Epub ahead of print October 20, 2016 - doi:10.1189/jlb.4A0416-196R

JLB

Article

Brucella abortus down-regulates MHC class II by the IL-6-dependent inhibition of CIITA through the downmodulation of IFN regulatory factor-1 (IRF-1)

Lis N. Velásquez, M. Ayelén Milillo,* M. Victoria Delpino,† Aldana Trotta,* Pablo Fernández,† Roberto G. Pozner,* Roland Lang,‡ Luciana Balboa,* Guillermo H. Giambartolomei,† and Paula Barrionuevo*,1*

*Instituto de Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas-Academia Nacional de Medicina, Buenos Aires, Argentina; †Instituto de Inmunología, Genética y Metabolismo, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina; and ‡Institut für Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen, Freidrich Alexander Universität Erlangen-Nürnberg, Germany

RECEIVED APRIL 21, 2016; REVISED SEPTEMBER 1, 2016; ACCEPTED SEPTEMBER 27, 2016. DOI: 10.1189/jlb.4A0416-196R

ABSTRACT

Brucella abortus **is an intracellular pathogen capable of surviving inside of macrophages. The success of *B. abortus* as a chronic pathogen relies on its ability to orchestrate different strategies to evade the adaptive CD4⁺ T cell responses that it elicits. Previously, we demonstrated that *B. abortus* inhibits the IFN-γ-induced surface expression of MHC class II (MHC-II) molecules on human monocytes, and this phenomenon correlated with a reduction in antigen presentation. However, the molecular mechanisms, whereby *B. abortus* is able to down-regulate the expression of MHC-II, remained to be elucidated. In this study, we demonstrated that *B. abortus* infection inhibits the IFN-γ-induced transcription of MHC-II, transactivator (CIITA) and MHC-II genes. Accordingly, we observed that the synthesis of MHC-II proteins was also diminished. *B. abortus* was not only able to reduce the expression of mature MHC-II, but it also inhibited the expression of invariant chain (Ii)-associated immature MHC-II molecules. Outer membrane protein 19 (Omp19), a prototypical *B. abortus* lipoprotein, diminished the expression of MHC-II and CIITA transcripts to the same extent as *B. abortus* infection. IL-6 contributes to these down-regulatory phenomena. In addition, *B. abortus* and its lipoproteins, through IL-6 secretion, induced the transcription of the negative regulators of IFN-γ signaling, suppressor of cytokine signaling (SOCS)-1 and -3, without interfering with STAT1 activation. Yet, *B. abortus* lipoproteins via IL-6 inhibit the expression of IFN regulatory factor 1 (IRF-1), a critical regulatory transcription factor for CIITA induction. Overall, these results indicate that *B. abortus* inhibits the expression of MHC-II molecules at very early points in their synthesis and in this way, may prevent recognition by T cells establishing a chronic infection. *J. Leukoc. Biol.* 101: 000–000; 2017.**

Introduction

B. abortus is a stealthy bug that lives inside macrophages and establishes a chronic infection [1]. Infection with *B. abortus* activates both innate and adaptive immune responses and generates a proinflammatory environment that favors the differentiation of CD4⁺ T cells toward a Th1 phenotype [2–5]. These Th1 cells secrete IFN-γ, a cytokine that not only increases the microbicidal potential of macrophages but also induces the expression of many important molecules on their surface, such as MHC-I and MHC-II, costimulatory molecules, and FcγRs, among others [6–9]. All of these effects ultimately lead to the killing of macrophage-dwelling bacteria and the resolution of the infection. However, *B. abortus* can persist inside macrophages and establish a chronic infection [10]. Thus, an important question that remains to be answered is what strategies this bacterium uses to evade the robust adaptive CD4⁺ T cell response that it elicits. Previous results from our laboratory demonstrated that *B. abortus* infection inhibits the IFN-γ-induced surface expression of MHC-II molecules and the consequent antigen presentation in human monocytes. This effect is mediated by *B. abortus* lipoproteins and depends on TLR2, and the secretion of I-6 is partially involved [11]. However, the molecular mechanisms that explain the

Abbreviations: 7-AAD = 7-aminoactinomycin D, BCA = bicinchoninic acid, BM = bone marrow, BMM = bone marrow-derived macrophage, Cat S = cathepsin S, CBP = CREB-binding protein, CIITA = MHC class II, transactivator, CLIP = class II-associated invariant chain peptide, GAS = γ activation site, HLA-DR = HLA-antigen D related, HLA-DRA = HLA-antigen D related α, *hprt* = hypoxanthine phosphoribosyltransferase, Ii = invariant chain,

(*continued on next page*)

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Instituto de Medicina Experimental (CONICET-Academia Nacional de Medicina), Pacheco de Melo 3081, C1425AUM, Buenos Aires, Argentina. E-mail: pbarrion2004@yahoo.com.ar

diminished surface expression of MHC-II mediated by *B. abortus* remained unexplored.

Stimulation with IFN-γ results in activation of the JAK/STAT signal transduction pathway [12, 13]. IFN-γ binding to its cell-surface receptor triggers the recruitment of STAT1 and its phosphorylation [12–17]. Tyrosine p-STAT1 homodimerizes and translocates to the nucleus, where it binds to GAS of IFN-γ-inducible genes, including MHC-II, FcγR type I (CD64), CIITA, and IRF-1 genes [16–19].

The regulation of MHC-II gene expression is primarily at the level of transcription, and the CIITA transcription factor is the master regulator required for both constitutive and IFN-γ-inducible MHC-II expression [20–25].

Newly synthesized class II α- and β-chains associate with the Ii, giving rise to immature MHC-II; afterwards, these molecules exit the endoplasmic reticulum, reaching the cell surface, and then recycle to an acidic endosomal/lysosomal compartment referred to as the MIIC [26, 27]. In the MIIC, removal of Ii and peptide loading are believed to be critical for appropriate export of the peptide-loaded class II molecule to the cell surface [28]. The processing of Ii has been shown to involve a coordinated action of different proteases generating Ii intermediates, down to the CLIP [26, 27, 29]. Cat S is one of the proteases responsible for the late steps in Ii processing to CLIP in human APCs. In macrophages, Cat S activity predominates upon exposure to proinflammatory stimuli, such as IFN-γ [30].

According to details mentioned above, several mechanisms may explain the diminished surface expression of MHC-II mediated by *B. abortus* infection. These mechanisms include the following: abnormal maturation and transport of MHC-II molecules to the cell surface, inhibition at the transcriptional/translational level of CIITA and/or MHC-II, and interference with the IFN-γ signaling pathway, among others.

In this study, we characterize the possible mechanisms whereby surface MHC-II expression is inhibited by the infection of *B. abortus*. The results obtained provided evidence indicating that *B. abortus*, through its lipoproteins and via IL-6 secretion, inhibits the IFN-γ-induced transcription of CIITA and MHC-II genes, resulting in reduced expression of mature as well as immature MHC-II molecules.

MATERIALS AND METHODS

Ethics statement

Human monocytes were isolated from healthy blood donors in accordance with the guidelines of the Ethical Committee of the IMEX Institute (Buenos Aires, Argentina). A written consent was obtained from all blood donors.

(*continued from previous page*)

IRF-1 = IFN regulatory factor 1, ISRE = IFN-stimulated response element, L-Omp19 = lipidated *B. abortus* outer membrane protein 19, LysMcre = lysozyme M promoter, MFI = mean fluorescence intensity, MHC-I/II = MHC class I/II, MIIC = MHC class II compartment, MOI = multiplicity of infection, NLR = nucleotide-binding oligomerization domain-like receptor, Omp19 = outer membrane protein 19, p-STAT1 = phosphorylated STAT1, PFA = paraformaldehyde, q = quantitative, SOCS = suppressor of cytokine signaling, SOCS-3$^{fl/fl}$ = suppressor of cytokine signaling 3 flox/flox, U-Omp19 = unlipidated *Brucella abortus* outer membrane protein 19

Bacteria

B. abortus strains 2308 and 2308-GFP [31] were cultured in tryptose-soy agar, supplemented with yeast extract (Merck, Darmstadt, Germany). The number of bacteria on stationary-phase cultures was determined by comparing the OD at 600 nm with a standard curve. All live *Brucella* manipulations were performed in Biosafety Level 3 facilities, located at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (Buenos Aires, Argentina).

Expression and purification of recombinant L-Omp19 and U-Omp19 from *B. abortus*

Lipoproteins were expressed and purified, as previously described [32]. To eliminate LPS contamination, recombinant Omps were adsorbed with Sepharose-polymyxin B (Sigma-Aldrich, St. Louis, MO, USA). Both proteins contained <0.25 endotoxin U/μg protein, as assessed by *Limulus* amebocyte lysate assay (Lonza, Basel, Switzerland). The protein concentration was determined by the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) using BSA as standard. The purified proteins were aliquoted and stored at −70°C until used.

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, 2 mM L-glutamine, 10% heat-inactivated FBS (Thermo Fisher Scientific), 100 U penicillin/ml, and 100 μg streptomycin/ml. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured, as previously described [32]. To induce maturation, cells were cultured in 0.05 μM 1,25-dihydroxyvitamin D3 (EMD Millipore, Billerica, MA, USA) for 72 h. PBMCs were obtained by Ficoll-Hypaque (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) gradient centrifugation from human blood collected from healthy adult individuals. Monocytes were then purified from PBMCs by Percoll gradient (GE Healthcare Bio-Sciences) and resuspended in standard medium. Purity of the isolated $CD14^+$ monocytes was >80%, as determined by flow cytometry. Viability of cells was >95% in all of the experiments, as measured by Trypan blue exclusion test. Mouse BMMs were generated by differentiation of BM progenitors from C57BL/6 mice in L cell conditioned media as a source of M-CSF, as previously described [33]. To delete SOCS-3 conditionally in macrophages, SOCS-3$^{fl/fl}$ mice [34] (provided by Dr. Akihiko Yoshimura, Kyushu University, Fukuoka, Japan) were backcrossed to C57BL/6 for 10 generations and crossed with mice expressing Cre under the control of the LysMcre [35] (provided by Dr. Irmgard Förster, Technical University Munich, Germany). BM from SOCS-3$^{fl/fl}$ mice, positive or negative for LysMcre, was used to differentiate SOCS-3-deficient or control macrophages, respectively.

In vitro infection

THP-1 cells or human monocytes at a concentration of 0.5×10^6/ml were infected in round-bottom polypropylene tubes (Falcon Scientific, Whitley Bay, UK) with different MOIs of *B. abortus* in the presence of 150 U/ml IFN-γ (Pierce Endogen, Rockford, USA) for 2 h in standard medium containing no antibiotics. Then, cells were washed extensively to remove uninternalized bacteria, and infected cells were maintained in culture in the presence of IFN-γ, 100 μg/ml gentamicin, and 50 μg/ml streptomycin for an additional 4, 24, or 48 h, as indicated. At different times postinfection, cells were washed 3 times with PBS before processing. To monitor *Brucella* intracellular survival, 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 tryptose-soy agar plates to enumerate CFUs.

Flow cytometry

THP-1 cells or human monocytes were infected with *B. abortus* at different MOIs. Additionally, THP-1 cells were incubated with L-Omp19, U-Omp19, or recombinant human IL-6 at the indicated concentrations in the presence of 150 U/ml IFN-γ for 48 h. To determine surface expression, cells were then washed and stained with FITC-labeled anti-human HLA-DR (clone L243; BD PharMingen, San Diego, CA, USA) or anti-human CD74 (clone By2; Santa

Cruz Biotechnology, Dallas, TX, USA) mAb or isotype-matched control mAb. Alternatively, cells were fixed with 2% PFA and permeabilized with 0.1% of saponin before labeling to determine total expression. To determine intracellular expression, cells were infected with *B. abortus* for 4 or 24 h, washed, and stained with PE-labeled anti-human p-STAT1 (Tyr 701; clone KIKSI0803; eBioscience, San Diego, CA, USA) mAb, according to the manufacturer's instructions. For viability assays, THP-1 cells were infected with different MOIs of *B. abortus* or treated with L-Omp19, U-Omp19, IL-6, L-Omp19 + anti-IL-6, or L-Omp19 + isotype control in the presence or absence of IFN-γ. THP-1 cells treated with 2% PFA were also included as a positive control. At 48 h, cells were stained with FITC-labeled anti-human HLA-DR, washed, and incubated with 7-AAD (BD Biosciences, San Jose, CA, USA) for 10 min on ice in darkness. MHC-II expression was evaluated gating on viable cells (7-AAD-negative cells). In all cases after labeling, cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences), and data were processed using CellQuest software (BD Biosciences). In the experiments with murine macrophages, SOCS-3-deficient and control BMMs were incubated with L-Omp19 in the presence of 10 ng/ml recombinant murine IFN-γ for 24 or 48 h. To determine MHC-II surface expression, cells were stained with APC-labeled anti-IA/IE (clone M5/114.15.2; eBioscience) and were analyzed on a FACSCanto II using FACSDiva software (both BD Biosciences). Results were expressed as MFI values and represent the arithmetic means ± SEM of the indicated experiments.

Western blot

THP-1 cells at a concentration of 1×10^6 cells/ml were used. Cells were left untreated or treated with IFN-γ, or they were infected with different MOIs of *B. abortus* in the presence of IFN-γ, as described. Following the different experimental treatments, cells were lysed in ice-cold buffer consisting of 150 mM NaCl, 10 mM Tris, 5 mM EDTA, 1% SDS, 1% Triton X-100, 1% sodium deoxycholate, gentamicin/streptomycin, and 0.2% azide, plus a protease inhibitor cocktail (Sigma-Aldrich). Lysates were incubated on ice for 2 h and cleared by centrifugation for 15 min at 14,000 rpm at 4°C. Protein concentrations were determined using the BCA protein assay (Thermo Fisher Scientific). Equal amounts of protein (20 μg) were then resolved on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences) for 1 h at 300 mA and blocked with 5% milk protein–0.1% Tween 20 overnight at 4°C. Membranes were then probed with primary anti-human MHC-II antibody (1:20 dilution; clone L243; purified from murine hybridoma culture supernatants), anti-human Cat S antibody (1:100 dilution; clone B-12; Santa Cruz Biotechnology), anti-human IRF-1 antibody (1:200 dilution; clone 13H3A44; BioLegend, San Diego, CA, USA), or anti-human p-STAT1 (Tyr701) antibody (1:1000 dilution; clone D4A7; Cell Signaling Technology, Danvers, MA, USA) overnight. After washing 3 times with TBS–0.05% Tween 20, blots were incubated for 1 h with an HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody (Santa Cruz Biotechnology). Immunoreactivity was detected using ECL Western blotting substrate (Thermo Fisher Scientific). Protein bands were visualized using Kodak Medical X-Ray General Purpose Film. For internal loading controls, membranes were stripped by incubating for 10 min twice in buffer consisting of 1.5% glycine, 0.1% SDS, 1% Tween 20, pH 2.2, and then reprobed with anti-β-actin antibody (1:2000 dilution; clone AC-15; Sigma-Aldrich). Results from the Western blot were analyzed by densitometric analysis (ImageJ software, U.S. National Institutes of Health, Bethesda, MD, USA).

Confocal microscopy

THP-1 cells were incubated in chambers slides (2×10^5 cells/well) with 10 ng/ml PMA (Sigma-Aldrich) for 24 h to promote adherence. Then, cells were infected with *B. abortus* or GFP-*B. abortus* (MOI 100:1) in the presence of IFN-γ for 48 h, fixed with 2% PFA, permeabilized with 0.1% saponin, and incubated with anti-human MHC-II antibody L243 (purified from murine hybridoma culture supernatants) or anti-human IRF-1 (clone 13H3A44; BioLegend) and Alexa 546-labeled anti-mouse IgG2a (Thermo Fisher Scientific). For nuclear staining, TO-PRO-3 (Thermo Fisher Scientific) was used. Slides were mounted with Poly-Mount (Polysciences, Hirschberg an der Bergstrasse, Germany) and analyzed using an Olympus FV1000 confocal microscope with an oil-immersion Plan Apochromatic 60× NA1.42 objective.

mRNA preparation and qPCR

THP-1 cells or human monocytes, at a concentration of 1×10^6 cells/ml, were infected with *B. abortus* (MOI 100:1), as described above. Additionally, THP-1 cells were incubated with L-Omp19, U-Omp19, or recombinant human IL-6 at the indicated concentrations in the presence of 150 U/ml IFN-γ for 48 h. Total RNA was extracted using the Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), and 1 μg RNA was subjected to reverse transcription using Improm-II Reverse Transcriptase (Promega, Madison, WI, USA). PCR analysis was performed with a Stratagene Mx3000P real-time PCR detection system using SYBR Green as a fluorescent DNA-binding dye. The primer sets used for amplification included the following: GAPDH sense 5′-cgaccactttgtcaagctca-3′, GAPDH antisense 5′-ttactccttggaggccatgt-3′; HLA-DRA sense 5′-agacaagttcaccccaccag-3′, HLA-DRA antisense 5′-agcat-caaactcccagtgct-3′; CIITA sense 5′-ccgacacagacaccatcaac-3′, CIITA antisense 5′-ttttctgcccaacttctgct-3′; Cat S sense 5′-ttatggcagagaagatgtcc-3′, Cat S antisense 5′-aagagggaaagctagcaatc-3′; SOCS-1 sense 5′-cttccgcacattccgttcg-3′, SOCS-1 antisense 5′-ggctgccatccaggtgaaag-3′; SOCS-3 sense 5′-ctact-gaaccctcctccg-3′, SOCS-3 antisense 5′-tctcataggagtccagg-3′. The amplification cycle for GAPDH, HLA-DRA, CIITA, and Cat S primers was 95°C for 30 s, 55°C for 60 s, and 72°C for 60 s. For SOCS-1, primers were 95°C for 60 s, 68°C for 40 s, and 72°C for 40 s. For SOCS-3, primers were 95°C for 60 s, 58°C for 60 s, and 72° for 45 s. All primer sets yielded a single product of the correct size. Relative expression levels were normalized against GAPDH. In the experiments with murine macrophages, BMMs were incubated with L-Omp19 in the presence of 10 ng/ml recombinant murine IFN-γ for 24 h. Total RNA was prepared using TriFast (Peqlab, Erlangen, Germany), and cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). For qRT-PCR, primers and probes for *Hprt*, *Socs3*, *Iab1*, and *CIIta* were selected from the Universal Probe library (Roche Diagnostics, Indianapolis, IN, USA) and are available upon request. *Hprt* was used as housekeeping control, and fold changes were calculated by the change in comparative threshold method using the unstimulated condition as calibrator.

Neutralization of IL-6

THP-1 cells, at a concentration of 0.5×10^6 cells/ml, were treated with L-Omp19 (100 ng/ml) plus IFN-γ in the presence of a neutralizing mAb against human IL-6 (clone MQ2-13A5; eBioscience) or its respective isotype control at a concentration of 40 μg/ml for 48 h. Then, total RNA was isolated as described previously.

Statistical analysis

Results were analyzed with 1-way ANOVA, followed by post hoc Tukey test, using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

B. abortus infection downmodulates the IFN-γ-induced surface and intracellular expression of mature MHC-II molecules

We have previously demonstrated that *B. abortus* infection inhibits the IFN-γ-induced surface expression of mature MHC-II on human monocytic cells [11]. With the aim of corroborating this phenomenon and to investigate whether it extends not only to the cell surface, we infected the THP-1 monocytic cell line with different MOIs of *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 time of 48 h. After this, the total and surface-exposed expression of MHC-II molecules was evaluated by flow cytometry with the mAb L243, which recognizes only mature MHC-II molecules. Staining of fixed and nonpermeabilized cells was used to determine membrane-associated mature MHC-II, whereas staining of fixed and saponin-permeabilized cells was used to evaluate the total amount of mature MHC-II. *B. abortus* infection inhibited the IFN-γ-induced surface expression of mature MHC-II molecules in a dose-dependent manner (**Fig. 1A** and **B**), as well as its total expression (Fig. 1C and D). However, *B. abortus* infection by itself (without IFN-γ treatment) was unable to inhibit the basal expression of mature MHC-II molecules (Supplemental Fig. 1E). Inhibition of IFN-γ-induced MHC-II expression was not a result of a loss of cell viability in infected cultures. By means of the dead-stain 7-AAD, we distinguished live from dead cells by flow cytometry (Supplemental Fig. 1A) and confirmed that the percentage of viable cells did not change among the different experimental groups at the time of analysis (Supplemental Fig. 1B and D). Moreover, we observed no differences in our results when excluding dead cells, by gating only on viable cells (7-AAD-negative cells; Supplemental Fig. 1C). *B. abortus* infection in THP-1 cells and human primary monocytes shows similar kinetics (Supplemental Fig. 2). Nevertheless, we successfully corroborated our results in human monocytes purified from peripheral blood (Fig. 1E and F). We also confirmed our observations about MHC-II down-regulation by confocal microscopy. For this, THP-1 cells were infected with *B. abortus* in the presence of IFN-γ, and mature MHC-II expression was evaluated with an Alexa 546-labeled (red) anti-human HLA-DR (clone L243) antibody. After 48 h of culture, cells treated only with IFN-γ showed mature MHC-II expression, confined predominantly to the cellular membrane. However, *B. abortus*-infected monocytes showed a drastic reduction of mature MHC-II expression (**Fig. 2A** and **B**). A detailed observation using cells infected with GFP-*B. abortus* indicated that infection causes the loss of mature MHC-II expression on the surface, as well as on the inside of the cells (Fig. 2C). Expression of mature MHC-II molecules was evaluated by Western blot in whole-cell lysates as well. IFN-γ increased total mature MHC-II protein expression, and this increase was significantly inhibited by *B. abortus* infection in a dose-dependent manner (Fig. 2D and E). Overall, these results demonstrate that *B. abortus* infection down-modulates the IFN-γ-induced surface and intracellular expression of mature MHC-II molecules.

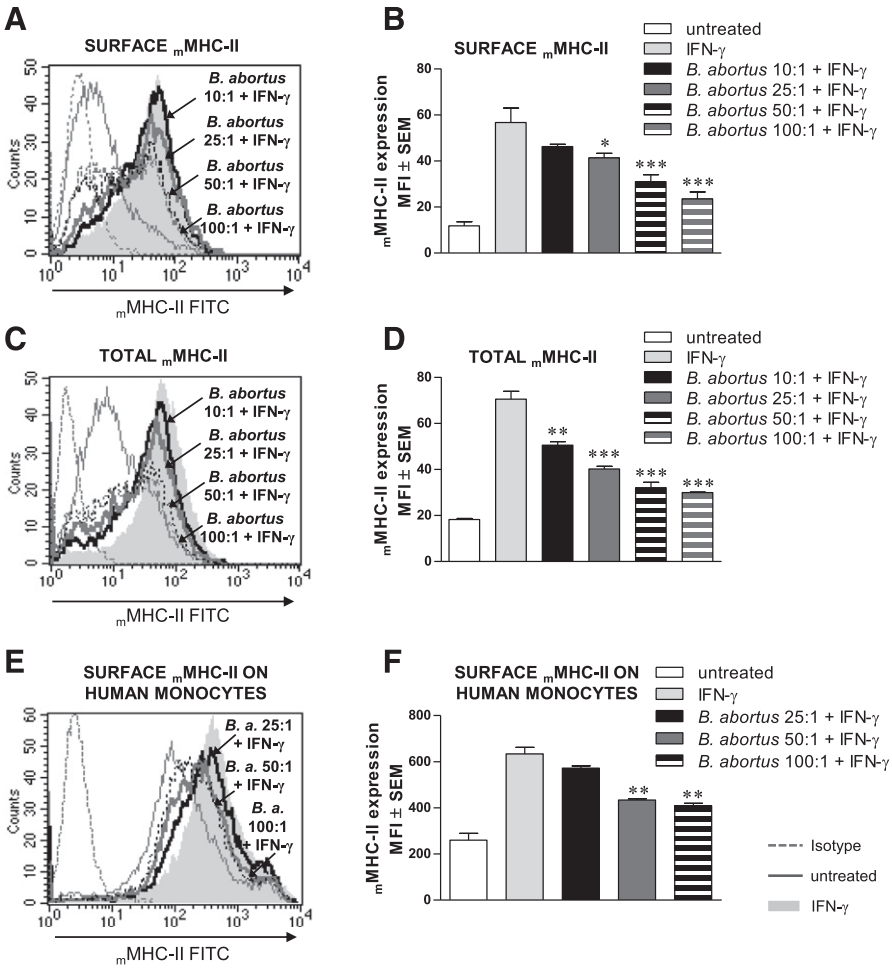


Figure 1. *B. abortus* infection down-modulates the IFN-γ-induced expression of mature MHC-II ($_m$MHC-II) molecules on THP-1 cells and human monocytes. (A–D) THP-1 cells were infected with *B. abortus* at different MOIs in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. $_m$MHC-II expression was assessed by flow cytometry. $_m$MHC-II surface expression was determined on nonpermeabilized cells (A and B), and $_m$MHC-II total expression was determined in saponin-permeabilized cells (C and D). (E and F) Peripheral blood-isolated human monocytes were infected with *B. abortus* (*B. a.*), as before for 24 h. $_m$MHC-II surface expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of 5 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. IFN-γ.

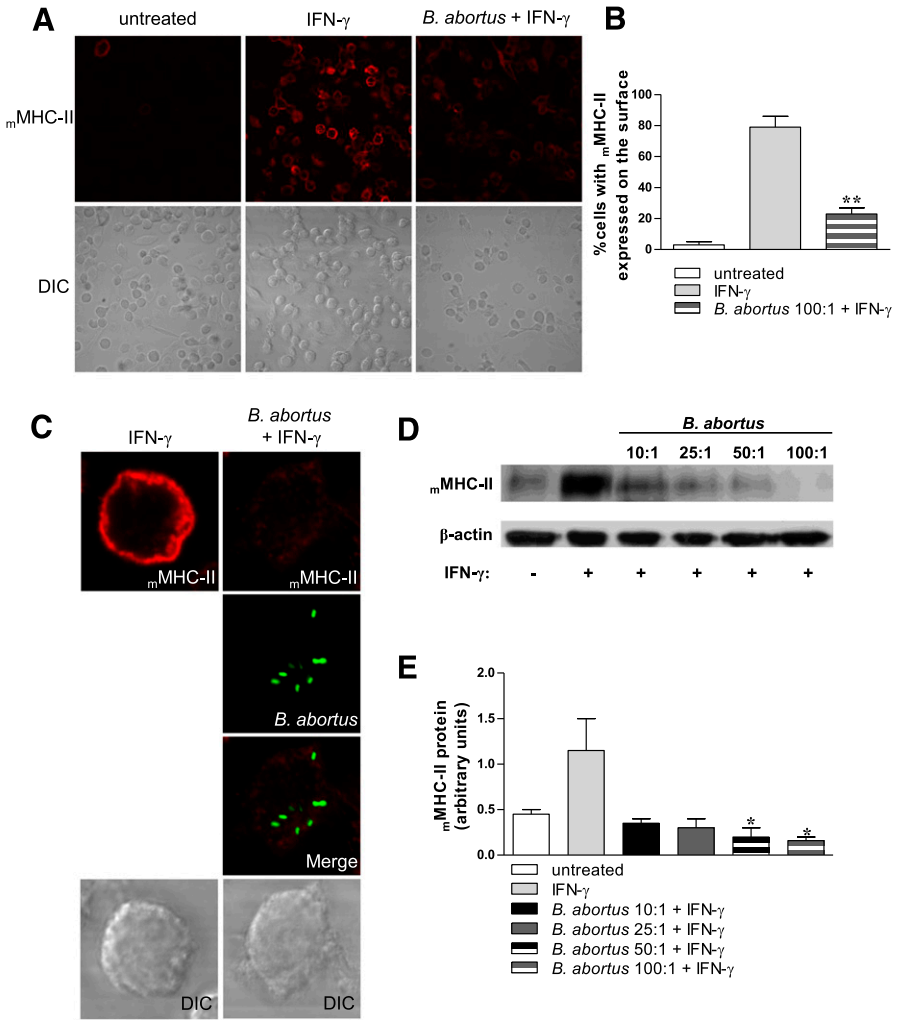


Figure 2. Infection with *B. abortus* results in diminished protein levels of mature MHC-II ($_m$MHC-II). (A–C) Confocal micrographs of THP-1 cells infected with *B. abortus* (A) or GFP-*B. abortus* (C; MOI 100:1) in the presence of IFN-γ and then stained with a primary anti-human MHC-II antibody (L243) and Alexa 546-labeled secondary antibody (red). DIC, Differential interference contrast. (B) Quantification of $_m$MHC-II surface expression. Data are expressed as percentage of cells with $_m$MHC-II expressed on the cell surface ± SEM of 2 independent experiments. $^{**}P < 0.01$ vs. IFN-γ. (D and E) THP-1 cells were infected with *B. abortus* at different MOIs in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. (D) Total cell extracts were obtained, and $_m$MHC-II expression was determined by Western blot. β-Actin expression was also determined as a control. (E) Densitometric analysis of $_m$MHC-II protein from 3 independent experiments performed as described above. Bars indicate the arithmetic means ± SEM of 3 independent experiments. $^{*}P < 0.05$ vs. IFN-γ.

The inhibition of mature MHC-II molecule expression mediated by *B. abortus* is not a result of an accumulation of immature MHC-II molecules

The diminished surface expression of mature MHC-II mediated by *B. abortus* could be the result of an abnormal maturation and transport to the cell membrane [36, 37]. Therefore, we hypothesized that this phenomenon could lead to an accumulation of immature molecules. In turn, accumulation of immature MHC-II molecules could be a result of defective maturational processing by inhibition of Cat S, the principal protease involved in the late steps of Ii cleavage [38]. To address this, we evaluated the expression of Ii-associated MHC-II molecules (immature MHC-II) in THP-1 cells infected with *B. abortus*. Cells were treated, as mentioned above, and the total and surface-exposed expression of immature MHC-II molecules was evaluated by flow cytometry using an antibody anti-Ii (CD74) that recognizes Ii. *B. abortus* infection inhibited the IFN-γ-induced surface expression of immature MHC-II (**Fig. 3A** and **B**). Moreover, the infection with *B. abortus* was capable of reducing the total expression of immature MHC-II (Fig. 3C and D). In addition, with the use of an antibody that recognizes both mature and immature MHC-II molecules (clone Tu36), we confirmed that infection with *B. abortus* is able to inhibit the surface (**Fig. 4A** and **B**) and total expression (Fig. 4C and D) of both MHC-II subpopulations. Corroborating our results, the expression of Cat S was not modified in cells infected with *B. abortus* with respect to cells treated with IFN-γ alone, as determined by Western blot (Fig. 4E and F) and real-time PCR (Fig. 4G). Overall, these results indicate that the attenuated surface expression of MHC-II molecules is not a result of inhibition of Cat S expression in infected cells and the subsequent intracellular accumulation of immature MHC-II molecules. On the contrary, *B. abortus* was not only able to reduce the expression of mature MHC-II molecules, but it also inhibited the expression of Ii-associated immature MHC-II molecules.

The decrease in total MHC-II protein levels induced by *B. abortus* infection correlates with diminished HLA-DR and CIITA transcription

The inhibition of mature and immature MHC-II molecule expression mediated by *B. abortus* could be a result of augmented shedding, increased degradation, and/or reduced synthesis of MHC-II molecules. To elucidate this, we first assessed whether MHC-II expression was affected at the transcriptional level. For

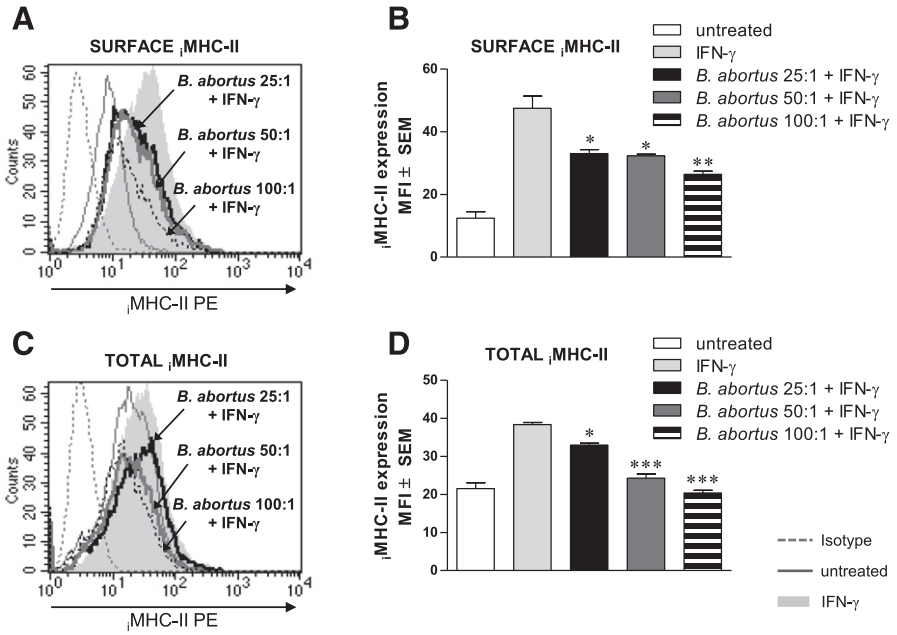


Figure 3. The inhibition of mature MHC-II molecule expression mediated by *B. abortus* is not a result of an accumulation of immature MHC-II (¡MHC-II) molecules. (A–D) THP-1 cells were infected with *B. abortus* at different MOIs in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. ¡MHC-II expression was assessed by flow cytometry. ¡MHC-II surface expression was determined on nonpermeabilized cells (A and B), and ¡MHC-II total expression was determined in saponin-permeabilized cells (C and D). Bars indicate the arithmetic means ± SEM of 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. IFN-γ.

this, HLA-DR mRNA level was measured by real-time qPCR under the same conditions in which MHC-II expression was assessed by flow cytometry. As expected, IFN-γ induced up-regulation of HLA-DR mRNA. *B. abortus* infection inhibited the induction of HLA-DR mRNA by IFN-γ after 48 h (**Fig. 5A**). By taking into account that CIITA is the master regulator of MHC-II expression, we evaluated whether decreased HLA-DR mRNA synthesis mediated by *B. abortus* infection, in turn, correlates with reduced transcription of CIITA. As shown in Fig. 5B, IFN-γ induced up-regulation of CIITA mRNA, whereas *B. abortus* infection was capable of inhibiting the IFN-γ-induced CIITA mRNA after 48 h. Once again, these results were corroborated in human monocytes purified from peripheral blood. *B. abortus* infection significantly inhibited the expression of both MHC-II and CIITA mRNA (Fig. 5C and D). Overall, these results indicate that *B. abortus* infection inhibits the IFN-γ-regulated CIITA transcriptional factor mRNA synthesis and thus, decreases HLA-DR mRNA. This may represent a major mechanism for decreased MHC-II surface expression in human monocytes infected with *B. abortus*.

B. abortus lipoproteins are the bacterial component involved in the MHC-II down-regulatory mechanisms

We have previously demonstrated that *B. abortus* lipoprotein Omp19 inhibited both MHC-II surface expression and antigen processing in human monocytes [11]. Moreover, tripalmitoyl-*S*-glyceryl cysteine (a synthetic lipohexapeptide that mimics the structure of the lipoprotein lipid moiety) was able to reproduce this phenomena [11], indicating that it could be brought about by any *B. abortus* lipoprotein. Thus, we hypothesized that *Brucella* lipoproteins could be the structural components involved in MHC-II down-regulatory mechanisms observed in *B. abortus* infection. To test this hypothesis, we used recombinant L-Omp19 as a *Brucella* lipoprotein model. THP-1 cells were incubated with IFN-γ in the presence of L-Omp19 for 48 h, and then the IFN-γ-induced MHC-II surface expression was evaluated by flow cytometry and HLA-DR and CIITA mRNA, by real-time qPCR. With the recapitulation of the phenomena observed upon infection, L-Omp19 down-modulated the IFN-γ-induced surface expression, but not the basal expression of MHC-II, in a dose-dependent fashion (**Fig. 6A** and **C** and Supplemental Fig. 3). L-Omp19 also significantly inhibited the HLA-DR and CIITA mRNA (Fig. 6D and E). The MHC-II down-regulatory mechanisms observed with L-Omp19 were dependent on the lipidation of the lipoprotein, as U-Omp19 failed to generate such inhibitory mechanisms on MHC-II expression (Fig. 6B–E). Overall, these results indicate that *B. abortus* lipoproteins are the component implicated in the regulatory mechanisms that explain the decreased expression of MHC-II molecules on the cell surface.

IL-6 participates in MHC-II down-regulatory mechanisms

We have previously demonstrated that the inhibition of IFN-γ-induced expression of MHC-II by *B. abortus* infection and their lipoproteins is mediated, at least in part, by IL-6 [11]. In addition, we have ruled out the involvement of IL-10 in this phenomenon [11]. Therefore, we next investigated the contribution of IL-6 in the diminished HLA-DR and CIITA transcription. For this, THP-1 cells were incubated with IFN-γ in the presence of IL-6 for 48 h, and then, the IFN-γ-induced MHC-II surface expression and HLA-DR and CIITA mRNA were determined the same way as above. IL-6 down-modulated the IFN-γ-induced surface expression but not the basal expression of MHC-II (**Fig. 7A** and **B** and Supplemental Fig. 4) and significantly inhibited the IFN-γ-induced HLA-DR and CIITA mRNA (Fig. 7C and D). Furthermore, THP-1 cells were cultured with L-Omp19 plus IFN-γ for 48 h in the presence or absence of neutralizing antibodies to IL-6 or the respective isotype control. The level of HLA-DR and CIITA mRNA was then evaluated by real-time qPCR. Neutralization of IL-6

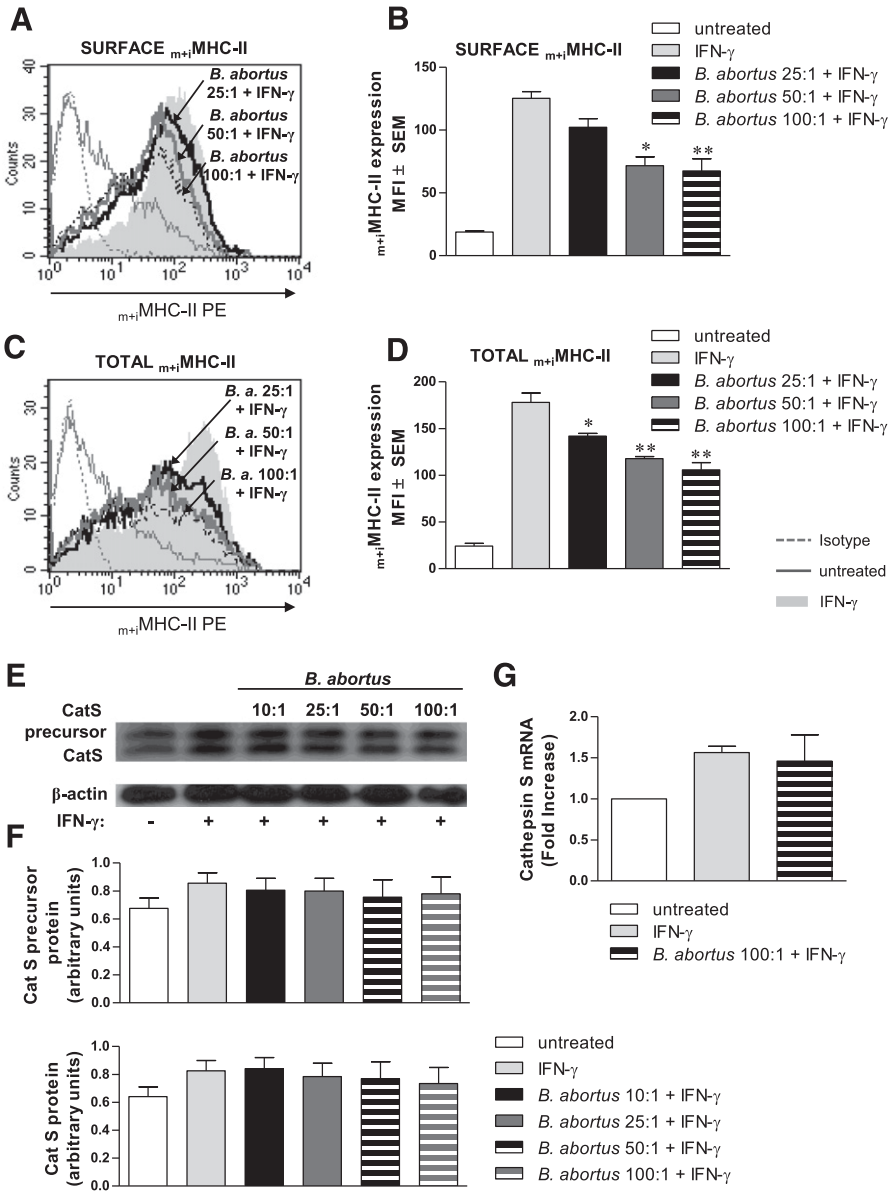


Figure 4. The inhibition of MHC-II surface expression mediated by *B. abortus* is not a result of defective maturational processing by inhibition of Cat S. (A–G) THP-1 cells were infected with *B. abortus* at different MOIs in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. Mature and immature MHC-II ($_{m+i}$MHC-II) expression was assessed by flow cytometry. $_{m+i}$MHC-II surface expression was determined on nonpermeabilized cells (A and B), and $_{m+i}$MHC-II total expression was determined in saponin-permeabilized cells (C and D). (E) Total-cell extracts were obtained, and Cat S or its precursor expression was determined by Western blot. β-Actin expression was also determined as control. (F) Densitometric analysis of Cat S protein from 3 independent experiments performed as described above. (G) Total RNA was isolated and analyzed for Cat S mRNA expression by real-time qPCR. Data are represented as fold increase in expression relative to GAPDH. Bars indicate the arithmetic means ± SEM of 3 independent experiments. $*P < 0.05$; $**P < 0.01$ vs. IFN-γ.

resulted in significant ($P < 0.001$) recovery of the inhibition of IFN-γ-induced HLA-DR and CIITA mRNA mediated by L-Omp19 (Fig. 7E and F). Therefore, these results indicate that the inhibition of HLA-DR and CIITA mRNA and consequent down-regulation of MHC-II molecules on the cell surface induced by *B. abortus* lipoproteins are mediated by IL-6.

B. abortus lipoproteins induce SOCS-1 and SOCS-3 via IL-6 secretion but do not prevent p-STAT1

After IFN-γ binds to its receptor, JAK-1 and -2 mediate p-STAT1. This leads to STAT1 homodimerization, translocation to the nucleus, DNA binding, and transcriptional activation [39]. In some systems, SOCS proteins can inhibit the JAK-mediated phosphorylation of substrates (e.g., STAT1), thereby inhibiting cytokine signaling [40–42]. Specifically, SOCS-1 and SOCS-3 were reported to be able to interfere with IFN-γ signaling [43, 44], and in particular, they were reported to be responsible for attenuating the IFN-γ-induced CIITA expression in some biologic systems [45, 46]. Furthermore, it was demonstrated that several pathogen-associated molecular patterns (e.g., LPS and CpG DNA) can induce SOCS proteins [47, 48]. Therefore, we evaluated the effect of *B. abortus* infection and L-Omp19 on SOCS-1 and SOCS-3 expression. For this, THP-1 cells were infected with *B. abortus* or treated with L-Omp19 in the presence of IFN-γ for 48 h, and then, the expression of SOCS-1 and SOCS-3 mRNA was evaluated by real-time PCR. *B. abortus* infection and L-Omp19, but not U-Omp19, significantly induced the expression of both SOCS-1 and SOCS-3 mRNA (**Fig. 8A–D**).

By taking into account that IL-6 contributes to the regulatory MHC-II mechanisms and that it has been reported that IL-6

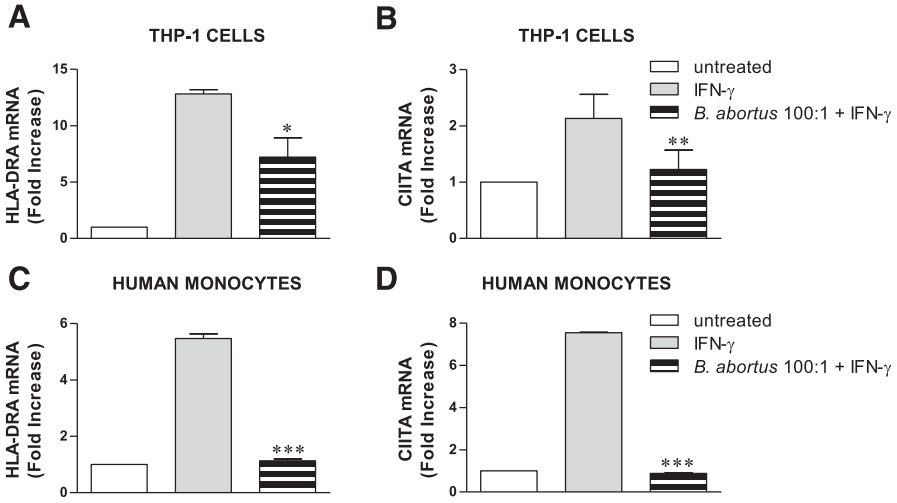


Figure 5. The decrease in total MHC-II protein levels induced by *B. abortus* infection correlates with decreased HLA-DR and CIITA mRNA. (A and B) THP-1 cells were infected with *B. abortus* (MOI 100:1) in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. Total RNA was isolated and analyzed for HLA-DRA (A) and CIITA (B) mRNA expression by real-time qPCR. (C and D) Peripheral blood-isolated human monocytes were infected with *B. abortus*, as before for 24 h. Total RNA was isolated and analyzed for HLA-DRA (C) and CIITA (D) mRNA expression by real-time qPCR. Data are represented as fold increase in expression relative to GAPDH. Bars indicate the arithmetic means ± SEM of 3 independent experiments.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. IFN-γ.

increases the expression of SOCS-1 and SOCS-3 [49, 50], we investigated the role of IL-6 in SOCS-1 and SOCS-3 induction mediated by *B. abortus* lipoproteins. For this, THP-1 cells were cultured with L-Omp19 plus IFN-γ for 48 h in the presence or absence of neutralizing antibodies to IL-6 or the respective isotype control. The level of SOCS-1 and SOCS-3 mRNA was then evaluated by real-time qPCR. Neutralization of IL-6 resulted in significant ($P < 0.05$) reversion of the induction of SOCS-1 and SOCS-3 mRNA mediated by L-Omp19 (Fig. 8E and F).

If inhibition of IFN-γ signaling were mediated by SOCS-1 and/or SOCS-3, then IFN-γ-induced p-STAT1 should be impaired. Therefore, we evaluated whether the SOCS-1 and SOCS-3 induction mediated by *B. abortus* correlated with decreased p-STAT1. For this, THP-1 cells were infected with *B. abortus* in the

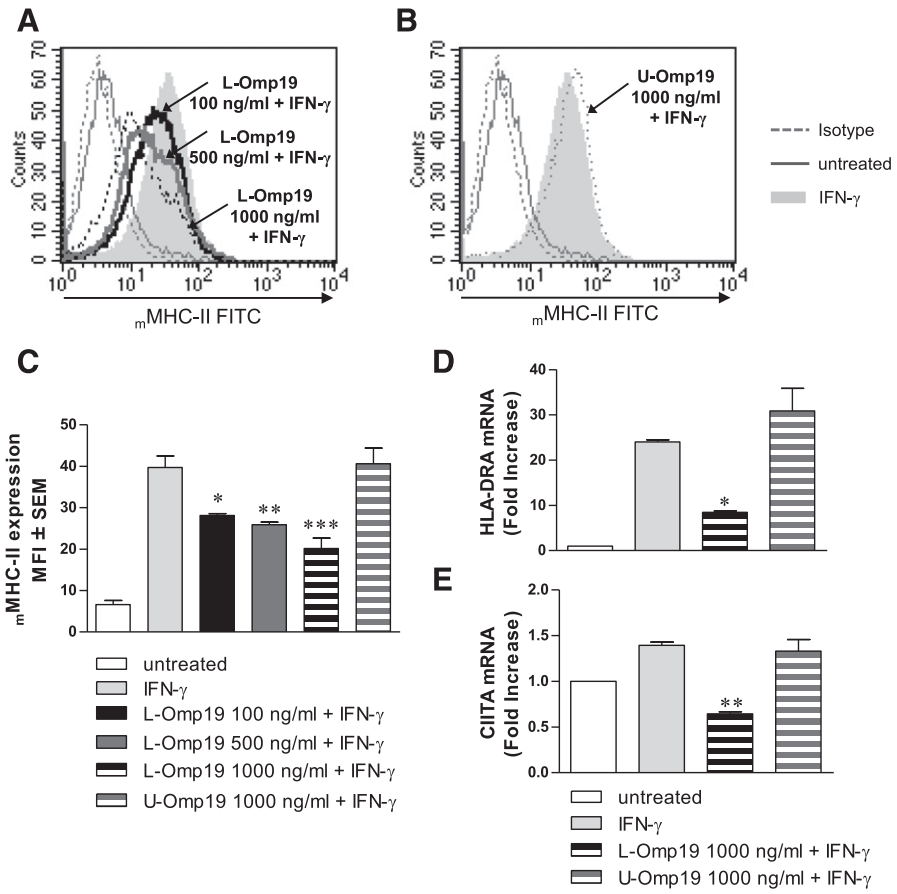


Figure 6. *B. abortus* lipoproteins are the bacterial component involved in the MHC-II down-regulatory mechanisms. (A–C) THP-1 cells were treated with L-Omp19 (A) at different concentrations or U-Omp19 (B) (1000 ng/ml) in the presence of IFN-γ for 48 h. Mature MHC-II ($_m$MHC-II) surface expression was assessed by flow cytometry. (C) Bars indicate the arithmetic means ± SEM of 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. IFN-γ. (D and E) THP-1 cells were treated with L-Omp19 (1000 ng/ml) or U-Omp19 (1000 ng/ml) in the presence of IFN-γ for 48 h. Total RNA was isolated and analyzed for HLA-DRA (D) and CIITA (E) mRNA expression by real-time qPCR. Data are represented as fold increase in expression relative to GAPDH. Bars indicate the arithmetic means ± SEM of 3 independent experiments. *$P < 0.05$; **$P < 0.01$ vs. IFN-γ.

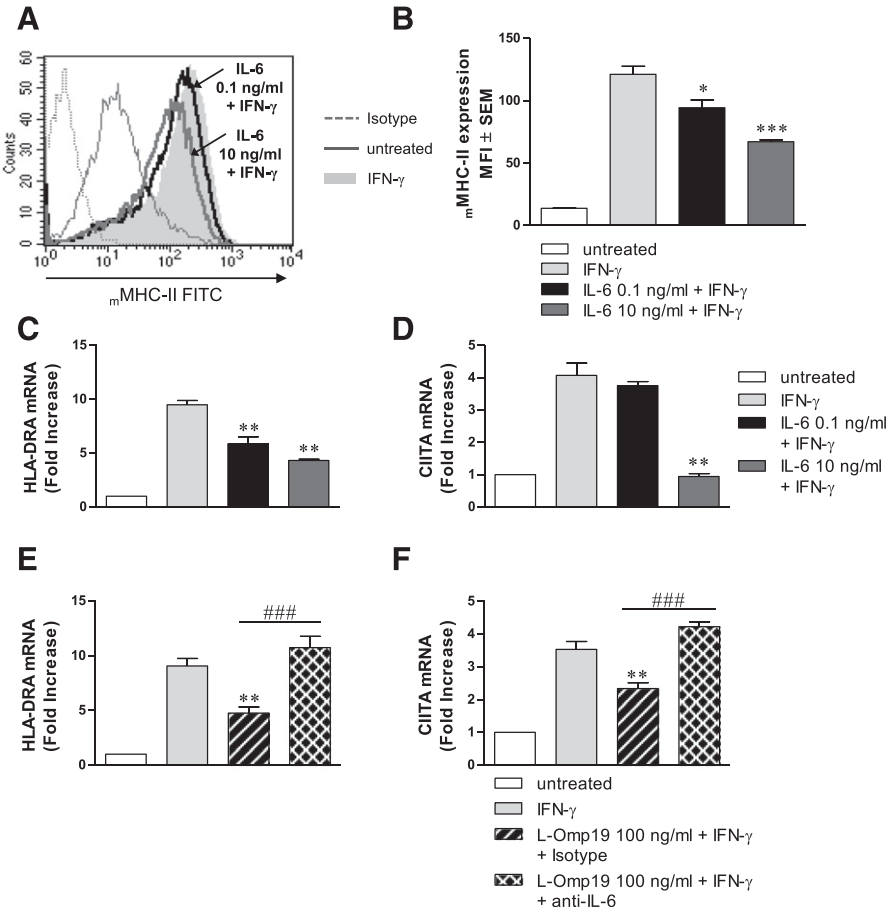


Figure 7. IL-6 participates in MHC-II down-regulatory mechanisms. (A–D) THP-1 cells were treated with IL-6 at different concentrations in the presence of IFN-γ for 48 h. (A and B) Mature MHC-II ($_m$MHC-II) surface expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of 3 independent experiments. $*P < 0.05$; $***P < 0.001$ vs. IFN-γ. (C and D) Total RNA was isolated and analyzed for HLA-DRA (C) and CIITA (D) mRNA expression by real-time qPCR. Data are represented as fold increase in expression relative to GAPDH. Bars indicate the arithmetic means ± SEM of 3 independent experiments. $**P < 0.01$ vs. IFN-γ. (E and F) THP-1 cells were treated with L-Omp19 (100 ng/ml) plus IFN-γ for 48 h in the presence or absence of 40 μg/ml anti-IL-6 or an isotype-matched control. Total RNA was isolated and analyzed for HLA-DRA (E) and CIITA (F) mRNA expression by real-time qPCR. Data are represented as fold increase in expression relative to GAPDH. Bars indicate the arithmetic means ± SEM of 3 independent experiments. $**P < 0.01$ vs. IFN-γ; $###P < 0.001$ vs. isotype.

presence of IFN-γ for 4 and 24 h, and then p-STAT1 was evaluated by flow cytometry (**Fig. 9A–D**) and by Western blot in whole-cell lysates (Fig. 9E and F). IFN-γ induced p-STAT1 at 4 and 24 h, but this induction was not inhibited by *B. abortus* infection at any of the times studied (Fig. 9A–F). In accordance with these results, L-Omp19 was also incapable of inhibiting IFN-γ-induced p-STAT1 (not shown). In summary, macrophages/monocytes infected with *B. abortus* or exposed to L-Omp19 maintain the function of the IFN-γ signaling pathway, including JAK-mediated p-STAT1. Thus, despite increased expression of mRNA for SOCS-1 and -3 via IL-6, SOCS proteins do not appear to block JAK activity or IFN-γ signaling in macrophages.

To examine further the role of SOCS proteins, we tested the ability of L-Omp19 lipoprotein to inhibit MHC-II expression in SOCS-3-deficient macrophages. With the confirmation and extension of our results in human monocytes, experiments in murine BMMs from wild-type mice demonstrated that L-Omp19 significantly down-regulated the IFN-γ-induced surface expression of MHC-II molecules (Supplemental Fig. 5A) and inhibited the expression of MHC-II and CIITA mRNA (Supplemental Fig. 5B and C). L-Omp19 efficiently induced expression of SOCS-3 in murine control macrophages, whereas the levels were, as expected, strongly reduced in SOCS-$3^{fl/fl}$ and LysMcre macrophages, indicating efficient Cre-mediated deletion of *Socs3* (Supplemental Fig. 5D). However, L-Omp19 also inhibited the expression of MHC-II molecules (Supplemental Fig. 5A) and MHC-II and CIITA mRNA (Supplemental Fig. 5B and C) in SOCS-3-deficient BMMs to the same extent as in control macrophages. Although we were unable to perform experiments with SOCS-1-deficient mice, it has been demonstrated already that a *Mycobacterium tuberculosis* lipoprotein mediates inhibition of MHC-II antigen processing in the absence of SOCS-1 [39]. Thus, inhibition of MHC-II expression by *B. abortus* and L-Omp19 is not mediated by a SOCS-mediated blockade of JAK/STAT1 activity.

B. abortus lipoproteins through IL-6 inhibit the expression of IRF-1, a critical factor for CIITA induction

After tyrosine phosphorylation, STAT1 is transported into the nucleus where it binds GAS sequences present in the promoter regions of CIITA and IRF-1 genes, among others [16–19]. In addition to a GAS, CIITA promoter contains an IRF-1-binding site, and both sites must be occupied to obtain maximal activation of this transactivator [45]. Thus, we tested the ability of *B. abortus* to modulate IRF-1 induction by IFN-γ. For this, THP-1 cells were infected with *B. abortus* in the presence of IFN-γ for 4 and 24 h, and then, IRF-1 expression was evaluated by Western blot in whole-cell lysates (**Fig. 10A** and **B**). *B. abortus* infection significantly inhibited the IFN-γ-increased IRF-1 expression at 4 h (Fig. 10A) and 24 h (Fig. 10B). Furthermore, THP-1 cells were cultured with L-Omp19 plus IFN-γ for 24 h in the presence or absence of neutralizing antibodies to IL-6 or the respective

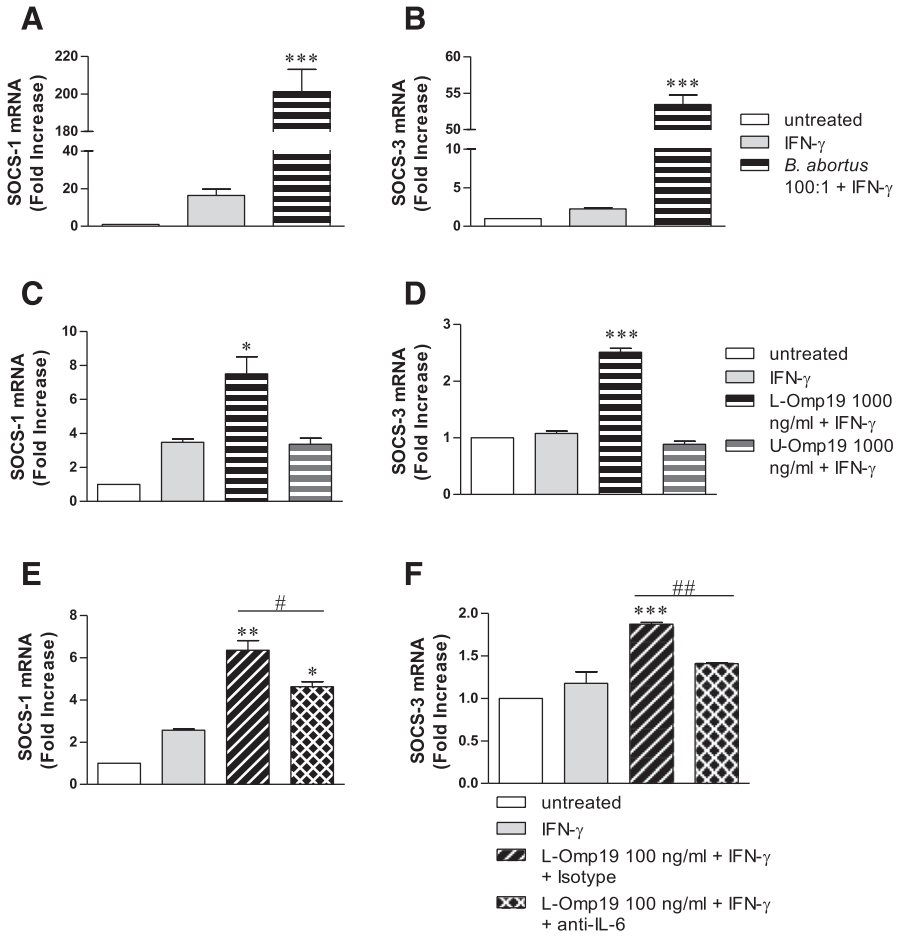


Figure 8. *B. abortus* infection induces the transcription of negative regulators of IFN-γ signaling, SOCS-1 and SOCS-3. (A and B) THP-1 cells were infected with *B. abortus* (MOI 100:1) in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 48 h. (C and D) THP-1 cells were treated with L-Omp19 (1000 ng/ml) or U-Omp19 (1000 ng/ml) in the presence of IFN-γ for 48 h. (E and F) THP-1 cells were treated with L-Omp19 (100 ng/ml) plus IFN-γ for 48 h in the presence or absence of 40 μg/ml anti-IL-6 or an isotype-matched control. Total RNA was isolated and analyzed for SOCS-1 (A, C, and E) and SOCS-3 (B, D, and F) mRNA expression by real-time qPCR. Data are represented as fold increase in expression relative to GAPDH. Bars indicate the arithmetic means ± SEM of 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. IFN-γ; $\#P < 0.05$; $\#\#P < 0.01$ vs. isotype.

isotype control. Then, IRF-1 expression was evaluated by Western blot (Fig. 10C). L-Omp19 significantly inhibited the IFN-γ-increased IRF-1 expression at 24 h, and neutralization of IL-6 resulted in significant ($P < 0.05$) recovery of the inhibition mediated by L-Omp19 (Fig. 10C). Finally, we evaluated whether this reduction of IRF-1 protein level mediated by *B. abortus* correlated with an impaired IRF-1 activation. For this purpose, we assessed IRF-1 translocation to the nucleus by confocal microscopy. THP-1 cells were infected with *B. abortus* in the presence of IFN-γ, and IRF-1 expression was evaluated with an Alexa 546-labeled (red) anti-human IRF-1 antibody. As expected, after 24 h of culture, IFN-γ induced the translocation of IRF-1 to the nucleus (Fig. 10D). However, *B. abortus* infection impaired this translocation (Fig. 10D).

Overall, these results indicate that *B. abortus* infection not only inhibits total IRF-1 but also, IRF-1 translocation to the nucleus. This contributes to the decreased expression of CIITA and consequent down-regulation of MHC-II molecules mediated by *B. abortus* and its lipoproteins.

DISCUSSION

Brucella is an elusive, furtive, and insightful bacterium that uses a variety of strategies to subvert the immune system to persist chronically in the host [10, 51]. One of the most effective strategies used by these bacteria is to hide within infected macrophages, to remain unnoticed against the recognition of T lymphocytes [10, 52]. In this regard, our laboratory has shown that *B. abortus* infection inhibits the IFN-γ-induced surface expression of MHC-II molecules and antigen presentation to $CD4^+$ T cells [11]. However, the molecular mechanisms involved in the inhibition of MHC-II expression on the macrophage surface remained to be elucidated. In this study, we demonstrate that *B. abortus* and its lipoproteins, through the induction of IL-6, downmodulates CIITA transcription, which results in reduced mRNA, protein, and surface expression levels of MHC-II in human monocytes. Although *B. abortus* lipoprotein-mediated IL-6 induction up-regulates SOCS-1 and SOCS-3 proteins, MHC-II down-regulation is independent of SOCS proteins, and it is not a result of the inhibition of p-STAT1. Conversely, *B. abortus* lipoproteins, through IL-6, inhibit the IFN-γ-increased expression and activation of IRF-1, a critical factor for CIITA induction, thus contributing to the down-regulation of CIITA mRNA transcription and resulting in reduced MHC-II surface expression on human monocytes with the consequent reduction in antigen presentation to $CD4^+$ T cells (**Fig. 11**).

The inhibition of MHC-II surface expression and antigen presentation is a strategy used by several pathogens to subvert the immune surveillance and to persist inside of the host [53, 54]. Like many other microorganisms, *B. abortus* infection could be affecting several steps in the synthesis, maturation, and transport

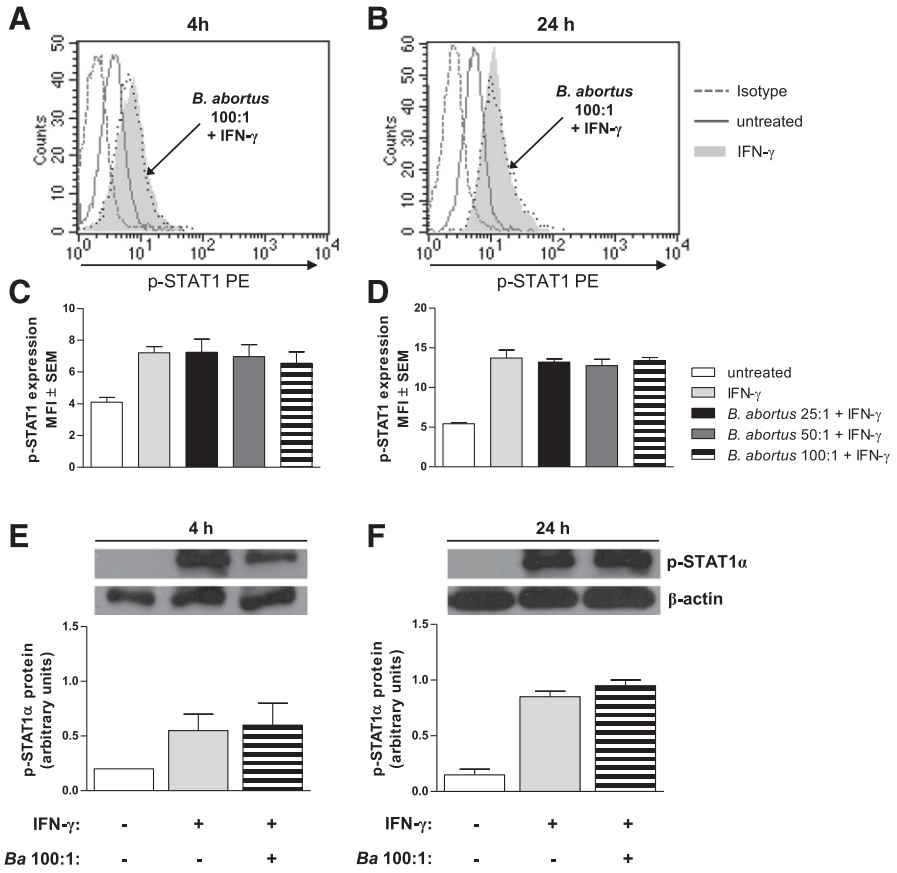


Figure 9. *B. abortus* infection does not prevent p-STAT1. (A–F) THP-1 cells were infected with different MOIs of *B. abortus* in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 4 and 24 h. (A–D) Expression of p-STAT1 was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of 5 independent experiments. (E and F) Total-cell extracts were obtained, and p-STAT1α expression was determined by Western blot. β-Actin expression was also determined as control. *Ba*, *B. abortus*. Bars correspond to the densitometric analysis and indicate the arithmetic means ± SEM of 3 independent experiments.

to the cell membrane of MHC-II molecules [36–38]. However, our results indicate that the attenuated surface expression of mature MHC-II molecules is not a result of a defective maturational processing, as *B. abortus* is not only able to reduce the expression of mature MHC-II molecules, but it also inhibits the expression of Ii-associated immature MHC-II molecules. In fact, here, we demonstrate that the infection generates a defect in the earlier steps of MHC-II synthesis, and thus, the observed decrease in total MHC-II protein levels induced by *B. abortus* correlates with decreased HLA-DR and CIITA mRNA transcription.

CIITA not only acts as a master switch for the constitutive and inducible expression of all MHC-II genes and their accessory genes (which encode the Ii, HLA-DO, and HLA-DM) [20, 55, 56], but also, CIITA was found to have a role in the regulation of MHC-I gene transcription [57–59]. This would suggest that *B. abortus* is also able to down-regulate the transcription of genes for MHC-I. However, we have recently demonstrated that although *B. abortus* also inhibits the IFN-γ-induced MHC-I cell-surface expression in human macrophages, this reduction is not a result of decreased MHC-I mRNA transcription and consequent inhibition of MHC-I protein synthesis (ref. [60] and unpublished results). Conversely, the decrease of MHC-I surface expression is a result of intracellular sequestration of MHC-I molecules already synthesized inside of the Golgi apparatus [60]. This may seem paradoxical if CIITA were the only transcription factor that regulates the transcription of MHC genes. However, NLRC5, which is a member of the NLR protein family, has been described recently as a specific transactivator of MHC-I genes in both humans and mice [61–63]. Thus, NLRC5 and CIITA (2 NLR proteins) are required for the transactivation of MHC-I and MHC-II genes, respectively. Therefore, the existence of the NLRC5 transactivator permits the explanation of why the decrease of CIITA transcription mediated by *B. abortus* does not down-regulate MHC-I mRNA expression.

One issue that merits discussion is how *B. abortus* decreases the expression of CIITA. By taking into account that *B. abortus* infection inhibits the IFN-γ-induced CIITA expression, a possible hypothesis is that *B. abortus* induces this phenomenon through induction of certain proteins that act negatively regulating the IFN-γ signaling pathway. In this respect, SOCS-1 and SOCS-3, 2 members of the SOCS protein family, have been reported to act as negative-feedback regulators of IFN-γ signaling [43, 44, 46, 64]. Particularly, it was reported that SOCS-1 attenuates IFN-γ-induced CIITA and MHC-II expression in macrophages [45]. By taking into account these evidences, we investigated the ability of *B. abortus* to induce both SOCS proteins. Our results indicate that *B. abortus* and its lipoproteins induced the expression of SOCS-1 as well as SOCS-3. Not only that, but we demonstrate that SOCS-1 and SOCS-3 induction is mediated through the secretion of IL-6, a cytokine that is involved in the increased transcription of these proteins [49, 50]. In accordance with these results, it was demonstrated that *M. tuberculosis* up-regulates SOCS-1, SOCS-3, and IL-6, and this is associated with the clinical severity of

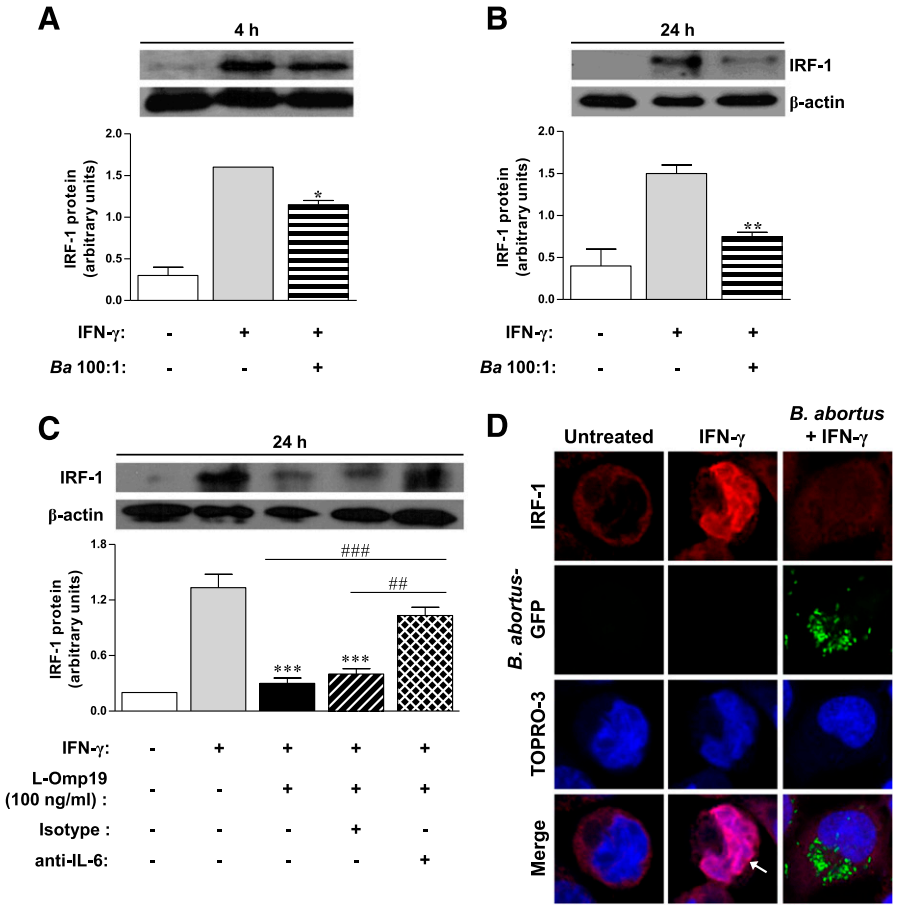


Figure 10. *B. abortus* lipoproteins, through IL-6 infection, inhibit IRF-1 expression. (A and B) THP-1 cells were infected with MOI 100:1 of *B. abortus* in the presence of IFN-γ for 2 h, washed, and cultured in the presence of IFN-γ for 4 and 24 h. (C) THP-1 cells were treated with L-Omp19 (100 ng/ml) plus IFN-γ for 24 h in the presence or absence of 40 μg/ml anti-IL-6 or an isotype-matched control. Total-cell extracts were obtained, and IRF-1 expression was determined by Western blot. β-Actin expression was also determined as control. Bars correspond to the densitometric analysis and indicate the arithmetic means ± SEM of 3 independent experiments. (D) Confocal micrographs of THP-1 cells infected with GFP-*B. abortus* (MOI 100:1) in the presence of IFN-γ for 24 h and then stained with a primary anti-human IRF-1 and Alexa 546-labeled secondary antibody (red). Nucleus was detected using TO-PRO-3 (blue). White arrow shows IRF-1 translocated to the nucleus and colocalizing with TO-PRO-3. Results are representative of 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. IFN-γ; $\#\#P < 0.01$ vs. isotype; $\#\#\#P < 0.001$ vs. L-Omp19 + IFN-γ.

tuberculosis [65]. Moreover, it was reported that SOCS-1 is regulated by TLR2 during *M. tuberculosis* infection [66]. The inhibition of the IFN-γ signaling pathway, by affecting STAT1 activation, may be another possible explanation for the inhibition of the IFN-γ-induced CIITA and MHC-II expression mediated by *B. abortus*. In fact, it has been reported that SOCS-1 and SOCS-3 can inhibit JAK-mediated p-STAT1, thereby preventing IFN-γ signaling [42]. However, our results demonstrate that *B. abortus*,

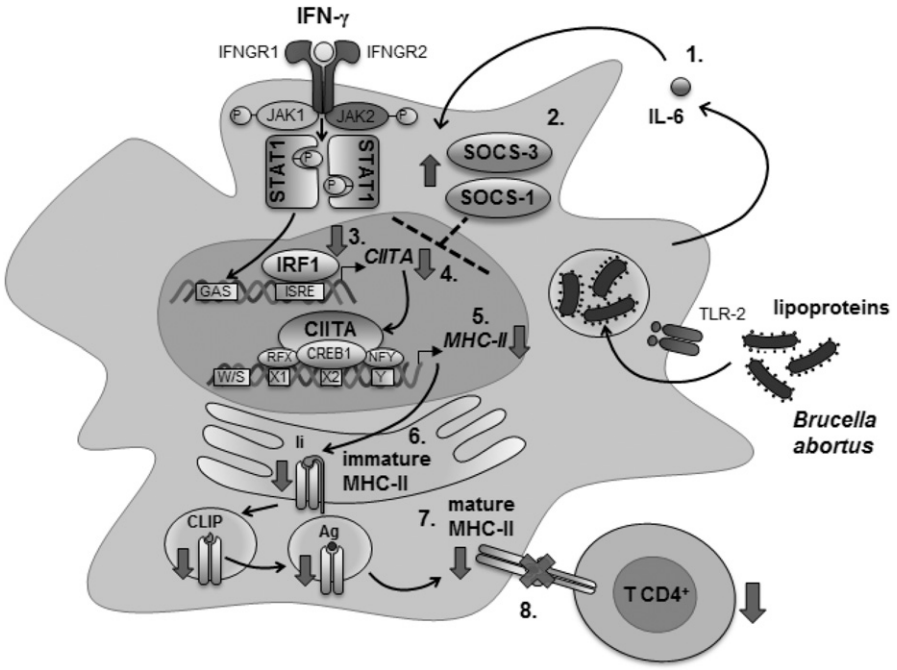


Figure 11. Proposed model for the MHC-II down-regulation mechanism mediated by *B. abortus*. (1) *B. abortus*, through its lipoproteins and via TLR2, induces the secretion of IL-6. (2) IL-6 is involved in the increased transcription of SOCS-1 and SOCS-3, but the up-regulation of these proteins does not interfere with p-STAT1. (3 and 4) *B. abortus* lipoproteins, through IL-6, inhibit the IFN-γ-induced expression of IRF-1, decreasing the transcription of the master regulator of MHC-II, CIITA. (5) The inhibition of CIITA mRNA expression results in a diminished transcription of MHC-II genes. (6 and 7) A diminished MHC-II mRNA causes a decrease in the expression of immature as well as mature MHC-II molecules. (8) This results in a reduction in antigen presentation to $CD4^+$ T cells, which enable the bacteria to hide inside macrophages and evade immune surveillance. IFNGR, IFN-γ receptor, RFX, Regulatory factor X; NFY, nuclear transcription factor Y, W/S, X1, X2 and Y, boxes in MHC-II promoter.

despite up-regulating the expression of SOCS-1 and SOCS-3, does not impair IFN-γ-induced p-STAT1. The lack of inhibition of STAT1 activation goes along with a study in which the mechanisms involved in the disruption of IFN-γ responses mediated by *Brucella suis* infection were investigated [67]. Even though no decrease in p-STAT1, dimerization, or translocation to the nucleus was observed, the authors demonstrated that IFN-γ-mediated association of p-STAT1 with CBP/P300 transactivators was markedly reduced in *B. suis*-infected macrophages, indicating that p-STAT1 was unable to recruit these transactivators normally [67]. In turn, STAT1-CBP/P300 disruption could also explain the inhibition of MHC-II expression, as it could lead to a diminished transcription of CIITA.

The inhibition of the IFN-γ-induced CIITA expression mediated by *B. abortus* could also be explained by the induction of negative regulators or the repression of positive regulators that bind to promoter sequences present in the CIITA gene. In line with this, IFN-γ-activated transcription factor IRF-1 binds to the ISRE present in the CIITA type IV promoter and is crucial for its transcriptional regulation [45]. Our results demonstrate that *B. abortus* significantly inhibits IRF-1 expression and its translocation to the nucleus, and this phenomenon could contribute to the decreased expression of CIITA and consequent down-regulation of MHC-II molecules mediated by *B. abortus*. In agreement with these results, it was reported that the 19 kDa lipoprotein from *M. tuberculosis* signaling via TLR2 inhibits IFN-γ-induced MHC-II expression by preventing the induction of IRF-1 mRNA and the consequent induction of CIITA [39].

It is not clear why expression of SOCS-1 and SOCS-3 was not associated with inhibition of p-STAT1. In line with this, it was recently reported that an important induction of SOCS-1 was accompanied by increased p-STAT1 in macrophages infected with Group A *Streptococcus* [68]. Furthermore, STAT1 was demonstrated to be able to induce SOCS-1 directly, and this phenomenon might be a novel and direct negative-feedback mechanism to block cytokine expression in bacterial infections and evasion of host immunity [68]. Moreover, it was demonstrated that *M. tuberculosis* 19 kDa lipoprotein induces SOCS-1 and SOCS-3 expression but does not inhibit IFN-γ-induced p-STAT1 [39]. In this context, it does not appear odd that the induction of SOCS-1 and SOCS-3 does not prevent p-STAT1. On the other hand, our experiments, using SOCS-3-deficient macrophages, along with a study demonstrating that an *M. tuberculosis* lipoprotein inhibits MHC-II antigen processing in the absence of SOCS-1 [39], indicate that *B. abortus* and L-Omp19 down-regulate MHC-II by a SOCS-independent mechanism. The latter suggests that induction of SOCS-1 and SOCS-3 proteins could be an epiphenomenon in our system and reinforces that IRF-1 inhibition is the relevant mechanism involved in *B. abortus*-mediated MHC-II down-regulation.

Overall, the results obtained in this study, along with our recent evidence indicating that *B. abortus* has the ability to induce intracellular retention of MHC-I molecules downmodulating cytotoxic $CD8^+$ T cell responses [60] and to induce apoptosis of $CD4^+$ T lymphocytes [69], reveal that *B. abortus* uses a broad spectrum of strategies to control host immune responses to its advantage. With either the reduction of antigen presentation to T lymphocytes or the direct attack of these cells, bacteria can use these tactics to hide inside of macrophages and evade immune surveillance.

AUTHORSHIP

L.N.V., G.H.G., and P.B. conceived of and designed the experiments. L.N.V., M.A.M., M.V.D., A.T., and L.B. performed the experiments. M.V.D. performed the infections with viable *B. abortus*. P.F. provided essential materials and technical assistance with the qRT-PCR. R.G.P. provided technical assistance with the confocal microscopy assays. R.L. and G.H.G. supported the work with key suggestions and helped with interpretation of the data. P.B. and G.H.G. interpreted the data and wrote the manuscript. All authors reviewed the manuscript.

ACKNOWLEDGMENTS

This work was supported by Grants PICT 2011-1420, 2011-1200, 2012-2252, 2013-0162, and 2014-1925 from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina), by Grants PIP 1390 and 0373 from CONICET (Argentina), by Grants UBACYT 20020130200030 and 20020120100128, by a grant from the Fundación Alberto J. Roemmers (2012–2014; Argentina), and by the Deutsche Forschungsgemeinschaft (SFB 796, TP B6; to R.L.). L.N.V. and M.A.M. are recipients of a fellowship from CONICET (Argentina). M.V.D., P.F., R.G.P., L.B., G.H.G., and P.B. are members of the Research Career of CONICET. The authors thank Horacio Salomón and the staff at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (Universidad de Buenos Aires) for their assistance with Biosafety Level 3 laboratory use. The authors also thank Dr. M. Eugenia Rodriguez (Centro de Investigación y Desarrollo de Fermentaciones Industriales, Universidad Nacional de La Plata) for the cession of the HLA-DRA and CIITA primers.

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

1. Köhler, S., Michaux-Charachon, S., Porte, F., Ramuz, M., Liautard, J. P. (2003) What is the nature of the replicative niche of a stealthy bug named *Brucella*? *Trends Microbiol.* **11,** 215–219.
2. Dornand, J., Gross, A., Lafont, V., Liautard, J., Oliaro, J., Liautard, J. P. (2002) The innate immune response against *Brucella* in humans. *Vet. Microbiol.* **90,** 383–394.
3. Zhan, Y., Cheers, C. (1995) Differential induction of macrophage-derived cytokines by live and dead intracellular bacteria in vitro. *Infect. Immun.* **63,** 720–723.
4. Zhan, Y., Cheers, C. (1993) Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. *Infect. Immun.* **61,** 4899–4901.
5. Zhan, Y., Cheers, C. (1995) Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. *Infect. Immun.* **63,** 1387–1390.
6. Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., Orme, I. M. (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* **178,** 2243–2247.
7. Flynn, J. L., Chan, J. (2001) Immunology of tuberculosis. *Annu. Rev. Immunol.* **19,** 93–129.
8. Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A., Bloom, B. R. (1993) An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* **178,** 2249–2254.
9. Schneeberger, E. E., DeFerrari, M., Skoskiewicz, M. J., Russell, P. S., Colvin, R. B. (1986) Induction of MHC-determined antigens in the lung by interferon-gamma. *Lab. Invest.* **55,** 138–144.

10. Celli, J. (2006) Surviving inside a macrophage: the many ways of *Brucella*. *Res. Microbiol.* **157,** 93–98.
11. Barrionuevo, P., Cassataro, J., Delpino, M. V., Zwerdling, A., Pasquevich, K. A., García Samartino, C., Wallach, J. C., Fossati, C. A., Giambartolomei, G. H. (2008) *Brucella abortus* inhibits major histocompatibility complex class II expression and antigen processing through interleukin-6 secretion via Toll-like receptor 2. *Infect. Immun.* **76,** 250–262.
12. Darnell, J. E., Jr., Kerr, I. M., Stark, G. R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264,** 1415–1421.
13. Bach, E. A., Aguet, M., Schreiber, R. D. (1997) The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu. Rev. Immunol.* **15,** 563–591.
14. Briscoe, J., Rogers, N. C., Witthuhn, B. A., Watling, D., Harpur, A. G., Wilks, A. F., Stark, G. R., Ihle, J. N., Kerr, I. M. (1996) Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *EMBO J.* **15,** 799–809.
15. Igarashi, K., Garotta, G., Ozmen, L., Ziemiecki, A., Wilks, A. F., Harpur, A. G., Larner, A. C., Finbloom, D. S. (1994) Interferon-gamma induces tyrosine phosphorylation of interferon-gamma receptor and regulated association of protein tyrosine kinases, Jak1 and Jak2, with its receptor. *J. Biol. Chem.* **269,** 14333–14336.
16. Greenlund, A. C., Farrar, M. A., Viviano, B. L., Schreiber, R. D. (1994) Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *EMBO J.* **13,** 1591–1600.
17. Shuai, K., Stark, G. R., Kerr, I. M., Darnell, J. E. Jr. (1993) A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. *Science* **261,** 1744–1746.
18. Muhlethaler-Mottet, A., Otten, L. A., Steimle, V., Mach, B. (1997) Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *EMBO J.* **16,** 2851–2860.
19. Pine, R., Canova, A., Schindler, C. (1994) Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. *EMBO J.* **13,** 158–167.
20. Steimle, V., Otten, L. A., Zufferey, M., Mach, B. (1993) Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). *Cell* **75,** 135–146.
21. Steimle, V., Siegrist, C. A., Mottet, A., Lisowska-Grospierre, B., Mach, B. (1994) Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science* **265,** 106–109.
22. Otten, L. A., Steimle, V., Bontron, S., Mach, B. (1998) Quantitative control of MHC class II expression by the transactivator CIITA. *Eur. J. Immunol.* **28,** 473–478.
23. Fontes, J. D., Kanazawa, S., Nekrep, N., Peterlin, B. M. (1999) The class II transactivator CIITA is a transcriptional integrator. *Microbes Infect.* **1,** 863–869.
24. Collawn, J. F., Benveniste, E. N. (1999) Regulation of MHC class II expression in the central nervous system. *Microbes Infect.* **1,** 893–902.
25. Masternak, K., Muhlethaler-Mottet, A., Villard, J., Zufferey, M., Steimle, V., Reith, W. (2000) CIITA is a transcriptional coactivator that is recruited to MHC class II promoters by multiple synergistic interactions with an enhanceosome complex. *Genes Dev.* **14,** 1156–1166.
26. McCormick, P. J., Martina, J. A., Bonifacino, J. S. (2005) Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. *Proc. Natl. Acad. Sci. USA* **102,** 7910–7915.
27. Dugast, M., Toussaint, H., Dousset, C., Benaroch, P. (2005) AP2 clathrin adaptor complex, but not AP1, controls the access of the major histocompatibility complex (MHC) class II to endosomes. *J. Biol. Chem.* **280,** 19656–19664.
28. Loss, G. E., Jr., Sant, A. J. (1993) Invariant chain retains MHC class II molecules in the endocytic pathway. *J. Immunol.* **150,** 3187–3197.
29. Pieters, J. (1997) MHC class II restricted antigen presentation. *Curr. Opin. Immunol.* **9,** 89–96.
30. Hsing, L. C., Rudensky, A. Y. (2005) The lysosomal cysteine proteases in MHC class II antigen presentation. *Immunol. Rev.* **207,** 229–241.
31. Bonomi, H. R., Marchesini, M. I., Klinke, S., Ugalde, J. E., Zylberman, V., Ugalde, R. A., Comerci, D. J., Goldbaum, F. A. (2010) An atypical riboflavin pathway is essential for *Brucella abortus* virulence. *PLoS One* **5,** e9435.
32. Giambartolomei, G. H., Zwerdling, A., Cassataro, J., Bruno, L., Fossati, C. A., Philipp, M. T. (2004) Lipoproteins, not lipopolysaccharide, are the key mediators of the proinflammatory response elicited by heat-killed *Brucella abortus*. *J. Immunol.* **173,** 4635–4642.
33. Hammer, M., Mages, J., Dietrich, H., Schmitz, F., Striebel, F., Murray, P. J., Wagner, H., Lang, R. (2005) Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. *Eur. J. Immunol.* **35,** 2991–3001.
34. Yasukawa, H., Ohishi, M., Mori, H., Murakami, M., Chinen, T., Aki, D., Hanada, T., Takeda, K., Akira, S., Hoshijima, M., Hirano, T., Chien, K. R., Yoshimura, A. (2003) IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol.* **4,** 551–556.
35. Clausen, B. E., Burkhardt, C., Reith, W., Renkawitz, R., Förster, I. (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* **8,** 265–277.
36. Hmama, Z., Gabathuler, R., Jefferies, W. A., de Jong, G., Reiner, N. E. (1998) Attenuation of HLA-DR expression by mononuclear phagocytes infected with *Mycobacterium tuberculosis* is related to intracellular sequestration of immature class II heterodimers. *J. Immunol.* **161,** 4882–4893.
37. Sendide, K., Deghmane, A. E., Reyrat, J. M., Talal, A., Hmama, Z. (2004) *Mycobacterium bovis* BCG urease attenuates major histocompatibility complex class II trafficking to the macrophage cell surface. *Infect. Immun.* **72,** 4200–4209.
38. Sendide, K., Deghmane, A. E., Pechkovsky, D., Av-Gay, Y., Talal, A., Hmama, Z. (2005) *Mycobacterium bovis* BCG attenuates surface expression of mature class II molecules through IL-10-dependent inhibition of cathepsin S. *J. Immunol.* **175,** 5324–5332.
39. Pai, R. K., Convery, M., Hamilton, T. A., Boom, W. H., Harding, C. V. (2003) Inhibition of IFN-gamma-induced class II transactivator expression by a 19-kDa lipoprotein from *Mycobacterium tuberculosis*: a potential mechanism for immune evasion. *J. Immunol.* **171,** 175–184.
40. Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., Hilton, D. J. (1997) A family of cytokine-inducible inhibitors of signalling. *Nature* **387,** 917–921.
41. Yasukawa, H., Sasaki, A., Yoshimura, A. (2000) Negative regulation of cytokine signaling pathways. *Annu. Rev. Immunol.* **18,** 143–164.
42. Alexander, W. S. (2002) Suppressors of cytokine signalling (SOCS) in the immune system. *Nat. Rev. Immunol.* **2,** 410–416.
43. Alexander, W. S., Starr, R., Fenner, J. E., Scott, C. L., Handman, E., Sprigg, N. S., Corbin, J. E., Cornish, A. L., Darwiche, R., Owczarek, C. M., Kay, T. W., Nicola, N. A., Hertzog, P. J., Metcalf, D., Hilton, D. J. (1999) SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* **98,** 597–608.
44. Marine, J. C., Topham, D. J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A., Ihle, J. N. (1999) SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* **98,** 609–616.
45. O'Keefe, G. M., Nguyen, V. T., Ping Tang, L. L., Benveniste, E. N. (2001) IFN-gamma regulation of class II transactivator promoter IV in macrophages and microglia: involvement of the suppressors of cytokine signaling-1 protein. *J. Immunol.* **166,** 2260–2269.
46. Kim, H., Suh, J. M., Hwang, E. S., Kim, D. W., Chung, H. K., Song, J. H., Hwang, J. H., Park, K. C., Ro, H. K., Jo, E. K., Chang, J. S., Lee, T. H., Lee, M. S., Kohn, L. D., Shong, M. (2003) Thyrotropin-mediated repression of class II trans-activator expression in thyroid cells: involvement of STAT3 and suppressor of cytokine signaling. *J. Immunol.* **171,** 616–627.
47. Dalpke, A. H., Opper, S., Zimmermann, S., Heeg, K. (2001) Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. *J. Immunol.* **166,** 7082–7089.
48. Stoiber, D., Kovarik, P., Cohney, S., Johnston, J. A., Steinlein, P., Decker, T. (1999) Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. *J. Immunol.* **163,** 2640–2647.
49. Kolliputi, N., Waxman, A. B. (2009) IL-6 cytoprotection in hyperoxic acute lung injury occurs via suppressor of cytokine signaling-1-induced apoptosis signal-regulating kinase-1 degradation. *Am. J. Respir. Cell Mol. Biol.* **40,** 314–324.
50. Minogue, A. M., Barrett, J. P., Lynch, M. A. (2012) LPS-induced release of IL-6 from glia modulates production of IL-1β in a JAK2-dependent manner. *J. Neuroinflammation* **9,** 126.
51. Letesson, J. J., Lestrate, P., Delrue, R. M., Danese, I., Bellefontaine, F., Fretin, D., Taminiau, B., Tibor, A., Dricot, A., Deschamps, C., Haine, V., Leonard, S., Laurent, T., Mertens, P., Vandenhaute, J., De Bolle, X. (2002) Fun stories about *Brucella*: the "furtive nasty bug". *Vet. Microbiol.* **90,** 317–328.
52. Gross, A., Terraza, A., Ouahrani-Bettache, S., Liautard, J. P., Dornand, J. (2000) In vitro *Brucella suis* infection prevents the programmed cell death of human monocytic cells. *Infect. Immun.* **68,** 342–351.
53. Baena, A., Porcelli, S. A. (2009) Evasion and subversion of antigen presentation by *Mycobacterium tuberculosis*. *Tissue Antigens* **74,** 189–204.
54. Harding, C. V., Boom, W. H. (2010) Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. *Nat. Rev. Microbiol.* **8,** 296–307.
55. Chang, C. H., Guerder, S., Hong, S. C., van Ewijk, W., Flavell, R. A. (1996) Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. *Immunity* **4,** 167–178.
56. Ting, J. P., Trowsdale, J. (2002) Genetic control of MHC class II expression. *Cell* **109** (Suppl), S21–S33.
57. Gobin, S. J., Peijnenburg, A., Keijsers, V., van den Elsen, P. J. (1997) Site alpha is crucial for two routes of IFN gamma-induced MHC class I

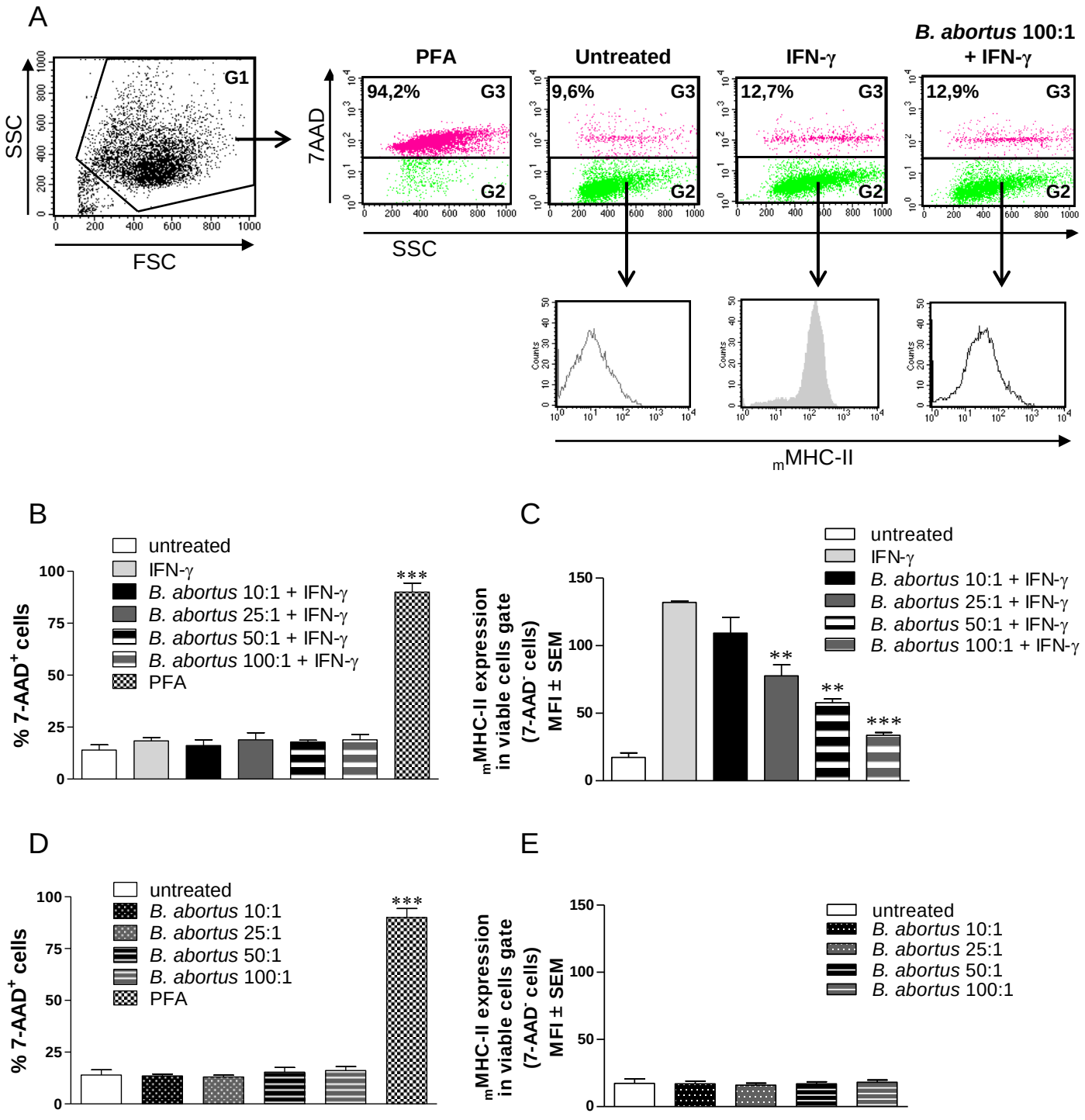
transactivation: the ISRE-mediated route and a novel pathway involving CIITA. *Immunity* **6,** 601–611.
58. Gobin, S. J., Peijnenburg, A., van Eggermond, M., van Zutphen, M., van den Berg, R., van den Elsen, P. J. (1998) The RFX complex is crucial for the constitutive and CIITA-mediated transactivation of MHC class I and beta2-microglobulin genes. *Immunity* **9,** 531–541.
59. Gobin, S. J., van Zutphen, M., Westerheide, S. D., Boss, J. M., van den Elsen, P. J. (2001) The MHC-specific enhanceosome and its role in MHC class I and beta(2)-microglobulin gene transactivation. *J. Immunol.* **167,** 5175–5184.
60. Barrionuevo, P., Delpino, M. V., Pozner, R. G., Velásquez, L. N., Cassataro, J., Giambartolomei, G. H. (2013) *Brucella abortus* induces intracellular retention of MHC-I molecules in human macrophages down-modulating cytotoxic CD8(+) T cell responses. *Cell. Microbiol.* **15,** 487–502.
61. Kobayashi, K. S., van den Elsen, P. J. (2012) NLRC5: a key regulator of MHC class I-dependent immune responses. *Nat. Rev. Immunol.* **12,** 813–820.
62. Meissner, T. B., Li, A., Biswas, A., Lee, K. H., Liu, Y. J., Bayir, E., Iliopoulos, D., van den Elsen, P. J., Kobayashi, K. S. (2010) NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci. USA* **107,** 13794–13799.
63. Yao, Y., Wang, Y., Chen, F., Huang, Y., Zhu, S., Leng, Q., Wang, H., Shi, Y., Qian, Y. (2012) NLRC5 regulates MHC class I antigen presentation in host defense against intracellular pathogens. *Cell Res.* **22,** 836–847.
64. Karlsen, A. E., Rønn, S. G., Lindberg, K., Johannesen, J., Galsgaard, E. D., Pociot, F., Nielsen, J. H., Mandrup-Poulsen, T., Nerup, J., Billestrup, N. (2001) Suppressor of cytokine signaling 3 (SOCS-3) protects beta-cells against interleukin-1beta- and interferon-gamma-mediated toxicity. *Proc. Natl. Acad. Sci. USA* **98,** 12191–12196.
65. Masood, K. I., Rottenberg, M. E., Salahuddin, N., Irfan, M., Rao, N., Carow, B., Islam, M., Hussain, R., Hasan, Z. (2013) Expression of *M. tuberculosis*-induced suppressor of cytokine signaling (SOCS) 1, SOCS3, FoxP3 and secretion of IL-6 associates with differing clinical severity of tuberculosis. *BMC Infect. Dis.* **13,** 13.
66. Srivastava, V., Manchanda, M., Gupta, S., Singla, R., Behera, D., Das, G., Natarajan, K. (2009) Toll-like receptor 2 and DC-SIGNR1 differentially regulate suppressors of cytokine signaling 1 in dendritic cells during *Mycobacterium tuberculosis* infection. *J. Biol. Chem.* **284,** 25532–25541.
67. Bouhet, S., Lafont, V., Billard, E., Gross, A., Dornand, J. (2009) The IFNgamma-induced STAT1-CBP/P300 association, required for a normal response to the cytokine, is disrupted in *Brucella*-infected macrophages. *Microb. Pathog.* **46,** 88–97.
68. Wu, J., Ma, C., Wang, H., Wu, S., Xue, G., Shi, X., Song, Z., Wei, L. (2015) A MyD88-JAK1-STAT1 complex directly induces SOCS-1 expression in macrophages infected with Group A Streptococcus. *Cell. Mol. Immunol.* **12,** 373–383.
69. Velásquez, L. N., Delpino, M. V., Ibañez, A. E., Coria, L. M., Miraglia, M. C., Scian, R., Cassataro, J., Giambartolomei, G. H., Barrionuevo, P. (2012) *Brucella abortus* induces apoptosis of human T lymphocytes. *Microbes Infect.* **14,** 639–650.

KEY WORDS:
bacteria · monocytes/macrophages · host immunity · immune-evasion strategies

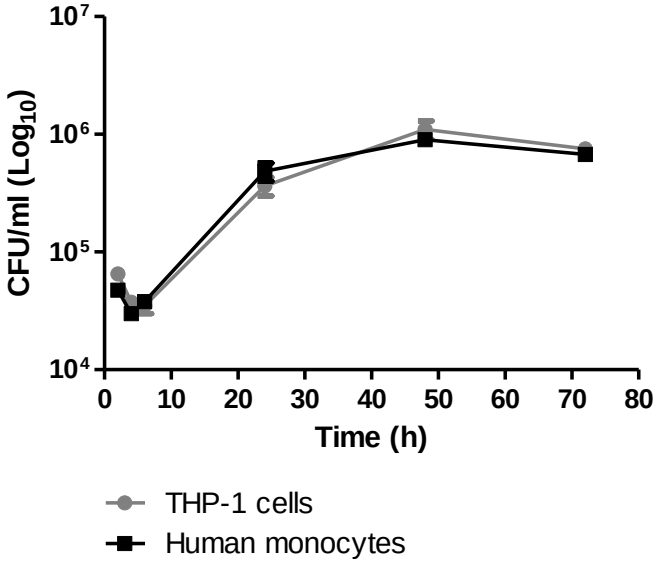
Brucella abortus down-regulates MHC class II by the IL-6-dependent inhibition of CIITA through the downmodulation of IFN regulatory factor-1 (IRF-1)

Lis N. Velásquez, M. Ayelén Milillo, M. Victoria Delpino, et al.

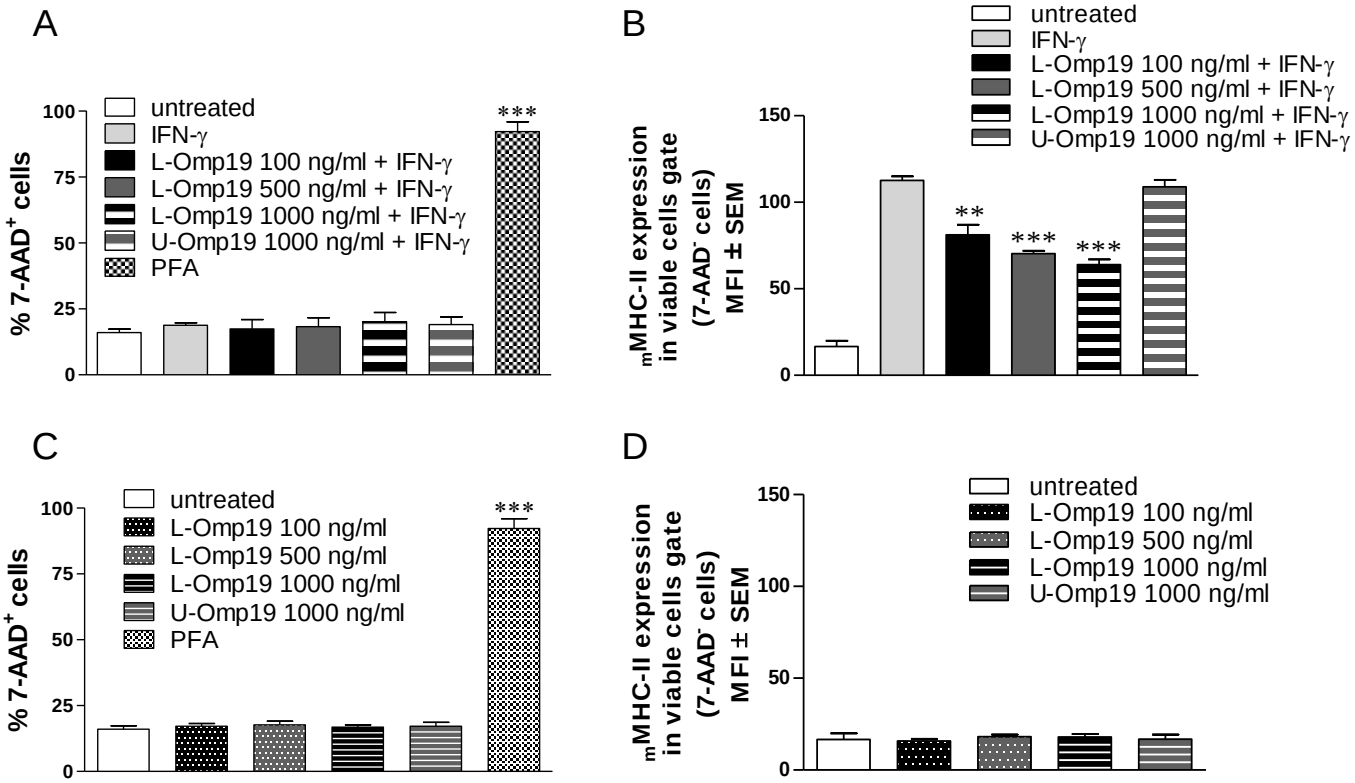
J Leukoc Biol published online October 20, 2016
Access the most recent version at doi:10.1189/jlb.4A0416-196R



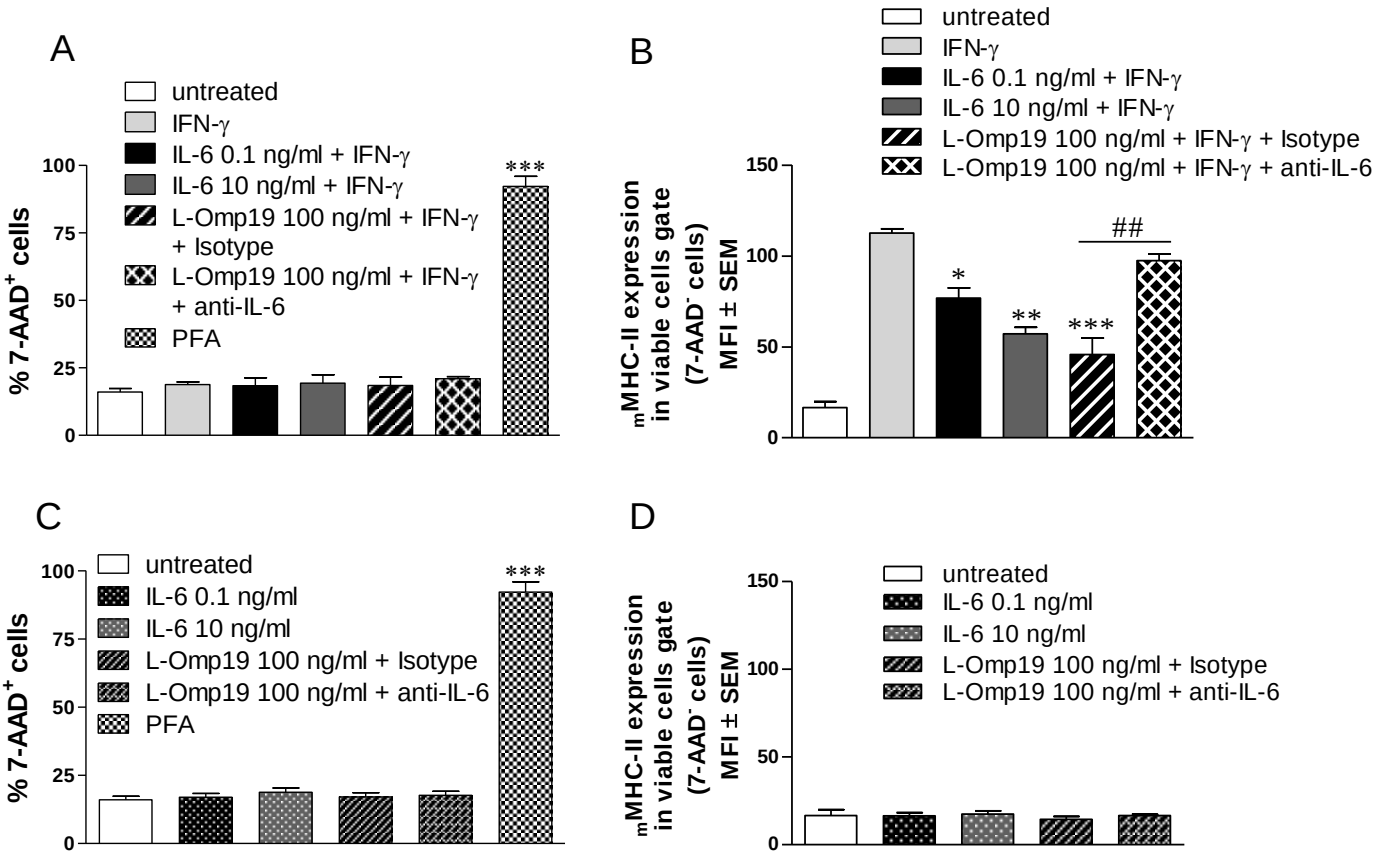
Supplemental Figure 1. The inhibition of IFN-γ-induced MHC-II expression mediated by *B. abortus* is not due to a loss of cell viability in infected cultures. THP-1 cells were infected with *B. abortus* at different MOI in the presence (B and C) or absence (D and E) of IFN-γ for 48 h. Then, cells were stained with anti-HLA-DR and 7-AAD (a dead stain). A. Gating strategy employed for analyzing FACS data. THP-1 cells were first gated (G1) on a FCS *vs*. SSC dot plot. Viable (G2) and nonviable (G3) cells were then separated using a 7AAD *vs*. SSC dot plot. $_{m}$MHC-II histograms were later performed on the viable (G2) population. Shown as example are 3 different treatments. THP-1 cells treated with 2% paraformaldehyde (PFA) were included as a nonviable positive control. B and D. Quantification of the percentage of nonviable cells (7AAD^{+} cells) ± SEM of three experiments. ***$P < 0.001$ *vs*. untreated. C and E. MHC-II surface expression was assessed by flow cytometry gating on viable cells (7-AAD^{-} cells). Bars indicate the arithmetic means ± SEM of three independent experiments. MFI, mean fluorescence intensity. ** $P<0.01$; *** $P<0.001$ *vs*. IFN-γ.



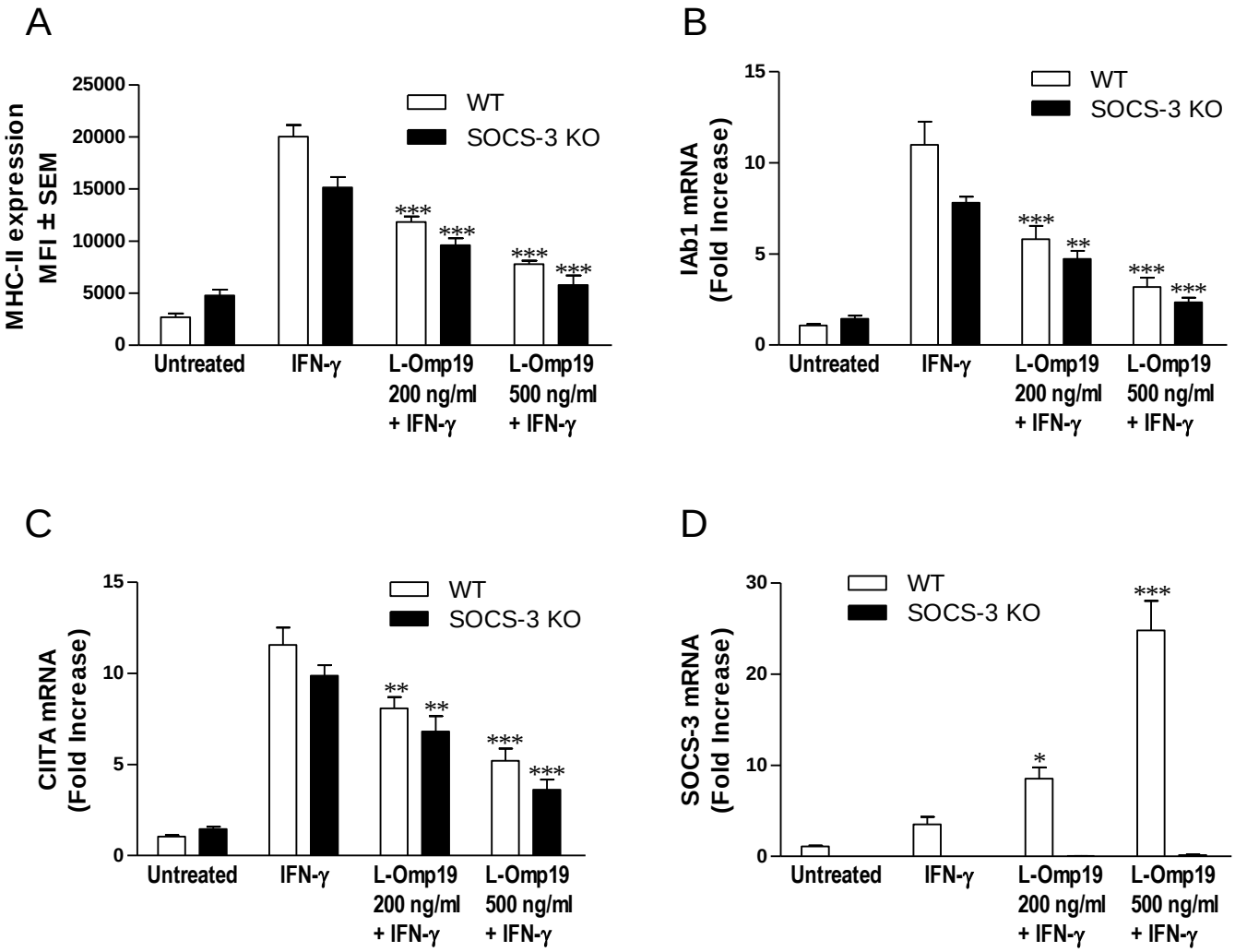
Supplemental Figure 2. THP-1 cells and human primary monocytes show similar kinetics of *B. abortus* infection. Human primary monocytes and THP-1 cells (5 x 10^5 cells) were infected with *B. abortus* (MOI 100:1), at 2, 4, 6, 24, 48 and 72 h post-infection cells were lysed and the number of viable intracellular bacteria was determined by plating lysates on tryptic soy agar.



Supplemental Figure 3. L-Omp19 down-modulates the IFN-γ-induced surface expression but not the basal expression of MHC-II. THP-1 cells were treated with L-Omp19 at different concentrations or U-Omp19 (1000 ng/ml) in the presence (A and B) or absence (C and D) of IFN-γ for 48 h. Then, cells were stained with anti-HLA-DR and 7-AAD (a dead stain). A and C. Quantification of the percentage of nonviable cells ($7AAD^+$ cells) ± SEM of three experiments. THP-1 cells treated with 2% paraformaldehyde (PFA) were included as positive controls. ***$P < 0.001$ *vs*. untreated. B and D. MHC-II surface expression was assessed by flow cytometry gating on viable cells ($7\text{-}AAD^-$ cells). Bars indicate the arithmetic means ± SEM of three independent experiments. MFI, mean fluorescence intensity. ** $P<0.01$; *** $P<0.001$ *vs*. IFN-γ.



Supplemental Figure 4. IL-6 down-modulates the IFN-γ-induced surface expression but not the basal expression of MHC-II. THP-1 cells were treated with different concentrations of IL-6, or L-Omp19 (100 ng/ml) with anti-IL-6 or an isotype matched control, in the presence (A and B) or absence (C and D) of IFN-γ for 48 h. Then, cells were stained with anti-HLA-DR and 7-AAD (a dead stain). A and C. Quantification of the percentage of nonviable cells ($7AAD^+$ cells) ± SEM of three experiments. THP-1 cells treated with 2% paraformaldehyde (PFA) were included as positive controls. ***$P < 0.001$ *vs*. untreated. B and D. MHC-II surface expression was assessed by flow cytometry gating on viable cells ($7\text{-}AAD^-$ cells). Bars indicate the arithmetic means ± SEM of three independent experiments. MFI, mean fluorescence intensity. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ *vs*. IFN-γ. ## $P<0.01$ *vs*. isotype.



Supplemental Figure 5. MHC-II down-regulation mediated by *B. abortus* lipoproteins is independent of SOCS-3. A. BMM from SOCS3$^{fl/fl}$ mice expressing LysMcre ("SOCS-3 KO") or not ("WT") were treated with L-Omp19 (200 and 500 ng/ml) in the presence of IFN-γ for 48 h. MHC-II surface expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of 4 mice with two biological replicates per mouse. MFI, mean fluorescence intensity. B and C. BMM from SOCS-3 KO or WT mice were treated with L-Omp19 (200 and 500 ng/ml) in the presence of IFN-γ for 24 h. Total RNA was isolated and analyzed for MHC-II (IAb1) (B), CIITA (C) and SOCS-3 (D) mRNA expression by quantitative real-time PCR. Data are represented as fold increase as described in Material and Methods. Bars indicate the arithmetic means ± SEM of 4 mice. * P<0.05; ** P<0.01; *** P<0.001 *vs*. WT IFN-γ or SOCS-3 KO IFN-γ respectively.