RESEARCH ARTICLE

Brucella invasion of human intestinal epithelial cells elicits a weak proinflammatory response but a significant CCL20 secretion

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Abstract

In spite of the frequent acquisition of *Brucella* infection by the oral route in humans, the interaction of the bacterium with cells of the intestinal mucosa has been poorly studied. Here, we show that different *Brucella* species can invade human colonic epithelial cell lines (Caco-2 and HT-29), in which only smooth species can replicate efficiently. Infection with smooth strains did not produce a significant cytotoxicity, while the rough strain RB51 was more cytotoxic. Infection of Caco-2 cells or HT-29 cells with either smooth or rough strains of *Brucella* did not result in an increased secretion of TNF-α, IL-1β, MCP-1, IL-10 or TGF-β as compared with uninfected controls, whereas all the infections induced the secretion of IL-8 and CCL20 by both cell types. The MCP-1 response to flagellin from *Salmonella typhimurium* was similar in *Brucella*-infected or uninfected cells, ruling out a bacterial inhibitory mechanism as a reason for the weak proinflammatory response. Infection did not modify ICAM-1 expression levels in Caco-2 cells, but increased them in HT-29 cells. These results suggest that *Brucella* induces only a weak proinflammatory response in gut epithelial cells, but produces a significant CCL20 secretion. The latter may be important for bacterial dissemination given the known ability of *Brucella* to survive in dendritic cells.

Introduction

Brucella spp. are facultative intracellular bacteria that cause brucellosis in a variety of animals and undulant fever in humans. The disease is one of the most widespread zoonoses in the world, especially in developing countries, where it causes important economic losses (Pappas *et al.*, 2005). In humans, brucellosis is a serious debilitating disease characterized by diverse pathological manifestations, which may lead to serious complications, including spondylitis, arthritis, endocarditis and meningoencephalitis. The consumption of *Brucella*-contaminated raw milk or unpasteurized cheese is one of the main ways of acquiring brucellosis in man as has been extensively documented by several epidemiological studies and case reports (Arnow *et al.*, 1984; Thapar & Young, 1986; al-Eissa *et al.*, 1990; Wallach *et al.*, 1994; Shaalan *et al.*, 2002; Méndez Martínez *et al.*, 2003). In addition, human brucellosis owing to the ingestion of meat, organs or blood from infected animals has also been reported (Syrjamaki *et al.*, 1984; Chan *et al.*, 1989; Mohd, 1989). While most cases of food-borne brucellosis have been caused by *Brucella melitensis*, cases owing to *Brucella abortus* and *Brucella suis* have also been reported (Rogers *et al.*, 1974; Galbraith & Pusey, 1984; Almér, 1985; Chan *et al.*, 1989; Priest *et al.*, 2008).

In spite of the importance of the digestive route for *Brucella* contagion in humans, the cellular and molecular mechanisms involved in this type of infection have been scarcely addressed. Moreover, the portion of the digestive tract that serves as a portal of entry for *Brucella* has been the subject of debate. While some experts consider

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Brucella as an enteroinvasive pathogen (Tsolis *et al.*, 2008), others sustain that the bacterium cannot survive to gastric juice and that the oral mucosa would be the normal site of entry after food ingestion (Gorvel *et al.*, 2009). However, it has been shown that systemic infection can be established in the mouse after *Brucella* inoculation through an intragastric tube (Bandara *et al.*, 2007; Delpino *et al.*, 2007; Paixão *et al.*, 2009). Moreover, we have shown that *Brucella* produces a bile salt hydrolase, which is important for the survival of the bacterium after intragastric infection (Delpino *et al.*, 2007).

Intestinal epithelial cells provide a physical barrier to prevent invasive infection by luminal microorganisms. Pathogenic bacteria that enter the host through the gastrointestinal tract must cross the epithelial barrier to gain access to the underlying mucosa. It has been shown that M cells of intestinal Peyer's patches are the gateway to the body for a number of enteric pathogens (Fujimora *et al.*, 1992; Clark *et al.*, 1994; Jones *et al.*, 1995), whereas other pathogens, such as *Campylobacter jejuni* and *Mycobacterium avium*, use enterocytes to penetrate the intestinal mucosa (Sangari *et al.*, 2001; Hu *et al.*, 2008). Whatever the type of intestinal cells used by pathogens to cross the intestinal barrier, the interaction of invading microbial pathogens with intestinal epithelial cells can trigger an immune response. Like professional immune cells, intestinal epithelial cells express receptors of the innate immune system such as Toll-like receptors (TLR) or nuclear oligomerization domain-like receptors (Lotz *et al.*, 2007; Abreu, 2010). Recognition of microbial structures leads to epithelial production of antimicrobial effector molecules and proinflammatory mediators.

The interaction of *Brucella* spp. with cells of the intestinal mucosa has been poorly studied. Using an intestinal loop model of infection in calves, Ackermann *et al.* (1998) demonstrated that infection with the vaccine strain *B. abortus* S19 occurs via Peyer's patches. Another study using a similar model of infection in mice showed that *B. abortus* can invade through epithelial cells of either Peyer's patches or villi and then associate with dendritic cells (DCs) located immediately beneath these structures (Salcedo *et al.*, 2008). However, as mice are not naturally infected with *Brucella*, the results obtained in the mouse model cannot easily be extrapolated to humans. A recent study showed that *B. melitensis* can translocate through a monolayer of human enterocytes differentiated to M cells, while the transit through monolayers of undifferentiated enterocytes is much lower (Paixão *et al.*, 2009). Except for these results, little is known about the interaction of *Brucella* with human intestinal epithelial cells and whether this interaction triggers any inflammatory response. In this study, we analyzed the interaction between smooth and rough *Brucella* species and human intestinal epithelial cells *in vitro*, including their ability to attach and invade these cells, and to stimulate their innate immune responses.

Materials and methods

Reagents

Lipopolysaccharides (LPS) from *B. abortus* 2308 and *Escherichia coli* O111 K58H2 were provided by Ignacio Moriyón (University of Navarra, Pamplona, Spain). The purity and the characteristics of these preparations have been published elsewhere (Velasco *et al.*, 2000). LPS-free cytoplasmic proteins from *B. abortus* (CP) were obtained as described previously (Goldbaum *et al.*, 1994). The synthetic bacterial lipohexapeptide (Pam3CSK4) was obtained from InvivoGen (San Diego). Flagellin from *Salmonella enterica* serovar Typhimurium was produced in recombinant form in the laboratory as described (Hiriart *et al.*, 2012).

Bacterial strains and growth conditions

The naturally smooth strains *B. abortus* 2308, *B. suis* 1330 and *B. melitensis* H38, the rough vaccine strain *B. abortus* RB51, a local isolate of *Brucella canis* (rough virulent strain), and a *virB10* polar mutant derived from *B. abortus* 2308 were grown in tryptic soy broth at 37 °C with agitation. At the LPS level, rough strains differ from smooth strains in that they lack the O-polysaccharide, which is involved in entry and survival in many cell types. Bacteria were washed twice with sterile phosphate buffered saline (PBS), and inocula were prepared in sterile PBS on the basis of the OD readings, but the actual concentration was later checked by plating on agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities.

Cell culture

The HT29 cell line (human colonic adenocarcinoma) was grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1/1) supplemented with 2 mM L-glutamine, 10% FBS (Gibco), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The Caco-2 cell line (human colonic adenocarcinoma) was grown in DMEM supplemented as above plus 25 mM HEPES. For infection assays, epithelial cell lines were seeded at 1×10^5 cells per well in 24-well plates and cultured in a 5% CO_2 atmosphere at 37 °C for 24 h in antibiotic-free culture medium. HT29 cells were used at early postconfluence (48 h after seeding), whereas Caco-2 cells were used at 10–14 days postconfluence when they acquire polarization and express several features of differentiated small intestinal enterocytes (Chantret *et al.*, 1988; Gaillard & Finlay, 1996). In some adhesion and invasion

experiments, Caco-2 cells were also used at subconfluence (nonpolarized) for comparison.

Cellular infections

Infections were performed at multiplicities of infection (MOI) of 200 bacteria per cell. After dispensing the bacterial suspension, the plates either were centrifuged (10 min at 300 ***g*** at room temperature) and then were incubated for 2 h at 37 °C under 5% CO_2 atmosphere, or were not centrifuged and were incubated for 16 h under the same conditions. At the end of incubation (time 0 p.i.), each well was washed three times with sterile PBS. For quantification of intracellular bacteria, the infected monolayers were incubated in the presence of 100 ug mL^{-1} of gentamicin (Sigma) and 50 ug mL^{-1} of streptomycin (Sigma) to kill extracellular bacteria. At different times after antibiotics addition (2, 24 or 48 h), cells were washed with sterile PBS and lysed with 0.2% Triton X100. Serial dilutions of the lysates were plated on agar to enumerate colony-forming units (CFU). The number of adherent bacteria was obtained by subtracting the number of intracellular bacteria from the number measured in the absence of antibiotics, both at 2 h p.i.

Evaluation of cytotoxic effects of *Brucella* infection

Vitality assay

Bacterial cytotoxicity was determined by flow cytometry using the LIVE DEAD Cell Vitality Assay Kit (Molecular Probes), which includes C12 resazurin (yields a red fluorescent product in metabolically active cells) and a cell-impermeant green fluorescent probe (SYTOX Green) that stains cells with compromised plasma membranes. Cells infected with *Brucella* and uninfected control cells were detached from the microplate wells with a 0.05% trypsin/0.02% ethylenediaminetetraacetic acid solution, treated with FBS to inactive trypsin and pooled with spontaneously detached cells. Cells were washed with PBS and treated with the probes according to the manufacturer's instructions. After washing, cell suspensions were fixed with 4% paraformaldehyde and analyzed in a flow cytometer (PAS III Model; Partec, Germany). Cells were classified as viable (red), dead (green) or injured (intermediate intensity of both red and green fluorescence).

Lactate dehydrogenase release

To further analyze the effect of infection on cell membrane permeability, the release of lactate dehydrogenase (LDH) from infected epithelial cells was also determined. Monolayers were infected with the different *Brucella* strains at a MOI of 200 as described above. Culture supernatants were harvested at 48 h p.i., and LDH levels were measured with a kinetic UV method (Roche Diagnostics GmbH, Germany). Results were expressed as the ratio between LDH levels measured in the samples (infected or uninfected cultures) and those corresponding to a 100% cell lysis (obtained by hypotonic lysis of the same number of cells).

Cell stimulation with *Brucella* antigens

Epithelial cells (5×10^5 cells per mL) cultured in 24-well plates were stimulated with different *B. abortus* antigens [heat-killed *B. abortus* (HKBA), LPS, CP] or with TLR agonists (*E. coli* LPS, Pam3CSK4, flagellin). Cultures were incubated for 24 h, except where indicated in the text. At the end of the culture, supernatants were harvested, aliquoted and stored at −70 °C until they were analyzed for cytokine content.

In other experiments, Caco-2 cells were infected during 14 h with *B. abortus* 2308 at MOI 200, washed, treated with antibiotics as described above and treated (or not) with flagellin (1 μg mL^{-1}). Cells were incubated for 2 h at 37 °C before harvesting supernatants for chemokine measurement.

Measurement of cytokine concentrations

Human IL-1β, IL-8, IL-10, MCP-1, TNF-α, GM-CSF, TGF-β and CCL20 were measured in culture supernatants of cells infected with *Brucella* or stimulated with *Brucella* antigens by sandwich ELISA (all from BD Biosciences, San Diego, except for CCL20, which was from R&D, Minneapolis) using paired cytokine-specific mAbs, according to the manufacturer's instructions.

Determination of ICAM-1 expression

ICAM-1 expression on the surface of epithelial cells infected or stimulated with *Brucella* antigens was determined by flow cytometry. Cells were incubated with an anti-CD54 monoclonal antibody (BD Biosciences) at 4 °C for 30 min, washed three times with PBS containing 1% bovine serum albumin and incubated as above with an anti-mouse IgG monoclonal antibody conjugated to fluorescein thiocyanate (Jackson Immuno Research, Laboratories Inc.). Cells were washed as above and were fixed with 4% paraformaldehyde in PBS for 15 min before reading in a flow cytometer (Partec).

Statistical analysis

Data were analyzed using analysis of variance (ANOVA). Comparisons between groups of data were made with the Tukey's post-test, and those against a control group

were performed with Dunnett's test. All the statistical analyses were performed using GRAPHPAD software (San Diego).

Results

Brucella adheres to and invades intestinal epithelial cells

The ability of the smooth and rough strains of *Brucella* to adhere to and invade the intestinal epithelial cell lines Caco-2 and HT-29 was investigated *in vitro*. For both cell lines, the adhesion of the rough strains *B. canis* and *B. abortus* RB51 to intestinal cells was significantly higher than that of the smooth strains *B. abortus* 2308, *B. suis* 1330 and *B. melitensis* H38 ($P < 0.001$ for both rough strains as compared with smooth strains) (Fig. 1a). Invasion (intracellular bacteria at time 0 p.i.) was also higher for rough strains than that for smooth strains (2–3 log higher, $P < 0.01$) (Fig. 1b).

For all the *Brucella* strains assayed, invasion levels tended to be lower for polarized cells than those for subconfluent Caco-2 cells (not shown), although differences were statistically significant only for *B. abortus* RB51 ($P < 0.01$). Nevertheless, the tendency of rough strains to invade epithelial cells more efficiently than smooth strains was observed for both subconfluent and polarized Caco-2 cells. Invasion levels of *B. abortus* RB51 and *B. abortus* 2308 were $1.2 \pm 0.8 \times 10^5$ and $1.8 \pm 0.4 \times 10^3$, respectively, in subconfluent Caco-2 cells, and the corresponding values in polarized Caco-2 cells were $4.5 \pm 0.5 \times 10^3$ and $5.0 \pm 1.8 \times 10^2$. In all the cell lines and conditions, percent invasion efficiency (intracellular bacteria/adhered bacteria $\times$ 100) was higher for the rough strain *B. abortus* RB51 than that for the smooth strain *B. abortus* 2308 (5.0 ± 1.4 vs. 2.0 ± 0.4 in subconfluent Caco-2 cells, 1.0 ± 0.06 vs. 0.23 ± 0.06 in polarized Caco-2 cells and 3.0 ± 0.2 vs. 0.04 ± 0.006 in HT-29 cells). Differences in adhesion and invasion between smooth and rough strains did not depend on the length of incubation, as they were observed for both

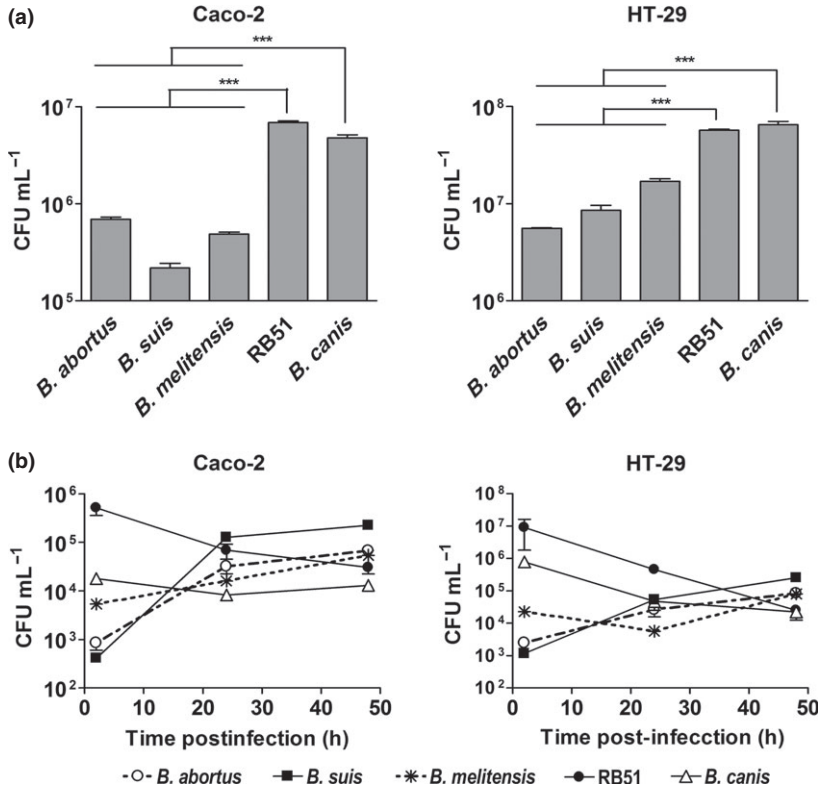

Fig. 1. Adhesion (a) and intracellular replication (b) of different strains of *Brucella* to human intestinal epithelial cells. The number of CFU of cell-associated bacteria was determined after the overnight infection of HT-29 cells and differentiated Caco-2 cells (time 0 p.i.). Intracellular bacteria were measured at different times p.i. by a gentamicin protection assay. The number of adhered bacteria was calculated as the difference between total and intracellular bacteria at time 0 p.i. Data represent mean ± SD of triplicate determinations and correspond to one experiment of three with similar results. Asterisks indicate significant differences (***$P < 0.001$; ANOVA followed by Tukey's test) between rough *Brucella* strains (RB51 and *Brucella canis*) and smooth strains. 2308: *Brucella abortus* 2308; RB51: *B. abortus* RB51.

overnight incubation (without centrifugation) and 2-h incubation (after centrifugation) (not shown).

Smooth strains of *Brucella* replicate efficiently in epithelial cells

To determine whether *Brucella* can replicate within intestinal epithelial cells, intracellular CFU were determined at different times p.i. As shown in Fig. 1b, after overnight infection all the smooth *Brucella* strains replicated efficiently in differentiated Caco-2 cells (at 48 h, the number of CFU per mL had increased 1.92 log for *B. abortus* 2308, 2.73 log for *B. suis* and 1.00 log for *B. melitensis* as compared with values measured at 2 h p.i.), and the same was true for HT-29 cells (1.52 log, 2.35 log and 0.55 log, respectively). In contrast, the rough strain *B. abortus* RB51 could not replicate in either cell line, and CFU declined with time (1.21 log in Caco-2 and 2.40 log in HT-29). *Brucella canis* behaved differently in Caco-2 than in HT-29 cells, as its CFU remained almost unchanged in the first case (0.15 log lower at 48 h) but diminished with time (1.54 log) in the second. These behaviors followed the same pattern for both differentiated and nondifferentiated Caco-2 cells and for overnight or short infection protocols (not shown); in every case, only the smooth strains exhibited an efficient intracellular replication.

To study the involvement of the type IV secretion system (T4SS) in the ability of *B. abortus* to replicate intracellularly in intestinal epithelial cells, HT-29 cells were infected with either *B. abortus* 2308 or an isogenic *virB10* polar mutant known to be unable to replicate in HeLa cells (Comerci *et al.*, 2001). Adherence was similar for both strains ($5.62 \pm 0.88 \times 10^6$ vs. $5.58 \pm 1.45 \times 10^6$ CFU per mL for the wild-type and mutant strains, respectively), and intracellular CFU at the end of the infection period was in the same order of magnitude ($2.42 \pm 0.39 \times 10^3$ vs. $6.40 \pm 2.4 \times 10^3$ CFU per mL, respectively). However, 24 h later, CFU counts were 2 log lower in cells infected with the *virB10* mutant than in those infected with the wild-type strain ($4.25 \pm 1.06 \times 10^2$ vs. $2.65 \pm 1.5 \times 10^4$ CFU per mL). At 48 h p.i., bacteria were undetectable in cells infected with the mutant, whereas CFU continued to increase in those infected with the wild-type strain.

Effect of *Brucella* infection on the viability of intestinal epithelial cells

One of the manifestations of cytotoxicity is the loss of the cell membrane barrier properties with the consequent release of cytosolic components to the culture medium. As a measure of membrane integrity, LDH activity was determined in supernatants of HT-29 and Caco-2 cells at 48 h after infection with the different strains of *Brucella* (Fig. 2). For the Caco-2 cell line, the levels of released LDH in cells infected with either strain of *Brucella* were comparable to those of uninfected controls, except for a significant increase in cells infected with *B. abortus* RB51. A similar trend was observed for HT-29 cells, in which a highly significant increase ($P < 0.001$) of LDH release was observed upon infection with *B. abortus* RB51. In addition, a small but statistically significant increase was observed in this cell line upon infection with *B. melitensis*.

Given the tendency of the rough strain of *B. abortus* (RB51) to produce a higher toxicity than its smooth counterpart (strain 2308), the potential damage or death induced in intestinal epithelial cells by infection with these strains was evaluated through the use of the fluorescent probe C12 resazurin (to detect metabolically active cells) and the cell-impermeant probe SYTOX Green (to detect cells with compromised plasma membranes). The results obtained using these probes indicated that infection with

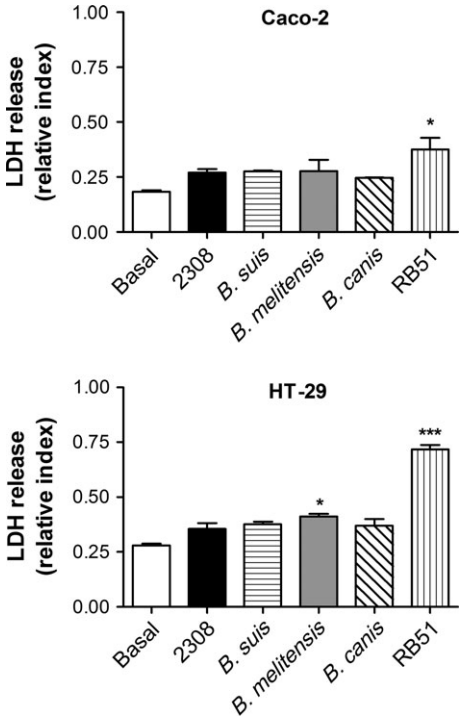

Fig. 2. LDH released by intestinal epithelial cells infected or not (basal) with different *Brucella* strains. Culture supernatants were collected at 48 h p.i. (MOI 200), and LDH activity was determined using a commercial kit. Results are expressed as the ratio between LDH measured in the samples and that corresponding to a 100% cell lysis (obtained by hypotonic lysis of a number of cells equal to that in infected cultures). Data represent mean ± SD of triplicate determinations and correspond to one experiment of three with similar results. Asterisks indicate significant differences as compared with the basal release of uninfected controls (*$P < 0.05$; ***$P < 0.001$, ANOVA followed by Dunnett's test).

either *B. abortus* 2308 or *B. abortus* RB51 does not increase significantly the percentage of damaged cells (reduced metabolic activity and membrane barrier function) of Caco-2 cells as compared with uninfected controls (11.2 ± 0.6% and 13.2 ± 0.7% vs. 8.5 ± 1.7%, respectively). Similarly, these infections did not increase significantly the percentage of dead cells (1.3 ± 0.2% and 1.5 ± 0.2% vs. 0.9 ± 0.1%, respectively). For HT-29 cells, in contrast, the percentage of damaged cells was significantly higher ($P < 0.01$) for infection with *B. abortus* 2308 or *B. abortus* RB51 than for uninfected cells (56.5 ± 0.7% and 60.2 ± 1.4% vs. 34.0%, 48 h p.i.). Notwithstanding, these infections did not lead to an increased percentage of dead HT-29 cells as compared with uninfected controls (6.2 ± 0.2% and 6.4 ± 0.5% vs. 7.7 ± 0.1%, respectively).

Brucella infection elicits a weak inflammatory response in intestinal epithelial cells

The potential of *Brucella* infection to induce the secretion of different cytokines and chemokines in intestinal epithelial cells was evaluated. Infection of Caco-2 cells (either differentiated or not) or HT-29 cells with either smooth or rough strains of *Brucella* did not result in an increased secretion of TNF-α, IL-1β, MCP-1, IL-10 or TGF-β as compared with uninfected controls (not shown). In contrast, all the infections induced IL-8 secretion both in differentiated Caco-2 cells and in HT-29 cells (Fig. 3). At 48 h p.i., IL-8 levels in culture supernatants from Caco-2 cells infected with smooth *Brucella* strains were 1.86- to 2.55-fold higher than in uninfected controls and were 5.12- and 5.03-fold higher in infections with *B. canis* and the RB51 mutant, respectively. In HT-29 cells, IL-8 levels were increased by 1.60- to 3.81-fold by smooth strains and by 3.63- and 5.09-fold by *B. canis* and the RB51 strain, respectively (values at 48 h p.i.). In addition, HT-29 cells produced low levels of GM-CSF in response to infection with all the *Brucella* strains (not shown). The ability of *B. abortus* to induce IL-8 and GM-CSF in HT-29 cells did not depend on a functional T4SS, as the secretion of these factors in response to infection with the *virB10* mutant was virtually identical to that elicited by the wild-type *B. abortus* 2308 strain (not shown).

The potential induction of CCL20 secretion by *Brucella* infection was also assessed taking into account the important role of this chemokine for the recruitment of monocytes and DCs, which are known to serve as replication niches for *Brucella* spp. (Billard *et al.*, 2005; Jimenez de Bagués *et al.*, 2005; Salcedo *et al.*, 2008). Notably, infections with all the *Brucella* strains assayed elicited a significant secretion of CCL20 at 24 h p.i. as compared with uninfected controls, and this was true for both differentiated Caco-2 cells (1.79- to 6.69-fold increase, $P < 0.01$ in all cases) and HT-29 cells (1.28- to 2.13-fold increase, $P < 0.01$ for all strains, except for *B. abortus*: $P < 0.05$) (Fig. 4). Similar trends were observed for values measured at 48 h p.i. for both differentiated Caco-2 cells (1.66- to 3.61-fold increase as compared with uninfected control, $P < 0.01$ in all cases) and HT-29 cells (1.18- to 2.13-fold increase, $P < 0.01$ for all strains except for *B. abortus*: $P < 0.05$). As in the case of IL-8, the concentration of CCL20 was higher in infections by rough *Brucella* strains than in those by smooth strains.

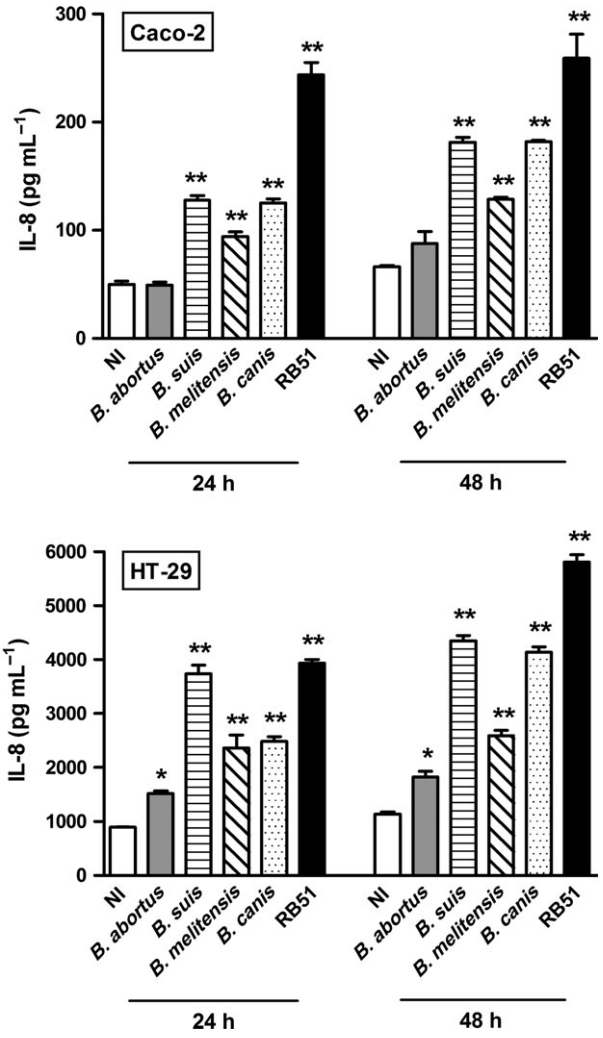

Fig. 3. Cytokine production by intestinal epithelial cells in response to infection with different *Brucella* strains. Cells were infected overnight at an MOI of 200, and culture supernatants were harvested at 24 or 48 h p.i. to measure cytokine levels by commercial ELISA assays. NI, noninfected. Data represent mean ± SD of triplicate determinations and correspond to one experiment of three with similar results. Asterisks indicate significant differences as compared with the uninfected controls (*$P < 0.05$; **$P < 0.01$, ANOVA followed by Dunnett's test).

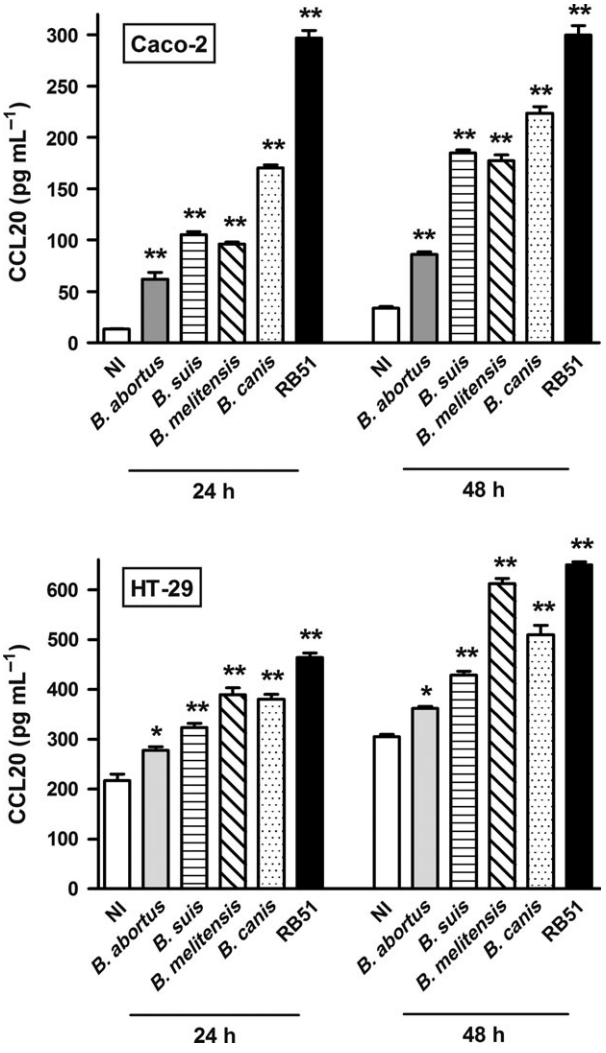

Fig. 4. CCL20 production by intestinal epithelial cells in response to infection with different *Brucella* strains. Cells were infected overnight at an MOI of 200, and culture supernatants were harvested at 24 or 48 h p.i. to measure CCL20 levels by a commercial ELISA assay. NI, noninfected. Data represent mean ± SD of triplicate determinations and correspond to one experiment of three with similar results. Asterisks indicate significant differences as compared with the uninfected controls (*$P < 0.05$; **$P < 0.01$, ANOVA followed by Dunnett's test).

Potential reasons for the weak proinflammatory activity of *Brucella*

Except for CCL20 and IL-8 production, Caco-2 cells were virtually unresponsive to *Brucella* infection. The lack of proinflammatory response did not seem to be due to the production of antiinflammatory mediators because, as mentioned above, *Brucella* infection did not result in an increased secretion of TGF-β or IL-10. To assess whether the lack of proinflammatory response may be due to an active inhibitory mechanism produced by the bacterium, cells were infected or not with *B. abortus* 2308 and subsequently stimulated for 2 h with flagellin from *Salmonella typhimurium* before measuring MCP-1 levels in culture supernatants. IL-8 levels were also measured for comparison. For both chemokines, the secretion induced by the antigenic stimulus was similar in either infected or uninfected cells, thus ruling out a *Brucella*-induced inhibitory mechanism on these chemokines (Fig. 5a).

A second potential explanation for the absent or reduced response of intestinal epithelial cells to *Brucella* infection may be a reduced capacity of *Brucella* antigens to stimulate the production of proinflammatory mediators in these cells. To test this possibility, HT-29 and Caco-2 cells were stimulated with antigens derived from *B. abortus*, including its LPS, its CP (depleted of LPS) and HKBA. As a control, cells were also incubated with the following TLR agonists: flagellin from *S. typhimurium* (TLR5 agonist), LPS from *E. coli* (TLR4 agonist) and the synthetic lipopeptide Pam3CSK4 (TLR2 agonist). Culture supernatants harvested at 24 h poststimulus were assessed for the presence of IL-1β, TNF-α, GM-CSF, IL-8 and MCP-1. Both cell lines responded differently to TLR agonists (Fig. 5b). HT-29 secreted high levels of IL-8 and very low levels of GM-CSF in response to flagellin and *E. coli* LPS (but not in response to Pam3CSK4) and did not produce IL-1β, TNF-α or MCP-1 in response to any antigen (not shown). In contrast, Caco-2 cells did not respond to *E. coli* LPS, but produced MCP-1 and IL-8 in response to flagellin and Pam3CSK4. The later cells were also unable to produce IL-1β or TNF-α in response to any antigen (not shown). Notably, none of the *Brucella* antigens assayed elicited the production of any of the cytokines and chemokines analyzed, which agrees with the low or absent response during infection. These results also demonstrate that Caco-2 and HT-29 cells can produce proinflammatory mediators in response to certain antigenic stimuli.

ICAM-1 induction by *Brucella* infection and antigen stimulation

Another feature of inflammatory reactions is the increased expression of adhesion molecules that contribute to the migration of phagocytic cells to the infection focus. It is known that intestinal epithelial cells increase the apical expression of ICAM-1 in response to infection by invasive bacteria, possibly contributing to maintain phagocytes in close contact with the intestinal epithelium, thus reducing mucosal invasion by pathogens (Huang *et al.*, 1996). Therefore, we decided to evaluate ICAM-1 expression in Caco-2 and HT-29 cells 24 h after infection with smooth and rough strains of *Brucella* or stimulation with HKBA. As shown in Fig. 6, Caco-2 cells constitutively expressed high levels of

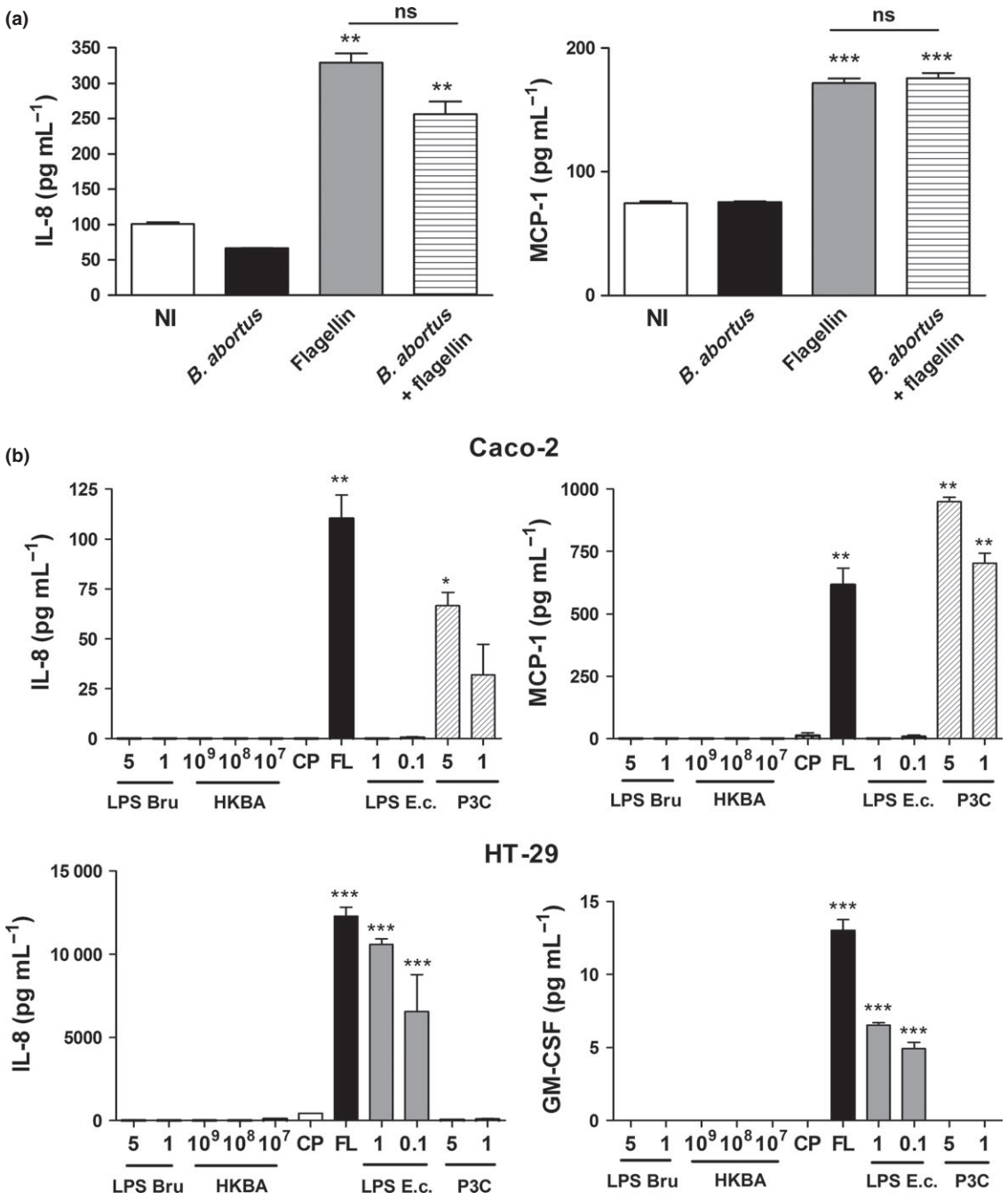

Fig. 5. (a) Effect of *Brucella abortus* preinfection on chemokine production by differentiated Caco-2 cells in response to flagellin. Caco-2 cells were either infected or not with *B. abortus* 2308 and were subsequently stimulated for 2 h with flagellin from *Salmonella typhimurium* (1 μg mL^{-1}) before measuring IL-8 and MCP-1 levels in culture supernatants. Data represent mean ± SD of triplicate determinations and correspond to one experiment of three with similar results. Asterisks indicate significant differences as compared with the uninfected control (**$P < 0.01$; ***$P < 0.001$, ANOVA followed by Dunnett's test). (b) Cytokine and chemokine production by intestinal epithelial cells in response to *Brucella* antigens or TLR agonists. Cells were stimulated for 24 h with different concentrations of HKBA, LPS from *B. abortus* or LPS-free cytosolic proteins from *B. abortus* (CP). As a control, cells were also stimulated in parallel with flagellin from *S. typhimurium* (FL, TLR5 agonist), LPS from *Escherichia coli* (TLR4 agonist) or the lipohexapeptide Pam3CSK4 (P3C, TLR2 agonist). MCP-1, IL-8 and GM-CSF levels were measured in supernatants by commercial ELISA assays. Data shown are mean ± SD of stimulation-specific values (basal secretion subtracted) measured in triplicate and correspond to one experiment of three with similar results. Asterisks indicate significant differences as compared with unstimulated controls (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; ANOVA followed by Dunnett's test).

ICAM-1, which were not modified by infection or by stimulation with either HKBA, flagellin or *E. coli* LPS. In HT-29 cells, in contrast, infection with both rough and smooth strains of *B. abortus* increased ICAM-1 expression as compared with uninfected cells, an effect not observed with HKBA.

Discussion

Ingestion of unpasteurized dairy products or raw meat has been widely shown to be a frequent means of acquiring brucellosis. Moreover, in several human populations, these practices continue to be the main mechanism for the

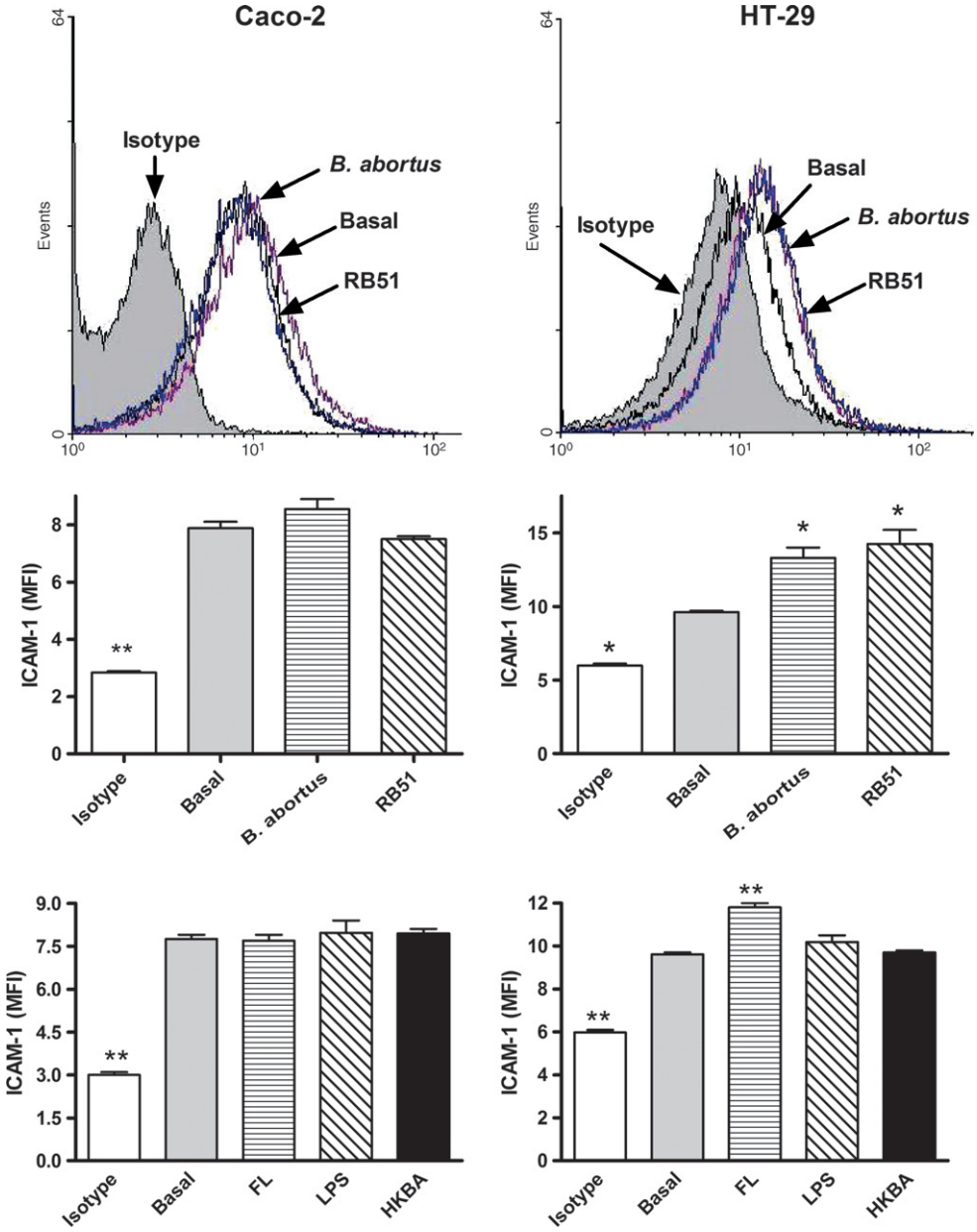

Fig. 6. ICAM-1 expression in intestinal epithelial cells in response to *Brucella* infection or stimulation with *Brucella* antigens. HT-29 cells (right panels) or Caco-2 cells (left panels) were infected with *Brucella abortus* 2308 or *B. abortus* RB51, and ICAM-1 expression was evaluated 24 h later by flow cytometry (upper and middle panels). In addition, HT-29 and Caco-2 cells were stimulated with HKBA, flagellin from *Salmonella enterica* serovar Typhimurium (1 ug mL^{-1}) or LPS from *Escherichia coli* (1 ug mL^{-1}) for 24 h before measuring ICAM-1 expression (bottom panels). Data represent mean ± SD of triplicate determinations and correspond to one experiment of three with similar results. Asterisks indicate significant differences (**$P < 0.01$) with uninfected or unstimulated controls (Basal) (ANOVA followed by Dunnett's test).

contagion of the disease. In spite of the importance of the oral route for *Brucella* infection in humans, the interaction of the bacterium with the digestive mucosa and the potential mechanisms of systemic bacterial dissemination from the digestive tract have been scarcely explored. In this study, we have assessed whether *Brucella* species can infect human intestinal epithelial cells and whether such infection elicits an inflammatory response that may be either beneficial or detrimental for *Brucella* dissemination.

We have found that both smooth and rough *Brucella* species can invade differentiated Caco-2 cells and HT-29 cells, although only the smooth species seem to undergo an efficient replication process. The capacity to adhere to and replicate within epithelial cells was highly concordant for each *Brucella* strain in each of the cell lines evaluated. The results on replication capacity are in line with similar observations made in other nonphagocytic human cells, including lung epithelial cells, endothelial cells and osteoblasts, in which only the smooth species were able to replicate efficiently (Delpino *et al.*, 2009; Ferrero *et al.*, 2009, 2011). It has been shown that the O-polysaccharide of *Brucella* LPS is involved in the entry and early survival stages of smooth brucellae in diverse cell types, so that rough strains lacking this polysaccharide cannot avoid fusion with lysosomes and are eliminated (Lapaque *et al.*, 2005). Also in agreement with previous studies, rough strains adhered to intestinal epithelial cells in higher numbers than the smooth strains tested. To our best knowledge, this is the first study to explore the ability of *Brucella* species to invade and replicate in human intestinal epithelial cells. While the ability of *B. melitensis* to translocate a polarized monolayer of Caco-2 cells was evaluated in a previous study (Paixão *et al.*, 2009), the presence of intracellular bacteria and their potential intracellular replication were not evaluated. In addition, that previous study included only *B. melitensis* and Caco-2 cells, whereas this study has included several *Brucella* species and a second epithelial cell line (HT-29). While the *in vivo* relevance of our *in vitro* results regarding the survival and replication of *Brucella* in human enterocytes is unknown at present, it is interesting to note that in the mice model viable *B. melitensis* was detected in the ileum, cecum and colon at 21 days p.i., while it was not detected in Peyer's patches (Paixão *et al.*, 2009).

As mentioned, while human infection through consumption of contaminated food has been documented for *B. melitensis*, *B. suis* and *B. abortus*, most reported cases are attributed to *B. melitensis.* Notably, experimental studies performed in human volunteers suggested that the acquisition of *B. abortus* through the consumption of contaminated cow's milk is rather difficult (Morales-Otero, 1929, 1933). In the second study, none of the three volunteers fed daily during 6 weeks with milk from a cow infected with *B. abortus* developed clinical brucellosis or agglutinins against *Brucella*. Interestingly, in the present study, the initial number of invading bacteria in enterocytes was lower for *B. abortus* than for *B. melitensis*, although at later time points both species showed similar kinetics of intracellular replication. This differential invasion capacity may explain, at least in part, the lower incidence of food-borne infections owing to *B. abortus*, although differences in other factors such as the concentration of bacteria in foods and the type of contaminated foods ingested may be also involved.

Our results show that *Brucella* infection does not induce a significant cytotoxicity in intestinal epithelial cells. Infection with *B. abortus* did not increase significantly the percentage of dead cells in either Caco-2 or HT-29 cultures, although a significant increase in metabolically compromised cells was observed for the later using fluorescent probes. However, the measurement of released LDH indicated that, except for *B. abortus* RB51 (rough vaccine strain), infection with the different *Brucella* strains did not alter the cell membrane integrity of these cell lines. These results agree with those of Paixão *et al.* (2009), who found that incubation of a polarized monolayer of Caco-2 cells with *B. melitensis* does not reduce the transepithelial electrical resistance (TEER). Nevertheless, it must be considered that these TEER measurements were taken at 2 h p.i. whereas our determinations of cytotoxic effects were performed at 48 h p.i.

A second goal of our study was to assess the cytokine and chemokine response of intestinal epithelial cells to *Brucella* infection. In this regard, it must be noted that, to our best knowledge, this is the first study to evaluate such response in human enterocytes. Infection of differentiated Caco-2 cells or HT-29 cells with either smooth or rough strains of *Brucella* did not stimulate the production of TNF-α, IL-1β, MCP-1, IL-10 or TGF-β as compared with uninfected controls. Further experiments demonstrated that, at least in the case of MCP-1, the lack of induction of the cytokine response was not due to an active inhibitory mechanism produced by the bacterium. Moreover, the two epithelial cell lines used were able to produce cytokines in response to flagellin (TLR5 agonist) and either Pam3CSK4 (TLR2 agonist) or *E. coli* LPS (TLR4 agonist) while all the *Brucella* antigens tested were not, suggesting an intrinsic inability of *Brucella* to elicit such responses. In contrast to the lack of response for the cytokines mentioned above, infection with natural *Brucella* strains produced a moderate induction of IL-8 secretion in both Caco-2 cells and HT-29 cells. The production of IL-8 seemed to depend on bacterial viability, as it was not induced by HKBA or the *Brucella* antigens tested (LPS and cytosolic proteins), but it did not seem

to depend on bacterial intracellular replication as it was induced by infections with the *virB10* mutant or the RB51 strain, both of which are unable to replicate in the intestinal cells tested. IL-8 levels tended to be higher for infections with the rough strains RB51 and *B. canis* than for infections with smooth strains, suggesting that the number of initially adhered or invading bacteria is an important determinant of IL-8 response magnitude. The potential *in vivo* relevance of this IL-8 response in human *Brucella* infections acquired through the oral route is unknown. In mice orally infected with *B. melitensis*, a few neutrophils have been observed in the ileal mucosa (Paixão *et al.*, 2009).

As another maker of inflammatory response, ICAM-1 expression levels were measured in *B. abortus*-infected cells. Infection with either the smooth or the rough strain of *B. abortus* did not elicit a significant induction of ICAM-1 in Caco-2 cells as compared with basal secretion in uninfected controls. These results agree with those reported by Huang *et al.* (1996) for Caco-2 cells infected with invasive bacteria such as *Yersinia enterocolitica* or enteroinvasive *E. coli*. In the case of HT-29 cells, *B. abortus* infection induced a low but statistically significant increase in ICAM-1 expression, with similar levels of induction for both the smooth strain and the rough strain (1.38-fold and 1.48-fold, respectively, relative to basal values). These values are much lower than those found by Huang *et al.* for the infection of this cell line with other invasive bacteria and again point to the comparatively low proinflammatory potential of *Brucella* spp. for intestinal epithelial cells. In agreement with that previous report, the induction of ICAM-1 in HT-29 cells in response to *E. coli* LPS was rather poor (1.06-fold increase).

In the present study, infection with natural *Brucella* strains also induced a significant secretion of CCL20, especially by differentiated Caco-2 cells (1.79- to 5.53-fold increase at 24 h p.i.). As in the case of IL-8, the magnitude of CCL20 secretion was higher in infections by rough *Brucella* strains than in those by smooth strains, again suggesting a direct relationship between the initial load of adhered or invading bacteria and the cytokine response. Interestingly, an increased CCL20 mRNA expression was detected in a previous study in the ileum of mice orally infected with *B. melitensis* (Paixão *et al.*, 2009). CCL20 is a chemoattractant for T and B lymphocytes and for immature DCs (Williams, 2006; Ito *et al.*, 2011). This later property may be especially relevant to *Brucella* dissemination from the gut after ingestion of contaminated food. Using a murine ileal loop model, Salcedo *et al.* (2008) demonstrated that early after inoculation in the gut lumen, *B. abortus* localizes to DCs of the follicle-associated epithelium. Given the known ability of *Brucella* to survive within DCs, it can be speculated that these cells may act as Trojan horses that carry the bacterium to regional lymph nodes, from where it can disseminate systemically. Therefore, *Brucella* seems to elicit a weak local inflammatory response, probably as a virulence strategy, but nevertheless elicits the secretion of CCL20 that may potentially contribute to bacterial dissemination to distant sites within infected DCs. The precise mechanism of translocation from the gut lumen has not been identified, but previous studies suggest that transcytosis through M cells may be involved (Ackermann *et al.*, 1998; Salcedo *et al.*, 2008; Paixão *et al.*, 2009). The potential role of human enterocytes in this translocation process has been assessed only at short postinfection times (Paixão *et al.*, 2009), so that an eventual transcytosis at longer times cannot be ruled out, especially taking into account the ability of *Brucella* species to invade and replicate within these cells as shown in the present study. Further studies will be required to address this issue. Regardless of their eventual role in bacterial translocation, human enterocytes are likely to contribute to *Brucella* dissemination from the gut by recruiting DCs to the infection site.

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