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Regulation of cytokine gene expression during *Brucella abortus* infection

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Toll-like receptors (TLR) play a key role in antimicrobial host defense. Bacterial cell wall components and lipopolysaccharide (LPS) are recognized by macrophages via TLR, resulting in activation of professional antigen-presenting cells, initiation of acquired immune responses and further elimination of the invasive bacteria. TLR2 and TLR4 have been shown to recognize bacterial components. TLR2 is required for signaling by numerous ligands from gram-negative and gram-positive bacteria such as lipoteichoic acids, peptidoglycan and lipoproteins. In contrast, TLR4 fails to confer responsiveness to gram-positive bacteria and their components, but it is the main LPS signaling receptor. LPS is a major constituent of the outer membrane of gram-negative bacteria, such as *Brucella*, and is known to activate neutrophils, monocytes, macrophages, and other cell types to up-regulate expression of adhesion molecules and produce a number of pro- and anti-inflammatory cytokines. This study demonstrates that the attenuated strain *Brucella abortus* RB51 can stimulate cells through TLR4 and MyD88, resulting in NF-κB activation. The virulent strain *B. abortus* 2308 can also stimulate the cells by a MyD88-dependent pathway without involving either TLR4 or TLR2. It also induced NF-κB activation and nuclear translocation, suggesting that *B. abortus* RB51 induces activation of the proinflammatory response by a TLR4-dependent pathway with the subsequent NF-κB activation and nuclear translocation; nevertheless, the 2308 strain induced NF-κB nuclear translocation that was activated by an alternative pathway, different from that induced by TLR.

Key words: *Brucella abortus*, RB51, TLR, NF-κB, transduction signals, cytokines.

INTRODUCTION

Brucellosis is a major zoonotic disease that causes a serious health and economic problem worldwide. In spite of the growing number of countries declared Brucella-free, the disease remains one of the main zoonotic infections throughout many parts of the world with major economical and public health implications. About 500,000 new cases occur annually worldwide with predominance in the Middle East, Mediterranean countries, South

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America and Central Asia. The causative organisms of brucellosis are gram-negative facultative intracellular pathogens that may affect a range of different mammals including man, cattle, sheep, goats, swine, rodents and marine mammals and in most host species, the disease primarily affects the reproductive system with concomitant loss in productivity of animals affected. In man, infection is associated with protean manifestations and characteristically recurrent febrile episodes that led to the description of this disease as undulant fever (Hisham et al., 2011). Resistance to intracellular pathogens such as *Listeria monocytogenes, Mycobacterium tuberculosis*, and *Brucella abortus* depends largely on the CD4+ T cells-mediated immunity able to activate macrophages through mediators like IFN-γ (Divekar et al., 2006; Flyn and Chan, 2001; Taylor et al., 2004; Thale and Kiderlen, 2005). Macrophage activation is an essential element to avoid chronicity of the infection caused by *B. abortus* (Pizarro et al., 1998; Sun et al., 2002). The cytokines produced during the early stages of immune response induction are essential to define the type of response to be developed (Eskra et al., 2003; Yingst and Hoover, 2003). The tumor necrosis factor (TNF-α) is a key cytokine in the control of the infectious process; it participates in the induction of the immune response and, together with IL-12, activates IFN-γ production by NK cells during the early phase of the innate immune response to a variety of pathogens. In addition, it plays an important role in the effector phase of the immune response by inducing either by itself or in synergy with other factors, cellular death of macrophages (Eskra et al., 2003; Huang et al., 2003).

The infection caused by several *Brucella* strains inhibits TNF-α production in macrophages; addition of exogenous TNF-α before infection favors restriction of intracellular multiplication of the bacterium, suggesting that the ability of *Brucella* to inhibit TNF-α production could be considered an attribute of its virulence (Jubier et al., 2001; Pappas et al., 2005). Bacterial recognition by host cells could be mediated by diverse receptors located in the membrane of the host cells; Toll-like receptors (TLR) constitute a family of membrane molecules that when recognizing bacterial ligands or from other infectious agents, induce signal transduction by means of a common pathway through adaptor molecules, such as the myeloid differentiation proteins MyD88, IRAK, and TRAF6. This signaling leads to activation of the pro-inflammatory response. Activation of the MyD88-dependent pathways gives rise to NF-κB release from its inhibitors (IκBs) and its translocation to the nucleus. Recently, other signal transduction pathways have been described involving the adaptor molecules TIRAP/Mal and TRIF (Campos et al., 2004; Dueñas et al., 2004; Huang et al., 2003; Liew et al., 2005). In this study we demonstrate that infection by the *B. abortus* RB51 vaccinal strain induces activation of IL-12 and IFN-γ pro-inflammatory cytokine genes and of the iNOS enzyme. Expression of these cytokines was induced by a MyD88-dependent pathway that led to the release of factor NF-κB from IκBs, leading to the pro-inflammatory response that favors T cells differentiation towards a Th1 profile. Infection by the virulent *B. abortus* 2308 strain did not induce activation of pro-inflammatory cytokine genes; neither did it induce expression of IFN-γ nor of the iNOS enzyme.

MATERIALS AND METHODS

Infection and mice vaccination

5- to 7-week old BALB/c female mice (CINVESTAV-IPN, Mexico, D.F.) were divided in three experimental groups consisting of 18 animals each. The first group of mice was intraperitoneally (i.p.) vaccinated with 0.4 ml PBS containing 4×10^8 CFU of *B. abortus* RB51. This high dose was applied because lower doses do not consistently colonize mice as established by Schurig et al. (1991). A second group of mice was infected under the aforementioned conditions with 5×10^4 CFU of *B. abortus* 2308 in 0.4 ml PBS, since this dose provides the most rigorous challenge (Montaraz and Winter, 1986) and higher doses are 100% lethal (unpublished data of our group). Unvaccinated negative control animals were injected with 0.4 ml PBS. Animals were kept under standard hygiene conditions and given food and water *ad libitum*.

Peritoneal exudate cells and macrophage cell line culture

Three mice per group were euthanized at 3 h, and 1, 2, 3 and 7 days after infection. Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity with 3 ml of minimal essential medium (MEM) containing 2 U/ml heparin and 1% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY). PEC's were placed in siliconized tubes, washed and resuspended in 1 ml RPMI 1640 (Gibco BRL) supplemented with 10% FBS and 2 mM glutamine (Sigma Chemical, St Louis, MO). PEC were counted by 'trypan blue' dye exclusion and the cell suspension was adjusted to 1×10^6 PEC/ml. Macrophages from the J774A.1 cell line were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 2 mM L-glutamine, 7% horse serum (Gibco BRL) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 250 µg/ml amphotericin B).

Analysis of cytokine and TLR genes expression

Total RNA was isolated from 1×10^6 PEC or J774A.1 macrophages at different post-infection times as indicated in each experiment with Trizol (Life Technologies, Gaithersburg, MD) and chloroform according to manufacturer's guidelines. cDNA was prepared from total RNA using 0.5 µg oligo-dT primers (Life Technologies) and 200 U Moloney murine leukemia virus reverse transcriptase (MMLVRT) enzyme (Life Technologies), following vendor's recommendations. The primers used and predicted sizes of the amplified products are shown in Table 1. To ensure that all RT reaction samples contained RNA, control RT-PCR with primers for mRNA of the 'housekeeping gene' glyceraldehyde 3-phospate dehydrogenase (G3PDH) were run in parallel. PCR products were analyzed by electrophoresis of 5 µl of the reaction mix at 95 V for 1.5 h in 1.5% agarose in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) containing 0.05 µg/ml of ethidium bromide; 1 Kb DNA ladder (Gibco BRL) was used as size marker. The difference in cytokine mRNA levels in PEC was estimated with an internal standard derived from PCR amplifications of G3PDH mRNA's.

Table 1. Sequence of primers used to amplify housekeeping gen, cytokines and iNOS mRNAs and predicted sizes of the amplified products.

Primer	Sequence	Predicted size (bp)
G3PDH	5´sense: 5´-ACC ACA GTC CAT GCC ATC AC-3´ 3´antisense: 5´-TCC ACC ACC CTG TTG CTG T-3´	452
TLR2	5´sense: 5´-TCT AAA GTC GAT CCG CGA CAT-3´ 3´antisense: 5´-TAC CCA GCT CGC TCA CTA CGT-3´	343
TLR-4	5´sense: 5´-CAA GAA CAT AGA TCT GAG CTT CAA-3´ 3´antisense: 5´-GCT GTC CAA TAG GGA AGC TTT CTA GAG-3´	277
IL-6	5´sense: 5´-ATG AAG TTC CTC TCT GCA AGA GAC T-3´ 3´antisense: 5´-CAC TAG GTT TGC CGA GTA GAT CT C-3´	638
IL12p40	5´sense: 5´-CAG AAG CTA ACC ATC TCC TGG TTT G-3´ 3´antisense: 5´-TCC GGA GTA ATT TGG TGC TCC ACA C-3´	394
TNF-α	5´sense: 5´-ATG AGC ACA GAA AGC ATG ATC CGC-3´ 3´antisense: 5´-CC AAA GTA GAC CTG CCC GGA CTC-3´	692
IFN-γ	5´sense: 5´-TGA ACG CTA CAC ACT GCA TCT TGG-3´ 3´antisense: 5´-CGA CTC CTT TTC CGC TTC CTG AG-3´	460
iNOS	5´sense: 5´-CAG CTC CAC AAG CTG GCT CG-3´ 3´antisense: 5´-CAG GAT GCT CTG AAC GTA GAC CTT G-3´	700

These calculations using a ChemiImager 4000 low light imaging system (San Leandro, CA) and the alpha ease software allowed us to obtain arbitrary units expressing the value of cDNA in each band.

The density of each cytokine band was normalized according to the corresponding G3PDH band from the same RT-PCR reaction mix. Experiments were repeated in four independent trials, but shown one.

Infection of macrophages with *B. abortus*

The vaccinal RB51 and the virulent 2308 strains of *B. abortus* were kindly donated by G. Schurig (Virginia Tech, USA) and maintained in trypticase soy agar (TSA). Macrophages from the J774A.1 cell line were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 2 mM L-glutamine and 7% horse serum (Gibco BRL) and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin and 250 µg/ml amphotericin). Cells (24×10^6) were seeded in each 144 × 21 mm petri dish for cell culture (Nunc, Denmark), they were infected with 24×10^8 CFU of *B. abortus* RB51 or of *B. abortus* 2308 and incubated for different time periods at 37°C in CO_2 atmosphere. For times over 30 min, macrophages were incubated for 30 min, then washed and supplemented with antibiotics-free RPMI medium and incubated for the remainder time. Cells were washed three times with PBS; then, the nuclear and cytoplasmic protein extracts were obtained.

Stimulation of macrophages with heat-inactivated *B. abortus* RB51 or 2308

Macrophages J774A.1 (24×10^6) were seeded in cell culture petri dishes adding 24×10^8 CFU of heat-inactivated *B. abortus* RB51 or of the 2308 strain at 70°C during 30 min and incubating for different times at 37°C in CO_2 atmosphere. For times longer than 30 min macrophages were incubated with dead bacteria during 30 min washed and supplemented with antibiotic-free RPMI and then incubated for the remainder time. Cells were washed three times with PBS and then the nuclear and cytoplasmic protein extracts were obtained.

Collection of cytoplasmic and nuclear protein extracts from macrophages

Macrophages were washed with cold PBS, released with a cell scraper and centrifuged at 1500 × *g* at 8°C during 5 min. Cells were resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT) and then frozen in a dry ice-acetone bath. Afterwards, they were thawed in ice bath and centrifuged at 1200 × *g* at 4°C during 10 min. The supernatants were collected as cytoplasmic extracts and the pellet containing the nuclei was resuspended in buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM EDTA, 1 mM DTT), supplemented with 0.5 mM PMSF proteases inhibitor. This mixture was incubated at 4°C during 30 min under gentle stirring in an angular platform and then centrifuged at 20,000 × *g* at 4°C for 20 min. The supernatant was collected as the nuclear extract. Buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 25% glycerol, 0.2 mM EDTA, 1 mM DTT), supplemented with 0.5 mM PMSF proteases inhibitor was added to the cytoplasmic and nuclear extracts which were stored and maintained at -70°C until needed. Protein concentration was measured by means of the reaction with bicinconinic acid (BCA, Pierce, Rockford, IL) using bovine serum albumin as

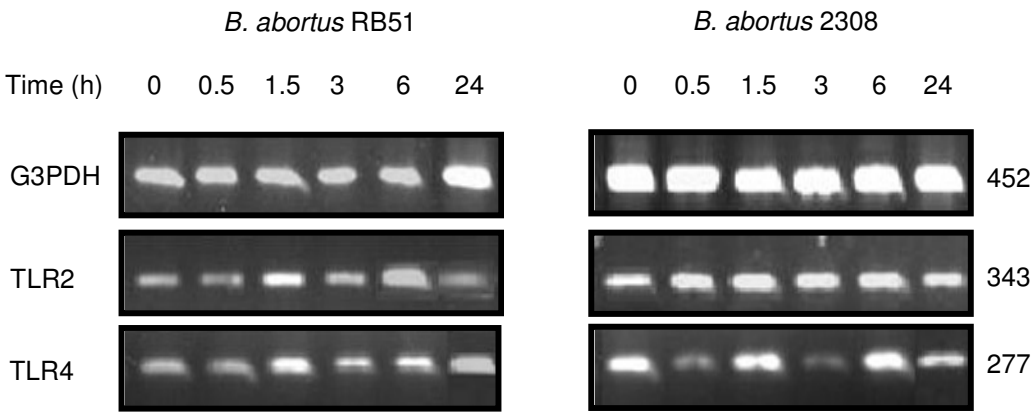

Figure 1. Expression of TLR2 and TLR4 genes. RT-PCR from J774A.1 macrophages cell line mRNA previously infected with live (a) or heat-killed (b) *Brucella abortus* RB51 or 2308 at 0, 1.5, 3, 6 and 24 h pos-tinfection. Numbers indicate products size.

standard.

Immunoblot analysis of proteins MyD88, plκBα and NF-κB

The cytoplasmic and nuclear protein extracts were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes that were blocked with 3% skim milk in TBS buffer (20 mM Tris, pH 7.6, 0.14 M NaCl) supplemented with 0.05% Tween 20 (TBS-T), during 1 h at room temperature. Membranes were washed with TBS-T and incubated overnight at 4°C with the polyclonal MyD88 antiserum, polyclonal plκBκα antiserum, polyclonal NF-κB antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000 dilution. After washing with TBS-T, membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology) during 1 h more at room temperature. The ECL plus kit (Amersham, Biosciences, Buckinghamshire, England) was used to develop the chemiluminescence for visualization on Kodak BioMax-MR film (Sigma-Aldrich, St Louis, MO).

RESULTS

Infection of macrophages with *B. abortus* RB51 and 2308 strains is independent from TLR2

Infection of J774A.1 macrophages with either *B. abortus* 2308 or RB51 did not induced great changes in TLR2 gene expression at any time post infection ($p = 0.423$ for strain RB51 and $p = 0.97$ for strain 2308). However, TLR4 gene showed a slight increase at 1.5 h after *B. abortus* RB51 infection; after that, gene expression showed a fluctuant behavior. After 3 h showed a decreased expression of genes, this condition continued during the next 6 h, later upon reaching 24 h it was observed an opposite effect, an increased expression of genes. The same increase was showed at 1.5 h in *B. abortus* 2308-infected cells and a significant decrease at 3 h. These results suggest that *B. abortus* RB51 is recognized by TLR4, whereas virulent 2308 strain is not recognized neither by TLR4 or TLR2 (Figure 1).

Modulation of expression of IL-6 and IL-12p40 genes in cells from *B. abortus* RB51-vaccinated mice

Vaccination with *B. abortus* RB51 produced a decrease in the expression of the IL-6 gene starting at 3 h post-vaccination. Expression of this gene was reestablished at 24 h, increased on day 3 post-infection and disappeared on day 7 post-infection. In PEC from *B. abortus* 2308-infected mice, the mRNA for IL-6 was not detected at any of the studied times (Figure 2). In PEC from *B. abortus* RB51, mRNA for IL-12 was also detected at 3 h and at least until 7 days after vaccination. In PBS-inoculated mice, no mRNA for IL-12p40 was detected, whereas PEC from mice infected with *B. abortus* 2308 showed a very low expression of the gene (Figure 2). This is of great importance because IL-12 activates macrophages and lymphocytes that are responsible for the destruction of bacteria.

TNF-α and IFN-γ genes were expressed in *B. abortus* RB51 vaccinated mice

As shown in Figure 2, *B. abortus* RB51 induced biphasic activation in TNF-α gene expression at 3 h and at 3 days post-vaccination, reaching the highest expression at day 1. In contrast, PEC from *B. abortus* 2308-infected mice revealed no TNF-α expression at any of the studied times. IFN-γ gene was detected at 3 h post-vaccination with *B. abortus* RB51, peaking at 2 days and was still detectable at day 7. It is worthwhile mentioning that IFN-γ expression occurred after IL-12p40 expression (from 3 h on, Figure 2). IFN-γ gene expression was not observed either in strain 2308-infected mice, at least not during the first 7 days p.i. or in PBS-inoculated mice (Figure 2). The

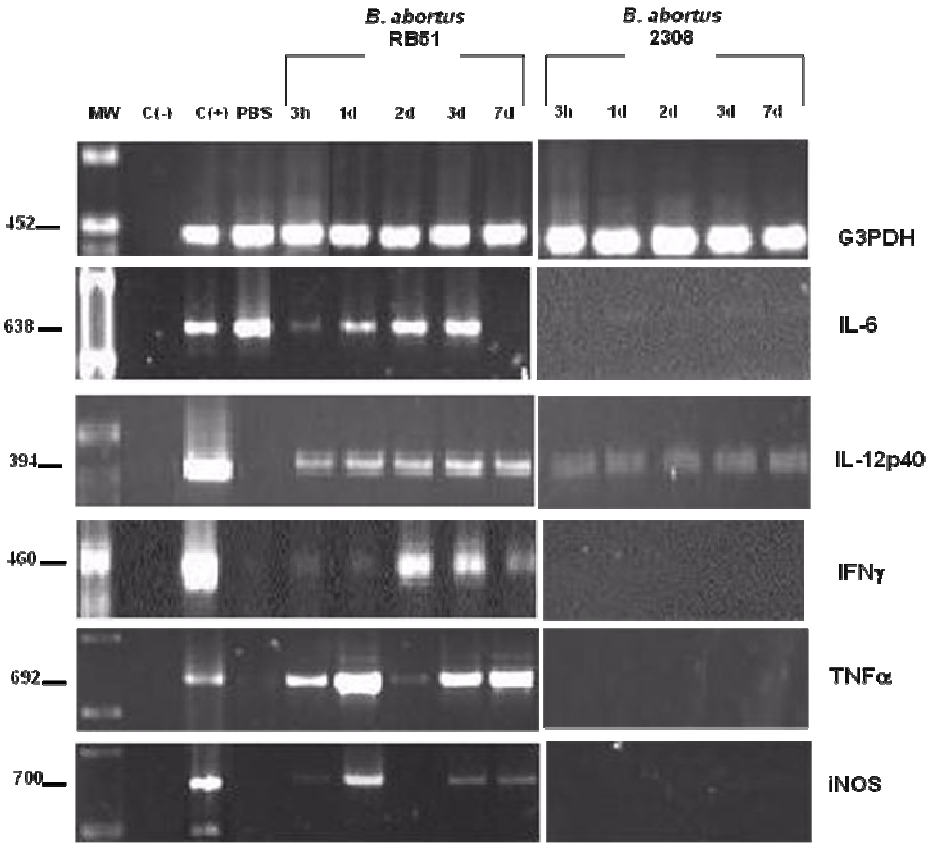

Figure 2. Expression of IL-6, IL-12p40, TNF-α, IFN-γ and iNOS cytokines. Cytokines and iNOS mRNA by BALB/c peritoneal exudate cells (PEC) were determined at 3 h and 1, 2, 3 and 7 days post vaccination with *B. abortus* RB51 or post infection with *B. abortus* 2308 analyzed by RT-PCR. Control group was inoculated with PBS. Total RNA was prepared from 106 PEC. Negative [lane C(-)] and positive controls [lane C(+)] were included. MW, molecular weight markers. The base-pair numbers of cDNA are indicated on the left.

absence of mRNA for IFN-γ correlates with the fact that strain 2308 does not induce protective immunity.

iNOS mRNA was expressed at day 1 post-vaccination

The gene for iNOS was expressed at 24 h post-vaccination with *B. abortus* RB51 (Figure 2). However, its expression ceased on the second day after vaccination, being re-expressed at days 3 and 7 at very low levels. The expression of this gene was not detected in either control or *B. abortus* 2308-infected mice.

Strains RB51 and 2308 of *B. abortus* induced activation of the NF-κB nuclear factor by means of the MyD88-dependent pathway

To assess the effect of infection with different *B. abortus* strains on the signaling that leads to activation of NF-κB, J774A.1 cell line macrophages were infected with either *B. abortus* RB51 or *B. abortus* 2308 and activation of proteins MyD88, NIK and IκBα was determined as well as the nuclear translocation of NF-κB. Infection with either bacterial strain induced activation of MyD88, pNIK and pIκBα, although each at different times (Figure 3a). In *B. abortus* RB51-infected cells, the activated proteins were detected at 5 min post-injection, whereas cells infected with *B. abortus* 2308 showed a delay in the activation of these proteins; MyD88 and pNIK were detected at 30 min post-injection and protein pIκBα at 15 min. These results indicate that both *B. abortus* strains induce nuclear release of NF-κB. While the two strains induces the activation of NF-κB through MyD88-dependent pathway, in the 2308 strain this induction is delayed in comparison with the one showed by the RB51 strain.

Heat-inactivated *B. abortus* RB51 and 2308 strains also activated NF-κB

Stimulation with heat-inactivated *B. abortus* RB51 induced nuclear release and translocation of NF-κB

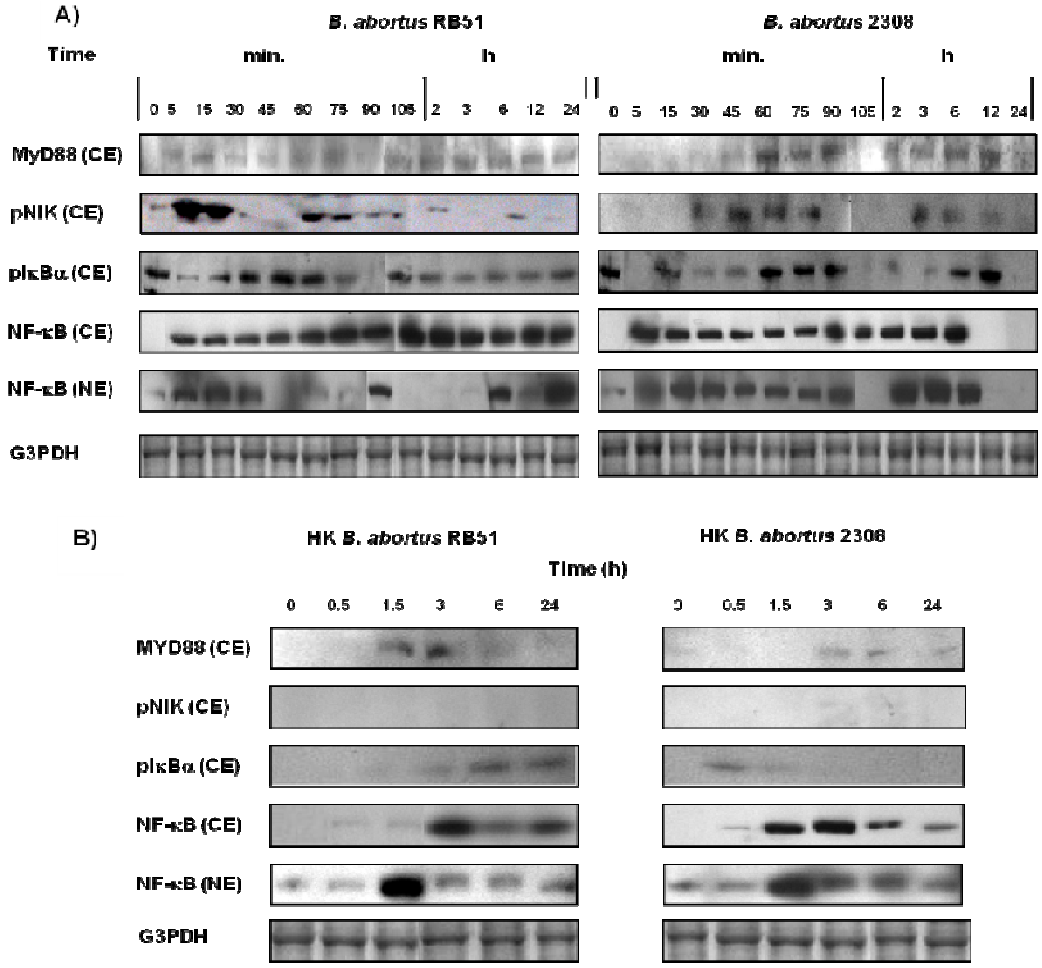

Figure 3. Activation of IκBα and NF-κB and translocation of NF-κB. Infection of macrophages with either strain (RB51 or 2308) of live *B. abortus* induced activation IκBα and the translocation of factor NF-κB towards the nucleus (a). Stimulation of macrophages with heat inactivated *B. abortus* RB51 or 2308 induced activation of IκBα leading to nuclear translocation of factor NF-κB (b). 60 µg of each extract (cytoplasmic and nuclear) were SDS-PAGE separated and transferred to nitrocellulose membranes. Membranes were incubated with the antibody for pIκBα, and NF-κB and the bound antibody was visualized by means of chemiluminescence. Results are from one of three different experiments with same results. CE = cytoplasmic extract, NE = nuclear extract.

(Figure 3b). The same behavior was observed in cells stimulated with heat-inactivated *B. abortus* 2308. In none of the experiments was the protein pNIK detected which could indicate the probable involvement of another pathway in the activation of factor NF-κB.

DISCUSSION

Macrophage activation is an essential element required to resolve an infection caused by *Brucella* spp. Macrophages phagocyte virulent and non-virulent *Brucella* strains with the same ability in a process involving the scavenger receptor A (SR-A) and components associated to lipid rafts such as cholesterol and the GM1 ganglioside. Internalization of the bacterium involves the formation of special intracellular membranal vesicles, some of them derived from the endoplasmic reticulum (Jimenez de Bagues et al., 2005; Kim et al., 2004; Naroeni and Porte, 2002). A key mechanism in cell-mediated immunity responsible for the protection against intracellular pathogens is the antimicrobial activity of macrophages stimulated by IFN-γ (Divekar et al., 2006; Huang et al., 2003; Thale and Kiderlen, 2005). Murphy et al. (2001b) reported that IFN-γ knockout C57BL/6 mice decreased their capacity to control the infection caused by *B. abortus*, in contrast to the wild strain resistant to infection. On the other hand, the number of CFU of *B. abortus* recovered from the IFN-γ gene knockout BALB/c mice was not different from the one observed in the wild

strain animals. Similar findings were reported by Baldwin and Parent (2002) when comparing murine strains resistant and susceptible to infection by *Brucella.* In humans, it has been demonstrated that T cells from patients with brucellosis produce IFN-γ when stimulated *in vitro* with *Brucella* antigens and this production is higher during the acute stage of the disease during which IFN-γ is produced by CD8+ T cells (Moreno et al., 2002). Therefore, IFN-γ added to the treatment of patients deficient in this cytokine could benefit the patient with chronic brucellosis or that does not respond to treatment (Akbulut et al., 2005). The effects of IL-12, produced by macrophages, and dendritic cells, on the resistance to *B. abortus* infection could be related with the production of IFN-γ (Baldwin and Parent, 2002; Huang et al., 2005; Paranavitana, 2005), since an important function of IL-12 is to stimulate NK cells and T cells to produce IFN-γ and in this way favor the Th1-type cellular response that mediates the protective effect (Dornand et al., 2004; Huang et al., 2005).

In the present study we observed that PEC from *B. abortus* RB51-vaccinated mice expressed the genes that encode cytokines IL-6, IL-12, TNF-α, IFN-γ and iNOS. However, no mRNA for cytokines TNF-α, IFN-γ and iNOS was detected in mice infected with *B. abortus* 2308 and in control non infected mice as well. Expression of IL-6 and IL-12 decreased in mice infected with *B. abortus* 2308 as compared with mice inoculated with *B. abortus* RB51. The lower or lack of expression of these genes could have been produced directly by the virulent bacterium either by a deficient signaling in the plasmatic membrane or by the inhibition or blockage of signals transduction during intracellular transit in the macrophage. Studies on the effect of bacterial surface on the organization of lipids in the lipid rafts suggest that there is an alteration in the organization of receptors and transduction molecules (Kim et al., 2004; Lapaque et al., 2006). The present results allow us to suggest that the protective immunity induced by *B. abortus* RB51 is related with the production of IL-12 by the macrophage during the first hours post-inoculation which in turn induces early expression of the IFN-γ gene, probably by NK cells. Although *B. abortus* 2308 seems to induce IL-12 expression, this is at a very low level and could therefore not reach the threshold to activate the IFN-γ gene and other genes. This could represent a key difference for the anti-*Brucella* response. Our results confirm that there is a very tight relation between IL-12 gene expression and the resistance to infection by *B. abortus* mediated by IFN-γ as reported by Huang et al. (2003, 2005) and Paranavitana et al. (2005), and for infections with other intracellular microorganisms such as *M. tuberculosis*, *Salmonella* or *Listeria* (Abebe et al., 2006; Kawakami et al., 2004; MacLennan et al., 2004; Puertollano et al., 2005). This phenomenon that involves IL-12 and IFN-γ responses could be relevant since the differences in the expression of these two cytokines occur at the moment in which T cells are being compromised in their differentiation. The presence of IL-12 and IFN-γ at the moment of antigenic presentation favors differentiation of these cells to Th1. Most papers analyze the late response in which there is also an association between the presence of IL-12 and IFN-γ and protection. The second stage of production of these cytokines completes a response model in which both cytokines form a positive feedback loop (Huang et al., 2003; Paranavitana et al., 2005). In turn, the role of TNF-α depends on the presence of IFN-γ during the first infection stages as shown by neutralizing TNF-α in IFN-γ knockout mice that developed an inflammatory process in the spleen but did not reduce the number of CFU (Murphy et al., 2001a, b).

As demonstrated in the present study, *B. abortus* RB51 induced activation of the TNF-α gene in PEC which was not achieved in mice infected with the virulent strain 2308 (Figure 2). This difference in TNF-α gene expression could be related with the difference in virulence between both strains. It is possible to assume that *B. abortus* 2308 inhibits TNF-α production during infection; this could be considered as a virulence mechanism. Jubier et al. (2001) demonstrated that protein Omp25 of *Brucella suis* regulates negatively TNF-α production in infected human macrophages and Ding et al. (2001) demonstrated that TNF-α, IL-10 and IL-12 production was inhibited in macrophages transfected with the gene of the protein HSP-70 from *Brucella melitensis.* Nitric oxide (NO) production catalyzed by the iNOS enzyme represents one of the bactericidal mechanisms of macrophages since it has been demonstrated that iNOS and NO production increases in macrophages infected with rough *B. abortus* strains (Jimenez de Bagues et al., 2005). The fact that expression of the iNOS gene was not observed in *B. abortus* 2308-infected mice correlated with the absence of IFN-γ gene expression, the latter being required for iNOS production. This could mean that there is no NO synthesis during *B. abortus* 2308 infection. Macrophages stimulated for IFN-γ production are resistant to intracellular infection by *L. monocytogenes* due to the release of large amounts of NO and TNF-α indicating a tight relation between both products. Similar results were found in infections caused by *Leishmania* (Ito et al., 2005; Kavoosi et al., 2006; Torres et al., 2005). In the present study, we demonstrated that infection of macrophages with *B. abortus* RB51 induced synthesis of the mRNA of iNOS which correlated with mRNA synthesis of TNF-α and IFN-γ in the same macrophages. Induction of NO synthesis by the iNOS enzyme and induction of IL-12 and IFN-γ could explain the anti-*Brucella* immunity conferred by the vaccination with *B. abortus* RB51. The lack of IL-6 mRNA in *B. abortus* 2308-infected mice suggests a very important participation of this cytokine in the protective mechanisms probably involving an acute response. Giambartolomei et al. (2004) demonstrated that IL-6 production by infected macrophages was not due to the presence of bacterial

LPS but due to lipoproteins. On the other hand, Zhan and Cheers (1995) showed that the uncontrolled growth of *Listeria* in culture produced low TNF-α and IL-6 responses. These findings are related with the present ones since *B. abortus* 2308 replicated much faster within the macrophage than the RB51 strain; hence, bacterial growth could have inhibited TNF-α and IL-6 production. On the other hand, LPS starts a complex cascade of events, particularly in monocytes and macrophages that lead to the production of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6 and IL-8 (Harju et al., 2001).

LPS is recognized by TLR2 and TLR4 in conjunction with MD2, an accessory extracellular protein that binds LPS. Binding of LPS to its receptor starts the cytoplasmic signaling cascade that leads to IκB degradation, nuclear translocation of NF-κB and transcriptional activation of the genes of the pro-inflammatory response (Akira and Takeda, 2004; Hornef et al., 2002; Liew et al., 2005). NF-κB regulates the expression of a large variety of genes that are critical for the innate immune response such as the genes that encode cytokines IL-1, IL-6, IL-12, TNF-α, adhesion molecules, acute stage proteins and inducible enzymes such as iNOS and COX-2 (Liew et al., 2005; Zhang and Ghosh, 2001). After LPS binding to the TLR4-CD14-MD2 complex, the adaptor MyD88 molecule is recruited and it binds to the cytoplasmic region of the receptor and interacts through its killing domain with protein kinase IRAK which in turn recruits the adaptor TRAF-6 towards the receptor complex. TRAF-6 binds to the TAK-TAB complex to activate IKK or signalosome that phosphorylates IκB for its ulterior degradation in proteasomes (Anderson, 2000; Liew et al., 2005). We investigated whether some of the steps in the NF-κB activation cascade could be affected in *B. abortus* 2308-infected cells as an explanation to the lack of pro-inflammatory cytokine genes expression. Both *B. abortus* RB51 and *B. abortus* 2308 induced activation of proteins MyD88, pNIK and pIκBα. However, strain RB51 achieved an earlier activation of these proteins than strain 2038.

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