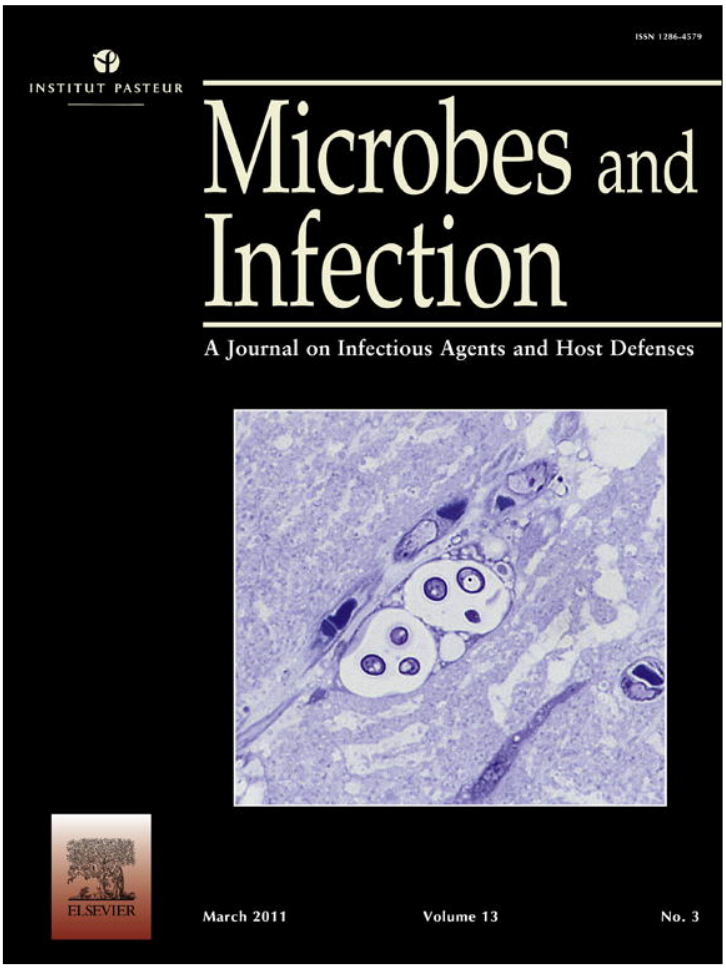

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Original article

Brucella abortus inhibits IFN-γ-induced FcγRI expression and FcγRI-restricted phagocytosis via toll-like receptor 2 on human monocytes/macrophages

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Abstract

The strategies that allow *Brucella abortus* to persist for years inside macrophages subverting host immune responses are not completely understood. Immunity against this bacterium relies on the capacity of IFN-γ to activate macrophages, endowing them with the ability to destroy intracellular bacteria. We report here that infection with *B. abortus* down-modulates the expression of the type I receptor for the Fc portion of IgG (FcγRI, CD64) and FcγRI-restricted phagocytosis regulated by IFN-γ in human monocytes/macrophages. Both phenomena were not dependent on bacterial viability, since they were also induced by heat-killed *B. abortus* (HKBA), suggesting that they were elicited by a structural bacterial component. Accordingly, a prototypical *B. abortus* lipoprotein (L-Omp19), but not its unlipidated form, inhibited both CD64 expression and FcγRI-restricted phagocytosis regulated by IFN-γ. Moreover, a synthetic lipohexapeptide that mimics the structure of the protein lipid moiety also inhibited CD64 expression, indicating that any *Brucella* lipoprotein could down-modulate CD64 expression and FcγRI-restricted phagocytosis. Pre-incubation of monocytes/macrophages with anti-TLR2 mAb blocked the inhibition of the CD64 expression mediated by HKBA and L-Omp19. These results, together with our previous observations establish that *B. abortus* utilizes its lipoproteins to inhibit the monocytes/macrophages activation mediated by IFN-γ and to subvert host immunonological responses.

Keywords: *Brucella abortus*; Lipoproteins; CD64 antigens; Macrophages

1. Introduction

Brucella abortus is a stealthy bug that lives inside macrophages and establishes a chronic infection [1]. Immunity against this bacterium relies in the capacity of IFN-γ to activate macrophages endowing them with the ability to destroy intracellular bacteria [2]. IFN-γ also induces the expression of many IFN-γ-inducible genes, crucial for the development of innate and adaptive immunity against this pathogen [3]. Despite of the importance of IFN-γ-induced responses to host defense against *Brucella*, it is important to consider that *B. abortus* have evolved mechanisms to inhibit or evade certain IFN-γ-induced responses. Among them, we have recently demonstrated that *B. abortus* inhibits the IFN-γ-induced expression of MHC-II molecules and antigen (Ag) presentation in human monocytes, and we have determined that the responsible components of these effects are their lipoproteins [4]. Yet, is unknown if *B. abortus* is capable to

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down-modulate other IFN-γ-induced responses on monocytes/macrophages to promote chronic infection.

The receptors for the Fc portion of immunoglobulin G (IgG) (FcγRs) are widely distributed on cells of the immune system and have been considered a link between cellular and humoral immunity by acting as a bridge between antibody specificity and effector cell functions. They also enable monocytes/macrophages and neutrophils to exert regulatory functions, as well as to trigger a variety of cytotoxic mechanisms [5,6]. Three different classes of FcγRs, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), have been identified on human leukocytes through the use of monoclonal antibodies (mAbs), functional analysis, and the molecular characterization of their primary structure. One of them, FcγRI, is a high-affinity receptor capable of the induction of phagocytosis, clearance of immune complexes, respiratory burst, antibody-dependent cell-mediated cytotoxicity, enhancement of Ag presentation, and secretion of inflammatory cytokines [7]. This receptor is expressed mainly in mononuclear phagocytes, and this expression is highly regulated by IFN-γ and it is a marker of macrophage activation [5].

The present study was undertaken to determine the effect of *B. abortus* on FcγRI expression and FcγRI-mediated phagocytosis in human monocytes/macrophages. As target cells we used human monocytes/macrophages. We first elucidated the ability of *B. abortus* to induce the down-modulation of FcγRI expression upon macrophage infection. Once the phenomenon was corroborated, we investigated the role of *Brucella* lipoproteins on the inhibition of FcγRI expression and FcγRI-mediated phagocytosis induced by *B. abortus*. For this purpose, we used purified recombinant outer membrane protein 19 (Omp19) from *B. abortus* as the model stimulant. Here, we present the results of this study.

2. Materials and methods

2.1. Bacteria

B. abortus S2308, *Brucella ovis* REO 198, *Brucella canis* and *B. abortus* RB51 were cultured in tryptose-soy agar supplemented with yeast extract (Merck). Bacterial numbers were determined as described [8]. To obtain heat-killed *B. abortus*, bacteria were washed five times for 10 min each in PBS and heat-killed by boiling for 20 min. Absence of *B. abortus* viability subsequent to heat-killing was verified by the absence of bacterial growth.

2.2. Lipoproteins and LPS

Lipidated Omp19 (L-Omp19) and unlipidated Omp19 (U-Omp19) from *B. abortus* were cloned and purified as previously described [8]. Both recombinant proteins contained less than 0.25 endotoxin U/μg proteins as assessed by *Limulus* amebocyte assay (Associates of Cape Cod). *B. abortus* 2308 LPS and *Escherichia coli* O111 K58H2 LPS were provided by Dr. I. Moriyón (University of Navarra, Pamplona, Spain). The purity and the characteristics of these preparations have been published elsewhere [9]. The lipohexapeptide tripalmitoyl-S-glyceryl-Cys-Ser-Lys4-OH (Pam_3Cys) was obtained from Boehringer Mannheim.

2.3. Cells and media

Unless otherwise specified, all experiments were performed at 37 °C in 5% CO_2 atmosphere and standard medium composed of RPMI-1640 supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 10% heat inactivated fetal bovine serum (Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (GE Bio-Sciences) gradient centrifugation from human blood collected from healthy adult individuals [10]. All volunteers gave informed consent prior to participating in the study. Monocytes were then purified from the PBMC by Percoll gradient (GE Bio-Sciences) and resuspended in standard medium. Viability of cells was more than 95% in all the experiments as measured by trypan blue exclusion test. Purity of the isolated $CD14^+$ monocytes was more than 90% as determined by flow cytometry.

2.4. Flow cytometry

Human monocytes at a concentration of 0.5×10^6/ml were incubated in round-bottom polypropylene tubes with 150 U/ml of recombinant human IFN-γ (Endogen) in the absence or the presence of HKBA, *B. abortus* LPS, L-Omp19, U-Omp19, Pam_3Cys, *E. coli* LPS or *Mycobacterium tuberculosis* lysate at the indicated concentration for 24 h. At the end of the culture, cells were washed and incubated with FITC-labeled anti-CD64, anti-HLA-DR, anti-CD16 (BD Biosciences), anti-human-CD11b (Beckman Coulter), anti-human-CD32 (CALTAG Laboratories) mAbs or isotype-matched control Ab. Cells were then washed and analyzed with FACScalibur flow cytometer (BD Biosciences), using CellQuest software (BD Biosciences). Results were expressed as mean fluorescence intensity (MFI) values and represent the arithmetic mean ± SEM of the indicated experiments.

In infection experiments, monocytes (0.5×10^6/ml) were infected with *B. abortus* (at different MOI), *B. ovis*, *B. canis* or *B. abortus* RB51 (MOI 100:1) in the presence of IFN-γ (150 U/ml, Endogen) for 2 h in standard medium containing no antibiotics. Then, cells were extensively washed to remove uninternalized bacteria and infection was maintained in the presence of IFN-γ for an additional 24 h. After culture, flow cytometry was conducted as indicated in the above paragraph. To monitor *Brucella* intracellular survival, concomitantly infected cells were lysed with 0.1% (vol/vol) Triton X-100 in H_2O after PBS washing and serial dilutions of lysates were rapidly plated onto TSB agar plates to enumerate CFUs.

For blocking of TLRs, monocytes (0.5×10^6/ml) were incubated with 20 μg/ml of anti-human TLR2 (clone TL2.1), anti-human TLR4 (clone HTA125) or isotype-matched control (eBioscience) 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), *E. coli* LPS (50 ng/ml) or Pam_3Cys

(10 ng/ml), in the presence of IFN-γ (150 ng/ml, Endogen), for 24 h as described above. After culture, flow cytometry was conducted as indicated.

2.5. Phagocytosis

After the different treatments, 0.5×10^6 human monocytes were washed and incubated with blocking anti-human-CD32 (FcγRII) and anti-human-CD16 (FcγRIII) mAbs or isotype-matched control Ab (Ancell) for 30 min at 37 °C. After this, phagocytosis was evaluated using a phagocytosis assay of *Bordetella pertussis* by flow cytometry, as previously described [11] with minor modifications. Briefly, GFP-expressing *B. pertussis* was grown overnight on BG agar plates and resuspended in RPMI-1640 medium containing 10% FCS. Bacteria were opsonized with anti-*B. pertussis* human IgG (200 μg/ml) for 30 min at 37 °C. After washing, opsonized bacteria were incubated with phagocytic cells (previously blocked with anti-CD32 and anti-CD16) in a 70:1 ratio for 20 min at 37 °C to allow binding of bacteria to monocytes/macrophages. After extensive washing to remove non-attached bacteria, cells were split in two aliquots: one aliquot was maintained on ice to determine monocyte surface-associated bacteria at this time point, while the other aliquot was further incubated for 1 h at 37 °C. Phagocytosis was stopped by placing monocytes on ice. Monocyte surface-bound bacteria in both samples were detected by incubation (30 min at 4 °C) with PE-conjugated goat $F(ab')_2$ anti-human IgG. After washing, samples were analyzed by flow cytometry. Five-thousand cells were analyzed per sample. Green and red fluorescence intensities of cells maintained at 4 °C throughout served as control for bacterial binding (i.e., 0% phagocytosis). The decrease in red fluorescence of green positive cells after incubation at 37 °C reflects bacterial phagocytosis. Phagocytosis rates were calculated from the difference in mean red fluorescence intensity of the cells incubated at 4 °C (bacterial binding) and cells incubated at 37 °C (bacterial phagocytosis) (Δ MFI PE), as described [11]. Controls to exclude auto-fluorescence of monocytes/macrophages or unspecific binding of F(ab′) of PE-secondary Abs to monocytes/macrophages were included in each experiment.

2.6. Statistical analysis

Statistical significance of results was calculated using the non-parametric Mann–Whitney *U* test with the InStat 2 Software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. B. abortus *infection down-modulates the IFN-γ-induced expression of FcγRI (CD64)*

We first evaluated the ability of *B. abortus* to inhibit the IFN-γ-induced expression of CD64 on human monocytes/macrophages. Cells were infected with *B. abortus* in the presence of IFN-γ for 2 h, washed to remove uninternalized bacteria and infection was maintained in the presence of IFN-γ for an additional 24 h. The expression of CD64 was evaluated by flow cytometry. *B. abortus* infection inhibited the IFN-γ-induced surface expression of CD64 in a dose-dependent manner (Fig. 1A and B). A significant ($P < 0.05$) CD64 down-modulation was detected in cultures infected with 25 bacteria/cell or higher. In parallel we determined the ability of *B. abortus* infection to inhibit the IFN-γ-induced expression of MHC-II molecules. This measure was used as positive control of our system. As described previously [4], the IFN-γ-induced expression of MHC-II also was down-modulated by *B. abortus* infection in a dose-dependent manner (Fig. 1C). In contrast, surface expression of CD11b did not differ between control and infected cells (Fig. 1D), indicating that *B. abortus* does 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 Annexin V assay, revealed no differences in the percentage of viable cells in infected or uninfected cells, even at the highest infection level (data not shown).

3.2. Infection with other Brucella *species also inhibits the CD64 expression*

The next experiments were designed to evaluate if the down-modulation of CD64 expression mediated by *B. abortus* can be extended to other *Brucella* species. For this purpose, human monocytes were infected with the rough *Brucella* species, *B. ovis* and *B. canis*; and with the rough *B. abortus* strain, *B. abortus* RB51, at MOI 100:1 in the presence of IFN-γ as described above. Then, the expression of CD64 or MHC-II was evaluated by flow cytometry. Infection of human monocytes with different *Brucella* species inhibited the CD64 (left panels, Fig. 2) and MHC-II (right panels, Fig. 2) expression; and the level of inhibition was similar to that obtained with *B. abortus* infection (Fig. 2). This indicates that different *Brucella* species are able to inhibit CD64 expression.

3.3. HKBA inhibits the IFN-γ-induced expression of CD64

To test whether viable bacteria were necessary to induce inhibition of CD64, the ability of HKBA to down-modulate IFN-γ-induced CD64 expression was examined. HKBA also inhibited the IFN-γ-induced expression of CD64. Inhibition was a function of the amount of bacteria present in the culture (Fig. 3A and B). Significant ($P < 0.01$) CD64 down-modulation was detected at similar bacteria/cell ratios that the ones used for infection; and the level of inhibition was similar to that obtained with *M. tuberculosis* lysate or *E. coli* LPS (Fig. 3C and D), two well known down-modulators of CD64 expression [12,13] used as controls. These results suggest that CD64 inhibition is caused by a structural component of *B. abortus*. Experiments were then conducted to evaluate the contribution of *B. abortus* LPS to the down-modulation of CD64 expression mediated by HKBA. For that purpose monocytes/macrophages were incubated with highly purified

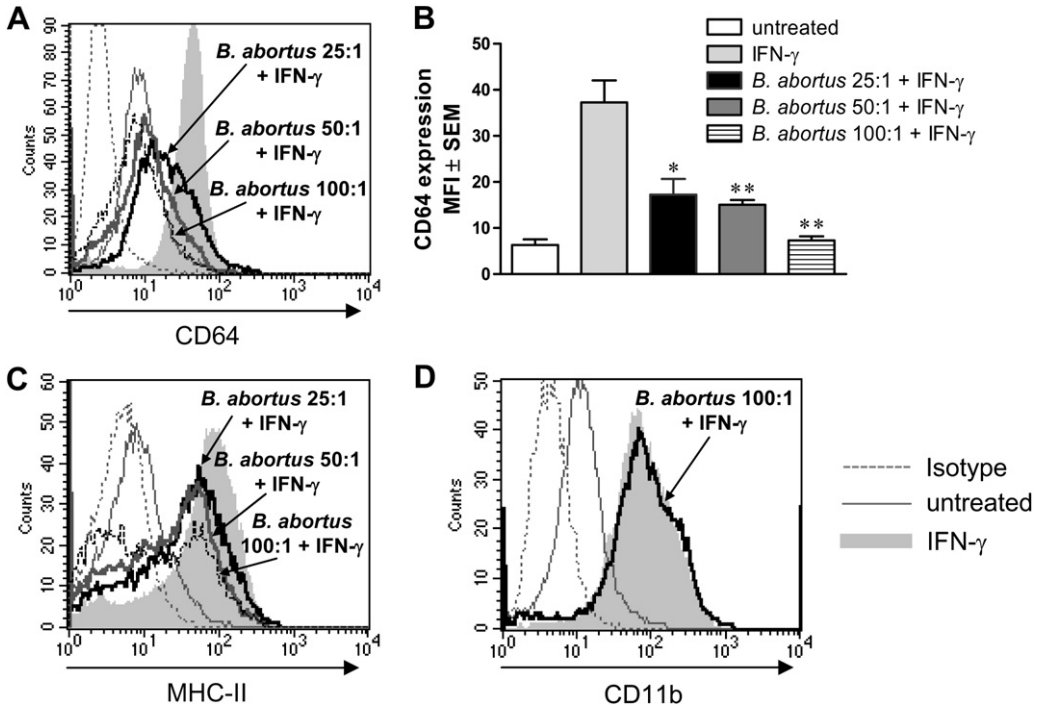

Fig. 1. *B. abortus* infection down-modulates the IFN-γ-induced expression of CD64. Human monocytes were infected with *B. abortus* at different multiplicities of infection (MOI) in the presence of IFN-γ (150 U/ml) for 2 h, washed, and culture in the presence of IFN-γ for 24 h (for CD64) or 48 h (for MHC-II and CD11b). CD64 (A and B), MHC-II (C) and CD11b (D) expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. The bars in panel B indicates 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. *$P < 0.05$ vs. IFN-γ; **$P < 0.01$ vs. IFN-γ.

B. abortus LPS and, after 24 h of culture, the expression of CD64 was evaluated by flow cytometry. High concentrations (1000 ng/ml) of highly purified *B. abortus* LPS were unable to reduce the IFN-γ-induced expression of CD64 (Fig. 3E). Our results indicate that *B. abortus* LPS does not contribute to the down-modulation of CD64 induced by HKBA.

3.4. B. abortus *lipoprotein Omp19 down-modulates the CD64 expression induced by IFN-γ*

As *B. abortus* LPS is not involved in the down-regulation of CD64 expression and taking into account that the lipoprotein Omp19 and other bacterial lipoproteins are capable of inhibiting IFN-γ-induced MHC-II expression [4,12,14,15], we hypothesized that *Brucella* lipoproteins could be the structural components involved in CD64 down-modulation. To test this hypothesis, we used recombinant lipidated Omp19 (L-Omp19) as a *Brucella* lipoprotein model. Monocytes/macrophages were incubated with IFN-γ in the presence or the absence of L-Omp19 and, after 24 h of culture, the IFN-γ-induced expression of CD64 was evaluated by flow cytometry. L-Omp19 down-modulated significantly the expression of CD64 in a dose-dependent fashion (Fig. 4A and B). Inhibition of CD64 expression was dependent on the lipidation of L-Omp19, as unlipidated Omp19 (U-Omp19) failed to down-modulate CD64 expression even at a concentration of 5000 ng/ml (Fig. 4C). To ascertain whether the effects elicited by L-Omp19 could be extended to all *B. abortus* lipoproteins, monocytes/macrophages were incubated with varying concentrations of a synthetic lipohexapeptide (Pam_3Cys) that mimics the structure of the lipoprotein lipid moiety, and the expression of CD64 was evaluated by flow cytometry after 24 h of stimulation. The range of Pam_3Cys concentrations used encompassed the value of the molar concentration of Omp19 at 1000 ng/ml. Pam_3Cys inhibited CD64 expression to a degree that was commensurate with that induced by L-Omp19 (Fig. 4D). These results indicate that the Pam_3-modified cysteine is the molecular structure that down-modulates the IFN-γ-induced expression of CD64 and thus, the latter phenomenon may be brought about by any *B. abortus* lipoprotein.

3.5. *The expression of FcγRII (CD32) and FcγRIII (CD16) is not modified by HKBA or L-Omp19*

The mononuclear phagocytes also express other classes of FcγRs, the FcγRII (CD32) and FcγRIII (CD16). However, the expression of these receptors is not up regulated by IFN-γ [16]. We decided to evaluate whether HKBA or L-Omp19 were capable to modulate the expression of other FcγRs resembling FcγRI. To test this, we determined the ability of HKBA, L-Omp19 or U-Omp19 to modulate the CD32 and CD16 expression. As described previously, the expression of these FcγRs is not induced by IFN-γ. The treatment with HKBA, L-Omp19 or U-Omp19 plus IFN-γ did not modified the expression of CD32 (MFI ± SEM. Untreated: 128.8 ± 3.4; IFN-γ: 114.3 ± 5.6;

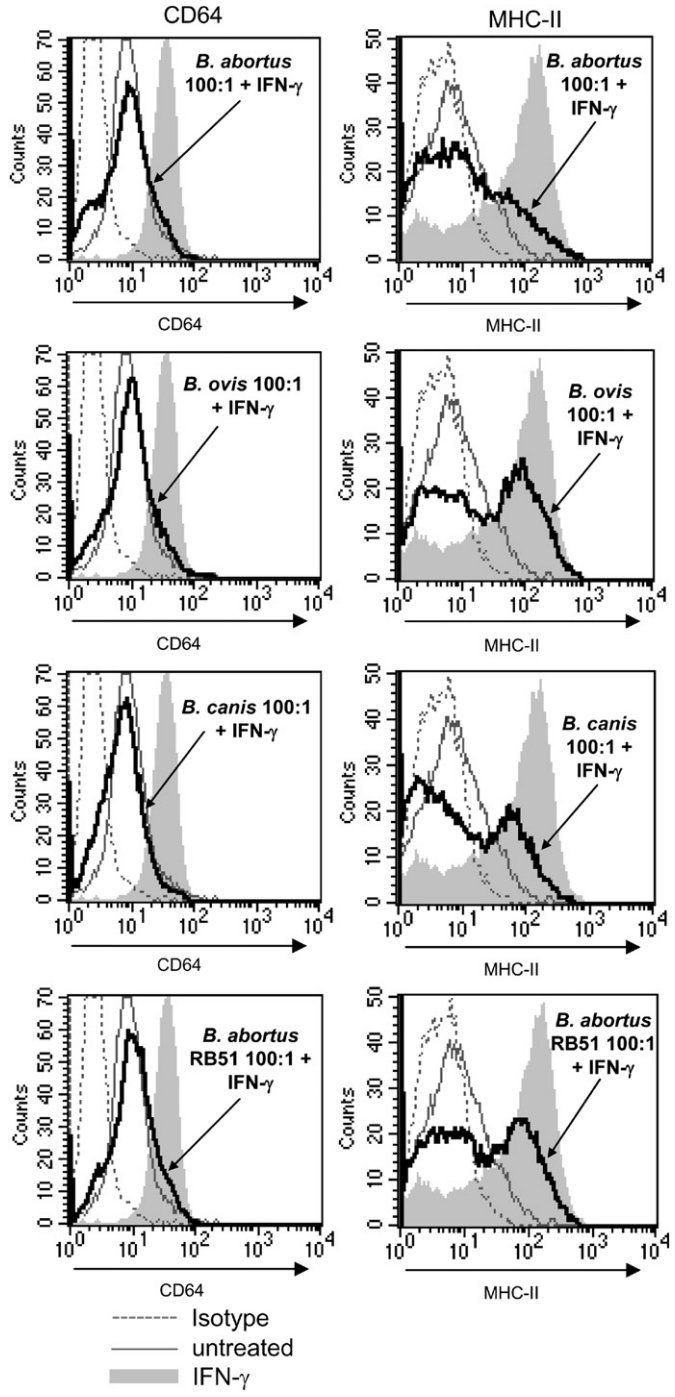

Fig. 2. The infection with different *Brucella* species also inhibits the CD64 expression. Human monocytes were infected with *B. abortus*, *B. ovis*, *B. canis* or *B. abortus* RB51 at MOI 100:1 in the presence of IFN-γ (150 U/ml) for 2 h, washed, and culture in the presence of IFN-γ for 24 h (for CD64) or 48 h (for MHC-II). CD64 (left panels) or MHC-II (right panels) expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. Nonspecific binding was determined using a control isotype Ab.

HKBA 10^9 + IFN-γ: 126.8 ± 14.9; L-Omp19 1000 ng/ml + IFN-γ: 132.1 ± 9.6; U-Omp19 1000 ng/ml + IFN-γ: 107.9 ± 8.7) and CD16 (MFI ± SEM. Untreated: 8.9 ± 1.5; IFN-γ: 7.6 ± 3.2; HKBA 10^9 + IFN-γ: 9.4 ± 1.9; L-Omp19 1000 ng/ml + IFN-γ: 8.2 ± 1.8; U-Omp19 1000 ng/ml + IFN-γ: 7.9 ± 1.4) respect to the expression on untreated cells. These results indicate that HKBA and L-Omp19 do not down-modulate all surface FcγRs, but specifically the FcγRI.

3.6. The inhibition of CD64 expression mediated by HKBA and L-Omp19 is TLR2-dependent

We have previously demonstrated that TLR2 mediates responses to HKBA and *B. abortus* lipoproteins in cells of the monocytic lineage [8,17]. Consequently, we further analyzed the role of TLR2 in the HKBA- and L-Omp19-induced CD64 down-regulation. Monocytes/macrophages were pre-incubated with anti-TLR2 Ab, anti-TLR4 Ab or their respective isotype controls and then cultured with L-Omp19 or HKBA. The expression of CD64 was evaluated by flow cytometry after culture. *E. coli* LPS, a TLR4 ligand, and Pam_3Cys, a TLR2 ligand, were used as controls. Pre-incubation of monocytes/macrophages with anti-TLR2 significantly blocked ($P < 0.05$) the L-Omp19-mediated inhibition of CD64 expression induced by IFN-γ (Fig. 5C and D). Anti-TLR2 also inhibited significantly ($P < 0.05$) the HKBA-mediated inhibition of CD64 expression (Fig. 5A and B). Anti-TLR4 Ab or isotype-control antibody had no effect on the response investigated (Fig. 5A–D). As expected, pre-incubation of monocytes/macrophages with anti-TLR4 significantly blocked the *E. coli* LPS-mediated CD64 down-regulation, whereas anti-TLR2 inhibited significantly the CD64 down-regulation induced by Pam_3Cys (Fig. 5E and F). These results indicate that the inhibition of CD64 expression induced by HKBA and L-Omp19 depends on TLR2.

3.7. HKBA and L-Omp19 inhibit FcγRI-restricted phagocytosis in human monocytes/macrophages

To determine if inhibition of IFN-γ-induced CD64 expression by HKBA and L-Omp19 is associated with changes in FcγRI-restricted phagocytosis, monocytes/macrophages were treated with IFN-γ in the presence of HKBA, L-Omp19 or U-Omp19 for 24 h. After different treatments, the cells were washed, incubated with anti-FcγRII and anti-FcγRIII blocking antibodies and the phagocytosis was evaluated using IgG opsonized *B. pertussis* as described in Materials and methods. Monocytes/macrophages treated with IFN-γ alone performed phagocytosis efficiently (Fig. 6A). Treatment with HKBA and L-Omp19 for 24 h significantly inhibited ($P < 0.05$) (Fig. 6E) the FcγRI-restricted phagocytosis of IFN-γ-treated monocytes/macrophages (Fig. 6B and C). U-Omp19 had no effect on FcγRI-restricted phagocytosis (Fig. 6D), indicating that acylation of lipoproteins is critical in the observed phenomenon. Thus, HKBA and L-Omp19 inhibited FcγRI-restricted phagocytosis regulated by IFN-γ in monocytes/macrophages.

4. Discussion

The cross-talk between the pathogen and the immune system of the host is a relatively unknown area of microbial pathogenesis. It is currently recognized that certain pathogens have

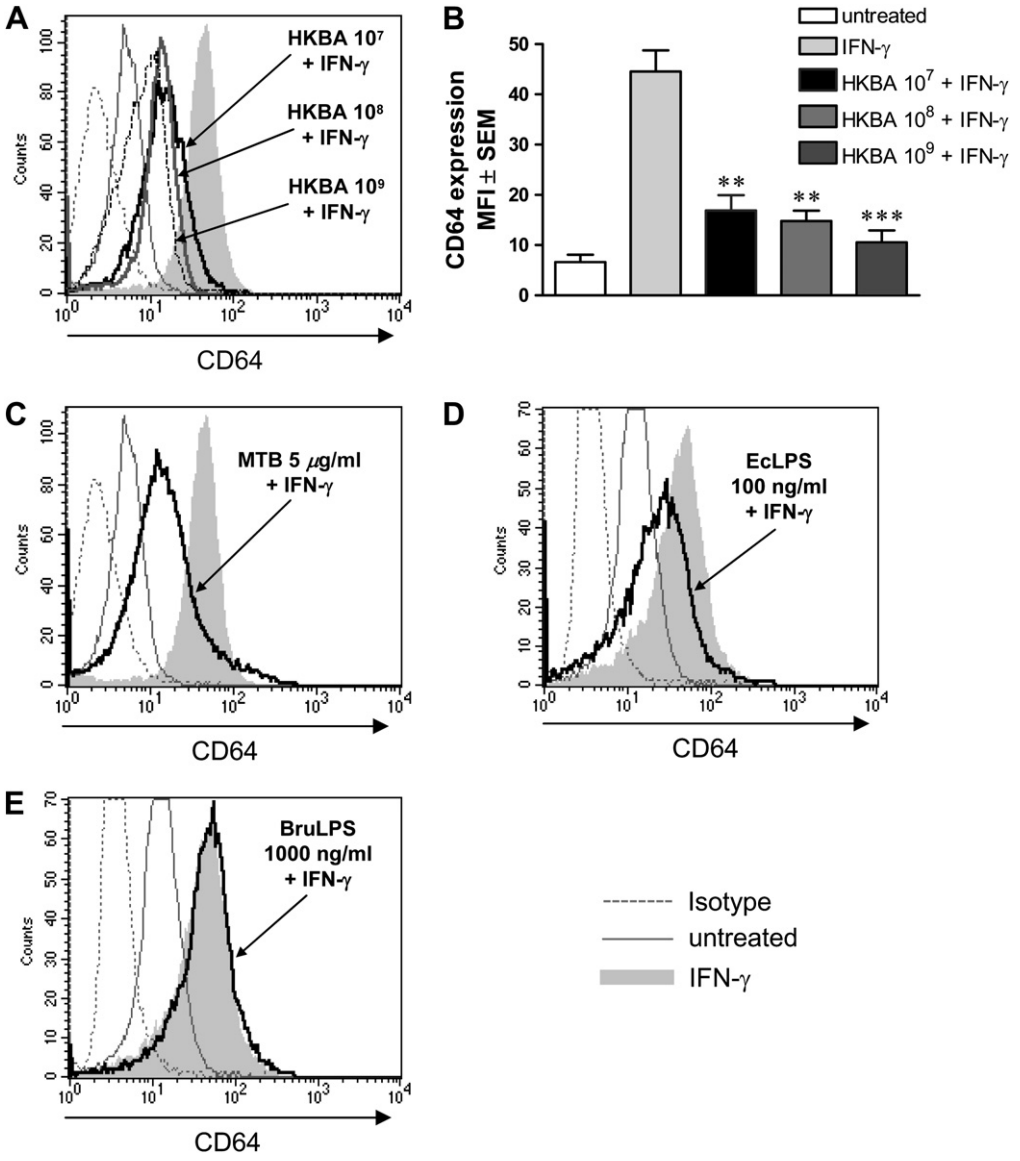

Fig. 3. HKBA inhibits the IFN-γ-induced expression of CD64. A and B, human monocytes were incubated with medium (untreated), IFN-γ (150 U/ml) or HKBA plus IFN-γ for 24 h. C and D, human monocytes were incubated with medium (untreated), IFN-γ (150 U/ml), *M. tuberculosis* lysate (MTB) plus IFN-γ (C) or *E. coli* LPS (EcLPS) plus IFN-γ (D) for 24 h. E, human monocytes were incubated with medium (untreated), IFN-γ (150 U/ml) or *B. abortus* LPS (BruLPS) plus IFN-γ for 24 h. CD64 expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. The bars in panel B indicates 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. $^{**}P < 0.01$ vs. IFN-γ; $^{***}P < 0.001$ vs. IFN-γ.

developed the capacity to actively modulate the immune response of the host to avoid its bactericidal effects [18]. Microorganisms capable of establishing chronic infections have not only the ability to initially escape the immune response of the host but also to persist in the host during its life span. These pathogens require a battery of virulence factors capable of modulating in a very precise manner the immune system of the host, primarily avoiding the innate immunity, preventing the efficient mounting of the acquired immunity and finally establishing a chronic phase of the infection. An important member of this group of organisms is *Brucella* spp. The study of *Brucella* pathogenicity has been focused mainly on identifying factors that affect the intracellular trafficking and multiplication of the bacterium within the host cell, an essential trait of *Brucella* virulence. It has been found that the type IV secretion system VirB is involved in controlling the maturation of the *Brucella*-containing vacuole into a replication permissive organelle [19,20], cyclic β 1−2-glucans help to prevent phagosome-lysosome fusion, allowing for bacterial intracellular replication [21], and the O-polysaccharide inhibits phagocytosis, protecting the bacteria from the phagolysosome and inhibiting host cell apoptosis [22,23]. Although information about *Brucellae* intracellular replication and trafficking has notably increased in the last years, little is known about how *Brucella* achieves a chronic state of infection. Recently, we demonstrated that *B. abortus* is capable to down-modulate the IFN-γ-induced expression of MHC-II on human monocytes. Moreover, *B. abortus* is able to inhibit Ag processing and presentation to

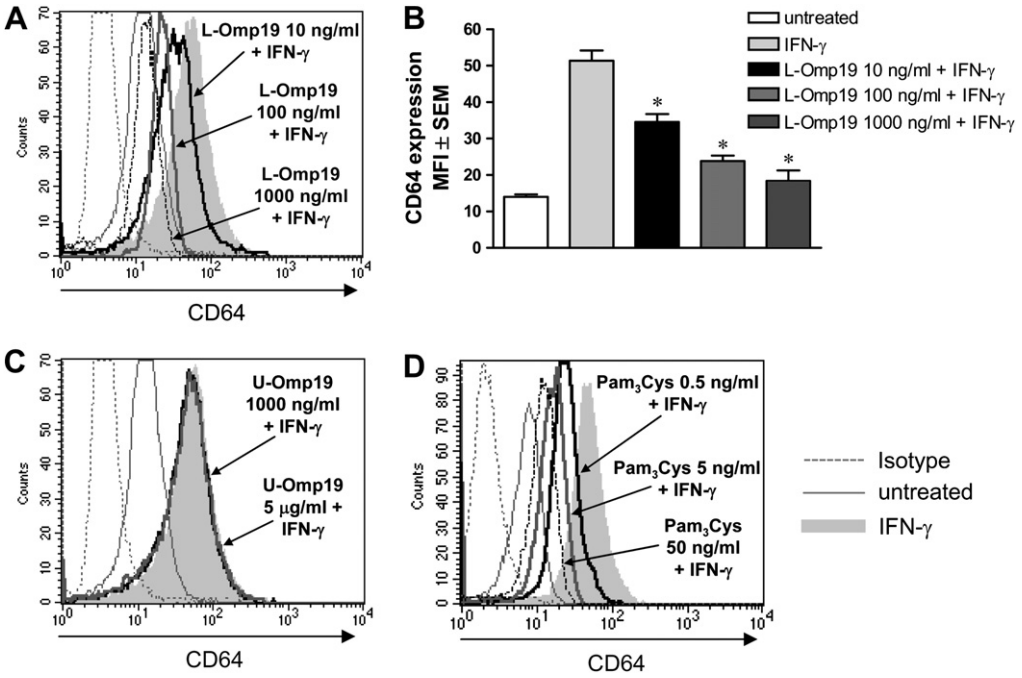

Fig. 4. *B. abortus* lipoprotein Omp19 down-modulates the CD64 expression induced by IFN-γ. A and B, human monocytes were incubated with medium (untreated), IFN-γ (150 U/ml) or L-Omp19 plus IFN-γ for 24 h. C, human monocytes were incubated with medium (untreated), IFN-γ (150 U/ml) or U-Omp19 plus IFN-γ for 24 h. D, human monocytes were incubated with medium (untreated), IFN-γ (150 U/ml) or Pam_3Cys plus IFN-γ for 24 h. CD64 expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. The bars in panel B indicates the arithmetic means of five experiments, and the error bars indicate the standard errors of the means. Nonspecific binding was determined using a control isotype Ab. $*P < 0.05$ vs. IFN-γ.

T lymphocytes [4]. This mechanism could explain how the *Brucella*-containing macrophages can avoid the immunological surveillance of MHC-II restricted IFN-γ-producing $CD4^+$ T lymphocytes, thus promoting chronic infection. However, it is unknown if *B. abortus* is capable to down-modulate other IFN-γ-induced responses on monocytes/macrophages to promote chronic infection.

In this paper we present evidence indicating that infection with *B. abortus* down-modulates expression of CD64 on human monocytes/macrophages. This effect can be extended to other *Brucella* species since rough *B. ovis*, *B. canis* and *B. abortus* strain RB51 was also capable to inhibit CD64 expression. These results suggest that this effect possibly could be also extrapolated to Brucella melitensis. This could be clinically important because the vast majority of chronic infections are caused by this Brucella species [24]. However, further experiments conducted to evaluate this should be conducted.

Down-modulation of CD64 expression correlates with inhibition of FcγRI-restricted phagocytosis regulated by IFN-γ in monocytes/macrophages. Both, inhibition of CD64 expression or FcγRI-restricted phagocytosis were not dependent on bacterial viability, since they were also induced by exposure to heat-killed *B. abortus* (HKBA), suggesting that they were elicited by a structural bacterial component. Among the factors possibly implicated *B. abortus* LPS was a probable candidate to play this role, since LPS from other bacteria were shown to diminish CD64 expression [13]. However, highly purified *B. abortus* LPS was unable to reduce CD64 expression. This result indicates that the inhibition of the CD64 expression induced by *B. abortus* is independent of its LPS.

B. abortus possesses lipoproteins [25]. Studies conducted in our laboratory have demonstrated that *B. abortus* lipoproteins can elicit not only inflammatory but also immunomodulatory responses, e.g., IL-10 and IL-6 from monocytes [8]. These findings have added support to the contention that lipoproteins, together with LPS, would be important virulence factors for *Brucella* survival and replication in the host. As other bacterial lipoproteins and the lipoprotein Omp19 of *B. abortus* have the ability of inhibiting IFN-γ-induced MHC-II expression and Ag presentation by murine and human macrophages [4,12,14,15], we hypothesized that *B. abortus* lipoproteins could be the structural components involved in the observed phenomena. L-Omp19, a prototypical *B. abortus* lipoprotein, inhibited both CD64 expression and FcγRI-restricted phagocytosis regulated by IFN-γ. In agreement with these results, Gehring et al. demonstrated that the 19-kDa lipoprotein of *M. tuberculosis* also inhibited IFN-γ-induced expression of FcγRI [12]. 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 CD64 expression. Since all brucellar lipoproteins likely share the Pam_3Cys modification, this entails that any lipoprotein should be able to down-modulate CD64 expression and FcγRI-restricted phagocytosis.

In a recent work, the mechanisms involved in the disruption of IFN-γ responses mediated by *Brucella* infection were investigated [26]. To decipher this, the authors measured IFN-γ-triggered signaling in *Brucella suis*-infected macrophages. They observed no decrease in STAT1 tyrosine or serine phosphorylation, or in dimerization of phosphorylated STAT1

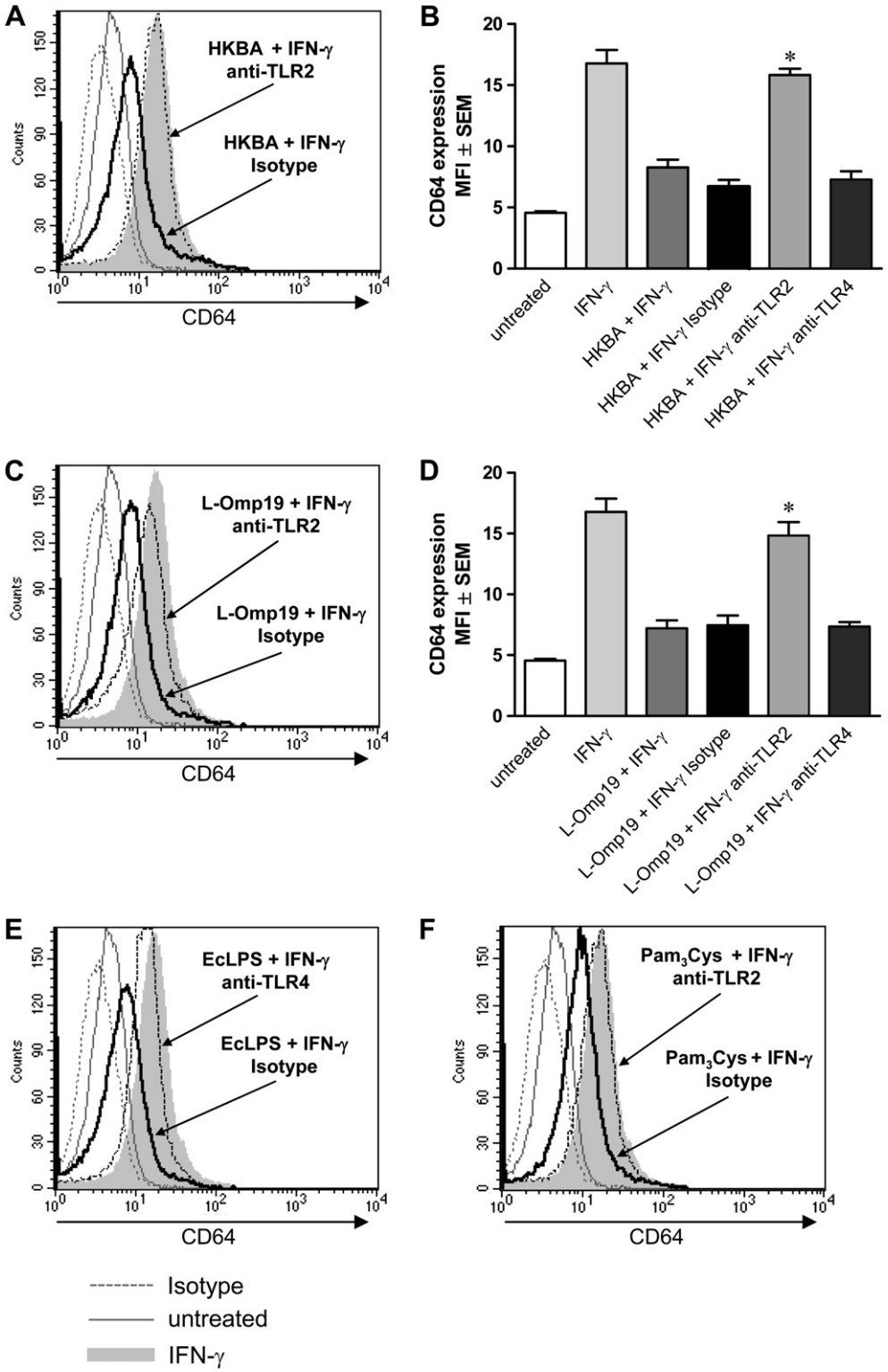

Fig. 5. The inhibition of CD64 expression mediated by HKBA and L-Omp19 is TLR2-dependent. Human monocytes were incubated with 20 μg/ml of anti-TLR2, anti-TLR4 or isotype-matched control for 30 min at 37 °C before the addition of (A and B) HKBA (1×10^8 bacteria/ml), (C and D) L-Omp19 (100 ng/ml), (E) *E. coli* LPS (EcLPS) (50 ng/ml) or (F) Pam_3Cys (10 ng/ml), in the presence of IFN-γ (150 U/ml). After 24 h of culture CD64 expression was assessed by flow cytometry. The histograms indicate the results of one representative of five independent experiments. The bars in panel B and D indicate the arithmetic means of five experiments. MFI, mean fluorescence intensity. Nonspecific binding was determined using a control isotype Ab. *$P < 0.05$ vs. HKBA plus IFN-γ (B) or L-Omp19 plus IFN-γ (D).

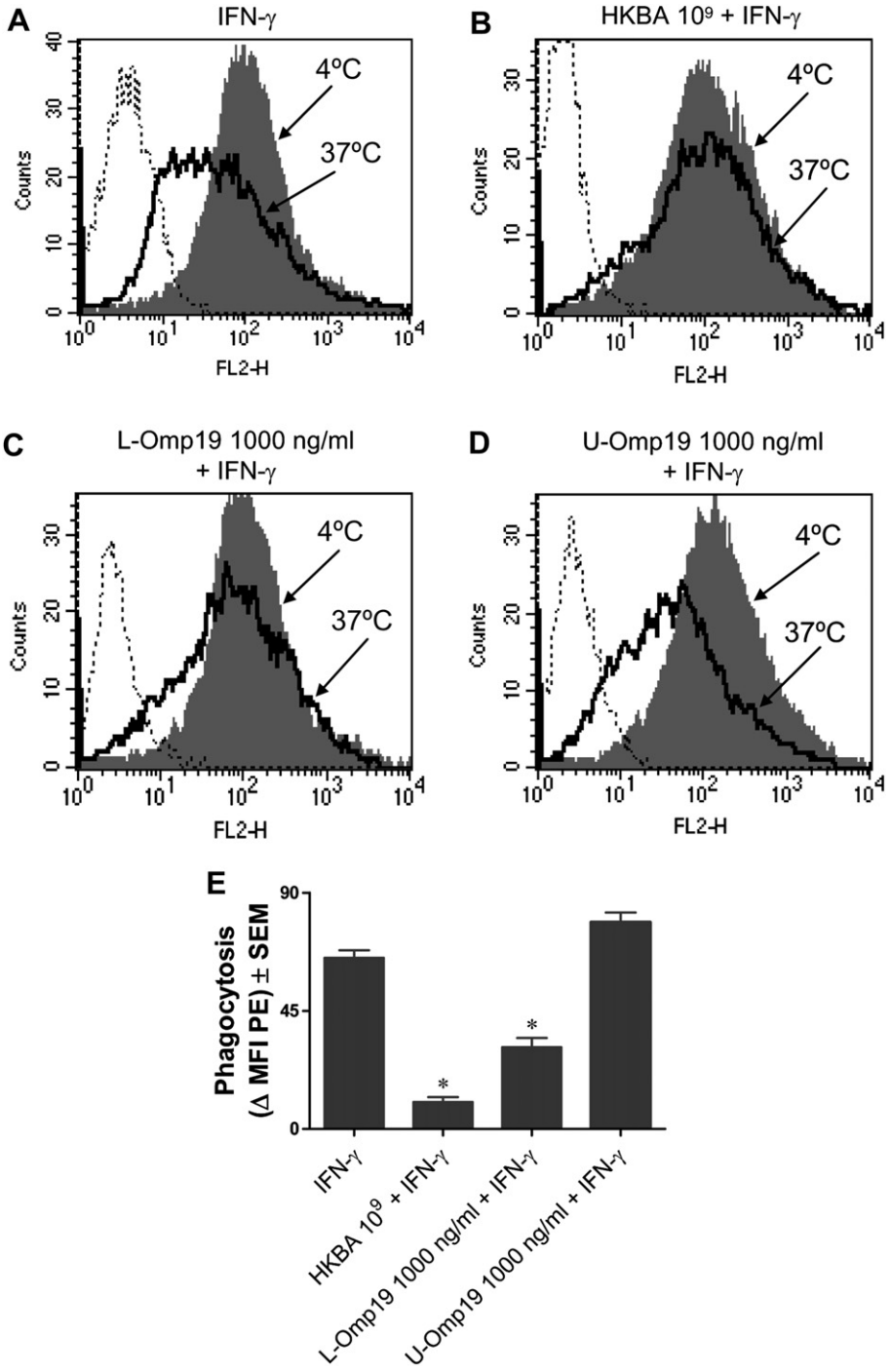

Fig. 6. HKBA and L-Omp19 inhibit FcγRI-restricted phagocytosis in human monocytes/macrophages. A-E, Human monocytes/macrophages were incubated with IFN-γ (150 U/ml) with or without HKBA, L-Omp19 or U-Omp19 for 24 h. After the different treatments, the cells were washed and incubated with anti-FcγRII and anti-FcγRIII blocking antibodies. After this, the cells were incubated with GFP-*B. pertussis* opsonized with human IgG. Then, cells were split in two aliquots and further incubated, either at 4 or 37 °C. Next, remaining cell surface-bound opsonized bacteria were detected by incubation with PE-conjugated goat $F(ab')_2$ of anti-human IgG and were analyzed by flow cytometry. The histograms (A–D) indicate the results of one representative of five independent experiments. The bars (E) indicate the phagocytosis rates. These rates were calculated from the difference in mean red fluorescence intensity of the cells incubated 20 min (bacterial binding) and cells incubated 1 h 20 min at 37 °C (bacterial phagocytosis) (Δ MFI PE) ± SEM (For details see Materials and methods). *$P < 0.05$ vs. IFN-γ.

(P-STAT1) and P-STAT1 translocation to the nucleus; or in P-STAT1 binding to GAS, a minimal IFN-γ-response DNA sequence. In contrast, immuno-precipitation experiments indicated that the IFN-γ-mediated association of P-STAT1 with CBP/P300 transactivators was markedly reduced in infected macrophages, demonstrating that P-STAT1 was unable to normally recruit these transactivators. In any case, the IFN-γ-induced STAT1-CBP/P300 association is required for a normal response of macrophages and its deficiency might contribute to impair macrophage responses to IFN-γ,

a mechanism supported by the inhibition of IFN-γ-induced expression of FcγRI (CD64) gene in *B. suis*-infected cells [26]. While the disruption of STAT1/CBP-P300 complexes was observed in *B. suis*-infected macrophages, this mechanism could also explain the inhibition of IFN-γ-induced expression of CD64 observed in macrophages infected with *B. abortus*. However, *Brucella* must have additional means to control the multiple effects of IFN-γ. In fact, we have already reported that one mechanism exploited by *B. abortus* to inhibit IFN-γ-induced MHC-II expression and Ag presentation is through the secretion of IL-6 by macrophages [4]. Therefore, other mechanisms involving IL-6 production [4,27,28] and/or TLR2 triggering [29] in infected macrophages should be considered.

The relative involvement of TLR2 and TLR4 in mediating *B. abortus*-induced CD64 down-modulation merits discussion. Our results indicate that HKBA inhibit CD64 expression via TLR2 and not TLR4. Since *B. abortus* LPS utilizes TLR4 [8] these results strengthen the contention that LPS is not the molecule employed by *B. abortus* to down-modulate CD64 expression and provide, in addition, proof of concept that *B. abortus* lipoproteins would be the TLR2 ligands employed by the bacterium to down-modulate CD64. As TLR2 can sample material present in phagosomal compartments [30,31], phagosomal-dwelling *Brucella* may chronically activate TLR2 in infected cells, leading to decreased CD64 expression and FcγRI-restricted phagocytosis.

Several reports were conducted to investigate the consequence of antibody opsonization, and thus the access through FcγRs, on entry and survival of *Brucella* in phagocytes. These studies have shown that antibody opsonization increases the entrance but decreases the survival of *B. suis* in the U937 human macrophage cell line, as would be expected according to conventional paradigms. As a result, there may be eventually similar numbers of live organisms/cells, with or without antibody opsonization. However, the subsequent intracellular growth rate of antibody-opsonized brucellae is slightly lower [32,33], and thus, overall, one would predict a decrease in infection with antibody opsonization based simply on the numbers of *B. suis* organisms per cell [34]. Other studies have shown that *Brucella* opsonized with a serum containing specific anti-*Brucella* antibodies (*ops-Brucella*) or with a control nonimmune serum (*c-Brucella*) was phagocytized and proliferated in murine J774A.1 cells [35]. As in human macrophages [32,36], bacterial opsonization substantially enhanced the phagocytosis process, promoted significant killing of the ingested bacteria and induced a slight diminution of their proliferation. Thus, 48 h after infection, the multiplication of the live bacteria was much lower in *ops-Brucella*-infected cells than in *c-Brucella*-infected cells [35]. In summary, opsonization with specific antibodies increases the number of phagocytized bacteria but lower their intra-macrophage development. Therefore, we can hypothesize that the CD64 inhibition could diminish the entry of *Brucella* but in contrast would increase their survival within macrophages. Thus, enhancing the intracellular growth and establishing chronic infection.

On the other hand, several studies investigated the relevance of FcγRs against intracellular pathogens using murine models of FcγRs deficiency. Maglione et al. demonstrated that Fcγ-chain-deficient C57BL/6 mice succumb to infection with *M. tuberculosis* quicker than wild-type controls [37]. Deficiency of the Fcγ-chain was previously reported to affect the progression of infection with other intracellular pathogens, including influenza and *Leishmania* species [38–41]. The inability to contain infection in Fcγ-chain$^{-/-}$ mice may be related to the increased IL-10 response in these mice, as IL-10 is an immunosuppressive cytokine that can subvert optimal *M. tuberculosis* containment [42,43].

These results demonstrate that FcγR are required for optimum immune activation in this model. Even though studies with FcγR-deficient mice had not been conducted with *B. abortus*, our results are a good approximation to predict the importance of CD64 in the contention of the bacteria during *Brucella* infection. In fact, other authors demonstrated that phagocytosis has been significantly reduced in polymorphonuclear and mononuclear phagocytes of active brucellosis patients. The alterations in phagocytic cell function were more pronounced in patients with bacteremia, with focal manifestations or with longer disease duration [44], suggesting that lipoproteins-mediated inhibition of phagocytosis is a potential mechanism that could be occurring in patients with chronic brucellosis.

Our results support a model in which the persistence of *Brucella* inside the macrophages for a prolonged period would allow chronic exposure to PAMPs (e.g., lipoproteins). Extended TLR2 signaling by lipoproteins may then reduce IFN-γ-induced CD64 expression and FcγRI-restricted phagocytosis of infected macrophages, which would then serve as niches in which *B. abortus* could persist. Thus, the results *shown* in this paper establish that the down-modulation of CD64 expression could be another possible mechanism employed by *B. abortus* to inhibit the monocytes/macrophages activation mediated by IFN-γ and to subvert host immunonological surveillance.

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