Cellular Microbiology (2013) **15**(4), 487–502
doi:10.1111/cmi.12058
First published online 21 November 2012

Brucella abortus induces intracellular retention of MHC-I molecules in human macrophages down-modulating cytotoxic CD8$^+$ T cell responses

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Summary

Brucella abortus **elicits a vigorous Th1 immune response which activates cytotoxic T lymphocytes. However, *B. abortus* persists in its hosts in the presence of CD8$^+$ T cells, establishing a chronic infection. Here, we report that *B. abortus* infection of human monocytes/macrophages inhibited the IFN-γ-induced MHC-I cell surface expression. This phenomenon was dependent on metabolically active viable bacteria. MHC-I down-modulation correlated with the development of diminished CD8$^+$ cytotoxic T cell response as evidenced by the reduced expression of the activation marker CD107a on CD8$^+$ T lymphocytes and a diminished percentage of IFN-γ-producing CD8$^+$ T cells. Inhibition of MHC-I expression was not due to changes in protein synthesis. Rather, we observed that upon *B. abortus* infection MHC-I molecules were retained within the Golgi apparatus. Overall, these results describe a novel mechanism based on the intracellular sequestration of MHC-I molecules whereby *B. abortus* would avoid CD8$^+$ cytotoxic T cell responses, evading their immunological surveillance.**

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Introduction

As a successful chronic and persistent pathogen *Brucella abortus* must overcome the many facets of the immune system designed to eliminate foreign invaders. To accomplish such a task *B. abortus* is endowed with a panoply of survival stratagems (Eisenschenk *et al.*, 1999; Velasco *et al.*, 2000; Giambartolomei *et al.*, 2002; Celli, 2006) that allows the bacterium to tilt in its favour this relentless 'tug of war'. Early in the infection *B. abortus* utilizes diverse evasion mechanisms (Hoffmann and Houle, 1983; Martinez de Tejada *et al.*, 1995; Lapaque *et al.*, 2005) that enhances its chances of survival before reaching its intracellular niche, the macrophage. Once inside the macrophage, *Brucella* dwells in an acidified compartment that fuses with components of the early endosomal pathway (Celli *et al.*, 2003). There, the vast majority of the ingested bacteria are rapidly killed. However, the establishment of a persistent infection relies on the ability of the bacterium to form a *Brucella*-containing vacuole (BCV) which traffics from the endocytic compartment to the endoplasmic reticulum (ER) (Gorvel and Moreno, 2002; Celli *et al.*, 2003; Roop *et al.*, 2004). Once inside the replicative BCV, bacteria are resistant to further attack and begin to multiply dramatically (Celli *et al.*, 2003; Roop *et al.*, 2004). *Brucella* also inhibits macrophage apoptosis (Gross *et al.*, 2000; Fernandez-Prada *et al.*, 2003), which in turn favours the pathogen's survival and replication.

Despite this refined escape mechanism *Brucella* has to face a second challenge if willing to persist in the host for a long time. It should be able to inhibit antigen (Ag) processing and presentation by *Brucella*-containing macrophages to avoid the immunological surveillance of T lymphocytes, thus promoting chronic infection. Presentation of *Brucella*-derived peptides on MHC class II (MHC-II) molecules is required to generate CD4$^+$ T cell responses. This is likely accomplished when the bacterium transits through the early acidic endosomal pathway where peptides derived from the degradation of *Brucella* proteins can be processed by the endocytic pathway (Braciale *et al.*, 1987). Previous studies in humans, mice and bovines have shown that cytokine-secreting CD4$^+$ T cells are developed during infection (Moreno-Lafont *et al.*, 2002; 2003; Baldwin and Goenka, 2006), confirming the ability of macrophage-dwelling *Brucella* to activate CD4$^+$ T cell responses. Also,

infection elicits $CD8^+$ T cell responses (Yingst and Hoover, 2003; Skendros *et al*., 2011), clearly indicating the ability of infected macrophages to present *Brucella* Ags on MHC-I molecules.

MHC-I molecules primarily present endogenous Ags that are synthesized and processed within the cytosol. Proteosomes cleave these Ags into peptides, which are then transported into the lumen of the ER. In the ER, peptides bind to nascent MHC-I molecules which transit through the Golgi apparatus (GA) to the cell surface to present their cargo to $CD8^+$ T cells (Braciale *et al*., 1987). Thus, cross-presentation of *Brucella* Ags from the endosome into the cytosol is required for the stimulation of *Brucella*-specific MHC-I restricted $CD8^+$ T cells. Alternatively, the ER nature of the BCV would be an optimal localization for peptide loading on MHC-I molecules (Oliveira *et al*., 1998).

Studies from our laboratory and from others have revealed that *B. abortus* is endowed with different strategies to avoid $CD4^+$ T cell scrutiny by inhibiting MHC-II expression and Ag processing (Forestier *et al*., 2000; Barrionuevo *et al*., 2008). However, although $CD8^+$ T cells are considered critical to resolution of *Brucella* infection (Oliveira and Splitter, 1995; Oliveira *et al*., 1998; He *et al*., 2001), few details are known as to how *Brucella* spp. persist in its hosts in the presence of $CD8^+$ T cells.

In this study we evaluated the effect of *B. abortus* on MHC-I expression and Ag presentation to cytotoxic $CD8^+$ T cells in human monocytes/macrophages. As model target cells we used the THP-1 human monocytic cell line. We first elucidated the ability of *B. abortus* to induce the down-modulation of MHC-I expression upon macrophage infection. Once the phenomenon was corroborated, we investigated if the MHC-I inhibition mediated by *B. abortus* infection correlated with a diminished MHC-I-restricted Ag presentation to cytotoxic $CD8^+$ T cells. Finally, we investigated the mechanism implicated in the downregulation of MHC-I cell surface expression. Here, we present the results of this study.

Results

B. abortus *infection down-modulates the IFN-γ-induced cell surface expression of MHC-I*

We determined the ability of *B. abortus* to inhibit the IFN-γ-induced expression of MHC-I molecules on human THP-1 cells. 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 48 h. The expression of MHC-I molecules (HLA-ABC) was evaluated by flow cytometry. *B. abortus* infection inhibited the IFN-γ-induced cell surface expression of MHC-I molecules in a dose-dependent manner (Fig. 1A and B). A significant ($P < 0.001$) MHC-I down-modulation was detected in cultures infected with 25 bacteria per cell or higher. Also, *B. abortus* infection inhibited the surface expression of MHC-I induced by different TLR ligands such as *Escherichia coli* LPS, Poly I:C, CpG and lipoproteins (Supplementary Fig. S1). In infected cells, *B. abortus* persists in only 5–10% of cells after 48 h of infection. Yet, down-modulation of IFN-γ-induced MHC-I expression was observed in all cells (Supplementary Fig. S2). In contrast, surface expression of CD11b, CD40 and the transferrin receptor (CD71) did not differ between uninfected and infected cells (Fig. 1C–E), indicating that *B. abortus* does not down-modulate all surface molecules globally. Next we evaluated whether the effect of *B. abortus* infection on MHC-I expression induced by IFN-γ could be extended to human monocytes. Purified human peripheral blood monocytes were infected with *B. abortus* in the presence of IFN-γ as described above and the expression of MHC-I was evaluated by flow cytometry. Infection with *B. abortus* downregulated the IFN-γ-induced expression of MHC-I on purified human monocytes (Fig. 1F). Inhibition was not due to a loss of cell viability in infected cultures. Two independent measures, trypan blue exclusion (data not shown) and Annexin V assay (Fig. 1G), revealed no differences in the percentage of viable cells in infected or uninfected cells, even at the highest infection level evaluated (500 bacteria per cell). On the contrary, high levels of apoptosis were obtained with the positive control paraformaldehyde (PFA).

To test whether viable bacteria were necessary to induce inhibition of MHC-I expression, the ability of heat-killed *Brucella abortus* (HKBA) to down-modulate IFN-γ-induced MHC-I expression was examined. HKBA was incapable to inhibit the IFN-γ-induced expression of MHC-I at any concentration tested (Fig. 1H). Moreover, *B. abortus* LPS (even at the concentration of 5000 ng ml^{-1}) was incapable to inhibit MHC-I expression. Also, a *B. abortus* LPS mutant (RB51) was capable to inhibit MHC-I expression (Supplementary Fig. S3). Altogether, these results indicate that the inhibition of MHC-I expression is not caused by *B. abortus* LPS or any other structural component of *B. abortus*. We also evaluated the effect of the *B. abortus* VirB type IV secretion system on MHC-I expression. Infection of THP-1 with a *vir*B10 mutant inhibited MHC-I expression; and the level of inhibition was similar to that obtained with wild-type *B. abortus* (Supplementary Fig. S4).

Taken together, these results demonstrate that infection with *B. abortus* down-modulates the cell surface expression of IFN-γ-induced MHC-I molecules on THP-1 cells as well as human monocytes and, that the inhibition of MHC-I expression requires metabolically active viable bacteria but is not mediated by the VirB type IV secretion system.

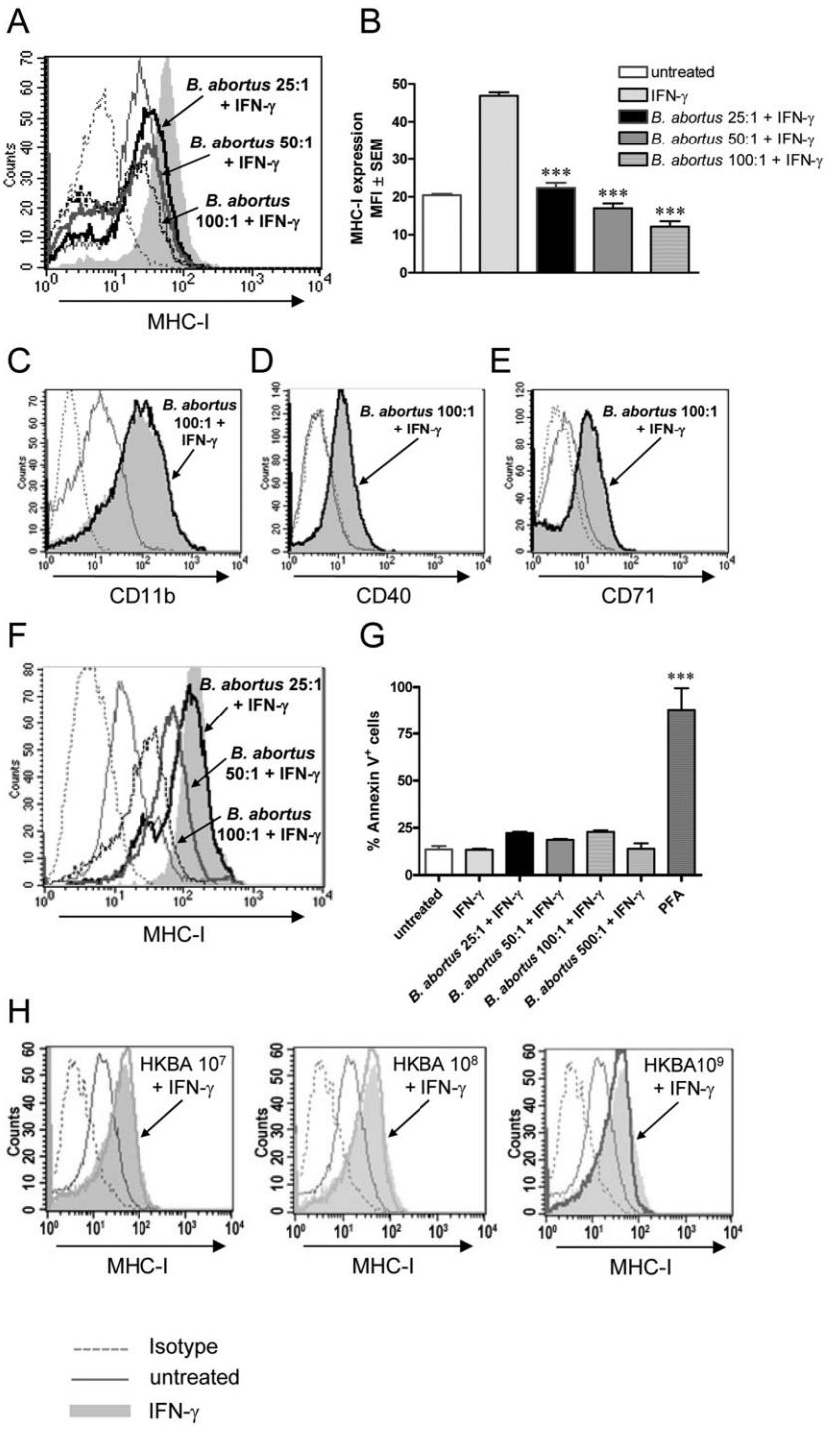

Fig. 1. *B. abortus* infection down-modulates the IFN-γ-induced surface expression of MHC-I. THP-1 cells (A–E) were infected with *B. abortus* at different moi in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. MHC-I (A and B), CD11b (C), CD40 (D) and CD71 (E) expression was assessed by flow cytometry. Purified human monocytes (F) from peripheral blood were infected with *B. abortus* as described, and the expression of MHC-I was assessed by flow cytometry. Bars in (B) represent the arithmetic means ± SEM of five experiments. MFI, mean fluorescence intensity. ***$P < 0.001$ versus IFN-γ-treated. THP-1 cells were infected with *B. abortus* as described, and apoptosis was determined by the Annexin V assay (G). Bars indicate the percentage of Annexin V-positive cells ± SEM of five experiments. ***$P < 0.001$ versus untreated. THP-1 cells were incubated with HKBA (10^7–10^9 bacteria ml^{-1}) (H) in the presence of IFN-γ for 48 h. MHC-I expression was assessed by flow cytometry. Histograms correspond to one representative of five independent experiments.

B. abortus *infection inhibits MHC-I-restricted Ag presentation by human monocytes/macrophages*

Next, we evaluated whether the diminished MHC-I surface expression induced by *B. abortus* was associated with changes in Ag presentation to MHC-I-restricted $CD8^+$ cytotoxic T cells. Purified human monocytes from mycobacterial purified protein derivative (PPD)-responder healthy individuals were infected with *B. abortus* in the presence of IFN-γ, pulsed with *Mycobacterium tuberculosis* lysate and incubated with autologous purified $CD8^+$ T cells. Macrophages treated with IFN-γ alone presented MHC-I-restricted epitopes recognized by autologous PPD-specific purified $CD8^+$ T cells as evidenced by the ability of these cells to upregulate the $CD8^+$ activation marker CD107a (McElroy *et al.*, 2007) and to produce IFN-γ when com-

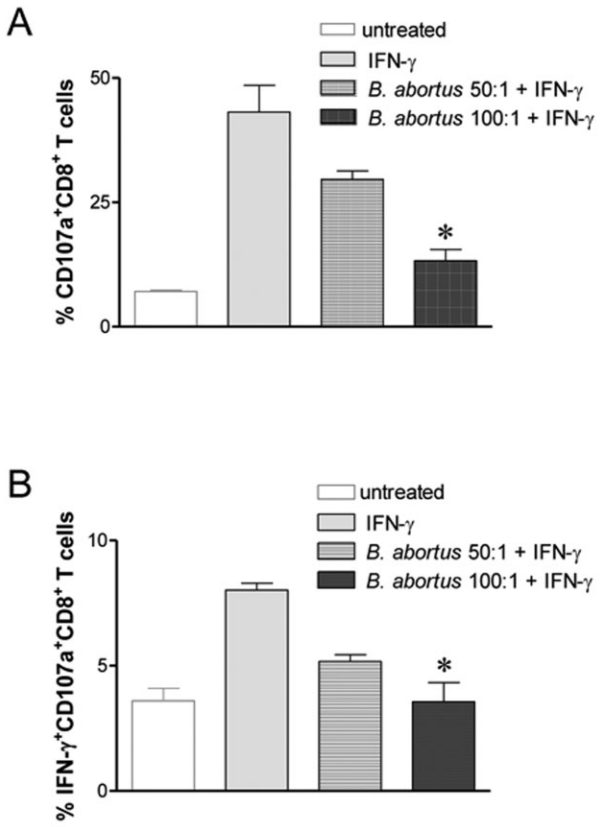

Fig. 2. *B. abortus* infection inhibits MHC-I-restricted Ag presentation. Monocytes isolated from PPD-responder healthy individuals were infected with *B. abortus* in the presence of IFN-γ. After 24 h, cells were incubated with *M. tuberculosis* lysate for another 24 h. Then, autologous purified $CD8^+$ T cells were added to the culture and cytotoxicity was evaluated by staining cells with CD107a, CD8 and IFN-γ mAbs and analysed by flow cytometry.
A. Quantification of the percentage of $CD107a^+$ $CD8^+$ T cells.
B. Quantification of the percentage of IFN-γ^+ $CD107a^+$ $CD8^+$ T cells.
Graphs show the results of one representative experiment of three performed. Bars express the mean ± SEM of duplicates. *$P < 0.05$ versus IFN-γ.

pared with untreated cells (Fig. 2A and B). *B. abortus* infection significantly ($P < 0.05$) inhibited Ag presentation since it diminished the activation of cytotoxic T lymphocytes as determined by CD107a expression (Fig. 2A). In addition, we observed a significant ($P < 0.05$) reduction in the production of intracellular IFN-γ by $CD107a^+$ $CD8^+$ T cells (Fig. 2B). Taken together, these results indicate that *B. abortus*-mediated inhibition of MHC-I expression correlates with a diminished Ag presentation to MHC-I-restricted $CD8^+$ cytotoxic T cells.

Inhibition of IFN-γ-induced surface expression of MHC-I by B. abortus *infection does not correlate with a decrease in MHC-I protein synthesis*

To gain insight into the mechanistic basis underlying the inhibition of MHC-I expression on the surface of human monocytes, we first explored whether infection with *B. abortus* resulted in changes in the regulation of MHC-I at the translational level. Total MHC-I protein load present in THP-1 cells was evaluated by Western blot analysis under the same experimental conditions in which surface MHC-I expression was assessed by flow cytometry. β-Actin and GAPDH were used as control of protein load. IFN-γ significantly ($P < 0.05$) increased total MHC-I protein expression and this increase was not altered by *B. abortus* infection even at the highest moi evaluated (100 bacteria/cell) (Fig. 3A and B). Total and surface-exposed expression of MHC-I was also evaluated using flow cytometry and fluorometry. For this purpose, THP-1 cells were infected with different moi of *B. abortus* in the presence of IFN-γ. Then, specific anti-HLA-ABC staining of fixed and non-permeabilized cells was used to determine membrane-associated MHC-I, while staining of fixed and saponin-permeabilized cells was used to evaluate the total amount of MHC-I. Both, flow cytometry and fluorometry indicated that infection with *B. abortus* inhibited the IFN-γ-induced surface expression of MHC-I (Fig. 3C and E), in agreement with the results shown in Fig. 1. However, infection with *B. abortus* was incapable to reduce the total level of MHC-I protein (Fig. 3D and F), corroborating the results obtained by Western blot. Overall, our results indicate that the inhibition of IFN-γ-induced surface expression of MHC-I by *B. abortus* infection does not correlate with decreased synthesis of total MHC-I protein.

B. abortus *infection induces intracellular retention of MHC-I molecules*

Taking into account that *B. abortus* infection inhibits MHC-I surface expression but did not modify the total amount of MHC-I protein, we hypothesized that infection with *B. abortus* could induce intracellular retention of MHC-I molecules. To evaluate this, the localization of MHC-I molecules was determined by confocal microscopy in cells infected with green fluorescence protein (GFP)-*B. abortus*. First, we confirmed by flow cytometry that GFP-*B. abortus* was equally able to down-modulate MHC-I expression than *B. abortus* without GFP (Fig. 4A). For confocal microscopy, THP-1 cells were infected with GFP-*B. abortus* in the presence of IFN-γ, and MHC-I expression was evaluated with a Cy3-labelled (red) anti-human MHC-I antibody. At 48 h of culture, 90 ± 10% of cells treated only with IFN-γ showed MHC-I expression confined predominantly to the cellular membrane (Fig. 4B and C). On the contrary, 84 ± 1% of *B. abortus*-infected monocytes showed MHC-I expression restricted to the cell interior concomitantly with a marked diminishing of MHC-I surface expression (Fig. 4B and C). Of note, *B. abortus* did not colocalized with MHC-I molecules

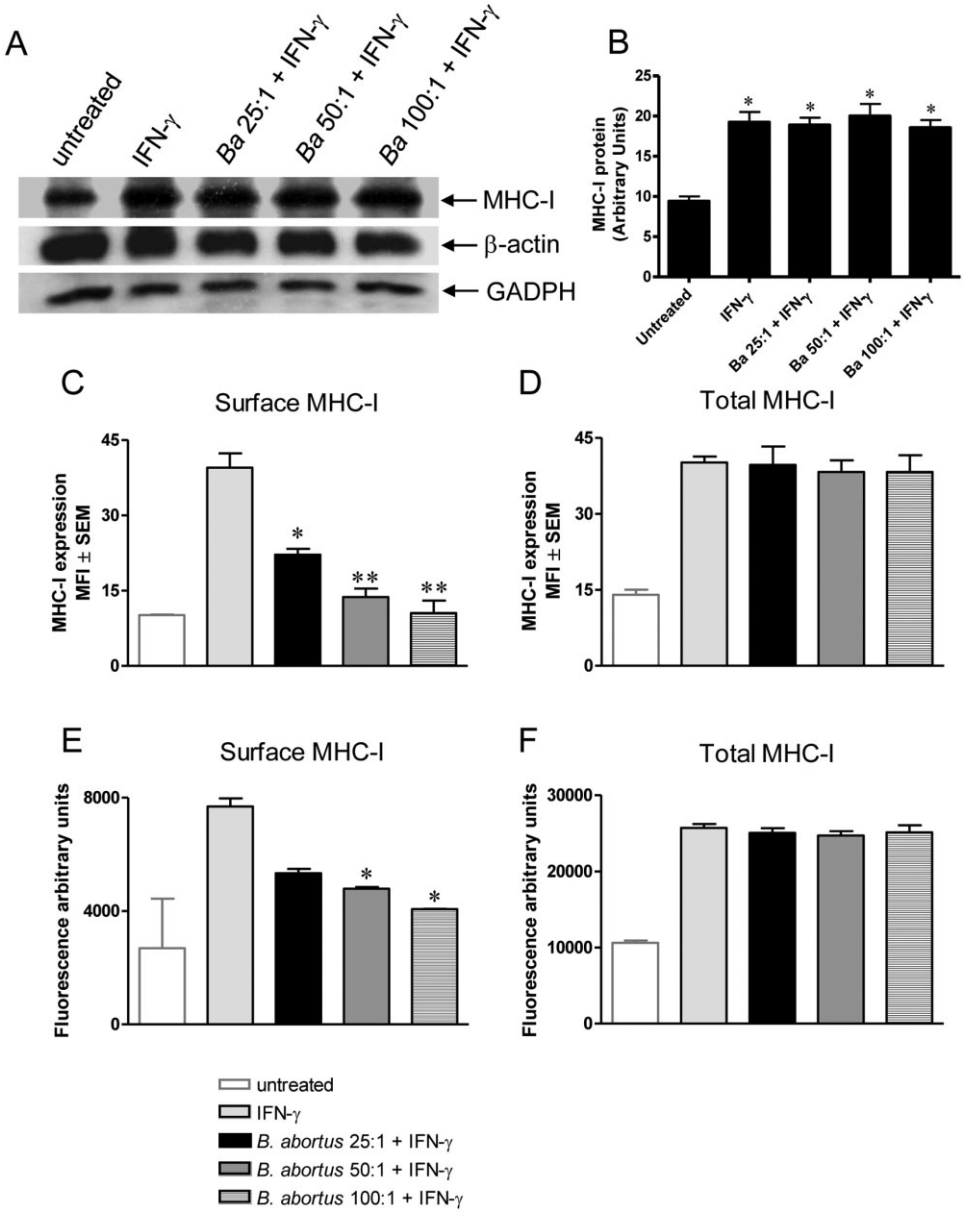

Fig. 3. *B. abortus* infection does not affect MHC-I protein synthesis. THP-1 cells were infected with *B. abortus* at different moi in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h.
A. Total-cell extracts were obtained and MHC-I expression was determined by Western blot. β-Actin and GADPH expression were also determined as control.
B. Densitometric analysis of MHC-I protein from two independent experiments performed as described in (A). $*P < 0.05$ versus untreated.
C–F. MHC-I expression was also assessed by flow cytometry (C and D) and fluorometry (E and F). MHC-I surface expression was determined on non-permeabilized cells (C and E) and MHC-I total expression was determined in saponin-permeabilized cells (D and F). Bars indicate the arithmetic means ± SEM of five experiments. MFI, mean fluorescence intensity. $*P < 0.05$; $**P < 0.01$ versus IFN-γ.

(Fig. 4B), being the percentage of cells showing colocalization between bacteria and MHC-I, in 200 cells infected with GFP-*B. abortus*, of 2 ± 1% (not shown). Together these results indicate that MHC-I molecules are retained within cells infected with *B. abortus*; however, MHC-I molecules are not confined to the vacuoles where *Brucella* replicates.

B. abortus *infection induce retention of MHC-I molecules in Golgi apparatus*

Next, we examined the subcellular localization of the retained MHC-I molecules. We designed experiments in which THP-1 cells were infected with *B. abortus* without GFP in the presence of IFN-γ and MHC-I was detected with

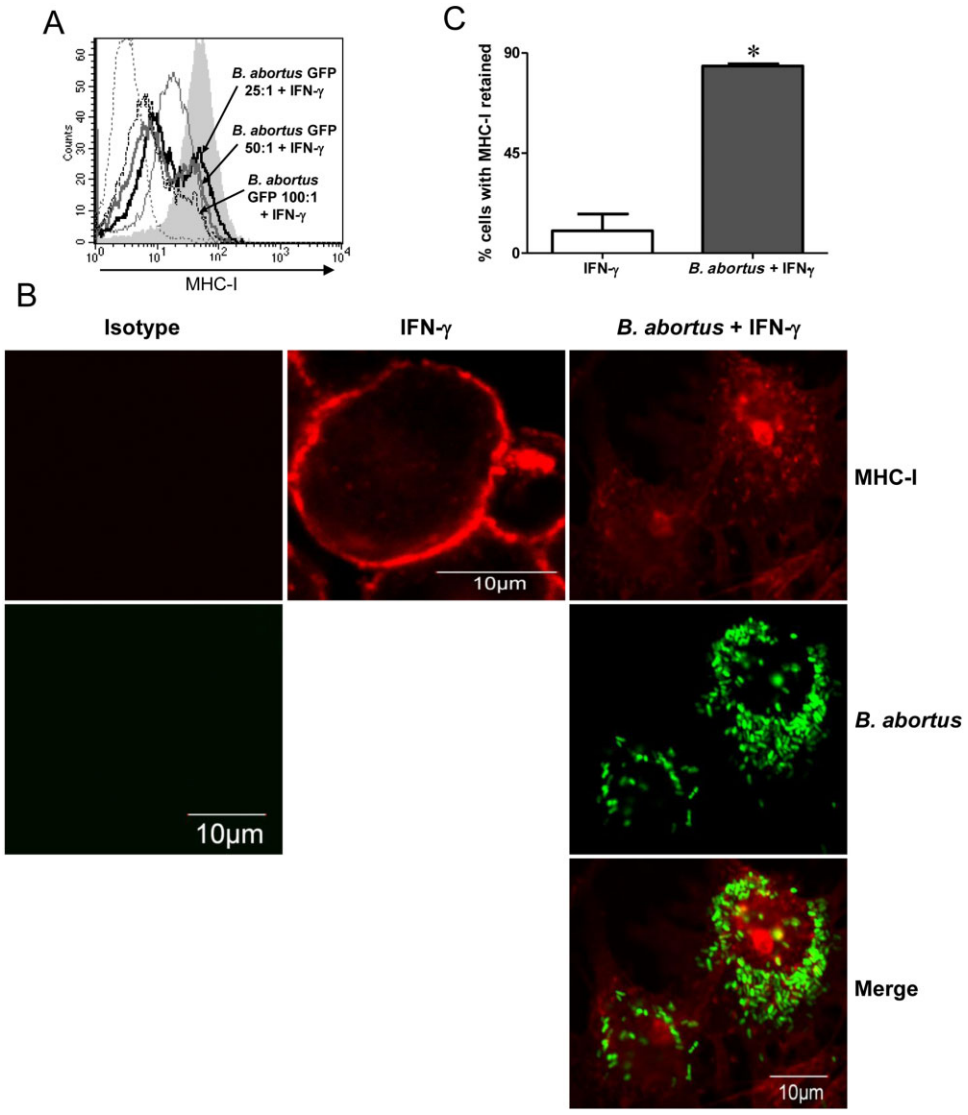

Fig. 4. *B. abortus* infection induces intracellular retention of MHC-I molecules.
A. THP-1 cells were infected with GFP-*B. abortus* at different moi in the presence of IFN-γ and the expression of MHC-I was assessed by flow cytometry.
B. Confocal micrographs of THP-1 cells infected with GFP-*B. abortus* (moi 100:1) in the presence of IFN-γ, and then stained with a primary anti-human MHC-I Ab (W6/32) and Cy3-labelled secondary Ab (red). Results are representative of three independent experiments.
C. Quantification of MHC-I retention. Data are expressed as percentage of cells with MHC-I retained ± SEM of two independent experiments. *$P < 0.05$ versus IFN-γ.

Alexa 546-labelled (red) anti-human MHC-I antibody and the subcellular compartments with specific primary mAbs followed by Alexa 488-labelled (green) secondary Ab. Again, when THP-1 cells were treated with IFN-γ for 48 h MHC-I was almost exclusively localized at the cellular surface. No colocalization was detected with either the early endosomal antigen 1 (EEA1), the lysosomal associated membrane protein 2 (LAMP-2) or the ER marker calnexin. Little colocalization of MHC-I molecules was observed in compartments positive for the Golgi matrix protein GM130, coherent with an active process of synthesis and membrane delivery of MHC-I molecules induced by IFN-γ (Fig. 5A). On the contrary, cells infected with *B. abortus* showed MHC-I expression restricted to the cell interior and in 93 ± 2% of *B. abortus*-infected monocytes that retained MHC-I molecules, they colocalize with the Golgi apparatus (GA) marker GM130 (Fig. 5B and C), and the nucleus was stained independently of this structure (Supplementary Fig. S5).

To test if the phenomenon mediated by *B. abortus* was unique to MHC-I, we investigated the expression of MHC-II, another molecule induced by IFN-γ which transit through GA. Cells treated only with IFN-γ showed MHC-II expression confined predominantly to the cellular mem-

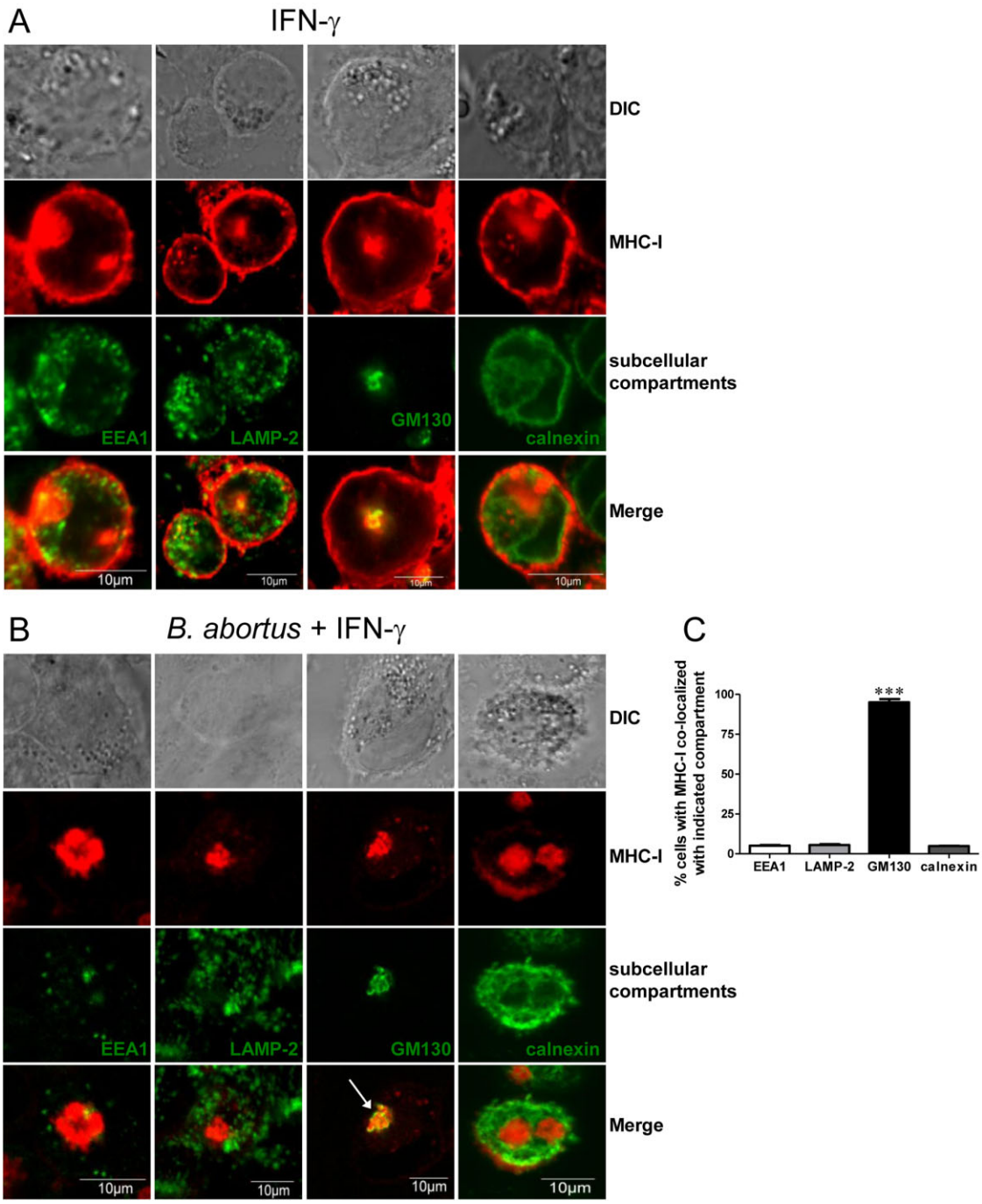

Fig. 5. *B. abortus* infection induces retention of MHC-I molecules in Golgi apparatus.
A and B. Confocal micrographs of THP-1 cells treated with IFN-γ (A) or infected with *B. abortus* without GFP (moi 100:1) in the presence of IFN-γ (B). MHC-I was detected using W6/32 mAb as a primary Ab and Alexa 546-labelled secondary Ab (red). Subcellular localization markers were detected using mAbs specific for EEA1 (early endosomes), LAMP-2 (late endosomes/lysosomes), GM130 (Golgi) and calnexin (ER) followed by Alexa 488-labelled secondary Ab (green). White arrow show colocalization (yellow staining). DIC, differential interference contrast. Results are representative of three independent experiments.
C. Quantification of cells with MHC-I retained colocalizing with the indicated subcellular compartment. Data are expressed as percentage of MHC-I colocalization with the different compartments markers evaluated and are means ± SEM of two independent experiments. $^{***}P < 0.01$ versus EEA1, LAMP-2 and calnexin.

brane. On the contrary, *B. abortus*-infected monocytes showed a drastic reduction of MHC-II expression either in the surface or within the cell and therefore MHC-II molecules did not colocalized with any of the compartments studied (Supplementary Fig. S6). These results indicates that the MHC-I retention in GA mediated by *B. abortus* infection is a mechanism specific for MHC-I molecules.

After deciphering the intracellular localization of MHC-I molecules we tried to find out the subcellular localization of *B. abortus*, which did not colocalized with MHC-I molecules after 48 h of infection. We used GFP-*B. abortus* and primary mAbs specific for subcellular compartments followed by Alexa 546-labelled (Red) secondary Ab. After 48 h of infection, *B. abortus* was enclosed within large LAMP-2-positive vacuoles, excluded from early endosomes, GA or ER (Fig. 6A and B). This was confirmed by ultrastructural studies and immunogold staining for LAMP-2 (Fig. 6C and D), indicating that *B. abortus* was enclosed in multi-membrane LAMP-2-positive vacuoles. These results are consistent with those of Starr *et al*. who, characterizing the post-replication stages of *B. abortus* infection in murine macrophages, observed that a population of bacteria were either engulfed by double-membrane crescents or delimited by multiple membranes associated with the lysosome-associated marker LAMP-1 beginning after 48 h of infection (Starr *et al*., 2012). Results were confirmed by using GFP-*B. abortus* and a three-colour confocal strategy. As shown in Fig. 7A, MHC-I molecules colocalized whit GA marker GM130 and GFP-*B. abortus* was excluded of this cellular localization, being surrounded by LAMP-2-positive vacuoles. Taking together, our results indicate that the infection with *B. abortus* induces intracellular retention of MHC-I molecules, and this retention is predominantly confined to GA.

The ionophore monensin mimics B. abortus*-induced retention of MHC-I molecules in Golgi apparatus*

The ionophore monensin is widely used to investigate the function of the GA and vesicular transport (Halaban *et al*., 2002; Marchetti *et al*., 2002). Monensin disperses the proton gradient across cell membranes and impedes the proper acidification of Golgi cisternae by displacing and inhibiting the H+-V-ATPase proton pump, leading to defective protein transport from the medial to the trans-Golgi (Zhang *et al*., 1996). To gain insight into the putative mechanism of MHC-I retention within the GA, we investigated whether treatment with monensin resulted in a down-modulation of surface MHC-I in IFN-γ-treated THP-1. Cells were treated with IFN-γ for 48 h and, 3 h before the end of culture, monensin was added. Then cells were analysed for surface MHC-I expression by flow cytometry. Monensin treatment caused a significant ($P < 0.05$) decrease in the IFN-γ-induced expression of MHC-I molecules on human THP-1 cells (Fig. 7B and C). The expression of other membrane glycoproteins upregulated by IFN-γ (CD11b, CD40 and CD71) was not affected in monensin-treated cells (Fig. 7D–F), indicating that the impeded transport of MHC-I molecules, as induced by *B. abortus* and monensin is not part of a generalized disturbance of intracellular traffic. Concomitantly with a marked diminishing of MHC-I surface expression, monensin-treated monocytes showed a MHC-I expression restricted to the GA (Fig. 7G). Altogether, these results indicate that, following inhibition of GA acidification by monensin, transport of MHC-I to the cell surface is obstructed, and support the contention that *B. abortus* prevents MHC-I transport through inhibition of GA acidification.

Discussion

Host control of brucellosis requires a set of cells and components of the immune system which together promote a complex response against *Brucella* spp. (Golding *et al*., 2001; Baldwin and Goenka, 2006). Yet, from the panoply of defensive resources that adaptive immunity brings into play, there are two components that are determinant to restrain *Brucella* infection. These are (i) IFN-γ produced by $CD4^+$ and $CD8^+$ T cells which activates the bactericidal action of macrophages to control intracellular *Brucella* infection (Zhan and Cheers, 1993; Murphy *et al*., 2001), and (ii) $CD8^+$ cytotoxic T cells which eliminate *Brucella*-infected target cells (Oliveira and Splitter, 1995; He *et al*., 2001). Importantly, IFN-γ upregulates MHC-I expression and Ag processing and presentation on cells (Zhou, 2009), vindicating the contention that when adaptive immunity is at play $CD8^+$ T cells are the main component in curbing infection (Oliveira and Splitter, 1995; Baldwin and Goenka, 2006). Nonetheless, *B. abortus* must own an effective strategy to survive and establish a chronic infection facing the obnoxious challenge of highly outfitted $CD8^+$ T cells.

In this manuscript we present evidence indicating that infection with *B. abortus* down-modulates the IFN-γ-induced expression of MHC-I on cells of the monocytic cell line THP-1, and on purified human monocytes. Inhibition of MHC-I expression was not due to a deleterious effect of the bacterium on cells (i.e. apoptosis) and required metabolically active viable bacteria. Down-modulation of MHC-I expression correlated with a diminished functional response that involved diminished cytotoxicity and IFN-γ secretion by Ag-specific $CD8^+$ T cells.

Brucella abortus might interfere with several steps of the MHC-I expression pathway, including transcription/translation of MHC-I heavy chain, degradation of the heavy chain and transport of the MHC-I complex to the cell surface (Ashrafi *et al*., 2002; Marchetti *et al*., 2002). Using

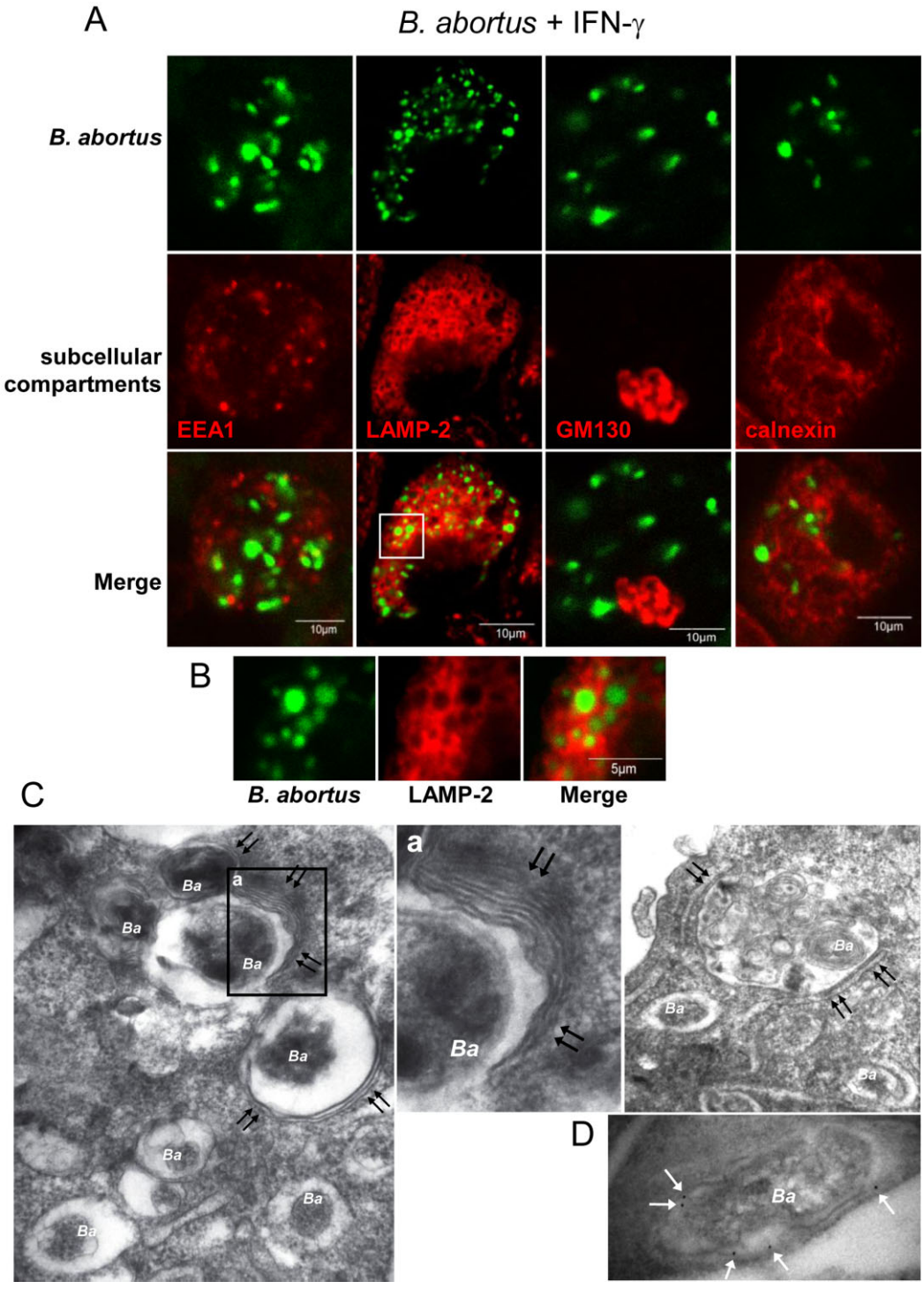

Fig. 6. *B. abortus* is enclosed within LAMP-positive vacuoles.
A. Confocal micrographs of THP-1 cells infected with GFP-*B. abortus* (moi 100:1) in the presence of IFN-γ, and then stained with the subcellular localization markers EEA1, LAMP-2, GM130 and calnexin followed by Alexa 546-labelled secondary Ab (red). Results are representative of three independent experiments.
B. Magnification of the inset in (A) showing *B. abortus* enclosed within LAMP-2$^+$ vacuoles.
C. Electronic microscopy images of THP-1 cells infected with *B. abortus* (moi 100:1) in the presence of IFN-γ for 48 h. Black arrows indicate double or multiple membranes structures around bacteria. Inset a shows multi-membrane *Brucella*-containing vacuoles.
D. Immuno-electron micrographs of THP-1 cells infected with *B. abortus* (moi 100:1) in the presence of IFN-γ for 48 h and processed for LAMP-2 immunogold staining. White arrows indicate LAMP-2 localized in association with membranes structures.
Ba: *B. abortus*.

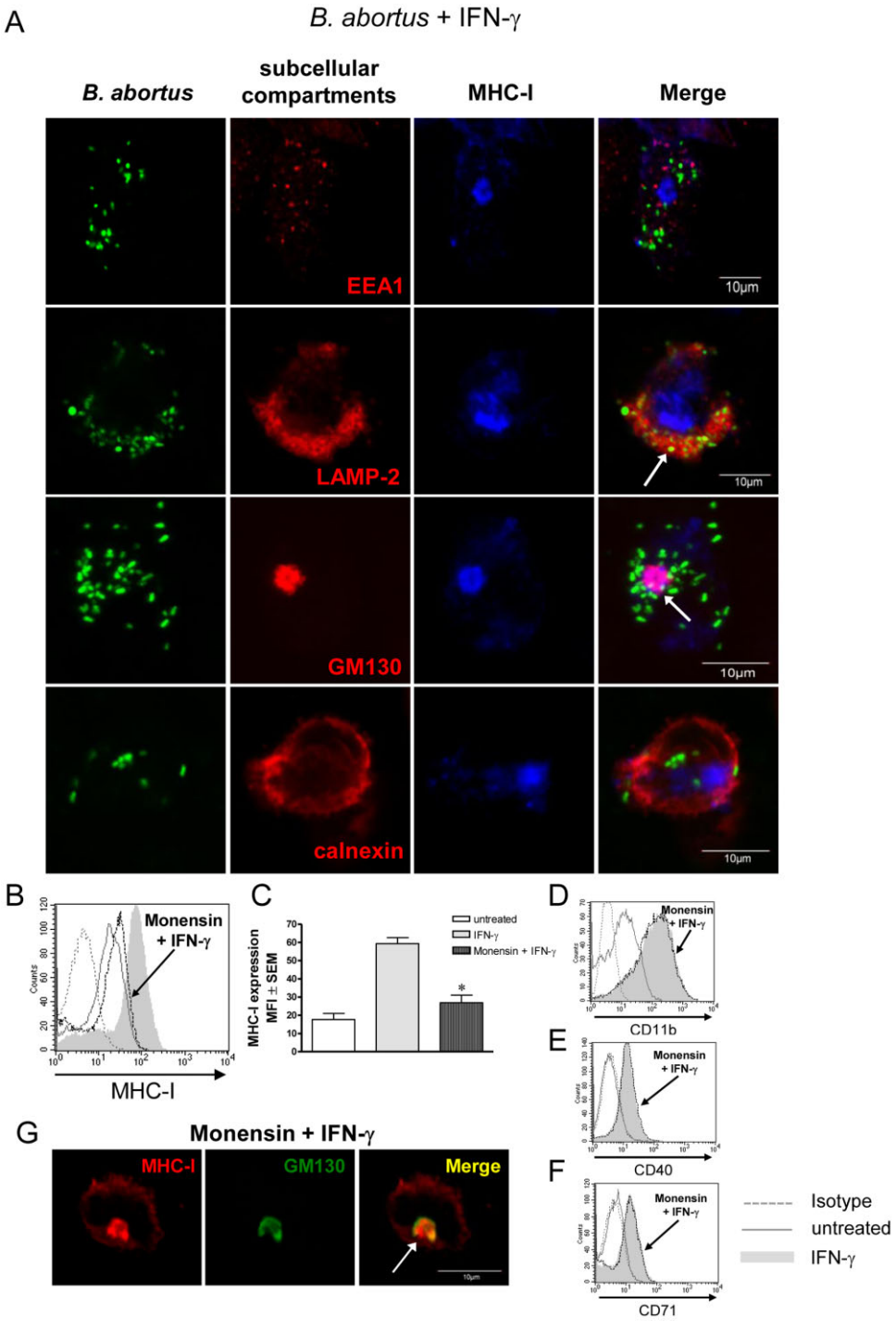

Fig. 7. Monensin mimics *B. abortus*-induced retention of MHC-I molecules in Golgi apparatus.
A. Confocal micrographs of THP-1 cells infected with GFP-*B. abortus* (moi 100:1) in the presence of IFN-γ. MHC-I was detected with the W6/32 mAb followed by Alexa 633-labelled secondary Ab (blue). The different subcellular compartments were detected using mAbs specific for EEA1, LAMP-2, GM130 and calnexin followed Alexa 546-labelled secondary Ab (red). White arrows show *B. abortus* enclosed within LAMP-2$^+$ vacuoles and MHC-I colocalizing with GM130 (pink staining). Results are representative of three independent experiments.
B–F. THP-1 cells were treated with monensin in the presence of IFN-γ. MHC-I (B and C), CD11b (D), CD40 (E) and CD71 (F) expression was assessed by flow cytometry. Histograms indicate the results of one representative of three independent experiments. Bars in (C) indicate the arithmetic means ± SEM of three experiments. *$P < 0.05$ versus IFN-γ.
G. Confocal micrographs of THP-1 cells treated with monensin plus IFN-γ. MHC-I was detected with the W6/32 mAb followed by Alexa 546-labelled secondary Ab (red). Golgi apparatus was detected using mAbs specific for GM130 followed by Alexa 488-labelled secondary Ab (green). White arrow shows colocalization (yellow staining). Results are representative of three independent experiments.

three different technical approaches (Western blot, fluorometry and flow cytometry) our results demonstrate that the inhibition of IFN-γ-induced surface expression of MHC-I by *B. abortus* infection does not correlate with decreased synthesis of total MHC-I protein, thus excluding the possibility of a decrease in MHC-I heavy chain transcription/translation or a putative degradation of the heavy chain. Rather, our results indicate that the inhibition of MHC-I surface expression by *B. abortus* infection is mediated by intracellular retention of these molecules.

Although reduction of MHC-I expression on host cells is a very well described immune evasion mechanism displayed by several viruses (Ploegh, 1998) and tumours (Rodriguez *et al*., 2007), regulation of expression of MHC-I expression during bacterial infections has also been characterized. Indeed, enterobacteria (Kirveskari *et al*., 1999), *Legionella pneumophila* (Neumeister *et al*., 2005), *Chlamydia pneumoniae* (Ibana *et al*., 2011) and *Mycobacterium avium* (Weiss *et al*., 2001) promote downregulation of MHC class I. Yet, our results demonstrate a novel mechanism of inhibition of MHC-I expression utilized by bacteria: the intracellular retention of these molecules, a mechanism that have been exploited and very well characterized in many viral infections (Andersson *et al*., 1985; Hill *et al*., 1994; Ashrafi *et al*., 2002; Marchetti *et al*., 2002).

The way in which *B. abortus* retains MHC-I molecules inside cells merits discussion. Considering the ER localization of the bacterium during the replicative phase of its life cycle, the most parsimonious explanation would be that *B. abortus* once inside the ER somehow interrupts MHC-I assembly and generates abortive molecules that cannot complete their intracellular traffic towards the cell surface. *B. abortus* could provoke stress inside the ER and consequently, alter the loading of peptides onto MHC-I molecules generating immature molecules that would be retained at the ER, as observed with other microorganisms (Andersson *et al*., 1985; Hill *et al*., 1994). Yet, *B. abortus* retention of MHC-I is confined to the GA. The detection of endogenous MHC-I within the GA with mAb W6/32, which recognizes properly folded MHC-I molecules in association with β2-microglobulin (Shields and Ribaudo, 1998), indicates that MHC-I molecules are properly assembled. This suggests that the arrest of MHC-I transport occurs following MHC-I complex formation in the ER and is instead a fault in cellular transport across the Golgi compartments. Indeed, treatment of control IFN-γ-stimulated cells with the ionophore monensin resulted in the acquisition of a phenotype remarkably similar to that of *B. abortus*-infected cells; MHC-I is retained in the GA, with a concordant downregulation of surface MHC-I. Monensin is a well-characterized inhibitor of Golgi acidification, which hinders cellular transport of newly synthesized proteins (Tartakoff, 1983), particularly interfering with the transit across Golgi compartments by impeding the correct glycosylation of proteins (Andersson *et al*., 1985). Taking into account the similar functional effect of monensin and *B. abortus*, we speculate that *B. abortus* causes distress and retention of the MHC-I complex within GA by altering pH. However, the impeded transport of the MHC-I complex in *Brucella*-infected cells is not part of a generalized disturbance of intracellular traffic as the transport of CD11b, CD40 and CD71 is not affected by *B. abortus* infection or by treatment with monensin. How then does *B. abortus* prevent only MHC-I from exiting the GA and reaching the plasma membrane? Although *B. abortus* never traffics through the GA in its life cycle, we speculate that when present in the ER in the replicative phase of the cycle, the bacterium could release proteins that by interacting with nascent MHC-I molecules could transit through the endomembrane compartments of the ER and GA and inhibit the vacuolar ATPase (Goldstein *et al*., 1991), causing in this way an alteration on the acidification of the GA and the retention of MHC-I molecules, as has been described for other pathogens (Marchetti *et al*., 2002). Probably, MHC-I molecules are more susceptible to imprecise glycosylation caused by pH alterations than CD11b, CD40 and CD71.

Recent results from our laboratory demonstrated the ability of *B. abortus* to infect and replicate in a variety of cell types different from macrophages, such as osteoblast, synoviocytes, bronchial epithelial cells, hepatocytes, endothelial cells, astrocytes and microglia, among others (Delpino *et al*., 2009; 2010; Ferrero *et al*., 2009; 2011; Garcia Samartino *et al*., 2010; Scian *et al*., 2011a,b; 2012). As any other mammalian nucleated cell, all these cells express MHC-I molecules. Despite the relevance of these alternative cellular niches in the natural history of the infection of *B. abortus*, if the mechanism we describe for MHC-I down-modulation upon infection will take place in all these cell environments; *B. abortus* would be able to successfully live long-term within the host avoiding the surveillance of $CD8^+$ T cells, virtually undisturbed. Additionally, Durward *et al*. have recently demonstrated that bacteria from the genus *Brucella* actively and persistently inhibit $CD8^+$ T cell killing of *Brucella* epitope-specific target cells in mice. This was achieved by the *Brucella* protein named TcpB, a novel effector of adaptive immune evasion that was proposed to act directly on T cells (Durward *et al*., 2012). Thus, *Brucella* possesses at least two possible mechanisms of blunting CTL responses: (i) down-modulating the expression of MHC-I molecules on the surfaces of cells and (ii) actively inhibiting $CD8^+$ T function through TcpB.

Since NK cells are able to kill cell targets with reduced levels of MHC-I molecules, our results would be paradoxical in that they would indicate that the down-modulation of IFN-γ-induced MHC-I by *B. abortus* would activate NK which would lead to the elimination of the bacterium. Yet, although it has been demonstrated *in vitro* that human NK

cells are able to kill *Brucella suis*-infected macrophages; this occurs independently of the down-modulation of MHC-I molecules (Dornand *et al*., 2004). Moreover, humans with acute brucellosis have suppressed NK activity (Salmeron *et al*., 1992) which, together with results in mice showing no role of NK in the control of infection (Fernandes *et al*., 1995), would support the contention that NK cells – as innate immune cells – would play no relevant function in controlling a slowly replicating organism such as *Brucella* spp., which causes chronic infections (Baldwin and Goenka, 2006).

Finally, our results together with the recent revealed capacities of *B. abortus* to actively inhibit $CD8^+$ T function (Durward *et al*., 2012) and to induce apoptosis of $CD4^+$ T lymphocytes (Velásquez *et al*., 2012), the main producers of IFN-γ during *Brucella* infection (He *et al*., 2001; Baldwin and Goenka, 2006), indicate that *B. abortus* would be able to control host immune responses in its advantage. May be the alleged furtive nature of this well-equipped parasite resides in its ability to manipulate the performance of its two main foes, $CD4^+$ and $CD8^+$ T lymphocytes.

Experimental procedures

Bacteria

Brucella abortus S2308, S2308-GFP (Bonomi *et al*., 2010), *B. abortus* RB51 and *B. abortus virB10* mutant were cultured in tryptose-soy agar supplemented with yeast extract (Merck). The number of bacteria on stationary-phase cultures was determined by comparing the OD at 600 nm with a standard curve. To obtain heat-killed *B. abortus* (HKBA), bacteria were washed five times for 10 min each in sterile phosphate-buffered saline (PBS), heat killed at 70°C for 20 min, aliquoted and stored at −70°C until used. Total absence of *B. abortus* viability subsequent to heat-killing was verified by the absence of bacterial growth in tryptose-soy agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities.

Cells and media

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 of penicillin ml^{-1} and 100 μg of streptomycin ml^{-1}. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as previously described (Giambartolomei *et al*., 2004). To induce maturation, cells were cultured in 0.05 μM 1, 25-dihydroxyvitamin D_3 (Calbiochem) for 72 h. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (GE Healthcare) gradient centrifugation from human blood collected from healthy adult individuals. All volunteers gave informed consent prior to participating in the study. The study was approved by the Ethical Committee of the IDEHU. Monocytes were obtained after centrifugation of PBMCs on a Percoll (GE Healthcare) gradient and resuspended in standard medium. Viability of cells was more than 95% in all the experiments as measured by trypan blue exclusion test.

In vitro *infection*

THP-1 cells or monocytes at a concentration of 0.5×10^6 ml^{-1} were infected in round-bottom polypropylene tubes (BD Falcon) with different multiplicities of infection (moi) of *B. abortus* in the presence of 150 U ml^{-1} IFN-γ (Endogen) or *E. coli* LPS (1 μg ml^{-1}), Poly I:C (InvivoGen) (100 μg ml^{-1}), CpG (IDT) (1.5 μM) and the lipohexapeptide Pam_3Cys (Boehringer) (100 ng ml^{-1}) for 2 h in standard medium containing no antibiotics. Then cells were extensively washed to remove uninternalized bacteria and infected cells were maintained in culture in the presence of IFN-γ or TLR agonists, 100 μg ml^{-1} gentamicin and 50 μg ml^{-1} streptomycin for an additional 48 h. To monitor *Brucella* intracellular survival, concomitantly infected cells were lysed with 0.1% (v/v) Triton X-100 in H_2O after PBS washing and serial dilutions of lysates were rapidly plated onto TSB agar plates to enumerate colony-forming units (cfu).

Flow cytometry

THP-1 cells or monocytes were infected with *B. abortus* at different moi. Additionally, THP-1 cells were incubated with HKBA in the presence of 150 U ml^{-1} IFN-γ for 48 h. Cells were then washed and stained with FITC-labelled anti-human HLA-ABC (clone G46-2.6; BD Pharmingen) or anti-human CD71 (clone M-A712; BD Pharmingen); or PE-labelled anti-human CD11b (ICRF44; eBioscience) or anti-human CD40 (clone 5C3; BD Pharmingen) mAbs or isotype-matched control mAbs. After labelling, cells were analysed on a FACSCalibur® flow cytometer (BD Biosciences) and data were processed using CellQuest software (BD Biosciences). Results were expressed as mean fluorescence intensity (MFI) values and represent the arithmetic mean ± SEM of the indicated experiments.

Apoptosis assay

THP-1 cells, at a concentration of 0.5×10^6 ml^{-1}, were infected with different moi of *B. abortus* in the presence of IFN-γ or were treated with 2% paraformaldehyde (PFA). At 48 h, cells were washed and incubated with Annexin V-FITC and Propidium Iodide (BD Biosciences) for 10 min on ice in darkness. Apoptotic cells were analysed on a FACSCalibur® flow cytometer (BD Biosciences) and data were processed using CellQuest software (BD Biosciences).

$CD8^+$ T lymphocyte cytotoxicity assay

PBMCs isolated from PPD responder healthy individuals were stimulated with 10 μg ml^{-1} *M. tuberculosis* lysate and 10 U ml^{-1} recombinant human IL-2 (BD Pharmingen) for 10 days. Then, $CD8^+$ T cells were purified using a negative isolation kit (BD Biosciences) following the manufacturer's instructions. Simultaneously, phorbol myristate acetate (PMA)-treated autologous purified monocytes at a concentration of 0.5×10^6 ml^{-1} were treated with IFN-γ or were infected with *B. abortus* (moi 50 and 100) in the presence of IFN-γ for 24 h. Following incubation,

medium was removed and cells were extensively washed prior to Ag exposure. Cells were then incubated with *M. tuberculosis* lysate (10 μg ml^{-1}). Following 24 h incubation, autologous CD8$^+$ T cells were added to the culture together with FITC-labelled anti-human CD107a mAb (clone H4A3; BD Pharmingen). After 1 h of incubation 1 μl per well of BD Golgi Stop (BD Pharmingen) was added and cells were culture for an additional 5 h. Cells were then surface-stained with PerCP-labelled anti-human CD8 mAb (clone SK1, BD Biosciences). After that, cells were permeabilized and stained with PE-labelled anti-human IFN-γ (clone 4SB3; BD Pharmingen) to determine intracellular cytokine production. Cells were analysed on a FACSCalibur® flow cytometer (BD Biosciences) and data were processed using CellQuest software (BD Biosciences).

SDS-PAGE and Western blot

THP-1 cells at a concentration of 2×10^6 cells ml^{-1} were used. Cells were left untreated, treated with IFN-γ or they were infected with different moi of *B. abortus* in the presence of IFN-γ as described. Following the different experimental treatments, cells were washed with PBS and lysed by incubation on ice for 1 h in 0.2 ml of 1% Triton X-100 in 150 mM NaCl, 25 mM Tris-HCl, pH 7.4 (TBS), gentamicin/streptomycin, 0.2% azide in the presence of an inhibitor mixture (Sigma-Aldrich). Lysates were then centrifuged for 15 min at 14 000 r.p.m. at 4°C. Protein concentrations were determined using the micro BCA protein assay (Pierce). Equal amounts of protein (30 μg) were then resolved on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes (GE Bio-Sciences) for 1 h at 300 mA and blocked with TBS 0.1% Tween 20 overnight at 4°C. Membranes were then probed with primary Ab in TBS 0.05% Tween 20 (1 μg ml^{-1} anti-human MHC class I rabbit polyclonal Ab, clone H-300; Santa Cruz Biotechnology) overnight. After washing three times with TBS 0.05% Tween 20, blots were incubated for 1 h with a HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Immunoreactivity was detected using the ECL Plus Western blot detection kit (GE Bio-Sciences). The chemiluminescent detection was done using ImageQuant (GE Bio-Sciences). Equal loading was checked by incubation of the blots with an anti-β-actin (clone C4; Santa Cruz Biotechnology) and anti-GAPDH Ab (clone A-.3; Santa Cruz Biotechnology). Results from Western blot were analysed by densitometric analysis (Image J software).

Fluorometry

THP-1 cells were left untreated, treated with IFN-γ or they were infected with different moi of *B. abortus* in the presence of IFN-γ as described. For surface MHC-I expression, cells were then washed, fixed and stained with FITC-labelled anti-human HLA-ABC (clone G46-2.6; BD Pharmingen) mAb. Alternatively, cells were fixed and permeabilized to determine total MHC-I expression. After labelling, cells were transferred to plates and were read in a fluorescence plate reader (Victor^3; Perkin-Elmer). Results were expressed as MFI values and represent the arithmetic mean ± SEM of the indicated experiments.

Confocal microscopy

THP-1 cells were incubated in chambers-slides with 10 ng ml^{-1} PMA (Sigma-Aldrich) for 24 h to promote adherence. Then, cells were infected with green fluorescence protein (GFP)-*B. abortus* (moi 100:1) in the presence of IFN-γ for 48 h, fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin and incubated with anti-HLA class I mAb W6/32 (purified from hybridoma culture supernatants) and Cy3-labelled anti-mouse IgG (red). For subcellular localization experiments, THP-1 cells were infected with *B. abortus* without GFP (moi 100:1) in the presence of IFN-γ for 48 h. MHC-I was detected with the mAb W6/32 and Alexa 546-labelled secondary Ab (Invitrogen). Colocalization markers were detected using mAbs specific for EEA1 (early endosomes), LAMP-2 (late endosomes/lysosomes), GM130 (Golgi) and calnexin (ER) (BD Biosciences) following Alexa 488-labelled secondary Ab (Invitrogen). For nuclear staining, TO-PRO®-3 (Invitrogen) was used. In other set of experiments, THP-1 cells were infected with GFP-*B. abortus* (moi 100:1) in the presence of IFN-γ. MHC-I was detected with the mAb W6/32 and Alexa 633-labelled secondary Ab (Invitrogen). The different compartments were detected using mAbs specific for EEA1, LAMP-2, GM130 and calnexin following Alexa 546-labelled secondary Ab. In all cases, slides were mounted with PolyMount (Polysciences) and analysed using FV-1000 confocal microscope with an oil-immersion Plan Apochromatic 60× NA1.42 objective (Olympus).

Electron microscopy

THP-1 cells were infected with *B. abortus* (moi 100:1) in the presence of IFN-γ for 48 h. For transmission electron microscopy, cells were fixed with 3% glutaraldehyde for 1 h at room temperature and overnight at 4°C, post-fixed for 2 h with 1.5% OsO4 in 0.1 M phosphate buffer, pH 7.5; dehydrated in ascending ethanol series and embedded in Epoxi resin (Epoxi Embedding Medium Kit-Sigma-Aldrich). Ultrathin sections were stained with 2% uranyl acetate and Reynold solution. For immuno-electron microscopy, cells were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde for 24 h. Quench-free aldehyde pellets were incubated in 0.1 M phosphate buffer, pH 7.5 for 20 min at room temperature and incubated in 0.2 M sucrose solution overnight at 4°C. The pellets were dehydrated through 70% ethanol and embedding in LR-White (Sigma-Aldrich) at 50°C. Ultrathin sections were collected in nickel grids. Sections were immunolabelled with a mouse monoclonal anti-LAMP-2 (Invitrogen) overnight at 4°C. Subsequently an anti-mouse IgG antibody conjugated with 10-nm-diameter colloidal gold particles (Sigma-Aldrich) was used according to the supplier's instructions. After immunolabelling, sections were post-stained with uranyl acetate and observed using a Zeiss EM109T transmission electron microscope equipped with digital camera with 11 Mega pixels of resolution for electron images.

Treatment of cells with monensin

Forty-eight-hour IFN-γ-treated THP-1 cells were incubated with 25 μM monensin (Sigma-Aldrich) 3 h before the end of culture. MHC-I expression was determined by flow cytometry or confocal microscopy as described above.

Statistical analysis

Results were analysed with one-way ANOVA followed by *post hoc* Bonferroni test and, when corresponding (Fig. 4) with Student's unpaired *t*-test, using the GraphPad Prism software.

Acknowledgements

We thank Horacio Salomón from the Centro Nacional de Referencia del Sida, University of Buenos Aires, for their assistance with biosafety level 3 laboratory use. We deeply appreciate the technical assistance of Margarita Lopez in conducting electron microscopy experiments. We also thank Dr Garcia for the session of *M. tuberculosis* lysate, Dr Comerci for *Brucella*-GFP and *vir*B10 mutant, and Dr Moriyón (University of Navarra, Pamplona, Spain) for providing *B. abortus* and *E. coli* LPS. This work was supported by Grants PICT 2010-0023, 2006-1335 and 2006-2180 from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina), PIP 5213 and 1390 from CONICET (Argentina) and UBACyT 20020090100083 from UBA (Argentina). L.N.V. is recipient of a fellowship from UBA (Argentina). P.B., M.V.D., R.P., J.C. and G.H.G. are members of the Research Career of CONICET.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *B. abortus* infection down-modulates the TLR agonists-induced surface expression of MHC-I. THP-1 cells were infected with *B. abortus* at different moi in the presence of IFN-γ (A), *E. coli* LPS (B), Pam_3Cys (C), CpG (D) and Poly I:C (E). MHC-I expression was assessed by flow cytometry. Bars represent the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. Histograms correspond to one representative of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus MHC-I upregulator.

Fig. S2. MHC-I expression was decreased in cells with and without *B. abortus* at 48 h. THP-1 cells were infected with GFP-*B. abortus* at different moi in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. MHC-I expression was assessed by flow cytometry, using W6/32 mAb as a primary Ab and PE-labelled secondary Ab. Bars represent the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. $^{**}P < 0.01$; $^{***}P < 0.001$ versus IFN-γ. MFI of IFN-γ: 51 ± 2.

Fig. S3. Downregulation of MHC-I surface expression is not mediated by *B. abortus* LPS. THP-1 cells were incubated with *B. abortus* LPS (BruLPS) (100 ng ml^{-1}, 1000 ng ml^{-1} and 5000 ng ml^{-1}) (A) or were infected with *B. abortus* or *B. abortus* RB51 at moi 100:1 (B), in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. MHC-I expression was assessed by flow cytometry. Histograms correspond to one representative of three independent experiments. Non-specific binding was determined using a control isotype Ab.

Fig. S4. Infection with a VirB mutant also inhibits MHC-I surface expression. (A) THP-1 cells were infected with *B. abortus* or the *vir*B10 mutant of *B. abortus* at moi 100:1 in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. MHC-I expression was assessed by flow cytometry. Histograms correspond to one representative of three independent experiments. Bars in (B) indicate the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. Non-specific binding was determined using a control isotype Ab. $^{**}P < 0.01$ versus IFN-γ.

Fig. S5. *B. abortus* infection induces retention of MHC-I molecules in Golgi apparatus. Confocal micrographs of THP-1 cells infected with *B. abortus* without GFP (moi 100:1) in the presence of IFN-γ. MHC-I was detected with the W6/32 mAb followed by Alexa 546-labelled secondary Ab (red). Golgi apparatus was detected using mAbs specific for GM130 followed by Alexa 488-labelled secondary Ab (green). Nucleus was detected using TOPRO-3 (blue). Results are representative of two independent experiments.

Fig. S6. *B. abortus* infection induces a drastic reduction of MHC-II expression.

A. THP-1 cells were infected with *B. abortus* at different moi in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. MHC-II expression was assessed by flow cytometry. Histograms correspond to one representative of three independent experiments. Non-specific binding was determined using a control isotype Ab.

B–D. Confocal micrographs of THP-1 cells treated with IFN-γ or infected with *B. abortus* without GFP (moi 100:1) in the presence of IFN-γ. MHC-II was detected using L243 mAb as a primary Ab and Alexa 546-labelled secondary Ab (red). Subcellular localization markers were detected using mAbs specific for EEA1 (early endosomes), LAMP-2 (late endosomes/lysosomes), GM130 (Golgi) and calnexin (ER) followed by Alexa 488-labelled secondary Ab (green) (C and D). DIC, differential interference contrast. Results are representative of three independent experiments.

E. Quantification of MHC-II expression. Data are expressed as percentage of cells with MHC-II expressed on the surface ± SEM of two independent experiments. $^{**}P < 0.01$ versus IFN-γ.