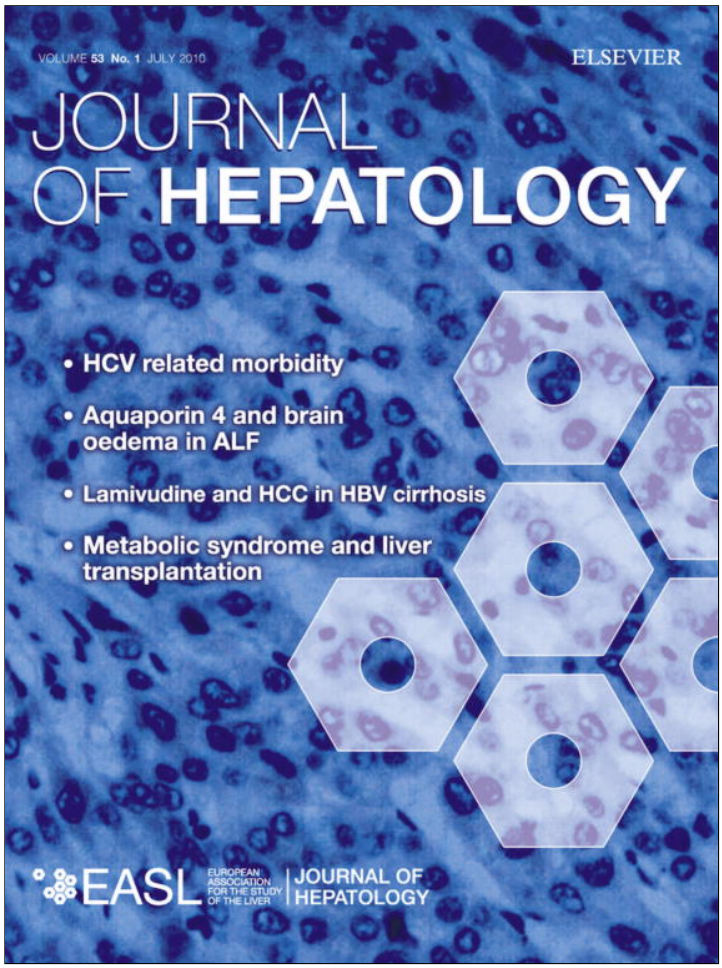

Research Article

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Brucella-infected hepatocytes mediate potentially tissue-damaging immune responses

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Background & Aims: Hepatic involvement is frequent in human brucellosis. While different histopathological lesions have been reported in these patients, the underlying cellular and molecular mechanisms have not been addressed.

Methods: This study assessed whether *Brucella abortus* can infect a human hepatoma cell line and induce a proinflammatory response in these cells.

Results: The bacterium not only infected the human hepatoma cell line HepG2 but also exhibited intracellular replication. The infection induced hepatoma cells to secrete IL-8, and supernatants from *Brucella*-infected hepatoma cells were shown to induce the migration of human neutrophils. The infection also induced the expression of the intercellular adhesion molecule ICAM-1 on hepatoma cells, and the adhesion of neutrophils to these cells was significantly higher than to uninfected hepatoma cells. ICAM-1 expression was also induced by stimulation of hepatoma cells with supernatants from *Brucella*-infected neutrophils. While *Brucella* infection did not induce the expression of matrix metalloproteinases (MMPs) in hepatoma cells, it significantly induced MMP-9 in neutrophils. Hepatoma cell apoptosis was significantly induced by *B. abortus* infection and also by stimulation with supernatants from *Brucella*-infected neutrophils.

Conclusions: The present study provides clues regarding potential mechanisms of tissue damage during liver brucellosis.

Introduction

Brucella spp. are gram-negative facultative intracellular bacteria that infect domestic and wild animals and can be transmitted to humans, in whom they produce a debilitating and eventually chronic disease. Human brucellosis remains endemic in many countries. The predilection of *Brucella* for the reticuloendothelial system is well characterized and is expressed clinically as hepatomegaly, splenomegaly, and peripheral lymphadenopathy [1,2]. The frequency of liver involvement in active brucellosis has ranged from 5% to 52% or more [1].

In a series reported by Akritidis et al. [3], all brucellosis patients with hepatic involvement, in which liver biopsy was performed, presented with granulomas, ranging from single parenchymal granulomatas to multiple localizations in both portal space and parenchymal tissue. Most of the patients presented with inflammatory infiltrations, and a half exhibited parenchymal necroses. With variations regarding frequency, these histological patterns have also been reported in other series. Intriguingly, there also have been speculations about a possible causal relationship between *Brucella abortus* infection and cirrhosis, which has not been definitively established [3]. In spite of several histopathological descriptions of liver brucellosis, the cellular and molecular mechanisms underlying the hepatic lesions have not been addressed.

The liver plays an important role in the innate immune response, providing the first line of defense against microbes and toxins crossing the intestinal barrier [4]. Kupffer cells are critical for the rapid clearance of microorganisms from the systemic circulation, and can facilitate the generation of a local inflammatory response leading to recruitment of inflammatory cells such as neutrophils, monocytes, and lymphocytes. On the other hand, hepatocytes can also produce proinflammatory cytokines and chemokines in response to bacterial invasion and/or cytokine activation [5–7]. The inflammatory microenvironment can then activate recruited neutrophils to release toxic oxygen radicals, cytokines, and matrix metalloproteases, all of which have been shown to mediate hepatocyte injury *in vitro* [8,9].

Matrix metalloproteinases (MMPs) are a family of enzymes that can degrade different extracellular matrices [10,11]. Many MMPs are not expressed in resting, healthy tissues, but are promptly induced in response to injury caused by trauma, infection, and toxins. Inflammatory cytokines, such as TNF-α and IL-1β, are the most potent inducers of many secreted MMPs [9,11]. In particular, the induction by IL-1 of locally produced MMP-9 was shown to be involved in liver damage in a model of acute liver failure [9].

In the present study, we investigated whether *Brucella* spp. can infect and survive within human hepatocytes and whether this

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infection elicits an inflammatory response that may be involved in the hepatic manifestations of brucellosis. The up-regulation of cytokines, adhesion molecules, and MMPs was in particular assessed.

Materials and methods

Bacterial culture

B. abortus 2308 were grown overnight in tryptic soy broth with constant agitation at 37 °C, harvested by centrifugation, and washed twice with phosphate-buffered saline (PBS). Bacterial numbers in cultures were estimated by comparing the optical densities at 600 nm with a standard curve. To prepare inoculum, cultures were diluted in sterile PBS to the desired bacterial concentration on the basis of optical density readings, but the precise concentration of inoculum was determined by plating cells on tryptic soy agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities.

Cell culture

The human hepatoma cell line HepG2 was cultured as monolayers in a 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco, Grand Island, NY). Human neutrophils were isolated from venous blood from healthy human volunteers by Ficoll–Paque (GE Healthcare, Uppsala, Sweden) gradient followed by sedimentation of erythrocytes in 6% dextran and hypotonic lysis. Neutrophils were harvested, washed twice with sterile PBS, and resuspended at a cell concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 5% FBS, 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell viability was >98%, as determined by trypan blue exclusion. The purity of the final neutrophil preparation was >95% as assessed by morphological examination with Giemsa staining and flow cytometry light scatter patterns. The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA) and was cultured as previously described [12].

Cellular infections

Cells were seeded at 5×10^5 cells/well in 24-well plates. *B. abortus* suspensions were added to get different multiplicities of infection (MOIs) (10:1, 100:1, 250:1, 500:1, and 1000:1 bacteria:cell). The plates were centrifuged for 10 min at 2000 rpm and then incubated for 2 h at 37 °C under a 5% CO_2 atmosphere. Cells were extensively washed with DMEM to remove extracellular bacteria and incubated in medium supplemented with 100 μg/ml gentamicin and 50 μg/ml streptomycin to kill extracellular bacteria. At different times post-infection (p.i.) (2, 24, 48, or 72 h), the supernatants from individual wells were harvested for measurement of cytokines, chemokines and metalloproteinases. To evaluate *Brucella* intracellular survival, cells were washed three times with sterile PBS and lysed with a sterile solution of 0.1% (vol/vol) Triton X-100 in H_2O. Serial dilutions of lysates were rapidly plated on tryptic soy agar plates to enumerate CFU. Infections of neutrophils were performed as described for HepG2 cells, and set up at MOI 100. At 24 h p.i. the supernatants from infected and non-infected cells (used as control) were harvested and sterilized by filtration through a 0.22 μm nitrocellulose filter, and used to stimulate non-infected HepG2 cells. Supernatants were used diluted 1/2 in complete medium. THP-1 cells were infected at MOI 100 as described for HepG2 cells.

ICAM-1 expression

HepG2 cells were infected at MOI 100 with *B. abortus* or stimulated with supernatants from *Brucella*-infected neutrophils for 24 h. As a positive control, some cells were stimulated with 200 mM of phorbol 12–myristate 13–acetate (PMA). At the end of culture, cells were washed and incubated with a fluorescein isothiocyanate (FITC)-labeled anti-human CD54 monoclonal antibody (BD Pharmingen, San Jose, CA), or an isotype-matched control antibody for 30 min on ice. Cells were then washed and analyzed with a FACScan flow cytometer using CellQuest software (both from Becton–Dickinson, Franklin Lakes, NJ). The results were expressed as mean fluorescence intensity (MFI).

Migration assay

Cell migration was quantified using 96-well microchemotaxis plates with 5 μm pore diameter polycarbonate filters (Corning, Corning, NY). Neutrophils (1×10^6 cells/ml) were placed in the upper well of the chambers and the indicated stimuli (dilutions of culture supernatants from *B. abortus*-infected hepatoma cells) were placed in the lower wells. Migration was scored by counting the number of cells that had reached the bottom well after 2 h. Migration toward *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1×10^{-7} M) (Sigma–Aldrich) served as a positive control.

Adhesion of neutrophils to hepatoma cells

HepG2 cells grown in 96-well plates were infected with *B. abortus* 2308 at different MOIs or stimulated with PMA as a positive control, and were washed to eliminate residual bacteria or stimulant before the addition of 1×10^6 human neutrophils previously labeled with calcein acetoxymethylester fluorescent dye (BD Biosciences). After incubating 1 h at 37 °C the non-adherent cells were washed away and plates were read in a fluorescence plate reader (Victor^3, Perkin Elmer, Waltham, MA) using 485/530 nm excitation/emission filters. Average percent adhesion was calculated as: relative fluorescence units (RFU) after wash/RFU before wash × 100.

Apoptosis assays

HepG2 cells were infected at an MOI of 100 with *B. abortus* 2308 or its isogenic *virB10* polar mutant or *virB1* polar mutant (kindly provided by Diego Comerci), or were treated with 4% paraformaldehyde (PFA), and were harvested 24 h later. Cells were washed and the percentage of apoptotic cells was assessed by the Annexin V-FITC (Sigma–Aldrich) assay with FACs analysis. Apoptosis was also assessed by FACs analysis using the TUNEL assay performed with the Fluorescein-FragEL™ DNA Fragmentation Detection Kit (Calbiochem, San Diego, USA). The percentage of apoptotic cells was assessed by fluorescence microscopy after labeling the cells by the TUNEL assay or by staining with the Hoechst 33342 dye.

LDH activity

As an indicator for cellular injury, the LDH activity present in the conditioned medium from HepG2 cells infected at different MOIs for 24 h was determined using a kinetic UV assay according to the manufacturer's instructions (LDH optimized, Roche, Mannheim, Germany). The non-specific release of LDH, as measured in supernatants from non-infected hepatoma cells, was subtracted from the LDH released in the medium of the infected cells. Total LDH activity in HepG2 cells was determined in sonicated cell samples. The percentage of specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release values were always <5% of the maximum release values.

Cytokine and metalloproteinase ELISA

MCP-1, IL-1β, TNF-α, GM-CSF, and IL-8 were quantified by ELISA (BD Pharmingen) as was also metalloproteinase-9 (MMP-9) (R&D Systems, Minneapolis, MN). All the protocols were performed according to manufacturer's instructions.

Zymography

A zymographic method [13] was used to measure gelatinase activity in culture supernatants from hepatoma cells or neutrophils infected or not with *B. abortus*. The samples were separated by SDS–PAGE in 10% gels containing 1 mg/ml gelatin (Sigma–Aldrich). Following electrophoresis, gels were serially washed with 50 mM Tris–HCl, pH 7.5 containing 2.5% Triton X-100 for 30 min, and with the same buffer added with 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for 30 min. Finally, gels were incubated for 24 h at 37 °C with 50 mM Tris–HCl, pH 7.5, containing 10 mM $CaCl_2$ and 200 mM NaCl. Sodium azide at 0.02% was included in all the buffers. Gelatinase activity was visualized by staining with 0.5% Coomassie Blue. Unstained bands indicated the presence of gelatinase activity, and their position indicated the molecular weight of the enzymes involved. The identity of the candidate MMP was confirmed by a specific ELISA as mentioned above.

Statistical analysis

Statistical analysis was performed with one-way ANOVA, followed by Post Hoc Tukey's Multiple Comparison test. Data are represented as mean ± SD.

Results

B. abortus infects and multiplies in hepatoma cells

As shown in Fig. 1A, *B. abortus* 2308 was internalized by the human hepatoma cell line HepG2 *in vitro*. The magnitude of the infection (intracellular UFC) was directly related to the MOI used, but infection and intracellular replication were observed even for MOIs as low as 100. The number of bacteria internalized into hepatoma cells after 2 h of infection was MOI-dependent (870 ± 98.994 UFC/ml for MOI 1000; 570 ± 42.426 UFC/ml for MOI 500; 375 ± 35.355 UFC/ml for MOI 250; 100 ± 42.426 UFC/ml for MOI 100). The number of intracellular bacteria had increased by more than one log at 48 h post-infection and continued growing thereafter.

For comparison, the THP-1 monocytic cell line, which is known to support *Brucella* intracellular growth, was infected in parallel under the same conditions used for hepatoma cells (MOI 100 in both cases). The magnitude of the infection was lower in hepatoma cells than in THP-1 cells (Fig. 1B). While CFU counts decreased by about 1 log during the first 6 h post-infection only in monocytic cells, the subsequent replication kinetics were similar in both cell lines.

Since the type IV secretion system (T4SS) encoded by the *virB* genes is involved in the capacity of *Brucella* to establish an intracellular replication niche in several cell types [14], we decided to test whether the T4SS is involved in the ability of *B. abortus* to replicate within human hepatoma cells. Hepatoma cells were infected (MOI 100) with wild type *B. abortus* 2308 or its isogenic *virB10* and *virB1* polar mutants, which cannot survive or replicate in HeLa cells [15]. At 48 h post-infection CFU counts were more than one log lower in hepatoma cells infected with the mutants than in those infected with the parental strain (Fig. 1C). While CFU from the latter had increased at 72 h post-infection, no CFU were recovered at this time point from cells infected with the mutant. Collectively, these results showed that *B. abortus* can infect and replicate in human hepatoma cells, and that replication depends on the T4SS.

B. abortus infection is cytotoxic for hepatoma cells

To determine whether *B. abortus* affects the viability of hepatoma cells, LDH activity was measured in supernatants of HepG2 cells at 24 h post-infection as an indicator of cytotoxicity. As shown in Fig. 2, infection induced a MOI-dependent specific release of LDH from hepatoma cells, which was significantly higher than that of uninfected cells. As shown in Fig. 2, LDH release also depended on a functional T4SS, since levels found after infection with *virB* mutants did not differ significantly from the spontaneous LDH release of uninfected cells.

B. abortus induces apoptosis of hepatoma cells in vitro

To analyze whether *B. abortus* is capable of inducing hepatocyte apoptosis, the HepG2 cell line was infected with *B. abortus* at MOI 100 or treated with paraformaldehyde (PFA) 4% as a positive control, and after 24 h cells were stained with annexin V-FITC (AV) and analyzed by flow cytometry. As shown in Fig. 3A, *B. abortus*-infected hepatoma cells exhibited a significantly higher AV binding than that of uninfected cells, suggesting a pro-apoptotic effect of *Brucella* infection. The occurrence of apoptosis was confirmed by a significantly higher TUNEL reaction of infected hepatoma cells than that of uninfected controls (Fig. 3A, lower panel). In the cell count performed by fluorescence microscopy (Fig. 3B and C) the mean percentage of apoptotic cells in infected cultures was 27% as determined by TUNEL and 30% as determined by Hoechst 33342 staining (versus 6% and 8%, respectively, for uninfected cells). Apoptosis depended on the expression of a functional T4SS, since the percentage of apoptotic cells did not differ significantly between hepatoma cells infected with *virB* mutants of *B. abortus* and uninfected controls.

Brucella infection induces IL-8 secretion in hepatoma cells

Supernatants from *Brucella*-infected hepatoma cells were analyzed for the presence of several proinflammatory cytokines. Infection resulted in a significant ($p < 0.05$) secretion of the chemokine IL-8 in a MOI-dependent fashion, with maximal levels at 48 h post-infection (Fig. 4A). In contrast, no specific secretion of IL-6, IL-1β, TNF-α, GM-CSF, and MCP-1 was detected.

Since the type IV secretion system (T4SS) encoded by the *virB* genes has been shown to be involved in the immune response to *B. abortus* infection [16], we decided to test whether IL-8

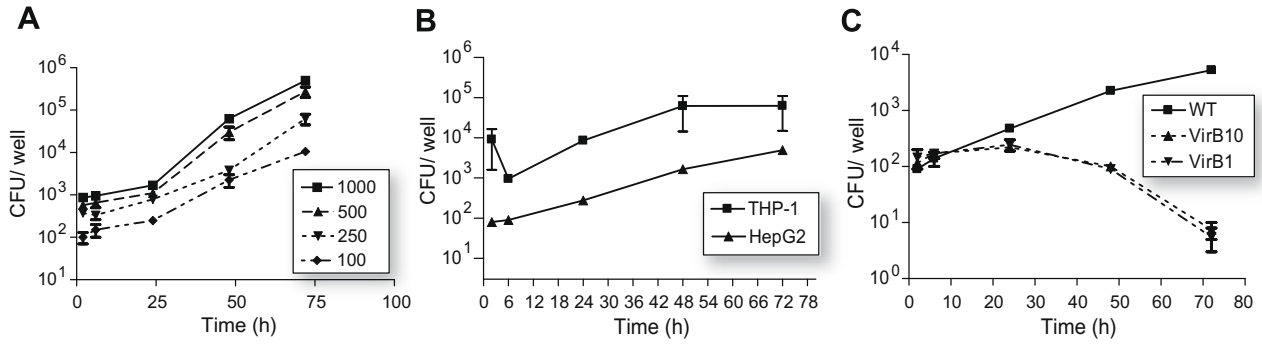

Fig. 1. Infection of human hepatocytes with *B. abortus*. (A) HepG2 cells were infected with *B. abortus* at different multiplicities of infection (MOI) and cells were lysed at different times post-infection to determine the number of viable intracellular bacteria by plating lysates on tryptic soy agar. (B) HepG2 cells and the monocytic cell line THP-1 were infected in parallel using a MOI of 100, and the number of intracellular bacteria at different times post-infection was determined as above. (C) HepG2 cells were infected with either wild type *B. abortus* or its isogenic *virB10* or *virB1* mutants, and intracellular replication was monitored as above.

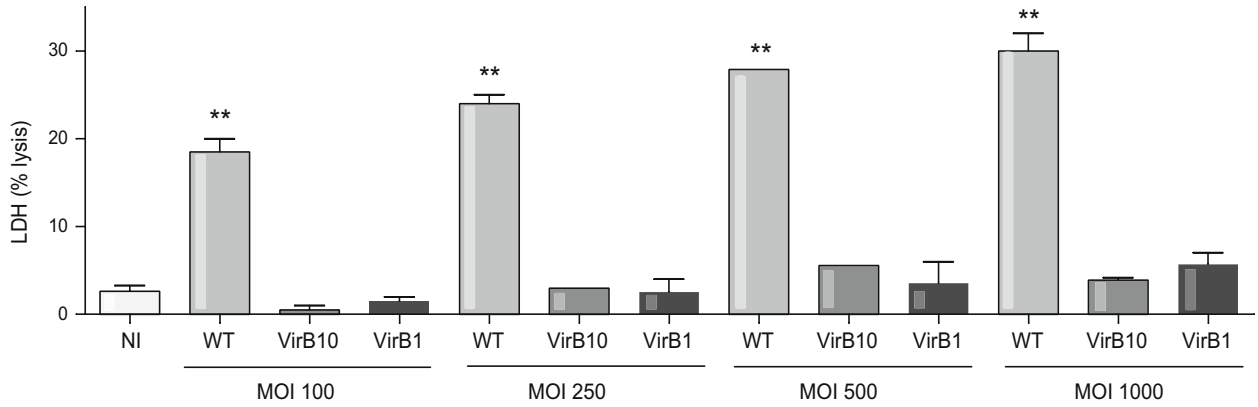

Fig. 2. Cytotoxic effect of *B. abortus* infection in human hepatocytes. HepG2 cells were infected at different MOI with either wild type *B. abortus* or its isogenic *virB10* or *virB1* mutants, and the activity of lactate dehydrogenase (LDH) was measured in culture supernatants. Values are expressed as percentages relative to the LDH activity in supernatants from the 100% lysis control. $^{**}p$ <0.01 as compared to uninfected cells.

secretion by infected hepatoma cells depends on T4SS. No significant difference in IL-8 secretion was found between hepatoma cells infected with the wild type strain and those infected with the *virB1* or *virB10* isogenic mutants, indicating that the T4SS is not implicated in IL-8 secretion (not shown).

B. abortus-infected hepatoma cells induce neutrophil migration

Considering that *B. abortus*-infected hepatoma cells secrete IL-8, experiments were conducted to evaluate if conditioned media from such cells can induce neutrophil migration. To this purpose, purified neutrophils were placed in the top well of a microchemotaxis plate and different dilutions of culture supernatants from infected or non-infected hepatoma cells were placed in the bottom well of the chamber. Migrated cells were counted 2 h later, and results were expressed as a chemoattraction index (CI) calculated as the number of cells that migrated to conditioned media divided by the number of cells that migrated to fresh culture medium (background migration). The CI was significantly higher (p <0.01) in wells containing conditioned media from infected hepatoma cells (and also in the fMLP control) than in those with conditioned medium from uninfected hepatoma cells (which was similar to background migration) (Fig. 4B).

B. abortus infection increases ICAM-1 protein expression in hepatoma cells

A key molecule involved in cellular recruitment during immune responses is the intercellular adhesion molecule-1 (ICAM-1, CD54), which is expressed by several cell types, including hepatocytes [17,18]. As shown in Fig. 5A, *B. abortus* infection stimulated ICAM-1 protein expression in HepG2 cells, yielding a MFI comparable to that of PMA-stimulated cells used as positive control. Since protein kinase C (PKC) signaling is usually involved in ICAM-1 induction in hepatocytes [19], we investigated if PKC is involved in ICAM-1 up-regulation by *B. abortus* infection. The stimulatory effect on ICAM-1 expression could be almost completely blocked by coincubation of *B. abortus* with the PKC inhibitor Calphostin C (Fig. 5A).

Neutrophils adhere more to Brucella-infected hepatoma cells

Neutrophilic infiltrates have been found in histologic studies on liver involvement in human brucellosis [3]. Since ICAM-1 expression was upregulated in *Brucella*-infected hepatoma cells, we investigated if neutrophils can adhere to such cells. Hepatoma cells were infected at different MOIs, and calcein-labeled uninfected neutrophils were added. While only 5% of neutrophils adhered to uninfected hepatoma cells, the percentage of adhered neutrophils increased significantly when hepatoma cells were infected with *B. abortus*, and this increase was MOI-dependent (Fig. 5B).

B. abortus infection increases MMP expression in neutrophils but not in hepatoma cells

Matrix metalloproteinases (MMPs) are involved in cellular migration to inflammatory foci, but if overexpressed they can also mediate tissue damage. Since parenchymal necroses have been observed in hepatic brucellosis, we decided to evaluate the production of MMPs by hepatoma cells and neutrophils in response to *Brucella* infection. Since most MMPs have gelatinase activity, such activity was determined in culture supernatants from infected cells by SDS–PAGE zymography. Exposure of hepatoma cells to PMA, used as a positive control, significantly induced the expression of a gelatinase with a molecular weight compatible with MMP-9. In contrast, these cells did not produce any MMP in response to *B. abortus* infection (data not shown). As shown in Fig. 6A, a gelatinase activity compatible with MMP-9 was detected by zymography in supernatants of neutrophils infected with *B. abortus* or stimulated with 10 ng/ml of *Escherichia coli* LPS (positive control). The increased production of this metalloproteinase in response to *Brucella* infection was confirmed by a specific ELISA test, which revealed a MOI-dependent production of MMP-9 in culture supernatants collected at 2 h post-infection (Fig. 6B). At 24 h post-infection the levels of MMP-9 were higher than those at 2 h post-infection (not shown). These results show that neutrophils, but not hepatoma cells, release high levels of MMP-9 after *B. abortus* infection.

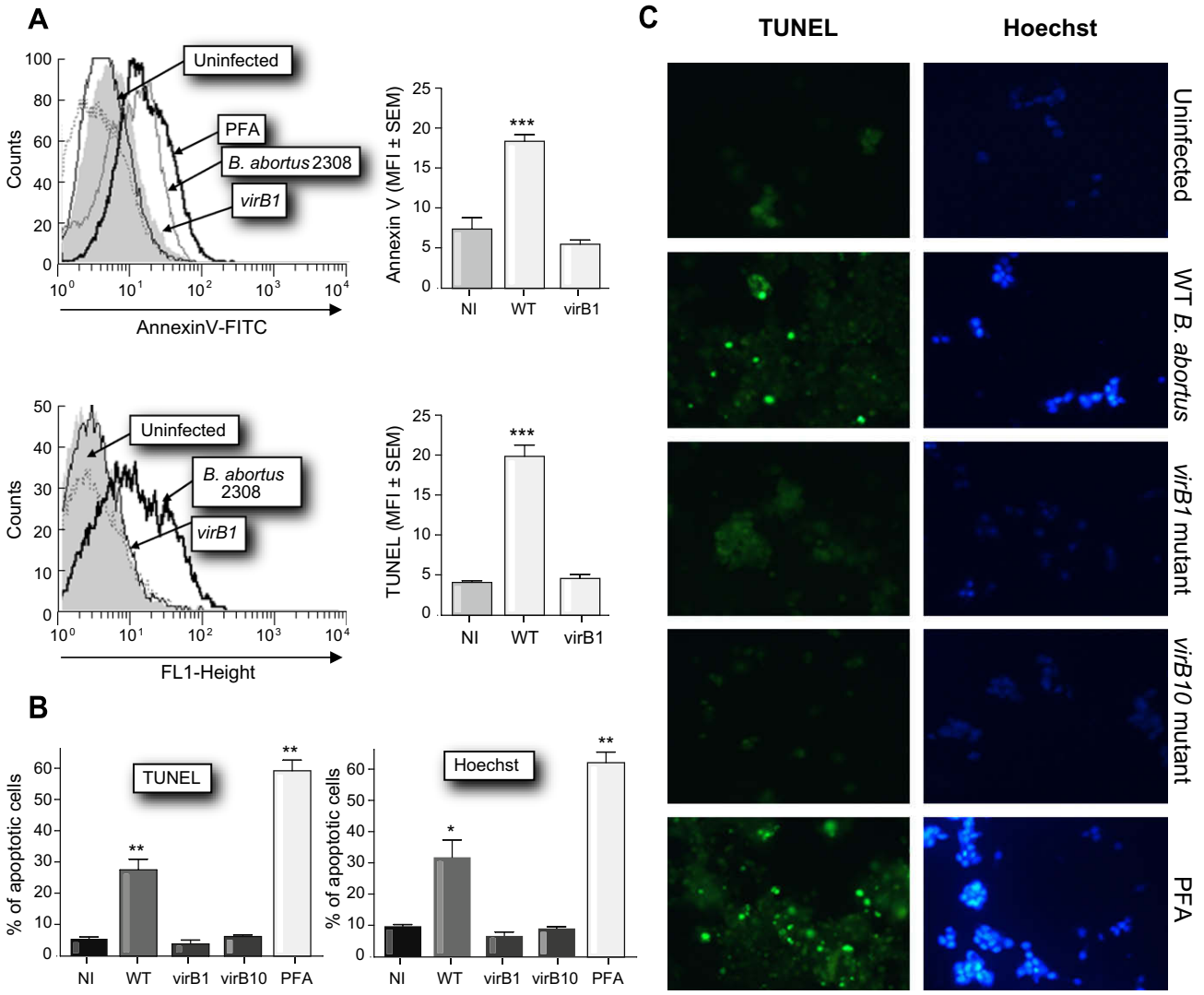

Fig. 3. Apoptosis induced by *B. abortus* infection in human hepatocytes. HepG2 cells were infected at MOI 100 with either wild type *B. abortus* (WT) or its isogenic *virB10* or *virB1* mutants; or were treated with paraformaldehyde (PFA) 4% as a positive control, and apoptosis was evaluated by the AnnexinV, TUNEL, and Hoechst 33342 techniques. (A) Flow cytometry analysis of apoptotic cells by AnnexinV-FITC binding and fluorescent TUNEL. The histograms show the results of one representative of three independent experiments. The bars represent the arithmetic means of three experiments, and the error bars indicate the standard errors of the means. MFI, mean fluorescence intensity. Non-specific binding was determined using a control isotype antibody. (B and C) Fluorescence microscopy analysis of apoptotic cells by TUNEL and Hoechst 33342 techniques [(B) quantitative analysis, (C) representative images]. ***p <0.001 for comparisons with uninfected controls. NI, non-infected cells.

Conditioned media from Brucella-infected neutrophils induce necrosis and apoptosis of hepatoma cells, and increase ICAM-1 expression

Neutrophils have been shown to induce tissue injury in different organs, including the liver, through the release of MMPs, reactive oxygen species, and TNF-α [20–21]. To analyze whether factors secreted by *Brucella*-infected neutrophils can induce hepatocyte injury we studied the effects of conditioned media from infected neutrophils (CMIN) on HepG2 cells. CMIN added at 1/2 dilution induced a significant LDH release by hepatoma cells, indicating a cytotoxic effect (Fig. 7A). Although CMIN already contained LDH levels equivalent to the lysis of 17.2 ± 0.2% of the hepatoma cells (8.6 ± 0.1% in the transferred diluted CMIN), levels found in supernatants from stimulated hepatoma cells were significantly higher (29.5 ± 0.1%), indicating a specific LDH release by hepatoma cells. In contrast, LDH levels in culture supernatants from hepatoma cells stimulated with conditioned media from uninfected neutrophils were very similar to those already present in the transferred media.

The potential of CMIN to induce hepatocyte apoptosis was also investigated. As revealed by flow cytometry analysis of the stimulated hepatoma cells subjected to Annexin V-FITC and TUNEL techniques, CMIN induced apoptosis of these cells (Fig. 7B). In the cell count performed by fluorescence microscopy (Fig. 8) the percentage of apoptotic cells in hepatoma cell cultures stimulated with CMIN was 42 ± 3% as determined by TUNEL and 36 ± 2% as determined by Hoechst 33342 staining (versus 8.5 ± 1.5% and 11 ± 1%, respectively, for cells stimulated with conditioned media from uninfected neutrophils, and 5 ± 1% in unstimulated cultures).

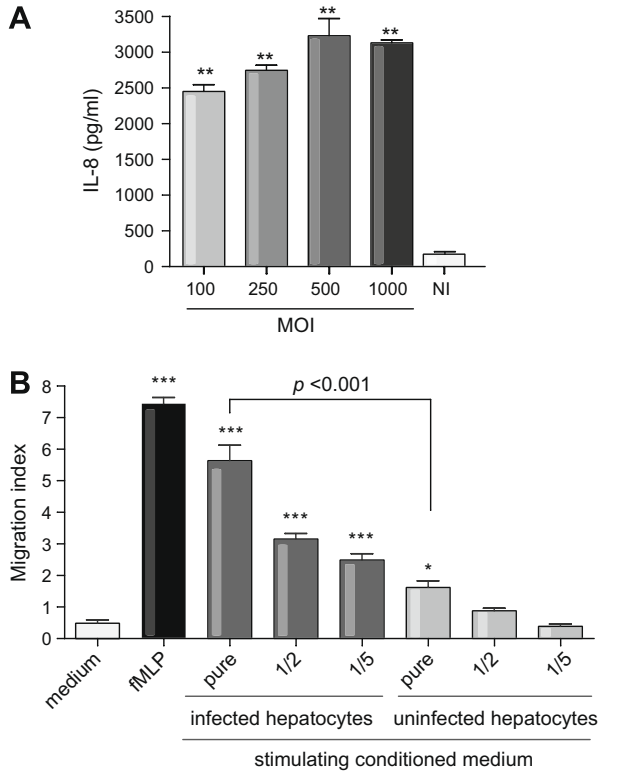

Fig. 4. Neutrophil migration induced by *Brucella*-infected hepatocytes. (A) IL-8 levels in culture supernatants from hepatocytes infected with *B. abortus* at different MOIs. $^{**}p$ <0.01 as compared to uninfected cells (ANOVA followed by Dunnett's multiple comparison test). (B) Migration of neutrophils induced by culture supernatants from *Brucella*-infected or uninfected hepatocytes, as measured in a microchemotaxis plate. Migrated cells were counted at 2 h, and results are expressed as a migration index (number of cells that migrated to conditioned medium divided by the number of cells that migrated to culture medium). Migration toward *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) served as a positive control. $^{***}p$ <0.001 and $^{*}p$ <0.05 as compared to spontaneous migration (medium).

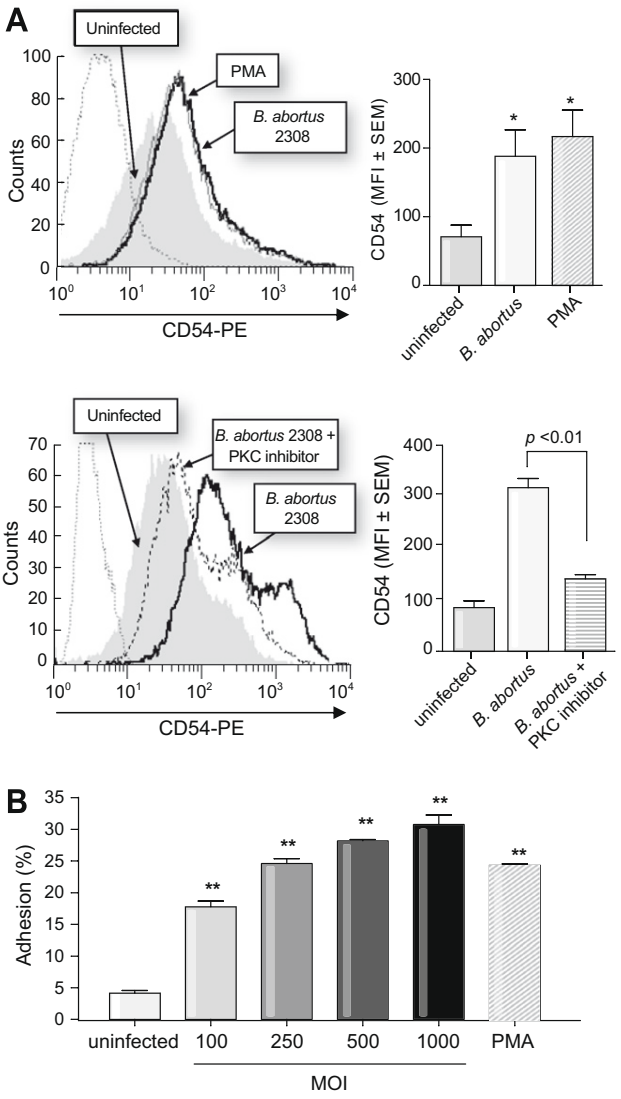

Fig. 5. Neutrophil adhesion to *Brucella*-infected hepatocytes. (A) Flow cytometry analysis of ICAM-1 expression in *Brucella*-infected hepatocytes, uninfected cells, and cells stimulated with phorbol–myristate–acetate (PMA, positive control). The effect of a PKC inhibitor on ICAM-1 expression is shown in the lower panel. Results are expressed as indicated for Fig. 3A. (B) Adhesion of calcein-labeled human neutrophils to hepatocytes infected with *B. abortus* at different MOIs, uninfected hepatocytes, or PMA-treated cells after coincubation for 1 h at 37 °C. Non-adherent cells were washed away, and plates were read in a fluorescence plate reader. Percent adhesion is calculated as: (relative fluorescence units (RFU) after wash/RFU before wash) × 100. $^{**}p$ <0.01 as compared to uninfected cells.

The addition of CMIN to hepatoma cells also resulted in a significant induction of ICAM-1 expression on the cell surface (Fig. 9). It has been demonstrated that IFN-γ, IL-1β, TNF-α, or combinations of such cytokines stimulate ICAM-1 expression in hepatocytes [17]. Interestingly we found that neutrophils secrete TNF-α, IL-1β, and IFN-γ in response to *B. abortus* infection (Fig. 10).

Discussion

The liver is frequently affected in patients with active brucellosis, with histopathological lesions such as granulomas, inflammatory infiltrations, and parenchymal necroses [3]. In the present study we have examined some potential mechanisms of liver damage in brucellosis.

To our best knowledge, the present study is the first to show that *B. abortus* can infect and multiply within hepatoma cells. This intracellular replication, and its dependence on a functional T4SS, are in line with the well known ability of *Brucella* to proliferate inside several cell types, including phagocytic and non-phagocytic cells [14].

Hepatocytes have been shown to respond to bacterial and viral infections with the secretion of proinflammatory cytokines and chemokines [5,6,22]. Therefore, hepatocytes may not only constitute a site for *Brucella* replication in the liver, but may also mediate the recruitment of inflammatory cells to the infectious focus. In line with this hypothesis, we found that hepatoma cells respond to *B. abortus* infection with a significant production of IL-8, a potent chemoattractant for neutrophils. In addition, we found that conditioned media from *Brucella*-infected hepatoma cells

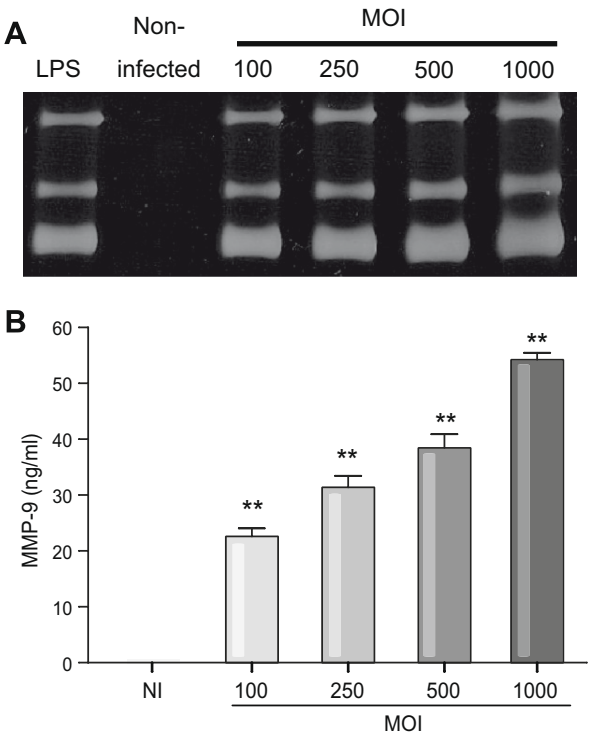

Fig. 6. Production of matrix metalloproteinases by *Brucella*-infected neutrophils. (A) Zymographic analysis of MMP activity in supernatants of neutrophils infected with *B. abortus* at different MOIs or stimulated with *E. coli* LPS (positive control). (B) MOI-dependent production of MMP-9 in culture supernatants of neutrophils at 2 h post-infection, as measured by a specific ELISA. $^{**}p$ <0.01 as compared to non-infected cells (NI).

induce neutrophil migration *in vitro*. Therefore, the presence of a neutrophilic infiltrate in the liver of *Brucella*-infected patients [23,24] may be related, at least in part, to the ability of hepatocytes to secrete neutrophil-attracting factors. There is growing evidence about the role of inflammation as a potential pathogenic factor in liver disease. Hepatocytes and neutrophils recruited to the liver are activated in microbial infections [5,6] and other hepatic pathological conditions [20].

The induction of ICAM-1 by infection and inflammation ensures the influx of neutrophils to the tissues were they are needed. Notably, we found that *B. abortus* infection induced ICAM-1 expression by hepatoma cells, which could facilitate the interaction of these cells with the infiltrating neutrophils. In agreement with this hypothesis, we found that neutrophils adhered more to *Brucella*-infected hepatoma cells than to uninfected cells. In addition, ICAM-1 expression on hepatoma cells was induced by factors secreted by *Brucella*-infected neutrophils. Interestingly, *B. abortus* infection has been shown to induce IL-8 secretion by neutrophils [25], and it has been demonstrated that IL-8 stimulates ICAM-1 expression in hepatocytes [18]. Globally, these interactions may constitute a positive feedback circuit by which neutrophils recruited to the infection focus in response to IL-8 produced by *Brucella*-infected hepatocytes engulf bacteria and respond to such phagocytosis by producing cytokines that in turn can induce ICAM-1 expression in proximal hepatocytes, thus favoring the recruitment of additional neutrophils.

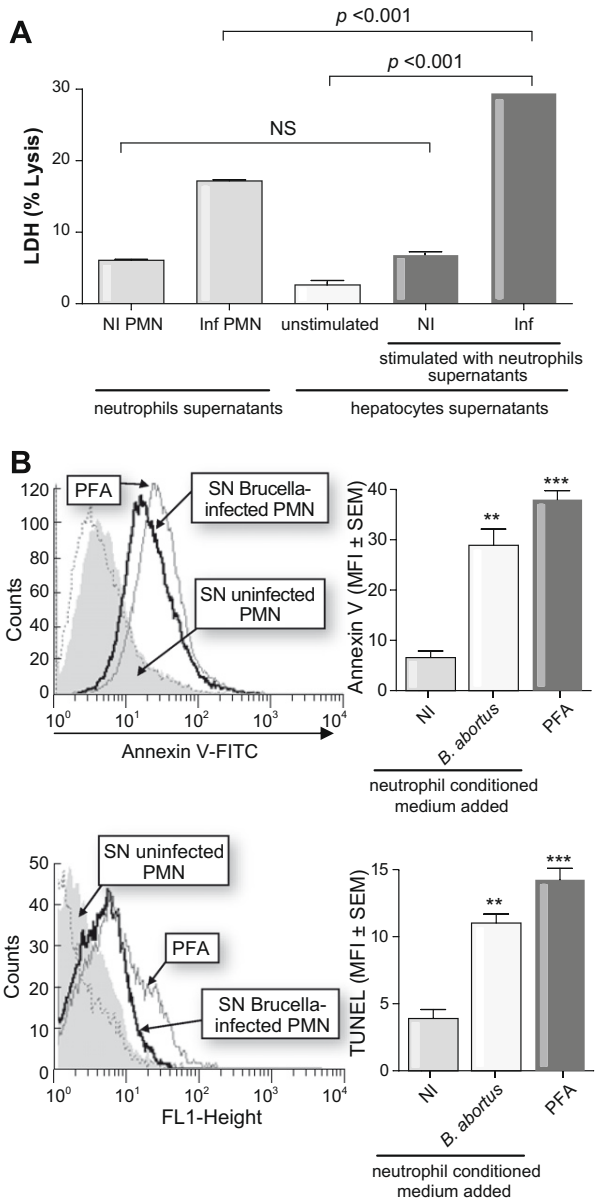

Fig. 7. Effects of *Brucella*-infected neutrophils on the viability of uninfected hepatocytes. (A) LDH release by hepatocytes stimulated with conditioned media from neutrophils infected or not with *B. abortus*, as compared to LDH already present in the transferred media. (B) Hepatocyte apoptosis induced by conditioned media from *Brucella*-infected or non-infected (NI) neutrophils, as revealed by flow cytometry analysis of cells stained using the Annexin V-FITC and TUNEL techniques. Results are expressed as indicated for Fig. 3A.

Leukocyte migration across the extracellular matrix also depends on matrix degradation, which also generates extracellular matrix derived fragments that can be highly chemotactic for leukocytes [26]. To promote their own transmigration, neutrophils can release matrix metalloproteinase-9 (MMP-9), which mediates the degradation of several extracellular matrix proteins. Notably, we found that neutrophils produce high levels of MMP-9 in response to *B. abortus* infection.

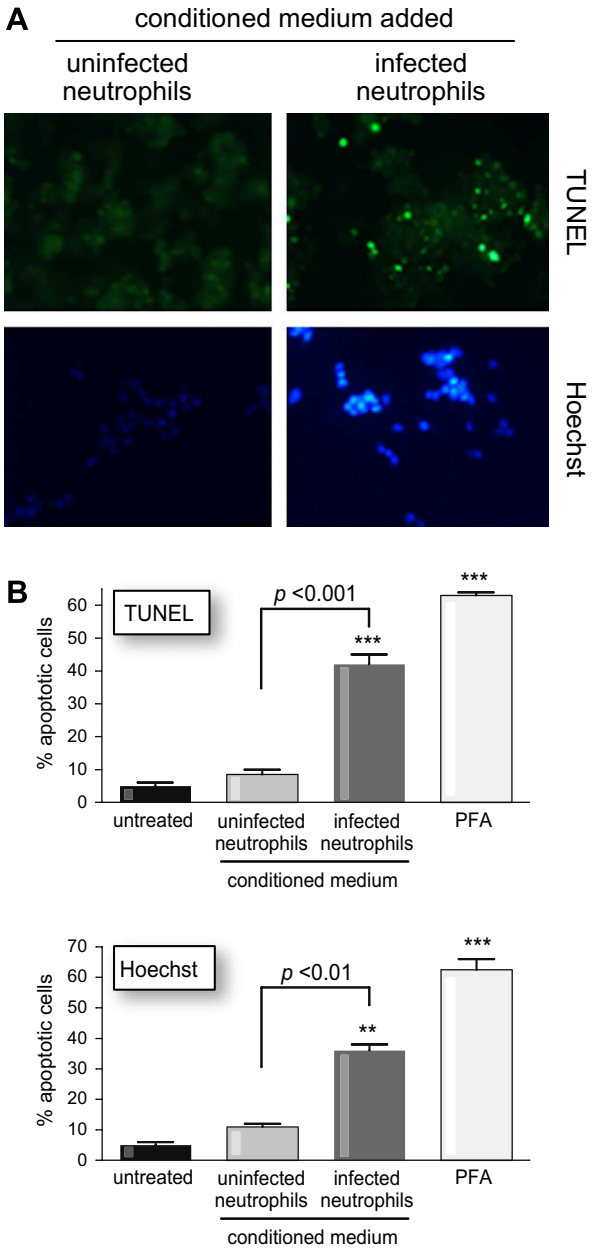

Fig. 8. Effects of *Brucella*-infected neutrophils on the viability of uninfected hepatocytes. Fluorescence microscopy analysis of apoptosis using TUNEL and Hoechst 33342 techniques [(A) representative images, (B) quantitative analysis] in hepatocytes stimulated with conditioned media from neutrophils infected or not with *B. abortus*.

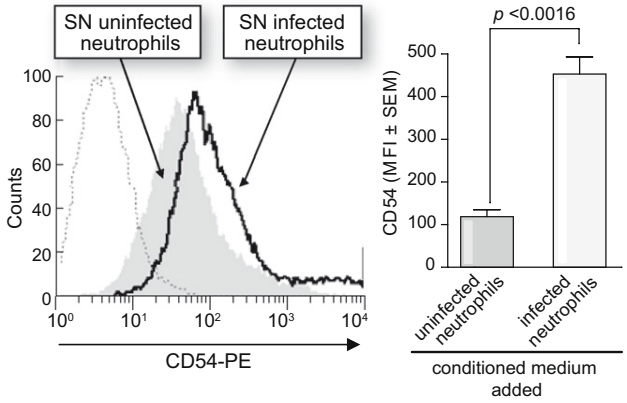

Fig. 9. Flow cytometry analysis of ICAM-1 (CD54) expression in hepatocytes after stimulation with conditioned media from *Brucella*-infected neutrophils or uninfected control cells. MFI, mean fluorescence intensity. Results are expressed as indicated in Fig. 3A.

Overall, our results suggest that *Brucella*-infected hepatocytes may be inducers of neutrophilic infiltration in the liver through both the secretion of IL-8 and the increased expression of ICAM-1. Recruited neutrophils can amplify this inflammatory reaction by secreting additional IL-8, inducing a further increase of ICAM-1 expression in hepatocytes, and degrading the extracellular matrix through MMP-9 secretion. The presence of neutrophils has been documented in active inflammatory hepatic lesions in patients with brucellosis [23], and in the liver of macaques experimentally infected with *Brucella* [27].

Liver parenchymal necroses have been described in hepatic brucellosis [3]. Such tissue damage may be due to a direct effect of *Brucella* infection on hepatocytes and/or to effects produced by the ongoing inflammatory response. In line with the first proposed mechanism, we found that *B. abortus* infection can induce apoptosis of hepatoma cells. Whether pathogen-induced apoptosis is harmful or beneficial to the host has been a considerable source of debate. Hepatocyte apoptosis may be responsible, at least in part, for the damage produced by *Brucella* infection to liver parenchyma. Apoptosis may also have an indirect damaging effect by triggering neutrophilic inflammation [28]. On the other hand, apoptosis might down-modulate the inflammatory response orchestrated by hepatocytes and may thus limit the tissue damage caused by proinflammatory mediators. It may seem contradictory that *B. abortus* is able to induce hepatocyte apoptosis when it has been reported that *Brucella* species are able to inhibit macrophage apoptosis [29]. However, this finding is not particularly surprising since *Brucella* spp. are preferentially adapted to establish chronic infections in macrophages [14]. Thus, our results suggest that hepatocytes do not constitute a survival niche for long-term *Brucella* persistence in the liver. We hypothesize that this role is probably played by Kupffer cells.

As mentioned above, parenchymal damage in liver brucellosis might be also due to collateral effects of the inflammatory reaction. While MMP-9 secretion may be important for neutrophil transmigration to the infectious focus, excessive MMP-9 activity can lead also to tissue injury. The expression of MMP-9 has been linked to numerous pathological conditions, including liver ischemia–reperfusion injury [30]. Therefore, the high levels of MMP-9 produced by *Brucella*-infected neutrophils may potentially be harmful to hepatic tissue. We have also found that neutrophils produce a variety of proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ) in response to *B. abortus* infection. Although these proinflammatory mediators could hypothetically play a role in host defense against *B. abortus* in the liver, they may also play an important role in the tissue damage observed during hepatic brucellosis. Previous studies have shown that proinflammatory mediators released by neutrophils and hepatocytes, including

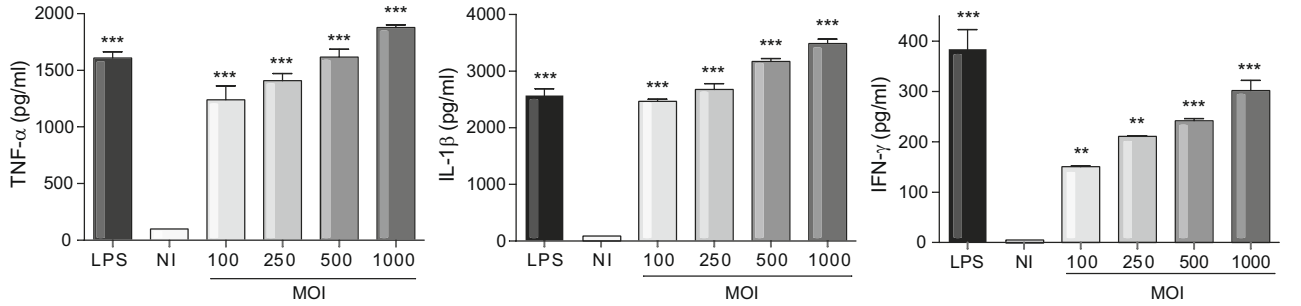

Fig. 10. Production of proinflammatory cytokines by *Brucella*-infected neutrophils. Cells were infected at different MOIs and the levels of TNF-α, IL-1β, and IFN-γ were measured by ELISA at 24 h post-infection.

IL-1β, IL-6, TNF-α, nitric oxide, reactive oxygen species, and chemokines, can worsen liver diseases [8].

Most of the cellular responses detected in the present study, such as IL-8 secretion by *Brucella*-infected HepG2 cells or the secretion of MMP-9 and proinflammatory cytokines by infected neutrophils, were MOI-dependent. This is in line with previous studies on *Brucella* infection in other cell types, either professional or non-professional phagocytes [31,32]. Therefore, the mechanisms of tissue damage proposed above may be more pronounced as the load of *Brucella* in the liver is higher.

In summary, the present study provides clues regarding potential mechanisms of tissue damage during liver brucellosis. As shown here, *Brucella* infection can induce apoptosis in human hepatocyte-like cells. In addition, in response to *Brucella* infection, these cells mount an inflammatory response that could contribute towards damaging liver cells via several mechanisms.

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