Key role of TLR2 in the inflammatory response and MHC-II down-regulation in *Brucella*-infected alveolar macrophages

Mariana C. Ferrero[a], M. Soledad Hielpos[a], Natalia B. Carvalho[b], Paula Barrionuevo[c], Patricia P. Corsetti[b], Guillermo H. Giambartolomei[c], Sergio C. Oliveira[b] and Pablo C. Baldi[a,#]

[a]Instituto de Estudios de la Inmunidad Humoral (CONICET/UBA), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

[b]Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil.

[c]Instituto de Inmunología, Genética y Metabolismo (CONICET/UBA), Hospital de Clínicas "José de San Martín," Buenos Aires, Argentina.

Running title: *Brucella abortus* interaction with alveolar macrophages

[#]Address correspondence to Pablo C. Baldi, pablobal@ffyb.uba.ar

ABSTRACT

Alveolar macrophages (AM) seem to constitute the main cellular target of inhaled brucellae. Here we show that *Brucella abortus* invades and replicates in murine AM without inducing cytotoxicity. *B. abortus* infection induced a statistically significant increase of TNF-α, KC, IL-1β, IL-6 and IL-12 in AM from C57BL/6 mice and Balb/c mice, but these responses were generally weaker and/or delayed as compared to those elicited in peritoneal macrophages. Studies using knockout mice for TLR2, TLR4 and TLR9 revealed that TNF-α and KC responses were mediated by TLR2 recognition. *Brucella* infection reduced in a MOI-dependent manner the expression of MHC-II molecules induced by gamma-interferon (IFN-γ) in AM. The same phenomenon was induced by incubation with heat-killed *B. abortus* (HKBA) or the lipidated form of the 19 kDa outer membrane protein of *Brucella* (L-Omp19), and was shown to be mediated by TLR2 recognition. In contrast, no significant down-regulation of MHC-II was induced by either unlipidated Omp19 or *Brucella* LPS. In a functional assay, treatment of AM with either L-Omp19 or HKBA reduced the MHC-II-restricted presentation of OVA peptides to specific T cells. One week after intra-tracheal infection viable *B. abortus* was detected in AM from both wild type and TLR2 KO mice, but CFU counts were higher in the latter. These results suggest that *B. abortus* may survive in AM after inhalatory infection in spite of a certain degree of immune control exerted by the TLR2-mediated inflammatory response. Both the modest nature of the latter and the modulation of MHC-II expression by the bacterium may contribute to such survival.

INTRODUCTION

Brucellosis is a worldwide distributed zoonotic disease caused by *Brucella* species, which affects over 500,000 people annually (1) and for which there is no approved efficacious human vaccine available. *Brucella melitensis, B. suis and B. abortus* are the most pathogenic species for humans and are responsible for the vast majority of human cases (2). The infection can be transmitted to humans by several ways, among which inhalation of infected aerosols is one of the most frequent. The easy aerosolization and airborne transmission of *Brucella* species has contributed to their consideration as potential biological weapons (1) and their classification by CDC and NIAID as category B bioterrorism agents. Outbreaks of human brucellosis due to airborne transmission have been reported in different settings, including abattoirs, vaccine production laboratories, and rural areas (3-5). Notably, aerosols have been implicated in most cases of laboratory-acquired brucellosis, which is considered the commonest laboratory-acquired infection (6).

Lung epithelial cells and alveolar macrophages (AM) are the first cells to be contacted by inhaled microorganisms. AM constitute the first line of pulmonary defense, and are capable of initiating a local immune response to pathogens, maintaining the functional integrity of pulmonary epithelium. Such immune response relies to a great extent on the recognition of foreign antigens by toll-like receptors (TLRs), which detect different pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, lipoproteins, flagellin, CpG DNA motifs, double-stranded RNA, etc., and trigger the activation of MyD88- and TRIF-dependent signaling pathways that lead to a wide range of cellular responses, including the secretion of proinflammatory cytokines and

chemokines and type I interferons, which contribute to the protective response to microbial pathogens (7). Previous studies have shown that TLR2, TLR4 and TLR9 are involved in the recognition of *Brucella* by macrophages and dendritic cells (8), but the importance of TLRs in the recognition of *Brucella* by AM has not been characterized.

The interaction of *Brucella* with the pulmonary cells has been scarcely studied. We have previously shown that *Brucella* species can infect and replicate within human lung epithelial cells, and can induce these cells to produce the monocyte chemoattractant MCP-1 (9-10). More recently, a study performed in mice after intranasal inoculation of *B. abortus* revealed that AM constitute the main cellular target of these inhaled bacteria. Moreover, confocal microscopy studies indicated that the number of bacteria per AM increased between days 2 and 5 p.i. (11). Notably, in another study in mice infected with aerosolized *B. abortus,* lung CFU counts increased steadily until week 4 p.i. and remained high at least until week 8 p.i. (12), suggesting that *B. abortus* can replicate and persist within the murine lung. It can be speculated that this long-term survival of *Brucella* in the lung may take place, at least in part, in AM.

The above speculation is also supported by the fact that several studies have shown that *Brucella* can survive and replicate inside human and murine macrophagic cells (13, 14). Key determinants of such survival are the ability of the bacterium to avoid the fusion of the *Brucella*-containing vacuole with lysosomes (15) and several physiologic adaptations of the brucellae to their intracellular niche (16). In addition, to avoid the TCR-mediated recognition of infected monocytes/macrophages, and an eventual T cell-mediated activation of such cells, the bacterium down-modulates the expression of MHC-II molecules on the cellular surface of these phagocytes (17).

To our best knowledge, all the studies regarding *Brucella* invasion, replication and immune evasion in monocytic/macrophagic cells have been performed using cell lines, peripheral blood monocytes or murine peritoneal or bone marrow-derived macrophages. No studies have been performed using isolated AM. This lack of knowledge is not trivial, since several studies have shown that AM differ from other monocytic/macrophagic populations in several ways, including the cytokine response to infection or to stimulation with microbial antigens (18-23) the susceptibility to infection or the intracellular replication kinetics for certain pathogens (22, 23), the signaling pathways or innate immunity receptors involved in responses to antigenic stimuli (24, 25), and several other aspects (26). Notably, a recent study showed that AM can constitutively carry pathogens from the lung to the draining lymph nodes, even before dendritic cells (27). Therefore, the main goals of the present study were to evaluate the time course of *Brucella* survival and replication in murine AM, to assess the cytokine response of these cells to infection, and to determine whether *Brucella* can also down-regulate MHC-II expression in AM as a strategy to avoid or delay specific adaptive immune responses. The role of TLRs in the *Brucella*-mediated effects under study was also evaluated.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The smooth strain *Brucella abortus* 2308 was grown in tryptic soy broth at 37ºC with agitation. Bacteria were washed twice with sterile phosphate buffered saline (PBS) and inocula were prepared in sterile PBS on the basis of the OD readings, but the actual concentration was later checked by plating on agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities. When indicated, *Brucella* organisms were washed five times for 10 min each in sterile PBS, heat-killed at 70°C for 20 min, aliquoted, and stored at −70°C until they were used. Absence of *B. abortus* viability after heat-killing was verified by the absence of bacterial growth on tryptose soy agar (TSA).

Reagents

B. abortus lipidated outer membrane protein 19 (L-Omp19) and unlipidated Omp19 (U-Omp19) were obtained as described previously (28). Both recombinant proteins contained less than 0.25 endotoxin U/μg of protein as assessed by Limulus Amebocyte Lysates (Associates of Cape Cod). Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL) using BSA as standard. *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 (29). The synthetic bacterial lipohexapeptide Pam3CysSerLys4 (Pam3Cys) was obtained from InvivoGen (San Diego, USA).

Mice

Specific pathogen-free, female BALB/c mice were purchased from University of La Plata, Argentina and used at 8 weeks of age. C57BL/6 mice, and TLR2, TLR4, TLR9 and MAL/TIRAP gene Knockout (KO) mice bred on C57BL/6 background were provided by Federal University of Minas Gerais (UFMG), Brazil. Animals were housed in groups of 5 animals, under controlled temperature (22°C ± 2°C) and artificial light under a 12 h cycle period. KO mice were kept under specific pathogen-free conditions in positive-pressure cabinets and provided with sterile food and water ad libitum. All animal procedures were performed according to the rules and standards for the use of laboratory animals of the National Institute of Health, USA. Animal experiments were approved by the Ethical Committee of the IDEHU Institute and the Institutional Animal Care and Use Committee of the UFMG.

Murine alveolar macrophages

Murine alveolar macrophages were isolated as previously described with some modifications (30). Briefly, mice were euthanized by an intraperitoneal injection of a lethal dose of ketamine and xylazine, their tracheas were cannulated, and the airways were perfused several times with 0.7 ml of sterile cold PBS containing 1 mM EDTA to provide 4 ml of broncho-alveolar lavage fluid (BAL). BAL samples were centrifuged at 400 x*g* for 10 min at 4ºC and the cells present in the pellet were resuspended in RPMI 1640 medium supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Gibco-BRL Life Technologies, Grand Island,NY), 100 U of penicillin per ml, and 50 ug of streptomycin per ml. Cell viability was routinely greater than 95% as determined by trypan blue exclusion.

Cells were dispensed in culture plates of 48 wells at $2x10^5$ or $5x10^5$ cells/well and incubated in a 5% CO_2 humidified atmosphere for 2 hour at 37 ºC for adhesion, before stimulation. After incubation culture supernatants were discarded and cells were washed several times with culture medium to remove non-adherent cells. The identity of adherent cells as alveolar macrophages was confirmed by flow cytometry using anti-CD11c and anti-F4/80 antibodies as described below.

Murine peritoneal macrophages

Peritoneal macrophages were extracted from BALB/c and C57BL/6 by peritoneal washing with ice cold PBS-EDTA 1mM, and were enriched by plastic adherence in 48-well plates incubated for 1 h at 37°C in a 5% CO_2 atmosphere. Cells (5×10^5 cells/well) were washed with fresh RPMI and then cultured overnight in RPMI, 2 mM glutamine, 100 U/ml of penicillin and 50 µg/mL streptomycin supplemented with 10% FBS before infection.

Cellular infections

Infections were performed at multiplicities of infection (MOI) of 100 bacteria/cell in culture medium containing no antibiotics. After dispensing the bacterial suspension the culture plates were either centrifuged (10 min at 300 x*g* at room temperature) and then incubated for 2 hours at 37°C under 5% CO_2 atmosphere. At the end of incubation (time 0 p.i.), each well was washed three times with sterile PBS. For quantification of intracellular bacteria, the infected monolayers were incubated in the presence of 100 ug/ml of gentamicin (Sigma, USA) and 50 ug/ml of streptomycin (Sigma, USA) to kill extracellular bacteria. At different times after antibiotics addition (2, 24 or 48 h) culture supernatants were harvested

178 for cytokine determinations, whereas cells were washed with sterile PBS and lysed with
179 0.2% Triton X100. Serial dilutions of the lysates were plated on TSA to enumerate colony
180 forming units (CFU).
181
182 ***Evaluation of cytotoxicity***
183 The reduction of the barrier function of the cell membrane is a marker of cytotoxicity. To
184 analyze the effect of infection on cell membrane permeability, the release of lactacte
185 dehydrogenase (LDH) from infected macrophages was determined. Cells were infected
186 with *B. abortus* 2308 at a MOI of 100 as described above. Culture supernatants were
187 harvested at 24 and 48 h p.i. and LDH levels were measured using the CytotTox 96 Non-
188 Radiactive Cytotocity Assay (Promega, USA). Results were expressed as the ratio between
189 LDH levels measured in the samples (infected or uninfected cultures) and those
190 corresponding to a 100% cell lysis (obtained by hypotonic lysis of the same number of
191 cells).
192
193 ***Measurement of cytokine concentrations.***
194 Murine KC, IL-6, IL-1β, MCP-1, IL-12 p40/p70, and TNF-α were measured in culture
195 supernatants of *Brucella*-infected AM by sandwich ELISA (all from BD Biosciences, San
196 Diego, USA, except for KC which was from R&D, Minneapolis, USA) using paired
197 cytokine-specific mAbs, according to the manufacturer's instructions.
198
199 ***Effect of infection or Brucella antigens on MHC-II expression***

Alveolar macrophages from wild-type and TLR KO mice (0.5 x 10^6 cells/well) were incubated with 150 U/ml of recombinant murine IFN-γ (Thermo Fisher Scientific, USA) in the absence or presence of heat-killed *B. abortus* (HKBA, 10^7 to 10^9 CFU/ml), L-Omp19, U-Omp19, Pam3Cys, CpG, *E. coli* LPS, or *M. tuberculosis* lysate for 48 h at the indicated concentrations. At the end of culture, cells were washed three times with sterile PBS and resuspended in Fc-block (at a concentration of 2 ug/ml in 0.1% BSA in PBS) for 15 min on ice. Then the cells were incubated for 30 min on ice with a phycoerythrin-labeled antibody against murine MHC-II (including I-A^b and I-A^d molecules; clone M5/114.15.2, eBioscience), a fluorescein isothiocyanate-labeled anti-mouse CD11c antibody (clone HL3, BD Bioscience), an allophycocyanin-labeled anti-mouse F4/80 antibody (clone CI:A3-1, Serotec), or an isotype-matched control antibody (BD Bioscience). The cells were then washed in BSA in 0.1% PBS and fixed with 1% paraformaldehyde in PBS (pH 7.4). Samples were stored in the dark at 4°C for subsequent analysis with a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ), using CellQuest software (Becton-Dickinson). A total of 10,000 to 20,000 ungated events were collected. The results were expressed as mean fluorescence intensities (MFI, arithmetic means ± standard errors of the means).

In infection experiments, 0.5 x 10^6 cells/ml were infected with *B. abortus* at MOIs 10, 50 or 100 in the presence of IFN-γ for 2 h in standard medium containing no antibiotics. Then the cells were extensively washed to remove non-internalized 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.

223 ***Antigen presentation assay***

224 Alveolar macrophages were harvested as described above, plated into flat bottom 96-well
225 flat-bottom plates (1.5 x 10^5 cells/well) in complete medium and allowed to adhere
226 overnight at 37°C. Cells were washed five times and treated with complete medium with or
227 without L-Omp19 or HKBA in the presence of IFN-γ. After 48 h of incubation, alveolar
228 macrophages were washed extensively with warm culture medium to remove residual IFN-
229 γ and antigens. Then cells were incubated with 50 to 5,000 μg/ml of ovalbumin (OVA;
230 Sigma) for 6 h, followed by incubation with BO97.10 T hybridoma cells (10^5 cells/well),
231 specific for the OVA323–339 peptide presented on I-Ab (31). Culture supernatants were
232 removed after to 24 h and stored at -70°C until they were assayed for IL-2 levels by
233 commercial ELISA (BD Biosciences).

234

235 ***Intratracheal infection and lung processing***

236 Mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and were
237 inoculated intra-tracheally with 100 ul of *B. abortus* suspension (1x10^6 CFU/ml) following
238 the procedure described by Revelli et al. (32), with minor modifications. At 7 days post-
239 infection mice were euthanized by intraperitoneal overdose of ketamine/ xylazine and BAL
240 samples were obtained as described above. BAL cells were resuspended in RPMI 10% FBS
241 without antibiotics, and aliquots were taken for cell counting. Cell suspensions were
242 adjusted to 5 x 10^5 cells/ml, and 1 ml per well was dispensed in 24-wells plates. Cells were
243 incubated 2 h at 37 °C in a 5% CO_2 atmosphere, and were washed 3 times with sterile PBS
244 to remove non-adherent cells. Adherent cells were lysed and plated for CFU determinations
245 as described for *in vitro* infections. After BAL, lungs were homogenized in sterile PBS

using a tissue grinder, and different dilutions of the resulting cell suspensions were plated on agar for CFU counting.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA). Comparisons between groups of data were performed with the Tukey's post-test, and those against a control group were performed with Dunnett's test. All the statistical analyses were performed using GraphPad software (San Diego, USA).

RESULTS

Brucella abortus invades and replicates within murine alveolar macrophages.

Experiments were carried out to determine whether *B. abortus* invades alveolar macrophages (AM) and establishes a replication niche within these cells. Murine AM were infected for 2 h at an MOI of 100 bacteria/cell, extracellular bacteria were killed by treatment with antibiotics, and intracellular CFU were determined at different times post-infection (p.i.) by plating cellular lysates in agar. As shown in **Figure 1** (upper and middle panel), *B. abortus* was able to invade and replicate within AM from both C57BL/6 and Balb/c mice, as CFU counts of intracellular bacteria increased steadily during the whole follow-up period. The detection of released lactate dehydrogenase (LDH) is widely used as a measure of cell damage or death for most cell types, including AM (33). To evaluate a potential cytotoxic effect of *Brucella* infection on AM, levels of LDH activity were measured in culture supernatants from *B. abortus*-infected AM at 24 and 48 h p.i. At both

time points no significant difference in LDH activity was detected between supernatants from *Brucella*-infected cells and those from uninfected AM from both Balb/c mice (**Figure 1**, lower panel) and C57BL/6 mice (not shown). Taken together these results indicate that virulent *B. abortus* can survive and replicate inside alveolar macrophages, and that the increasing load of intracellular bacteria does not result in cytotoxic effects on these cells.

Proinflammatory response of AM to B. abortus infection

The levels of several pro-inflammatory cytokines, including TNF-α, IL-1-β, KC, IL-6, MCP-1 and IL-12, were measured at different p.i. times in culture supernatants from *B. abortus*-infected AM. As shown in **Figure 2**, at 24 h p.i. cytokines were either not detected or were present at very low levels in supernatants from infected AM from C57BL/6 mice. Except for MCP-1, which was not detected at any p.i. time, cytokine levels increased at 48 h p.i. as compared to 24 h p.i. Nevertheless, levels were rather low for IL-6 and IL-12 whereas the highest levels corresponded to TNF-α, IL-1-β and KC.

A similar profile of cytokine response was observed for AM from Balb/c mice (**Figure 3**). Again, all the cytokines were low or absent at 24 h p.i., and all of them (except MCP-1 which was never detected) increased at 48 h p.i. with the highest increases for TNF-α and KC and, to a lesser extent, IL-1-β. As the cytokine response of AM to bacterial antigens usually differs from that of macrophages from other localizations, generally resulting in a milder proinflammatory profile (22, 23), cytokine levels were also measured in culture supernatants from *B. abortus*-infected peritoneal macrophages (PM) from Balb/c mice and compared with those obtained for AM from the same animals. As shown in **Figure 3**, cytokine responses were generally weaker and/or delayed as compared to those

292 elicited in PM. While, as mentioned, cytokines were either absent or low at 24 h p.i. in AM,
293 all were already produced by infected PM at this time point. For all the cytokines, levels
294 detected at 24 h p.i. were significantly higher for PM than for AM ($p<0.001$ in all cases).
295 MCP-1 was not detected in culture supernatants from *B. abortus*-infected AM at any time
296 point but was detected in *Brucella*-infected PM at both p.i. times.

297 Overall, these results indicate that murine AM respond to *B. abortus* infection with
298 a delayed and restricted panel of proinflammatory mediators (mainly represented by TNF-α
299 and KC) as compared with murine PM.

300

301 ***The proinflammatory response of AM to Brucella is mainly TLR2-mediated***

302 To determine whether Toll-like receptors (TLR) may be involved in the induction of
303 cytokine responses, infections were performed in parallel in AM from C57BL/6 mice
304 (wild-type) and from mice of the same background genetically deficient for TLR2, TLR4,
305 or TLR9. We choose to evaluate these receptors since they were demonstrated to be
306 important for cytokine production in response to *Brucella* in PM and dendritic cells (8).
307 Since KC and TNF-α were markedly induced by infection in AM from wild-type (wt)
308 mice, these cytokines were measured in experiments with AM from TLR knock-outs (KO).
309 As shown in **Figure 4A**, TNF-α and KC were greatly reduced in supernatants of AM from
310 TLR2 KO mice as compared to those from wt mice (92% and 97% reduction, respectively).
311 In contrast, the production of both cytokines was much less reduced in cells from TLR4 KO
312 mice (significant reduction only for TNF-α) and TLR9 KO mice (29% and 23% reduction,
313 both statistically significant). TNF-α production in response to infection was significantly
314 lower in AM from TLR2 KO mice than in those from wt, TLR4 KO or TLR9 KO mice

315 ($p<0.001$ in all cases), and the same was true for KC production ($p<0.001$ in all cases).
316 These results suggest that TNF-α and KC production by AM in response to *B. abortus*
317 infection depends mainly on TLR2 recognition, with significantly smaller contributions of
318 TLR4 and TLR9. In agreement with the results obtained with TLR2 and TLR4 KO mice,
319 the TNF-α and KC responses of AM from mice deficient in the adaptor protein
320 MAL/TIRAP were also greatly reduced (95% and 98% reduction, respectively) as
321 compared to wt mice.

322 To rule out that differences in TNF-α and KC production between AM from wt and
323 TLR2 KO mice were due to differences between these cells in the rate of *Brucella* infection
324 or replication, CFU counts in AM lysates were determined at 2 and 24 h p.i. As shown in
325 **Figure 4B**, no significant differences in *B. abortus* CFU counts were found between AM
326 from wt and TLR2 KO mice at both p.i. times.

327

328 ***Both live and heat-killed Brucella down-regulate MHC-II expression in AM***

329 As mentioned, previous studies have shown that *B. abortus* can replicate and survive for
330 several weeks in lungs of mice infected through the nasal route (12), suggesting that the
331 bacterium may display mechanisms of immune evasion at the pulmonary level. Since it has
332 been shown that mycobacteria can down-regulate the expression of MHC-II molecules in
333 macrophages (30, 34), we decided to test whether this evasion mechanism may also operate
334 in *Brucella* infections. AM from C57BL/6 and Balb/c mice were infected or not with *B.*
335 *abortus* at different multiplicities of infection for 2 h in the presence of IFN-γ (as an
336 inducer of MHC-II expression), washed extensively, and maintained in the presence of
337 IFN-γ for an additional 48 h. After this, the expression of MHC-II ($I\text{-}A^d$) was evaluated by

flow cytometry. As shown in **Figure 5**, the expression of MHC-II was significantly reduced in cells that had been incubated with live *B. abortus* during the first 2 hours of IFN-γ stimulation as compared with cells incubated with IFN-γ alone. For AM from both mouse strains the down-regulating effect of *B. abortus* infection was MOI dependent, with mean fluorescence intensity (MFI) reductions of 25%, 62% and 70% for MOIs 10, 50 and 100, respectively, in AM from C57BL/6 mice, and 25%, 35% and 44% in AM from Balb/c mice. To determine whether this down-regulation may be mediated by structural components of the bacterium, AM from Balb/c mice were stimulated with IFN-γ in the absence or presence of heat-killed *B. abortus* (HKBA) for 48 h, and the expression of MHC-II molecules was evaluated as described. As shown in **Figure 5** (lower panels), the heat-killed bacterium also induced a significant MHC-II down-regulation, suggesting that this effect was due to a structural component of *Brucella*. The down-regulating effect of HKBA was dose-dependent, with MFI reductions of 20%, 77% and 91% for HKBA doses of 10^7, 10^8 and 10^9 CFU/ml, respectively.

These results indicate that infection with *B. abortus* induces a down-regulation of MHC-II expression in AM, and that this phenomenon is mediated by a structural component of the bacterium.

Lipoproteins mediate the down-regulation of MHC-II by Brucella

Previous studies on MHC-II down-regulation by *M. bovis* have shown that such effect may be mediated by a 19 kDa lipoprotein of this bacterium (30). Interestingly, previous studies from our group have shown that *Brucella* lipoproteins, and in particular the lipidated form of a 19 kDa lipoprotein (L-Omp19) may mediate important biological activities in human

peripheral monocytes, such as the induction of proinflammatory responses and the down-regulation of FcγRI (CD64) and MHC-II expression (17, 28, 35). Therefore, we decided to test whether L-Omp19 may also be involved in the down-regulation of MHC-II molecules induced by *Brucella* in AM. To do this, AM were incubated with murine IFN-γ in the absence or presence of this lipoprotein. As shown in **Figure 6**, incubation with L-Omp19 induced a down-regulation of MHC-II as compared to cells treated only with IFN-γ. The down-regulating effect of L-Omp19 was dose-dependent, with MFI reductions of 44%, 79% and 92% for L-Omp19 doses of 10, 100 and 1000 ng/ml, respectively. The down-regulating effect of L-Omp19 on MHC-II expression depended on the lipid moiety of the lipoprotein since its unlipidated version (U-Omp19) did not decrease MHC-II expression even when used at high concentrations (5000 ng/ml). The requirement for lipidation was further supported by the fact that Pam3Cys, a synthetic lipohexapeptide that mimics the lipid moiety of lipoproteins, also down-regulated in a dose-dependent manner the expression of MHC-II induced by IFN-γ (**Figure 6**). These results suggest that L-Omp19 (and probably other Omps) is involved in the down-regulating effect of *B. abortus* on the IFN-γ-induced MHC-II expression in murine AM.

Since LPS from some bacteria have been shown to diminish MHC-II expression in peritoneal macrophages (36), the LPS from *B. abortus* was considered other candidate molecule potentially involved in the inhibition of MHC-II expression in AM. Thus, experiments were conducted to evaluate the contribution of *B. abortus* LPS to such effect. As shown in **Figure 6**, *B. abortus* LPS was unable to inhibit the expression of MHC-II molecules even when used at high concentrations (5000 ng/ml). In contrast, LPS from *E. coli* inhibited in a dose-dependent manner the IFN-γ-induced MHC-II expression, attaining

MFI reductions of 95.5% and 88.2% at doses 10 and 50 times lower than that used for *Brucella* LPS (1000 and 100 ng/ml, respectively) (data not shown).

Taken together, these results indicate that *B. abortus* lipoproteins, but not its LPS, contribute to the down-modulation of MHC-II in murine AM induced by HKBA stimulation or *B. abortus* infection.

MHC II down-regulation by Brucella is TLR2-mediated

Since TLR2, TLR4 and TLR9 have been shown to be involved in immune responses to *Brucella* infection and to *Brucella* antigens (8), experiments were carried out to determine if one or more of these receptors was involved in the down-regulation of MHC-II expression induced by *B. abortus* antigens in murine AM. To do this, AM from wild-type C57BL/6 mice or from TLR2, TLR4 and TLR9 KO mice of C57BL/6 background were incubated with murine IFN-γ in the absence or presence of HKBA, L-Omp19 or LPS from *B. abortus*. Pam3Cys*, E. coli* LPS, and CpG were included in parallel experiments since TLR2, TLR4 and TLR9 agonists have been shown to down-regulate MHC-II expression in murine peritoneal macrophages (36, 37).

As shown in **Figure 7**, the IFN-γ-induced MHC-II expression on AM from wild-type C57BL/6 mice was down-regulated by coincubation with HKBA and L-Omp19 but not by coincubation with LPS from *B. abortus,* in agreement with results obtained with AM from Balb/c mice (shown in **Figure 6**). Results obtained with AM from TLR4 and TLR9 KO mice were similar to those yielded by AM from WT mice, indicating that these receptors are not involved in the down-regulation of MHC-II expression by *Brucella* antigens. In contrast, the down-regulating effect of HKBA and L-Omp19 on MHC-II

expression was abolished in AM obtained from TLR2 KO mice, indicating that this receptor is involved in such effect. The down-regulating effect was also abolished in AM from MAL/TIRAP KO mice, in agreement with the involvement of TLR2 in MHC-II down-regulation by *Brucella* antigens.

These results indicate that TLR2 recognition mediates the down-regulating effect of *Brucella* antigens on MHC-II expression in murine AM.

Functional evidence of MHC-II down-regulation.

To examine whether MHC-II down-regulation by *Brucella* has a functional consequence on the ability of AM to present antigenic peptides to specific T cells, AM from C57BL/6 mice were treated with IFN-γ alone or with the addition of L-Omp19 or HKBA. After 48 h these cells were incubated with different amounts of ovalbumin and with a T cell hybridoma specific for an ovalbumin peptide presented in the context of I-A^b. As a measure of T cell activation, the levels of IL-2 were determined in culture supernatants at 24 h of coculture. As shown in **Figure 8**, IL-2 levels were significantly lower in wells in which AM had been pretreated with L-Omp-19 or HKBA than in non-pretreated cells. These results suggest that the reduction of MHC-II expression on AM induced by *Brucella* affects the ability of these cells to present MHC-II-restricted antigens to specific T cells, thus reducing the activation of the latter.

Impact of TLR2 absence on Brucella survival in AM

Our in vitro experiments revealed two TLR2-mediated phenomena that may have opposite effects on bacterial clearance: the production of proinflammatory cytokines (which may

favor clearance) and the downregulation of MHC-II (which may favor bacterial persistence). Probably, the net effect of TLR2 recognition on *Brucella* survival in AM results from a balance between these two TLR2-mediated phenomena. To test the *in vivo* effect of TLR2 absence on *Brucella* survival within AM, wild-type and TLR2 KO C57BL/6 mice were infected with *B. abortus* through the intratracheal route. AM were obtained at one week p.i., lysed and plated on agar to determine intracellular CFU counts. After bronchoalveolar lavage to obtain AM, lungs were processed and homogenates were also plated on agar. As shown in **Figure 9**, viable bacteria were recovered from AM and lung homogenates from both mouse strains. However, bacterial counts were significantly higher in AM and lung homogenates from TLR2 KO mice than in those from wild-type mice. These results suggest that although the TLR2-mediated inflammatory response is important for controlling *B. abortus* infection in AM, the bacterium may still survive in these cells for at least one week after inhalatory infection.

DISCUSSION

Airborne transmission is an important way for the acquisition of *Brucella* spp. infections by humans (4, 5) and may be also involved in animal cases. In spite of this, the interaction of brucellae with cells of the respiratory system has been scarcely documented and, in particular, the immunological aspects of such interaction have only recently begun to be studied (9, 11).

Alveolar macrophages (AM) and alveolar epithelial cells are the first cells contacted by inhaled bacteria immediately after reaching the lungs through infected aerosols. In the case of *B. abortus*, a recent study in mice has shown by confocal microscopy that AM

453 constitute the main cellular target of these bacteria after intranasal infection (11). While an
454 extensive body of literature has examined many aspects of the interaction between
455 brucellae and different types of human or animal monocytic/macrophagic cells, there are no
456 reports on the interaction between these bacteria and AM. Taking into account that AM
457 differ from other monocytic/macrophagic populations in several ways, including cytokine
458 response to infection or innate immunity receptors involved (19-21, 24, 25), we decided to
459 characterize these aspects in the case of the interaction between brucellae and murine AM.
460 In addition, since *Mycobacterium tuberculosis* has been shown to down-regulate MHC-II
461 expression in AM (37) we wondered whether a similar phenomenon may occur in *Brucella*-
462 infected AM.

463 Our results show that *B. abortus* invades and replicates in murine AM. The ability
464 of brucellae to replicate inside AM is in line with the findings of Archambaud et al. (11)
465 who observed the same phenomenon by confocal microscopy, and is also in line with
466 similar findings in several types of monocytic/macrophagic cells (13, 14). Notably, the
467 infection did not induce cytotoxicity as measured by LDH release, suggesting that the
468 viability of AM is not affected by *B. abortus* in spite of the marked increase in intracellular
469 bacterial load. These results suggest that AM may constitute a durable replicative niche for
470 brucellae in the lung.

471 Previous studies have shown that mouse peritoneal macrophages and macrophagic
472 cells lines respond to *Brucella* infection or stimulation with the production of the
473 proinflammatory cytokines TNF-α, IL-1β and IL-6 and the Th1-inducing cytokine IL-12
474 (38-43). In contrast, the cytokine response of AM to *Brucella* has not been reported. In the
475 present study we found that AM from both Balb/c and C57BL/6 mice respond to *Brucella*

infection with a marked increase in the secretion of TNF-α KC (neutrophil chemoattractant) and IL-1β at 48 h p.i., but only low levels of IL-12 and IL-6. In addition, these cells do not produce MCP-1 in response to *Brucella* infection. Therefore, upon *Brucella* recognition AM seem to elicit a relatively attenuated inflammatory response with preserved KC and TNF-α secretion. The significant production of TNF-α and KC by *Brucella*-infected AM suggests that these cells may contribute to the recruitment of neutrophils to the infection focus. A moderate amount of neutrophilic inflammation has been reported in the few histological studies performed in animal models of acute inhalational brucellosis (44, 45).

The profile of cytokine response of murine AM to *Brucella* infection differed from that found in PM. While infection elicited a marked increase of TNF-α in both AM and PM, the AM response was delayed as compared to that of PM. The significant increase of TNF-α levels in response to *Brucella* agrees with data previously reported for murine PM and J774.A1 cells (39, 43). Differences in the kinetics of AM and PM responses were even more marked for KC, which was produced at very high levels at 24 h p.i. by *Brucella*-infected PM but did not exhibit a significant increase in AM at that time. Whereas IL-6 has been shown to be induced by *Brucella* infection in PM and J774.A1 cells (41, 43) and was also significantly induced in PM at 24 and 48 h p.i. in the present study, this cytokine was not increased in *Brucella*-infected AM at 24 h p.i. and exhibited only a small increase at 48 h p.i. IL-12 was secreted by *B. abortus*-infected PM, in agreement with previous studies (40), but its production by infected AM was comparatively low and delayed. The monocyte chemoattractant MCP-1 has been shown to be induced by *Brucella* infection in the murine macrophagic cell line RAW 264.7 and microglia cells (46, 47). In the present study, MCP-1 was produced early (24 h p.i.) by PM in response to *B. abortus* infection, but was not

499 produced by infected AM at any time point. Taken together these results suggest that AM
500 produce a weaker and/or delayed cytokine response to *B. abortus* as compared to PM. This
501 restricted response may be beneficial for *Brucella* infection, since some of the affected
502 cytokines are known to be involved in the control of such infection. It is known that TNF-α
503 is important for the influx of phagocytes to the site of infection and for macrophage
504 activation, and is critically involved in immune responses to intracellular pathogens.
505 Previous studies have shown that the spleen load of *Brucella* is increased in mice treated
506 with neutralizing antibodies against TNF-α (48). Although studies on localized effects of
507 TNF-α deficiency at mucosal surfaces are lacking, it can be speculated that the
508 comparatively delayed TNF-α production by AM in response to *B. abortus* may favor the
509 bacterium. It is known that IL-12 promotes efficient immune responses against intracellular
510 pathogens not only by inducing a Th1 profile but also by promoting the migration of
511 dendritic cells to draining lymph nodes (49). A central role for IL-12 in the control of *B.*
512 *abortus* infection has been also demonstrated (42). Thus, a reduced local production of IL-
513 12 may contribute to the long-term persistence of *Brucella* in the lung.

514 In the present study, IL-1β was among the cytokines induced by *Brucella* infection
515 in AM, although its production by these cells was delayed as compared to PM responses.
516 While studies have shown that the genetic deficiency of IL-1β does not alter the splenic
517 load of *B. abortus* in mice infected through the intraperitoneal route (50), the importance of
518 this cytokine for the response to inhaled *Brucella* has not been explored. AM seemed to be
519 poor producers of IL-1β upon LPS stimulation, since this was the only cytokine not elicited
520 by the dose of *E. coli* LPS used as a control in cytokine assays (0.1 μg/ml). In line with
521 these findings, murine AM have been reported to respond to a ten-fold higher dose of LPS

522 than the one used here (1 μg/ml) with low levels of IL-1β (around 35 pg/ml) but high levels
523 of KC (around 10 ng/ml) (51).

524 Toll-like receptors (TLRs) are involved in the inflammatory response of
525 macrophages and other cell types to infection by different pathogens or to stimulation with
526 their antigens. In the case of *Brucella*, previous studies have shown that TLR2 recognition
527 is centrally involved in the inflammatory response of human or murine macrophages to
528 these bacteria (28, 39). In agreement with those previous reports, our studies using KO
529 mice for TLR2, TLR4, TLR9 and MAL/TIRAP revealed that TNF-α and KC responses of
530 AM to *Brucella* infection were mainly mediated by TLR2 recognition; both responses were
531 almost abolished in AM from TLR2 KO and MAL/TIRAP KO mice. In addition, a slight
532 but significant reduction of TNF-α and KC secretion was observed in AM from TLR9 KO
533 mice, and a slight diminution of TNF-α was also observed in AM from TLR4 KO mice.
534 The participation of multiple TLRs in the inflammatory response to *Brucella* infection or
535 antigens agrees with the published literature (8), although this study shows that TLR2
536 recognition has a preponderant role in the case of AM. Although not formally shown in the
537 present study, it can be inferred from the existing literature that the *Brucella* antigens
538 involved in the TLR2-mediated cytokine response of AM are outer membrane lipoproteins
539 (28).

540 Alveolar macrophages perform several protective functions in the lung, including
541 the phagocytosis and killing of invading pathogens, and the secretion of cytokines and
542 chemokines that contribute to the innate and adaptive immune response. In addition, like
543 macrophages located in other tissues, AM process microbial antigens and display antigenic
544 peptides in the context of MHC molecules for their recognition by specific T cells. Gamma

545 interferon (IFN-γ) activates macrophages and induces their expression of MHC-II
546 molecules, resulting in an enhanced antigen presentation to specific CD4+ T cells and the
547 consequent activation of the latter. This partially explains the central role of IFN-γ in the
548 protection against infections by intracellular pathogens. Therefore, pathogens that can
549 induce a down-regulation of the MHC-II expression induced by IFN-γ on macrophages
550 may hinder the recognition of infected cells by specific T lymphocytes thus evading some
551 adaptive immune responses. Interestingly, *Brucella* has been reported to persist for a long
552 time in the lungs of infected mice (12), a phenomenon that can be speculated to be due, at
553 least in part, to the ability of the bacterium to evade the immune recognition of infected
554 AM. Of note, it has been previously shown that *B. abortus* infection can down-regulate the
555 IFN-γ-induced expression of MHC-II molecules in human monocytes, leading to a reduced
556 antigen-specific T cell proliferation (17). Against this background we decided to evaluate
557 whether *Brucella* infection can also modulate MHC-II expression and antigen presentation
558 in AM. We found that infection of AM with *B. abortus* reduced in a MOI-dependent
559 manner the expression of MHC-II molecules induced by IFN-γ. These findings agree not
560 only with our previous studies in human monocytes but also with results from other groups
561 showing that *M. tuberculosis*-infected AM have decreased MHC class II molecule
562 expression and decreased antigen presentation to specific CD4+ T cells (30).

563 Of note, it has been shown that the effects of *Brucella* on MHC-II expression and
564 antigen presentation mentioned above do not require viable bacteria and can be similarly
565 induced by HKBA and also by L-Omp19, an outer membrane lipoprotein, in both cases
566 through TLR2-mediated recognition (17). Similarly, it has been reported that viable *M.*
567 *tuberculosis* is not required for inhibition of macrophage MHC class II expression, which

568 can be achieved by exposure of macrophages to *M. tuberculosis* lysate and also to *M.*
569 *tuberculosis* lipoproteins, which are recognized by TLR2 (52). In line with those previous
570 reports we found that MHC-II down-regulation was induced in AM by incubation with
571 HKBA or L-Omp19, and that for both stimulants the effect was mediated by TLR2
572 recognition. Moreover, we found that the presence of either L-Omp19 or HKBA during
573 IFN-γ stimulation reduced significantly the ability of AM to activate a MHC-II-restricted
574 OVA-specific T cell hybridoma. These results strongly suggest that AM infected with
575 *Brucella* may remain undetected by specific T cells thus ensuring the long-term survival of
576 such cells and, consequently, the long-term persistence of the bacterium in the lung. The
577 absence of a significant cytotoxic effect of *Brucella* on AM may also contribute to the
578 maintenance of these cells as a long-term reservoir of bacteria.

579 The results obtained in the present study indicate that TLR2 recognition is involved
580 in the cytokine/chemokine response of AM to *Brucella* infection but also in the down-
581 regulation of MHC-II expression and antigen presentation induced by *Brucella* and its
582 antigens in AM. This dual effect of TLR2 recognition has been also reported for the
583 interaction of *M. tuberculosis* (or its antigens) with macrophages. While activation of TLR2
584 by the 19 kDa lipoprotein of *M. tuberculosis* induces the killing of intracellular bacteria in
585 both NO-dependent and -independent pathways in murine macrophages and in human
586 monocytes and alveolar macrophages (53), prolonged TLR signaling by *M. tuberculosis* or
587 its 19 kDa lipoprotein has been shown to inhibit several IFN- induced genes (54). It has
588 been speculated that the later mechanism may provide homeostatic feedback regulation to
589 limit the extent of the induced responses (37). In particular, it has been postulated that the
590 down-regulation of antigen presentation might be especially pronounced during persistent

591 infection with intravacuolar pathogens that survive microbicidal mechanisms and that can
592 persistently colocalize with TLRs in phagosomes for prolonged TLR signaling (37). Like
593 *M. tuberculosis*, *Brucella* can establish a prolonged residence in the phagosomes of tissue
594 macrophages and can display TLR2 agonists (like L-Omp19 and other lipoproteins),
595 probably allowing a chronic stimulation of TLR2 that may lead to the inhibition of MHC-II
596 expression and antigen presentation.

597 As mentioned, the TLR2-mediated recognition of *Brucella* antigens induces the
598 production of proinflammatory cytokines, which may favor bacterial clearance, but also the
599 downregulation of MHC-II, which may favor bacterial persistence. Probably, the net effect
600 of TLR2 recognition on *Brucella* survival in AM results from a balance between these two
601 TLR2-mediated phenomena. Notably, we detected viable *B. abortus* in AM of mice that
602 had been infected one week earlier through the intratracheal route, indicating the ability of
603 *B. abortus* to survive *in vivo* for a prolonged post-infection time within these cells.
604 However, bacterial counts were significantly higher in AM from TLR2 KO mice, indicating
605 the involvement of TLR2-mediated inflammation in the control of lung infection by *B.*
606 *abortus*. A previous study in intranasally infected mice has shown that *Brucella*-infected
607 AM migrate to the draining lymph node, where they are detected as early as 1.5 days post-
608 infection (11). The fact that we detected *Brucella*-infected AM in the lungs at one week p.i.
609 suggests that, upon infection, some infected AM migrate early to lymph nodes whereas
610 others remain in the lung.

611 Collectively, these results suggest that *B. abortus* may survive in AM after
612 inhalatory infection in spite of a certain degree of immune control exerted by the TLR2-
613 mediated inflammatory response. Both the modest nature of the latter and the modulation of

MHC-II expression by the bacterium may contribute to such survival. Further studies will be needed to determine whether AM may contribute to the reported persistence of brucellae in the lungs or to a late dissemination of the bacterium from the lungs to distal tissues.

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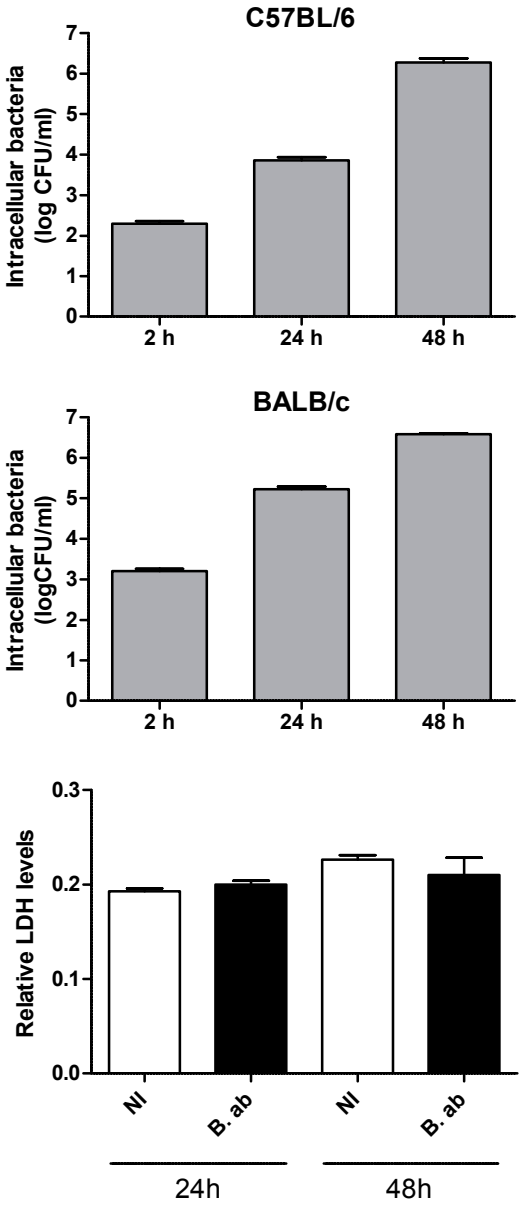


Figure 1. Invasion and intracellular replication of *B. abortus* in murine alveolar macrophages (AM). AM ($2x10^5$ cells/well) from C57BL/6 mice (upper panel) or Balb/c mice (middle panel) were infected at MOI 100 for 2 h, and intracellular colony-forming units (CFU) were measured at different times p.i. Results are expressed as mean ± SEM of values measured in triplicate in each experiment. Data are representative of an experiment from three performed with similar results. Lower panel: Effect of *Brucella* infection on the cell membrane permeability of AM from Balb/c mice. Cells were infected as above and culture supernatants were collected at 24 and 48 h p.i. to measure LDH levels. Results are expressed as the ratio between LDH levels found in infected or non-infected (NI) cells and those found in a whole cellular lysate (100% lysis). B. ab.: *B. abortus*.

Figure 2

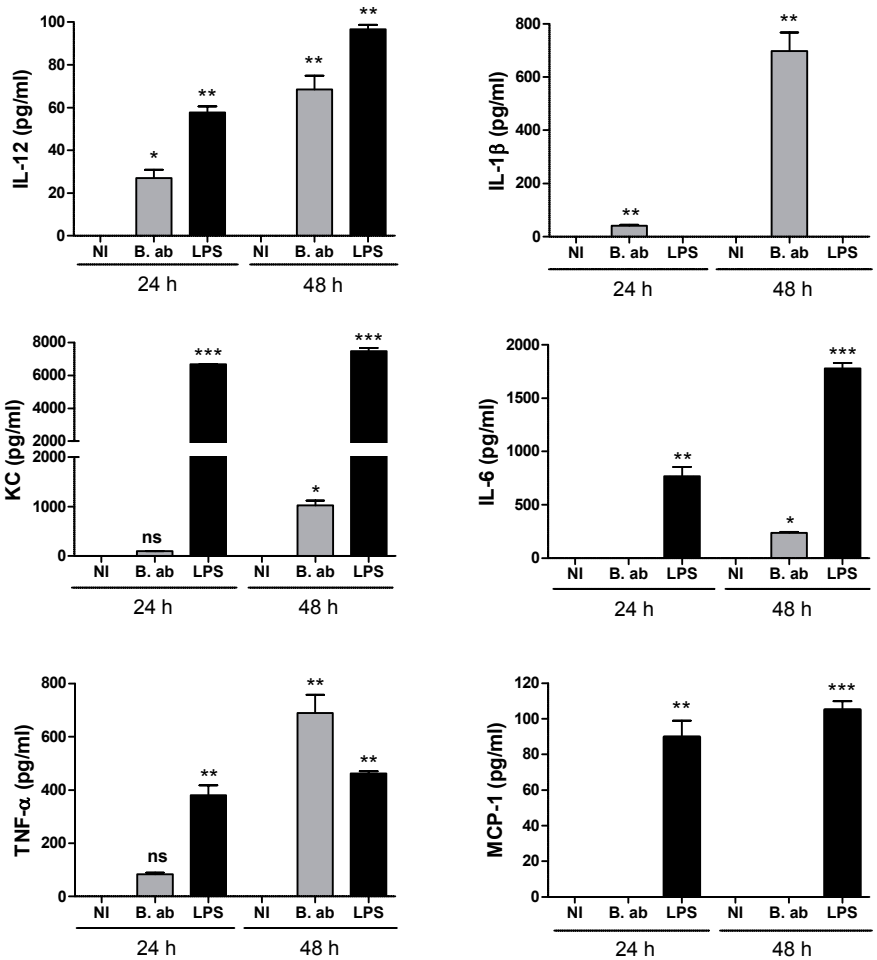


Figure 2: Secretion of proinflammatory cytokines by AM from C57BL/6 mice in response to *B. abortus* infection. Alveolar macrophages were infected as described for Figure 1, and culture supernatants were harvested at 24 and 48 h to measure cytokines by sandwich ELISA. NI: non-infected cells. B. ab.: *B. abortus*-infected cells. LPS: Cells stimulated with lipopolysaccharide from *E. coli* (100 ng/ml). Data represent mean ± SD from values measured in triplicate in each experiment. The figure shows a representative experiment from two performed with similar results. Asterisks indicate significant differences between infected and uninfected cells at each time point (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) (ANOVA followed by Dunnett's post-test).

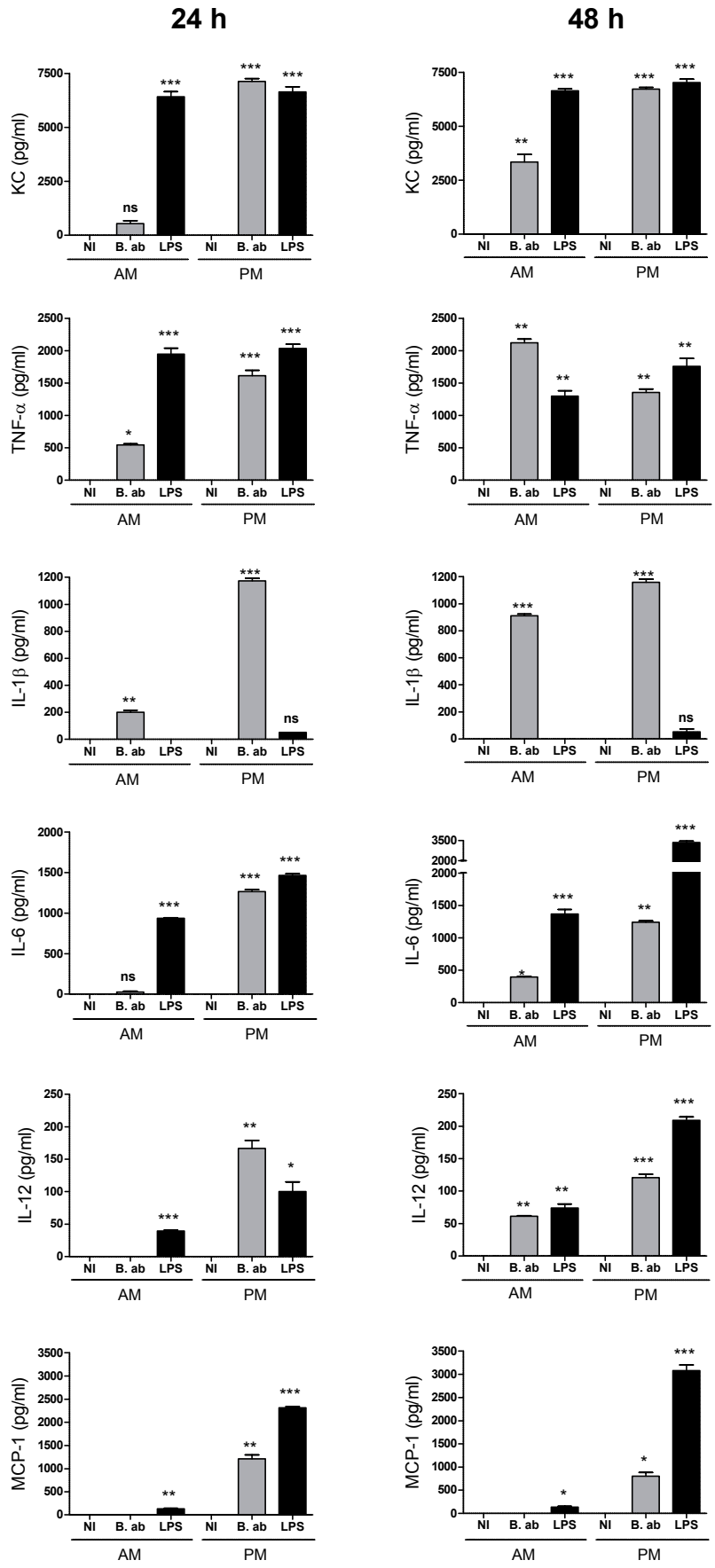


Figure 3: Secretion of proinflammatory cytokines by AM and peritoneal macrophages (PM) from Balb/c mice in response to *B. abortus* infection. Alveolar macrophages were infected as described for Figure 1, and culture supernatants were harvested at 24 and 48 h to measure cytokines by sandwich ELISA. NI: non-infected cells. B. ab.: *B. abortus*-infected cells. LPS: Cells stimulated with lipopolysaccharide from *E. coli* (100 ng/ml). Data represent mean ± SD from values measured in triplicate in each experiment. The figure shows a representative experiment from two performed with similar results. Asterisks indicate significant differences between infected and uninfected cells at each time point (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) (ANOVA followed by Dunnett's post-test).

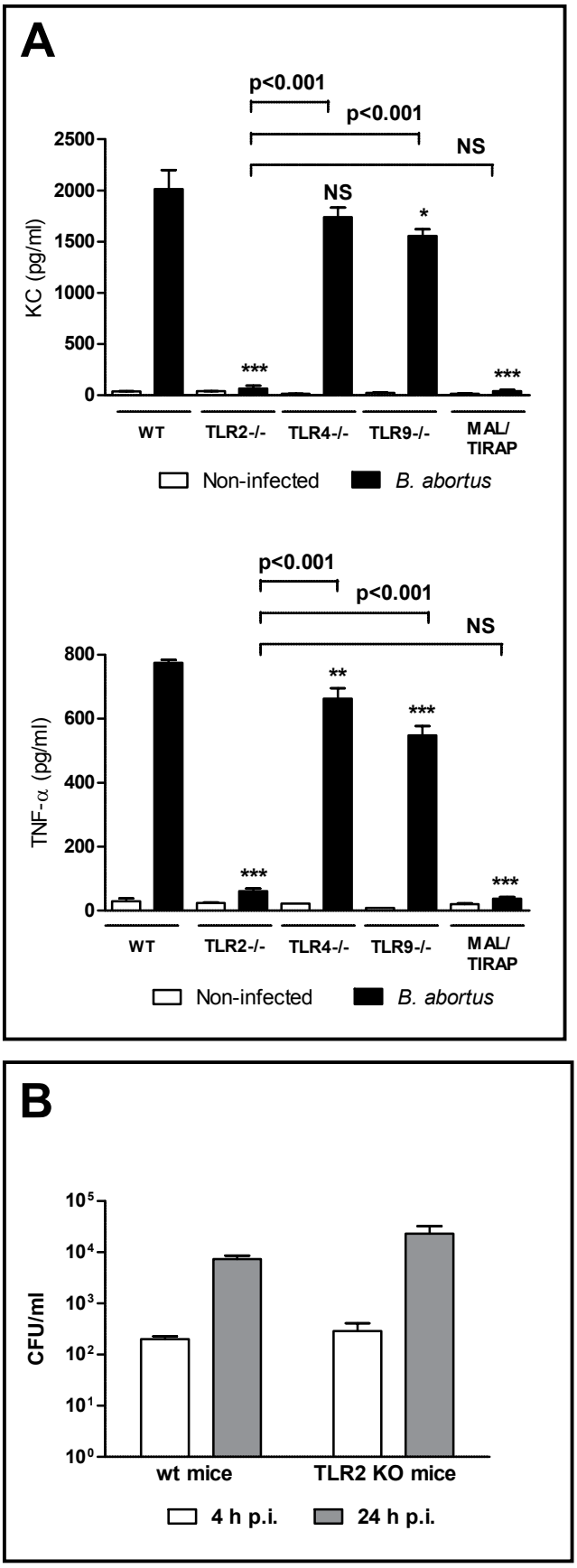


Figure 4. (A) Cytokine secretion by AM from C57BL/6 mice in response to *B. abortus* infection depends mainly on TLR2 recognition. Alveolar macrophages from wild-type (WT) and TLR or MAL/TIRAP mutant mice were infected as described for Figure 1, and culture supernatants were harvested at 48 h to measure TNF-α and KC by sandwich ELISA. NI: Non-infected. Data represent mean ± SD from values measured in triplicate in each experiment. The figure shows a representative experiment from two performed with similar results. Asterisks indicate significant differences between mutant mice and WT mice (*, p<0.05; **, p<0.01; ***, p<0.001, NS, non-significant) (ANOVA followed by Tukey's post-test). Bru: B. abortus. (B) TLR2 is not involved in *Brucella* invasion to AM. AM from WT and TLR2 KO mice were infected as described above and cell lysates were obtained at 4 and 24 h p.i. to determine intracellular CFU counts. Data represent mean ± SD from values measured in duplicate in each experiment. The figure shows a representative experiment from two performed with similar results.

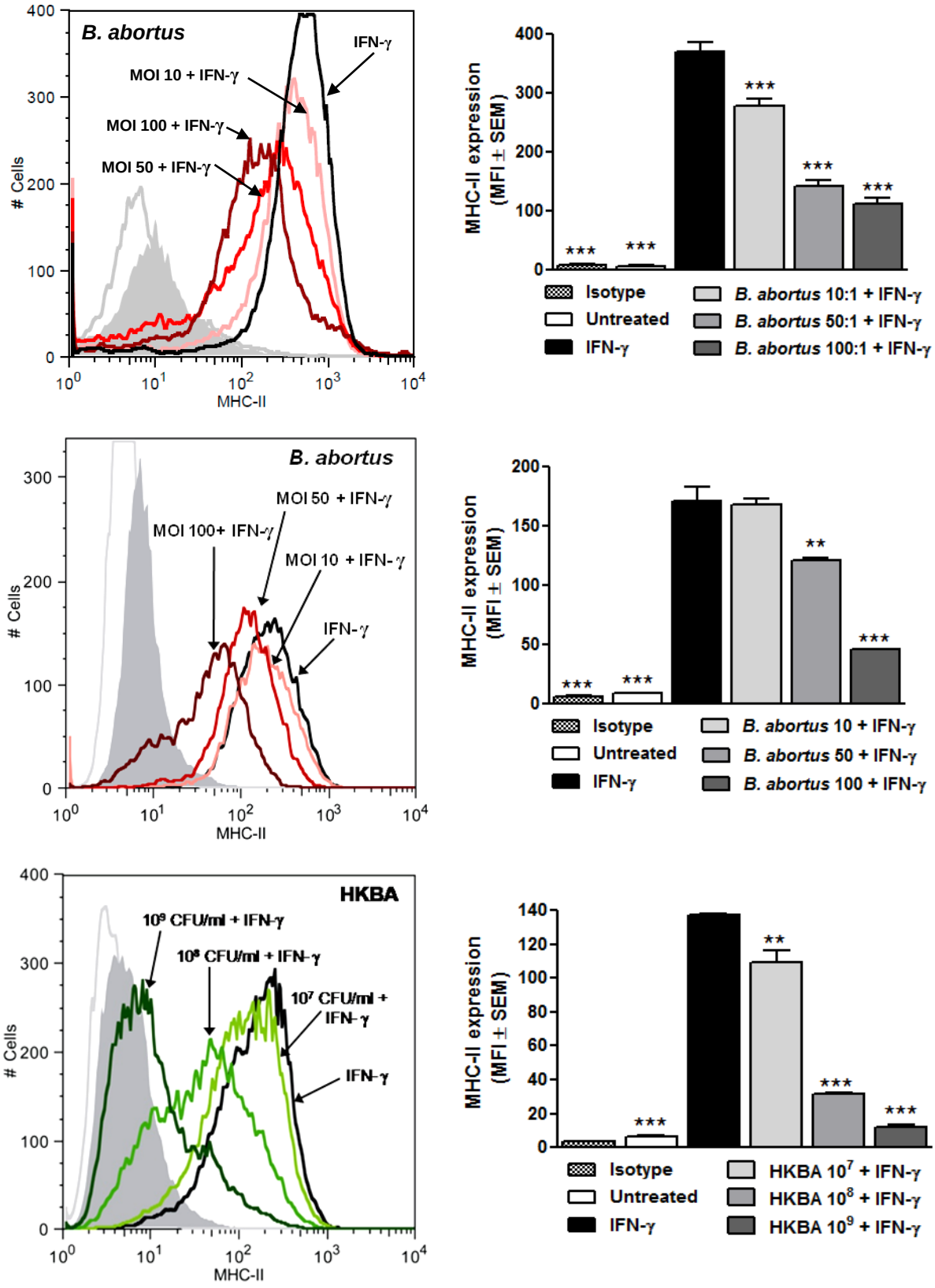
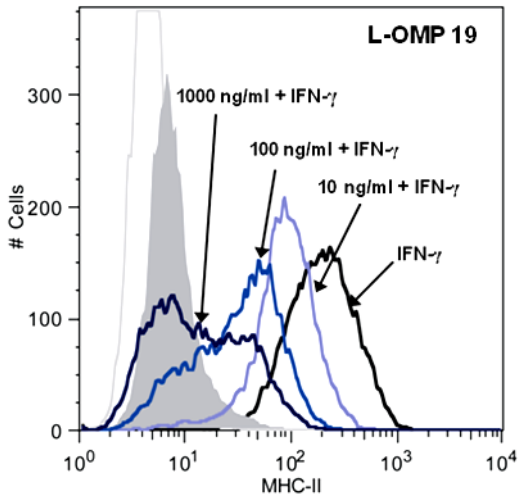
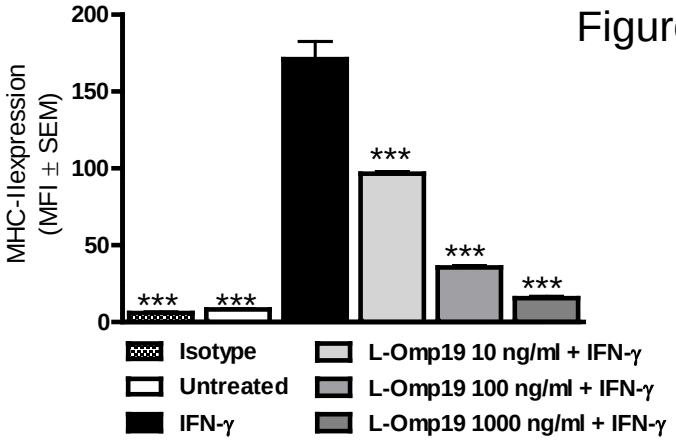
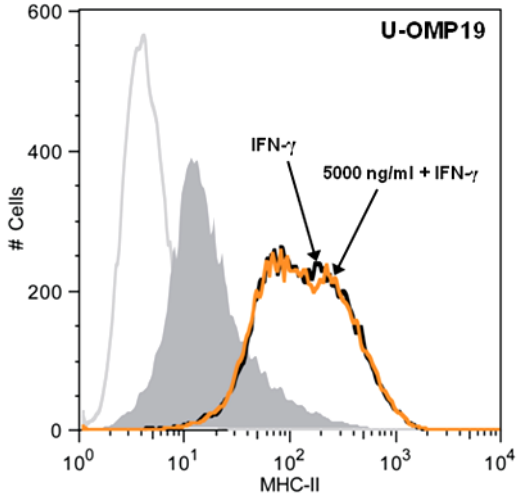


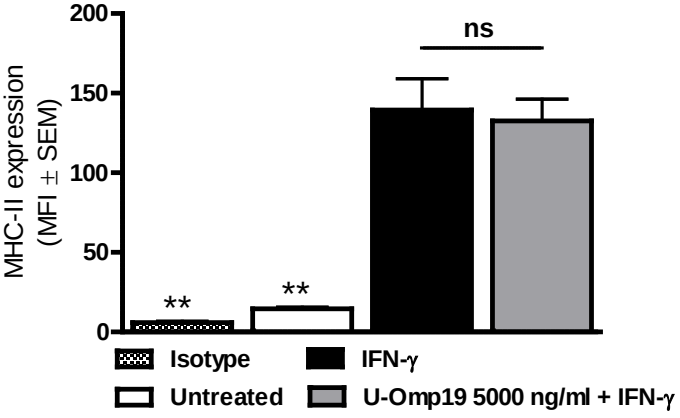
FIGURE 5. *Brucella* infection or stimulation with heat-killed bacteria (HKBA) down-regulates IFN-γ-induced MHC-II expression in murine AM. Alveolar macrophages from C57BL/6 (upper panels) or Balb/c mice (middle panels) were incubated for 2 h with IFN-γ in the presence or absence of different MOIs of live or *B. abortus*, and were then maintained in the presence of IFN-γ for an additional 48 h. The expression of MHC-II molecules (I-A^d) was measured by flow cytometry using a specific antibody. Similar experiments were performed with AM from Balb/c mice but replacing the infection with stimulation with HKBA (lower panels). Right panels show mean fluorescence intensity (MFI) from values measured in duplicate in each experiment. The figure shows a representative experiment from three performed with similar results. Asterisks indicate significant differences between untreated cells or cells stimulated with IFN-γ plus live bacteria or HKBA as compared to those stimulated with IFN-γ alone (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, NS, non-significant; ANOVA followed by Dunnett's post-test).

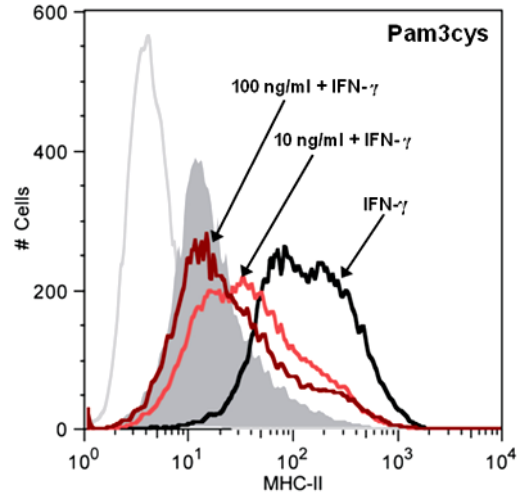
Figure 6

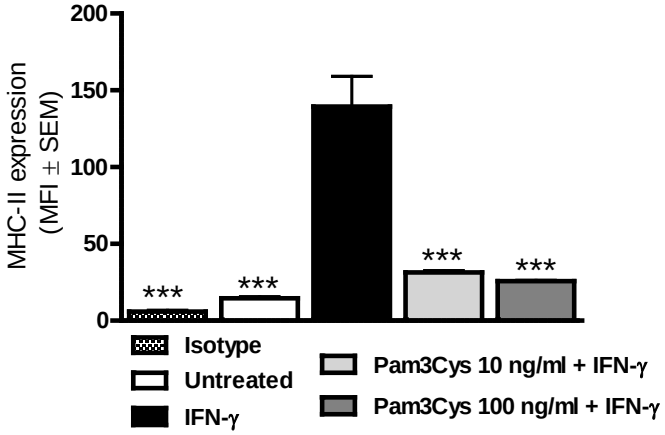

L-OMP 19
1000 ng/ml + IFN-γ
100 ng/ml + IFN-γ
10 ng/ml + IFN-γ
IFN-γ
Cells
MHC-II

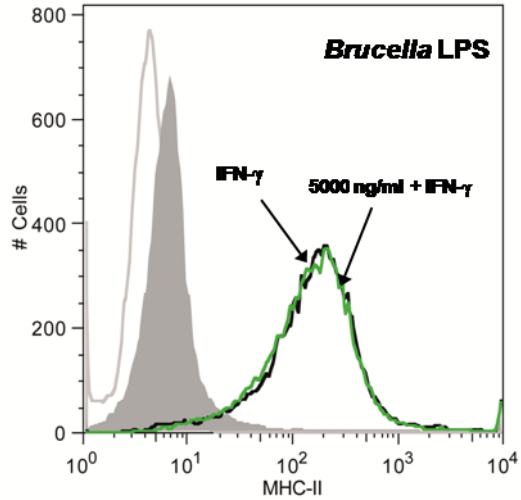

MHC-IIexpression
(MFI ± SEM)
***
Isotype
Untreated
IFN-γ
L-Omp19 10 ng/ml + IFN-γ
L-Omp19 100 ng/ml + IFN-γ
L-Omp19 1000 ng/ml + IFN-γ


U-OMP19
IFN-γ
5000 ng/ml + IFN-γ
Cells
MHC-II


MHC-II expression
(MFI ± SEM)
ns
**
Isotype
Untreated
IFN-γ
U-Omp19 5000 ng/ml + IFN-γ


Pam3cys
100 ng/ml + IFN-γ
10 ng/ml + IFN-γ
IFN-γ
Cells
MHC-II


MHC-II expression
(MFI ± SEM)
***
Isotype
Untreated
IFN-γ
Pam3Cys 10 ng/ml + IFN-γ
Pam3Cys 100 ng/ml + IFN-γ


Brucella LPS
IFN-γ
5000 ng/ml + IFN-γ
Cells
MHC-II

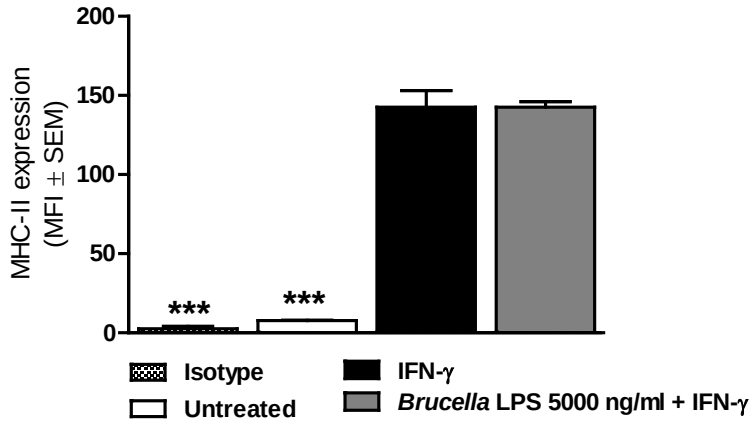

MHC-II expression
(MFI ± SEM)
***
Isotype
Untreated
IFN-γ
Brucella LPS 5000 ng/ml + IFN-γ

FIGURE 6. The down-regulation of the IFN-γ-induced MHC-II expression in murine AM is mediated by *Brucella* lipoproteins. Cells were incubated for 2 h with IFN-γ in the presence or absence of different doses of L-Omp19, U-Omp19, Pam3Cys or *Brucella* LPS, and were then maintained in the presence of IFN-γ for an additional 48 h. The expression of MHC-II molecules ($I\text{-}A^d$) was measured by flow cytometry using a specific antibody. Right panels show mean fluorescence intensity (MFI) from values measured in duplicate in each experiment. The figure shows a representative experiment from three performed with similar results. Asterisks indicate significant differences between untreated cells or cells stimulated with IFN-γ plus *Brucella* antigens or Pam3Cys as compared to those stimulated with IFN-γ alone (**, $p<0.01$; ***, $p<0.001$, NS, non-significant; ANOVA followed by Dunnett's post-test).

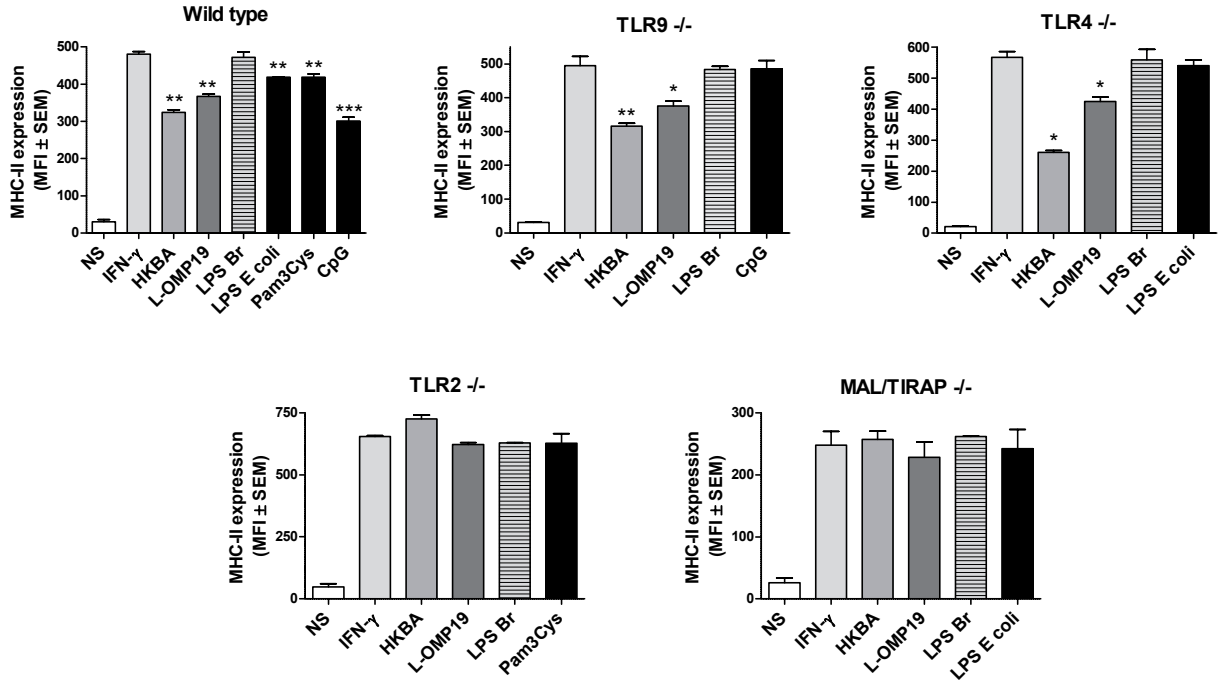


Figure 7: *Brucella*-induced down-regulation of MHC-II depends on TLR2 and MAL/TIRAP signaling. AM from wild-type C57BL/6 mice or from TLR2, TLR4, TLR9 and MAL/TIRAP KO mice from C57BL/6 background were incubated with murine IFN-γ in the absence or presence of HKBA, L-Omp19 or LPS from *B. abortus* (LPS Br) for 48 h. LPS from *E. coli*, Pam3Cys and CpG were included as control agonists for TLR4, TLR2 and TLR9, respectively. The expression of MHC-II was assessed by flow cytometry. Results are expressed as mean fluorescence intensity (MFI) with their corresponding SEM values. Data are representative of an experiment from three performed with similar results. (*) $p<0.05$, (**) $p<0.01$***, $p<0.001$ vs. IFN-γ alone (ANOVA followed by Tukey's post-test).

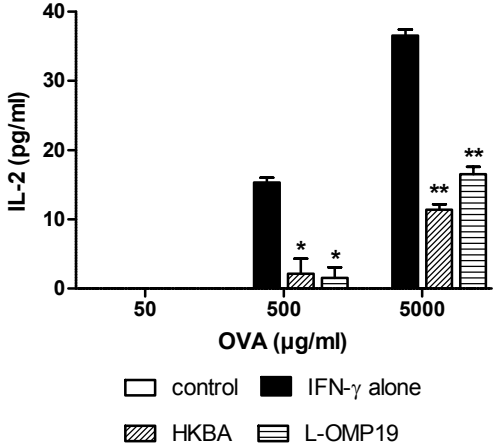


Figure 8 : MHC-II down-regulation by *Brucella* antigens impairs the ability of AM to stimulate OVA-specific T cells. AM from C57BL/6 mice were treated with IFN-γ alone or with the addition of L-Omp19 or HKBA. After 48 h these cells were incubated with different amounts of OVA and with a T cell hybridoma specific for an OVA peptide presented in the context of I-Ab (MHC-II). IL-2 levels were measured in the culture supernatants 24 h later. Data are presented as means ± SD of IL-2 concentrations assayed in triplicate in each experiment. The figure shows a representative experiment from three performed with similar results. (*) $p<0.05$, (**) $p<0.01$ vs. IFN-γ (ANOVA followed by Dunnet's post-test).

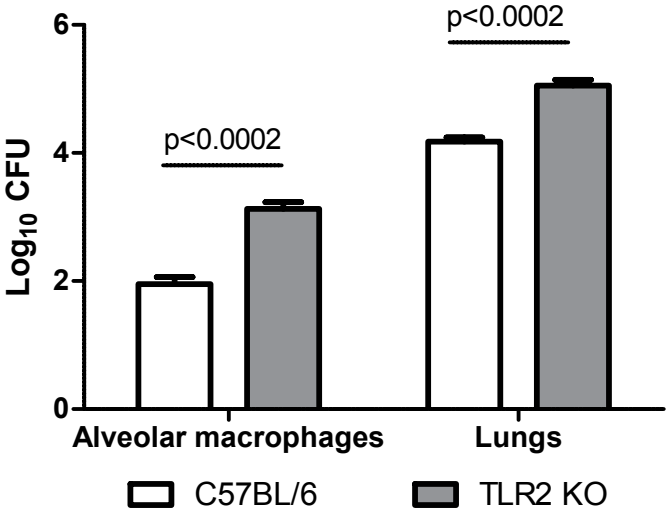


Figure 9. TLR2 absence impacts on the ability of AM to control *B. abortus* infection. Wild-type C57BL/6 mice and TLR2 KO mice were infected intra-tracheally with *B. abortus* and were euthanized one week later. Cells obtained by bronchoalveolar lavage (BAL) were adjusted to 5 x 10^5/ml and were dispensed in wells of a 24-well plate. Adherent cells were lysed and lysates were plated for CFU determination. After BAL, lungs were homogenized in sterile PBS and different dilutions of the resulting cell suspensions were plated on agar for CFU counting. Data represent mean ± SD of CFU/well (AM) or CFU/lungs determined in duplicate in one experiment from two performed with similar results. Data for each type of sample were analyzed using unpaired *t* test.