. Therefore, LPS-induced interference on MHC class II Ag presentation is likely to occur distal to intracellular events leading to the meeting of antigenic peptides and MHC class II molecules. Altogether, these results suggest that the functional failure of LPS-loaded macrophages to present exogenous Ags could be related to events involving the meeting of correctly formed peptide-MHC class II complexes with their specific TCR. One hypothesis to explain this inhibition would be that LPS embedded in the membrane of MHC class II-positive compartments would interact with already formed HEL peptide-MHC class II complexes, thus forming a ternary complex, which then recycles to the plasma membrane. In the presence of LPS, an important proportion of class II-peptide complexes seems to be sequestered in the macrodomains present at the cell surface, decreasing the number of functional MHC class II-peptide complexes able to activate T cells. We suggest that the LPS O-chain would prevent the correct interaction between the MHC class II-peptide complex and the TCR, leading to a nonactivation of the T cells.

Another interesting model is related to a superantigen (SAG)-like function in T cell activation. It is known that SAGs simultaneously bind the V $\beta$  domain of the TCR and MHC class II molecules on APC and consequently modify the geometry of TCR-peptide/MHC complexes, which may be less critical for T cell activation than certain other factors, in particular, those involved in the stability of the resulting complex (37, 38). In addition, the serial triggering (39–41) and kinetics proofreading models (42) of



T cell activation suggest that the short half-lives of TCR-peptide/MHC complexes are required for efficient T cell stimulation. It has been proposed that the ternary complex TCR-SAG-MHC has a short half-life similar to that of the TCR-peptide/MHC complex, which renders the complexes unable to serially engage a large number of TCRs (38). In this study, we detected the presence of LPS-MHC class II macromolecules in macrophages even after 60 days post-LPS injection, thus highlighting the remarkable stability of these surface LPS macromolecules. In this model, LPS could down-regulate T cell responses by stabilizing the MHC-peptide complexes at the cell surface of APC. Consequently, in contrast to SAG, LPS would be less efficient at triggering T cells because they form TCR-LPS-MHC complexes with a very long half-life. Because it remains to be established whether ligands that form complexes with very long half-lives are actually less efficient at triggering T cells, further molecular characterization of the surface MHC class II-LPS complexes will certainly be of great interest to better understand the mechanisms by which this molecule alters the activation of T lymphocytes and, thus, inhibits the cellular immune response.

Interestingly, the inhibition of immune response described here in vitro correlates with that observed in vivo upon infection by *Brucella*. Indeed, it is worth noting that chronic brucellosis is accompanied by a profound general immunosuppression, which can be revealed by using an IL-2 detection system (43, 44). Such mechanisms seem to be related to the location of *Brucella* within macrophages, the major effector of microorganism cell-mediated killing. For instance, infected macrophages may exert a negative feedback control, which diminishes lymphocyte proliferation in response to *Brucella* Ags (45, 46). Baldwin et al. hypothesized that chronically infected macrophages may fail to act as a target of T cells and may down-regulate T lymphocyte function (47). Our study using LPS-loaded macrophages is in agreement with the latter work leading to the conclusion that macrophages containing either bacteria or LPS are immunosuppressors. In contrast to enterobacteria such as *Salmonella* and *Shigella*, *Brucella* are able to survive and multiply inside phagocytic cells without provoking their apoptosis, a property partly due to the peculiar function and structure of *Brucella* LPS, which avoids the activation of the macrophage-killing systems and confers resistance for the pathogen to the microbicidal action of antibiotics (48, 49). Because it has been shown that LPS can be released from bacteria inside host cells and that LPS does not seem to be degraded by peritoneal macrophages (18), we can hypothesize that the *Brucella*-induced immunosuppression could be attributed to bacteria-associated or -released LPS.

In conclusion, this study affords evidence that *Brucella* LPS plays a central role in the immunosuppression observed upon brucellosis infection and may account for the presence of anergic T cells in infected patients (50).

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