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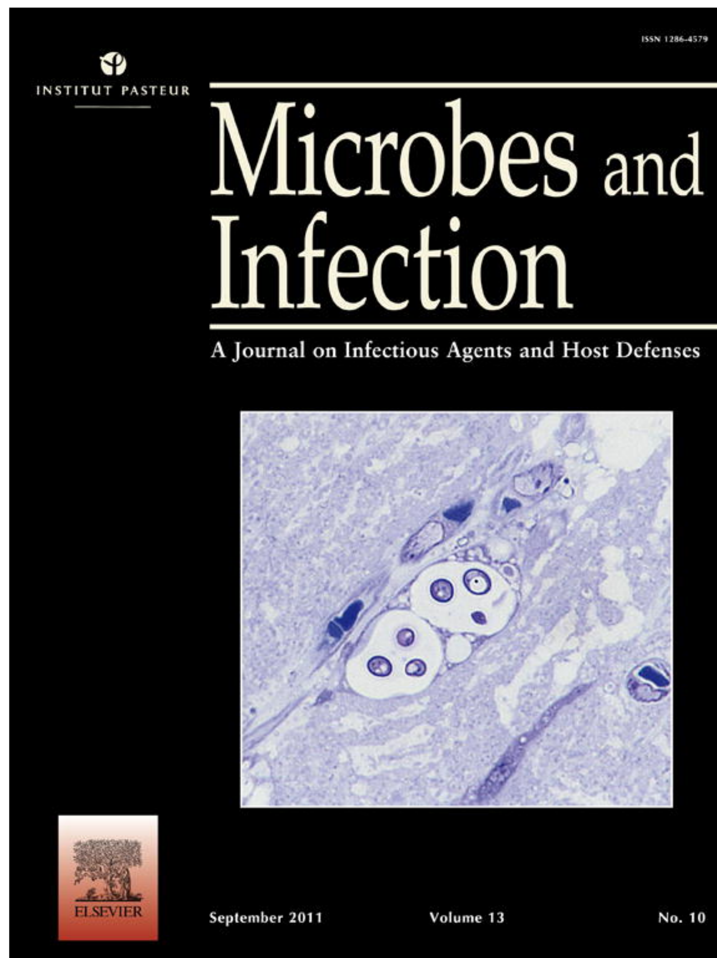

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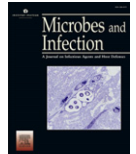

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Microbes and Infection 13 (2011) 852–861
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Original article

Proinflammatory response of human endothelial cells to *Brucella* infection

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Received 2 March 2011; accepted 19 April 2011
Available online 17 May 2011

Abstract

Although vascular pathologies such as vasculitis, endocarditis and mycotic aneurysms have been described in brucellosis patients, the interaction of *Brucella* with the endothelium has not been characterized. In this study we show that *Brucella abortus* and *Brucella suis* can infect and replicate in primary human umbilical vein endothelial cells (HUVEC) and in the microvascular endothelial cell line HMEC-1. Infection led to an increased production of IL-8, MCP-1 and IL-6 in HUVEC and HMEC-1 cells, and an increased expression of adhesion molecules (CD54 in both cells, CD106 and CD62E in HUVEC). Experiments with purified antigens from the bacterial outer membrane revealed that lipoproteins (Omp19) but not lipopolysaccharide mediate these proinflammatory responses. Infection of polarized HMEC-1 cells resulted in an increased capacity of these cells to promote the transmigration of neutrophils from the apical to the basolateral side of the monolayer, and the same phenomenon was observed when the cells were stimulated with live bacteria from the basolateral side. Overall, these results suggest that *Brucella* spp. can infect and survive within endothelial cells, and can induce a proinflammatory response that might be involved in the vascular manifestations of brucellosis.
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Keywords: Brucella; Endothelial cells; Inflammation mediators; Cytokines; Chemokines; Adhesion molecules

1. Introduction

Brucellosis is a debilitating disease caused by *Brucella* spp., which may affect different organs and may eventually progress to a chronic illness. Humans usually get the infection from contact with infected animals and animal products, particularly milk and cheese [1]. Brucellosis manifestations both in the acute and chronic phases of the disease are mainly due to inflammatory phenomena.

A characteristic feature of pathological lesions in human brucellosis is the presence of perivascular leukocytic infiltrates in all the affected organs, which can be seen both in the acute and chronic phase of the disease [2]. Possibly due to the dissemination of the bacterium through blood circulation, the inflammatory response elicited by *Brucella* is accompanied by vasculitis, which can be observed in diverse localizations including the skin [3,4] and the brain [5,6]. While some authors have speculated that the pathogenesis of cutaneous vascular lesions is due to immune complexes [7], such complexes have been detected only in a few cases. Moreover, the isolation of *Brucella* from disseminated skin lesions in patients with systemic brucellosis [8] suggests that infection of cutaneous tissues, including blood vessels, may occur through hematogenous spread and is involved in the pathogenesis of such lesions. Since lesions exhibit leukocytic infiltrates (neutrophils, monocytes, and lymphocytes) and tissue necrosis, an inflammatory response elicited by the bacterium appears as a likely explanation for the vasculitis. During an inflammatory response the adhesion molecules of endothelial cells mediate the attachment of circulating leukocytes and their subsequent extravasation to perivascular tissues to reach the inflammatory focus. Therefore, the formation of perivascular infiltrates depends, at least in part, on adhesion molecules whose expression is activated by the

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doi:10.1016/j.micinf.2011.04.010

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infection itself or by proinflammatory cytokines produced by other cells [9]. Chemokines produced by endothelial cells also assist in the extravasation process [10].

Besides their potential important role in the proinflammatory immune response to *Brucella* infection, endothelial cells may be also involved in specific and severe vascular complications of brucellosis, namely infective endocarditis and mycotic aneurisms. Endocarditis is a relatively rare, but potentially fatal complication of brucellosis. While this condition occurs in 1%–2% of brucellosis cases, it constitutes the main cause of mortality among brucellosis patients [1]. Aortic and/or mitral valves are involved in most cases, and *Brucella* has been frequently isolated from the affected valves [11–13]. Mycotic aneurysms due to *Brucella* have been reported in patients with or without concomitant endocarditis. These lesions have been found to affect the abdominal aorta [14], the cerebral arteries [15,16], the superior mesenteric artery [17], etc. In some instances the infecting organism has been isolated from the resected aneurysm [14]. In spite of the severity of endocarditis or mycotic aneurysms due to *Brucella* the pathogenic mechanisms leading to these complications have not been explored.

The surface of cardiac valves is covered by endothelial cells, which seem to play a central role in the pathogenesis of endocarditis caused by several bacteria. Studies with animal models have shown that direct bacterial invasion of the endothelium and the endothelial proinflammatory response, including the secretion of chemokines that recruit monocytes, are involved in the pathological process [18,19]. In addition, it has been shown that the expression of several adhesion molecules is upregulated in endothelial cells of valves from patients with acute endocarditis [20].

Based on the knowledge acquired about infections by other bacteria, it can be speculated that the activation of the endothelium in response to *Brucella* infection, with the consequent up-regulation of adhesion molecules and the secretion of proinflammatory chemokines, may be important for the occurrence of vasculitis, endocarditis and mycotic aneurysms in brucellosis patients. Previous studies have shown that *Brucella abortus* can induce an inflammatory profile in human monocytes/macrophages, dendritic cells and neutrophils. Notably, this response was induced by *Brucella* lipoproteins rather than by its lipopolysaccharide (LPS) [21–24].

In spite of the potential role of the endothelium in the pathological phenomena of brucellosis mentioned above, there are not published studies on the capacity of *Brucella* to invade endothelial cells and to induce an inflammatory response in these cells. The elucidation of these aspects constituted the goal of the present study.

2. Materials and methods

2.1. 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 [25]. The lipidated and unlipidated forms of the 19 kDa outer membrane protein from *B. abortus* (L-Omp19 and U-Omp19, respectively) were obtained as described previously [21]. Both recombinant proteins contained less than 0.25 endotoxin U per µg of protein as assessed by Limulus Amebocyte Lysate Test (Associates of Cape Cod, East Falmouth, MA). Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL) using BSA as standard.

2.2. Bacterial strains and growth conditions

B. abortus 2308, *Brucella suis* 1330 and *B. abortus* RB51 were grown overnight in tryptic soy broth (TSB), harvested by centrifugation, and washed twice in phosphate-buffered saline (PBS). Bacterial numbers in cultures were estimated by comparing the OD at 600 nm with a standard curve, but the actual concentration of inocula was checked by plating on tryptic soy agar (TSA). All live *Brucella* manipulations were performed in biosafety level 3 facilities located at the Centro Nacional de Referencia del Sida, School of Medicine, University of Buenos Aires.

2.3. Cell culture

The human microvascular endothelial cell line HMEC-1 was obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, USA). Cells were grown in MCDB131 medium (Invitrogen, Carlsbad, CA) containing 10 ug/ml hydrocortisone, 1 ng/ml epidermal growth factor (BD Pharmingen, San Jose, CA), 10% fetal calf serum, 2 mM L–glutamine, 100 U/ml penicillin and 100 mg/µl streptomycin.

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase perfusion as described by Jaffe et al. [26] with modifications. Briefly, the umbilical vein was cannulated and perfused with 0.1% collagenase, and the umbilical cord was incubated for 15 min at 37 °C. After a soft massage of the umbilical cord, the cell suspension was supplemented with 10% FCS, centrifuged and resuspended in culture medium (RPMI supplemented with 10% FCS, 2 mM glutamine, 45 µg/ml heparin, 19 µg/ml endothelial cell growth factor supplement, and antibiotics). The suspension was transferred to culture bottles coated with 0.2% gelatin and incubated at 37 °C in a 5% CO_2 atmosphere, with regular medium changes. After 3–5 days, cells from confluent cultures were trypsinized, pooled, and passaged onto microtiter plates. Cultured cells were identified as being endothelial by their morphology and the expression of von Willebrand Factor as assessed by flow cytometry. HUVEC were used in experiments at passages 1 to 3.

2.4. Cellular infections

For infection assays, endothelial cells were seeded at 5×10^5 cells/well in 24-well plates and cultured in a 5% CO_2 atmosphere at 37 °C for 24 h in antibiotic-free culture medium. *Brucella* infections were performed at a multiplicity of infection (MOI) of 100 and 1000. At the end of the incubation time (time

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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 of gentamicin (Sigma, USA) and 50 ug/ml of streptomycin (Sigma) to kill extracellular bacteria. At different times after antibiotics addition culture supernatants were harvested for cytokine measurement by ELISA. The infected cells were washed with sterile PBS and treated for 10 min with 0.2% Triton X-100 (Sigma). The lysates were serially diluted and plated on TSA to enumerate colony forming units (CFU).

2.5. Stimulation with antigens

Endothelial cells (5×10^5 cell/ml) cultured in 24-well plates were stimulated with different *B. abortus* antigens (LPS, L-Omp19 and U-Omp19) at the doses indicated in each figure, or with LPS from *E. coli* (1 ng/ml). Cultures were incubated for 24 h. At the end of the culture, supernatants were harvested, aliquoted and stored at −70 °C until they were analyzed for cytokine content.

2.6. Measurement of cytokine concentrations

The content of human IL-6, IL-8 and MCP-1 in culture supernatants from *Brucella*-infected or antigen-stimulated cells were measured by sandwich ELISA using paired cytokine-specific mAbs, according to the manufacturer's instructions (BD Biosciences).

2.7. Analysis of adhesion molecules expression

HMEC-1 and HUVEC cells were infected at MOI 100 or 1000 with *B. abortus* or stimulated with *B. abortus* antigens as described above. At the end of culture, cells were detached from culture wells with trypsin-EDTA, washed and incubated for 30 min on ice with phycoerythrin-labeled antibodies against human CD54 (ICAM-1), CD106 (VCAM-1) or CD62E (E-selectin), or with isotype-matched control antibodies (all from BD Pharmingen, San Jose, CA). Cells were then washed, fixed with 4% paraformaldehyde, 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).

2.8. Neutrophils isolation

Human neutrophils were isolated from venous blood of normal human volunteers by Ficoll−Paque (GE Healthcare) gradient, followed by sedimentation of erythrocytes in 6% dextran and hypotonic lysis. The harvested neutrophils were washed twice with sterile PBS and resuspended at 1×10^6 cells/ml in RPMI 1640 supplemented with 5% FBS and 1 mM glutamine (Gibco-BRL Life Technologies). Cell viability was >98%, as determined by trypan blue exclusion. The purity of the final neutrophil preparation was >95% as determined by morphological examination with Giemsa staining and flow cytometry light scatter patterns.

2.9. Transendothelial migration of neutrophils

HMEC-1 cells were cultured on fibronectin-coated Transwell inserts (6.5 mm diameter polycarbonate membrane with 3 um pores; Costar) at a density of 1×10^5 cells per insert. Monolayers formed within 2−3 days. The formation of a polarized confluent monolayer with adequate intercellular junctions was determined by measuring electrical resistance changes across the endothelial cell monolayer using a volt-ohmmeter (Millicell ERS-2, Millipore Corporation, Billerica, MA). The monolayers were used when the transendothelial electrical resistance was around 40 Ω cm^2. The monolayers were infected with *B. abortus* at MOI 100 and 1000 and then incubated with antibiotics as described above. At 48 h p.i. the medium in the upper chamber was removed and was replaced with a suspension of normal human neutrophils (1×10^6 cells in 100 μl) in complete culture medium. The plates were incubated for 2.5 h at 37 °C under a 5% CO_2 atmosphere. At the conclusion of the incubation, the medium in the lower chamber was harvested for counting migrated cells in a hemacytometer. Results were expressed as fold increase relative to the transmigration rate of neutrophils through uninfected endothelial cells, which was assigned a value of 1. As a positive control, endothelial cells were incubated with TNF-α (25 ng/ml) for 4 h before adding the neutrophils, with a washing step in between.

The ability of *B. abortus* to promote transendothelial migration of neutrophils through stimulation of the endothelial cells by the basolateral side was also examined, using a procedure similar to that of Moreland et al. [27]. Transwell filters with HMEC-1 monolayers were transferred to clean 24-well plates, and washed twice with PBS before adding 100 μl of supplemented MCDB131 medium to the upper compartment. Suspensions of live *B. abortus* at different concentrations were added to the lower compartment in a total volume of 600 μl and left in contact with the endothelial cells for 3 h at 37 °C. At the end of this incubation the medium of the upper chamber was replaced with a suspension of neutrophils (1×10^6 cells in a volume of 100 μl). After incubating for 2.5 h at 37 °C, migrated cells were collected from the lower chamber for counting in a hemacytometer. Results were expressed as described above.

2.10. Statistical analysis

Data were analyzed using analysis of variance (ANOVA). Multiple comparisons between groups were made with the Tukey's post-test, and those against a control group were made with the Dunnett's post-test. All the statistical analyses were performed with the GraphPad software (San Diego, CA).

3. Results

3.1. Brucella invades and replicates intracellularly in human endothelial cells

To determine whether *Brucella* can adhere to human endothelial cells, HMEC-1 cells were incubated for 2 h with smooth (*B. abortus* 2308 and *B. suis*) and rough strains

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(*B. abortus* RB51) of *Brucella* at MOI 100. After washing to eliminate unbound bacteria, some wells were treated with antibiotics to kill extracellular bacteria and some wells were not. Cells lysates were obtained and plated in each case, and adherence was calculated as the difference in CFU between wells not treated and those treated with antibiotics. As shown in Fig. 1A, both smooth and rough strains of *Brucella* adhered in high numbers to endothelial cells, although the adherence of the rough strain RB51 was higher.

All the *Brucella* strains assayed were also able to invade endothelial cells, but the number of intracellular bacteria at 2 h p.i. was significantly higher ($p < 0.01$) for the rough strain RB51 than for the smooth strains (mean values: 8.61×10^5, 1.32×10^5 and 1.01×10^5 CFU/well for *B. abortus* RB51, *B. abortus* 2308 and *B. suis*, respectively) (not shown). In addition, as shown in Fig. 1B, the number of bacteria internalized at 2 h p.i. increased with the MOI used. The invasion capacity of each *Brucella* strain was calculated as the percentage of adhered bacteria that was internalized by endothelial cells at 2 h p.i. In spite of the higher adherence of the rough strain, no significant differences in invasion capacity were found between this strain and the smooth ones ($4.6 \pm 0.8\%$, $4.4 \pm 1.1\%$, and $5.8 \pm 0.6\%$ for *B. abortus* RB51, *B. abortus* 2308 and *B. suis*, respectively). While both smooth and rough strains invaded endothelial cells, only the smooth strains replicated efficiently inside these cells (Fig. 1C). At 48 h p.i., the number of intracellular CFU of smooth strains was about 1000 times higher than the number recorded at 2 h p.i.

The ability of *Brucella* to invade and replicate in human endothelial cells was corroborated with primary cultures (HUVEC). As shown in Fig. 1D, in this case the number of intracellular CFU of *B. abortus* 2308 was also about 1000 times higher at 48 h p.i. than at 2 h p.i., evidencing replication.

3.2. Brucella infection induces cytokines, chemokines and adhesion molecules in human endothelial cells

Cell culture supernatants from endothelial cells were harvested at 24 h p.i. to measure the levels of proinflammatory cytokines and chemokines produced by these cells in response to *B. abortus* infection. As shown in Fig. 2, infection at MOI 100 or MOI 1000 elicited a significant production of IL-8, MCP-1 and IL-6 in both HUVEC and HMEC-1 cells. For both cell lines the levels detected in supernatants of *Brucella*-infected cells at 24 h p.i. were significantly higher than those measured in uninfected cells (except for MCP-1 in HUVEC infected at MOI 100).

The impact of *Brucella* infection on the expression of adhesion molecules in endothelial cells was also analyzed. As shown in Fig. 3, the expression of CD106, CD54 and CD62E was significantly increased in *Brucella*-infected HUVEC cells as compared to uninfected cells. The infection also induced

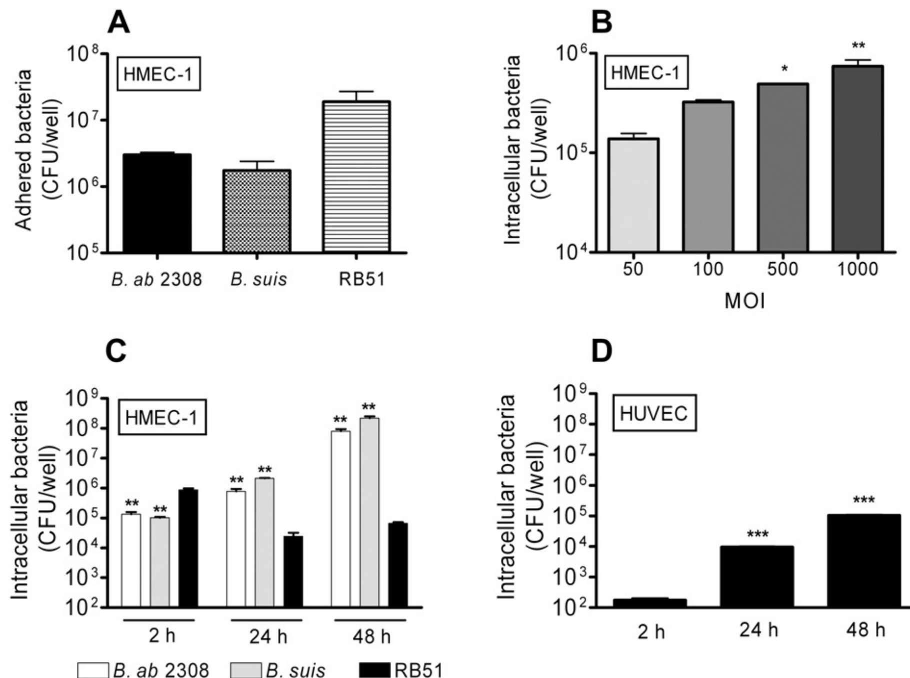

Fig. 1. Infection of human endothelial cells by *Brucella*. (A) Adherence of *B. abortus* 2308, *B. suis* and *B. abortus* RB51 (RB51) to the human endothelial cell line HMEC-1. Endothelial cells were infected with *Brucella* at a multiplicity of infection (MOI) of 100, and the number of viable intracellular bacteria was determined with the gentamicin protection assay. The number of adherent extracellular bacteria was calculated by subtracting the number of intracellular bacteria from the total count obtained in the absence of antibiotics. (B) Invasion of HMEC-1 by *B. abortus* 2308. Cells were incubated for 2 h with *B. abortus* at different MOI and intracellular bacteria were determined as described above. (C) Intracellular replication of different strains of *Brucella* in HMEC-1. After infection with the different strains at MOI 100, the number of intracellular bacteria was measured at different times post-infection. (D) Intracellular replication of *B. abortus* 2308 in HUVEC cells. After infection at MOI 100 intracellular bacteria were measured at different times p.i. Data presented in all panels are mean ± SEM of CFU. Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between groups, as follows: Panel B, all groups versus MOI 50; Panel C, *B. abortus* 2308 and *B. suis* versus *B. abortus* RB51; Panel D, 24 h and 48 h versus 2 h (ANOVA followed by Tukey's Multiple Comparison test).

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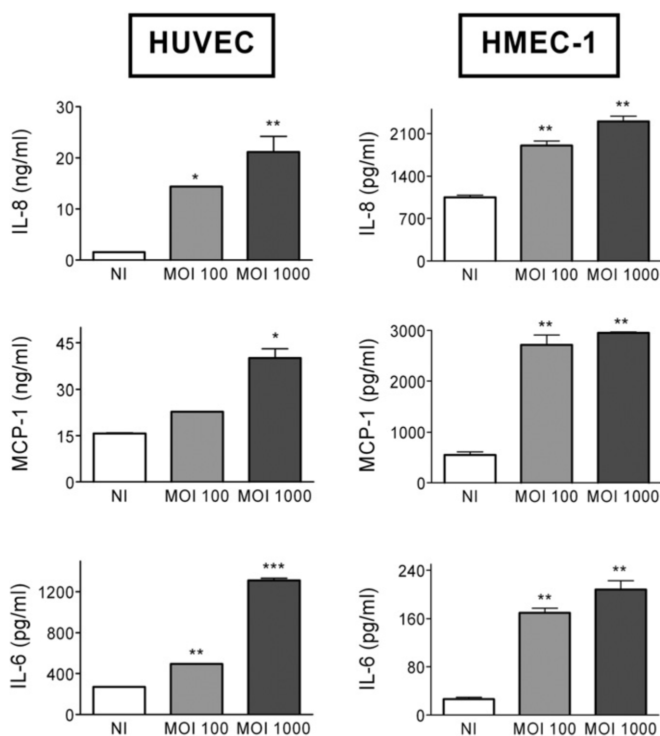

Fig. 2. Induction of cytokines in human endothelial cells in response to *Brucella* infection. HMEC-1 and HUVEC cells were infected with *B. abortus* 2308 at a MOI of 100 or 1000, and supernatants were harvested at 24 h p.i. to measure the levels of IL-8, MCP-1 and IL-6 by commercial capture ELISAs. Data are mean ± SEM of cytokine levels measured in triplicate in one experiment, which was repeated twice with similar results. Asterisks indicate levels significantly higher (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$) than those produced by uninfected endothelial cells (white bars) (ANOVA followed by Tukey's Multiple Comparison test).

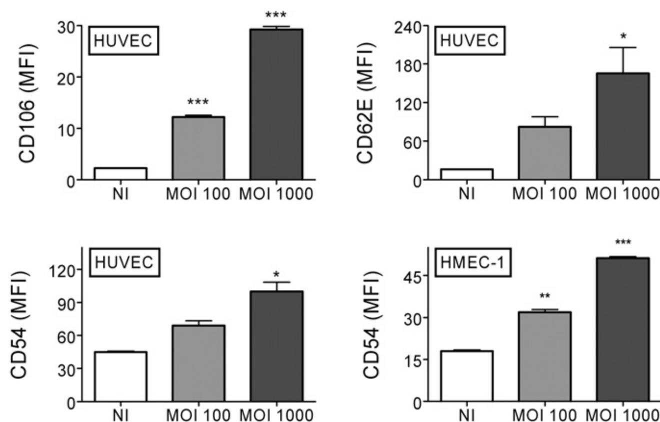

Fig. 3. Induction of adhesion molecules in human endothelial cells in response to *Brucella* infection. HMEC-1 and HUVEC cells were infected with *B. abortus* 2308 at a MOI of 100 or 1000, and the expression of adhesion molecules was determined by flow cytometry in cells harvested at 6 h p.i. (CD106 and CD62E) or 24 h p.i. (CD54). Results are expressed as mean fluorescence intensity (MFI). Data are mean ± SEM of expression levels measured in duplicate in one experiment, which was repeated twice with similar results. Asterisks indicate expression levels significantly higher (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$) than those of uninfected endothelial cells (white bars) (ANOVA followed by Tukey's Multiple Comparison test).

significantly the expression of CD54 in HMEC-1 cells (Fig. 3), but no significant increase of CD106 and CD62E was observed (not shown). When induction was observed, the magnitude of the expression was directly related to the MOI used. Overall, these results show that *B. abortus* infection induces a proinflammatory response in human endothelial cells, as evidenced by an increased secretion of cytokines and chemokines, and the up-regulation of adhesion molecules.

3.3. Brucella lipoproteins mediate proinflammatory effects on endothelial cells

Given the ability of *B. abortus* infection to induce a proinflammatory response in human endothelial cells, experiments were performed to determine whether such response could be elicited by structural components of the bacterium. Previous studies have shown that *Brucella* lipoproteins can induce proinflammatory responses in several cell types from human or murine origin [21−24]. These studies also showed that the lipidic motif of lipoproteins is necessary to mediate this effect, since the unlipidated proteins do not induce a proinflammatory response. In this study we used as a model the lipidated and unlipidated versions of the 19 kDa lipoprotein of *B. abortus* (L-Omp19 and U-Omp19) to examine whether lipoproteins are involved in the inflammatory response of endothelial cells to *Brucella*. As shown in Fig. 4 both HUVEC and HMEC-1 cells responded in a dose-dependent manner to stimulation with L-Omp19 with the secretion of IL-8, MCP-1 and IL-6. In contrast, these responses were not elicited by comparatively high doses of U-Omp19.

Stimulation of HUVEC with L-Omp19 also resulted in an increased expression of all the adhesion molecules analyzed, and this induction occurred in a dose-dependent manner (Fig. 5). This lipoprotein also induced the expression of CD54 in HMEC-1 cells. As shown in the figure, the unlipidated version of Omp19 did not elicit a significant increase of any adhesion molecule in either HUVEC or HMEC-1 endothelial cells. Overall, these results show that L-Omp19 is involved in the capacity of *Brucella* to induce the expression of cytokines and adhesion molecules in human endothelial cells.

3.4. Brucella LPS does not mediate the proinflammatory response of endothelial cells to the bacterium

Another abundant molecule in the surface of *Brucella* is the LPS. To assess the potential contribution of LPS to the proinflammatory response of endothelial cells during *Brucella* infection, HUVEC and HMEC-1 cells were incubated with LPS from *B. abortus*. LPS from *E. coli* was included as a comparator given its known ability to induce inflammatory responses in several cell types. As shown in Fig. 4, *E. coli* LPS used at 1 ng/ml induced a significant secretion of IL-8, MCP-1 and IL-6, whereas the LPS from *B. abortus* had no significant effect even at a dose 1000 times higher. Results were similar for both primary endothelial cells (HUVEC) and the endothelial cell line (HMEC-1). In agreement with the results obtained for the infection, the magnitude of the cytokine

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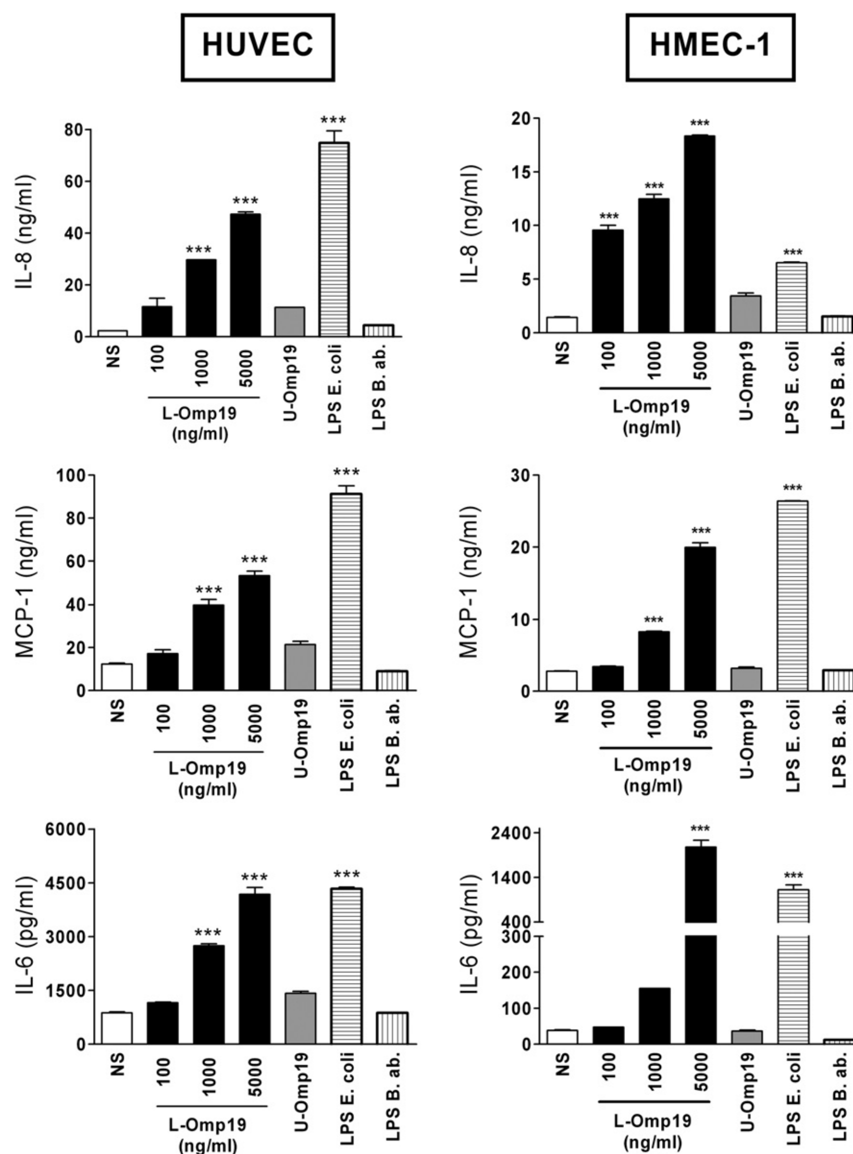

Fig. 4. Induction of cytokines in human endothelial cells in response to stimulation with *B. abortus* antigens. HMEC-1 and HUVEC cells were stimulated with either lipidated (L) Omp19 (100–5000 ng/ml), unlipidated (U) Omp19 (5000 ng/ml), LPS from *B. abortus* (1000 ng/ml) or LPS from *E. coli* (1 ng/ml) as a control. Supernatants were harvested at 24 h post-stimulation to measure the levels of IL-8, MCP-1 and IL-6 by commercial capture ELISAs. Data are mean ± SEM of cytokine levels measured in triplicate in one experiment, which was repeated twice with similar results. Asterisks indicate levels significantly higher (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$) than those produced by non-stimulated (NS) endothelial cells (white bars) (ANOVA followed by Tukey's Multiple Comparison test).

response tended to be higher for HUVEC than for HMEC-1 cells. As in the case of cytokines, the expression of all the adhesion molecules measured was significantly induced by *E. coli* LPS but not by LPS from *B. abortus* (Fig. 5). Overall, these results show that LPS is not involved in the capacity of *Brucella* to induce the expression of cytokines and adhesion molecules in human endothelial cells.

3.5. Endothelial activation by Brucella *promotes the transmigration of neutrophils through a polarized endothelium*

Proinflammatory endothelial activation usually contributes to the recruitment of phagocytes to the site of infection or inflammation. Taking into account that *Brucella* infection leads to an increased expression of adhesion molecules and proinflammatory chemokines in endothelial cells, experiments were carried out to determine whether the *Brucella*-infected endothelium has an enhanced capacity to promote the transmigration of neutrophils. Polarized monolayers of HMEC-1 cells were grown on Transwell membranes and were either infected with *B. abortus* from the apical side (upper chamber) or put in contact with live *B. abortus* through the basolateral side (lower chamber) before adding a suspension of normal human neutrophils on the apical side. The number of neutrophils that migrated to the lower chamber was measured 2.5 h later. Results were expressed as fold increase compared to untreated endothelial cells. As shown in Fig. 6A, *Brucella*

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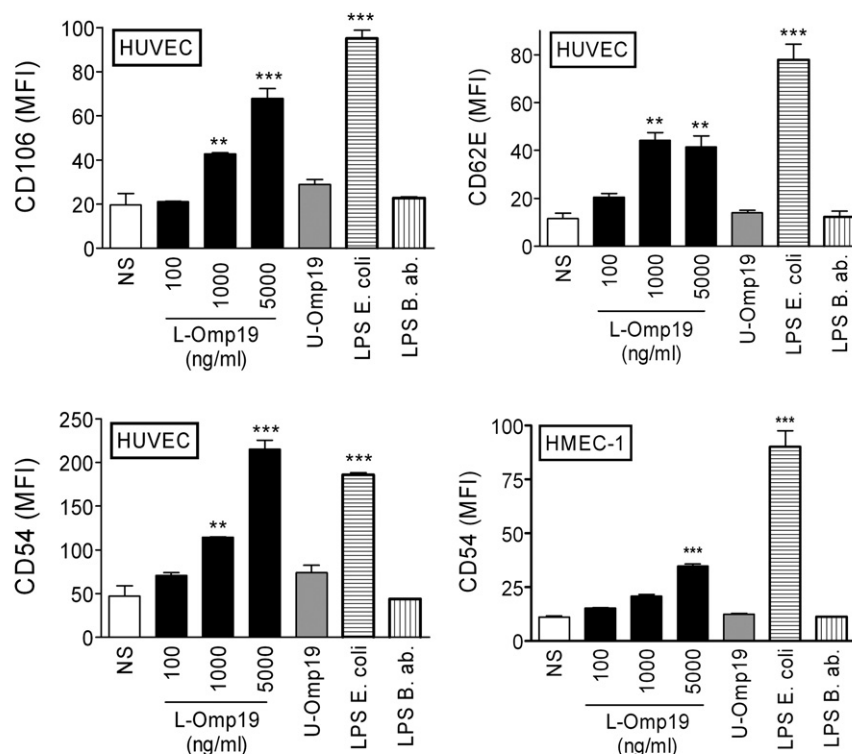

Fig. 5. Induction of adhesion molecules in human endothelial cells in response to stimulation with *B. abortus* antigens. HMEC-1 and HUVEC cells were stimulated with either lipidated (L) Omp19 (100–5000 ng/ml), unlipidated (U) Omp19 (5000 ng/ml), LPS from *B. abortus* (1000 ng/ml) or LPS from *E. coli* (1 ng/ml) as a control. The expression of adhesion molecules was determined by flow cytometry in cells harvested at 6 h (CD106 and CD62E) or 24 h (CD54) post-stimulation. Data are mean ± SEM of expression levels measured in duplicate in one experiment, which was repeated twice with similar results. Asterisks indicate expression levels significantly higher (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$) than those of non-stimulated (NS) endothelial cells (white bars) (ANOVA followed by Tukey's Multiple Comparison test).

infection of endothelial cells at MOI 100 and MOI 1000 increased the transmigration of neutrophils by about 5-fold and 8-fold, respectively, as compared to the uninfected endothelium. Similarly, the stimulation of the endothelium from the basolateral side with live *B. abortus* at 1×10^6, 1×10^7 and 1×10^8 CFU/ml (equivalent to MOI 10, 100 and 1000) increased the transmigration of neutrophils by about 2-fold, 16-fold and 20-fold, respectively (Fig. 6B). These results confirm that the increased expression of adhesion molecules and chemokines in endothelial cells upon *Brucella* infection or stimulation results in an increased transmigration of neutrophils through the polarized endothelium.

4. Discussion

The endothelium serves regulatory functions in hemostasis, vascular tone and permeability, angiogenesis, and inflammation. Regarding this last function, the activation of the endothelium in response to antigens from invading pathogens is essential to allow the transmigration of phagocytic leukocytes to the infection site. This activation is characterized by the increased surface expression of adhesion molecules and the increased secretion of chemokines, which work together to attract leukocytes to the infected tissue. Several *in vitro* studies have shown that human endothelial cells respond to bacterial infection or to stimulation with bacterial antigens with an increased secretion of proinflammatory cytokines and chemokines, and an augmented surface expression of cellular adhesion molecules [28–31]. Furthermore, in some cases this activation has been evidenced by the increased capacity of endothelial cells to mediate the transmigration of phagocytes [30,31]. In agreement with these studies, our results showed that HUVEC and HMEC-1 cells respond to *Brucella* infection, and also to some *Brucella* antigens, with the secretion of chemokines (IL-8 and MCP-1) and proinflammatory cytokines (IL-6), and the up-regulation of adhesion molecules (ICAM-1, VCAM-1, CD62E). Moreover, we found that these changes correlate with an increased capacity of endothelial cells to promote the transmigration of neutrophils. This suggests that the interaction of *Brucella* with the endothelium during *in vivo* infection may result in an increased transmigration of phagocytes to the infected tissues. This may explain the presence of perivascular leukocytic infiltrates in all the affected organs during both the acute and the chronic phase of human brucellosis [2].

While the physiological function of phagocytes is to eliminate the invading pathogen, a sustained recruitment and activation of these cells may result in tissue damage due to the action of lytic enzymes and other deleterious substances released by phagocytes. Such sustained recruitment and activation may occur when the infection cannot be eradicated and induces a long lasting proinflammatory response. When the

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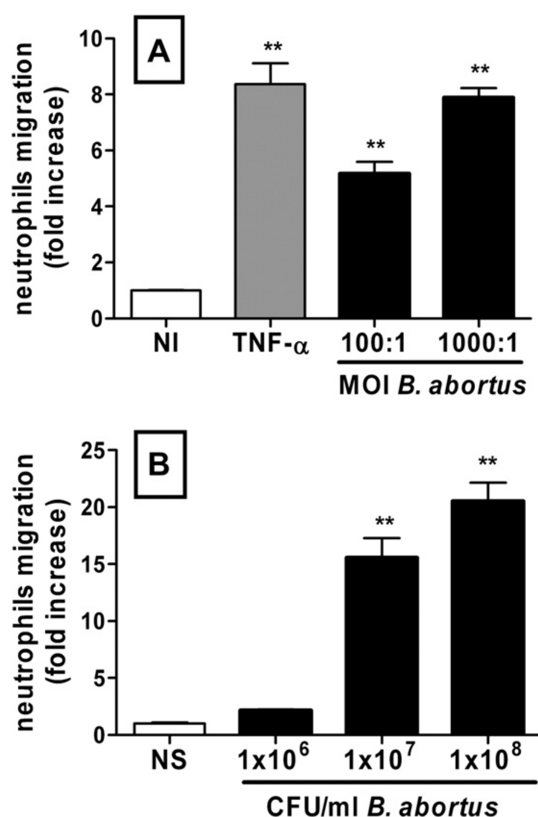

Fig. 6. Transmigration of human neutrophils through endothelial cells infected (A) or incubated (B) with *B. abortus*. Polarized monolayers of HMEC-1 cells grown on Transwell membranes were infected with *B. abortus* or incubated with 25 ng/ml of TNF-α (positive control) from the apical side (upper chamber), or were incubated with live *B. abortus* at the basolateral side (lower chamber) before adding a suspension of normal human neutrophils in the upper chamber. The number of neutrophils that migrated to the lower chamber was measured 2.5 h later. Results were expressed as fold increase compared to untreated endothelial cells. Data are mean ± SEM of values measured in duplicate in one experiment, which was repeated twice with similar results. Asterisks indicate a significant (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$) fold increase in migration compared to the migration through uninfected (A) or unstimulated (B) endothelial cells (white bars) (ANOVA followed by Dunnett's Multiple Comparison test).

permanently infected tissue is the endothelium itself, the sustained inflammatory process may derive in complications such as vasculitis or endocarditis. It has been shown that pathogens such as *Staphylococcus aureus, Rickettsia* spp., *Orientia tsutsugamushi*, *Bartonella henselae*, and *Mycobacterium tuberculosis* [28,29,32−34] can infect and in some cases replicate intracellularly in human endothelial cells. In the present study we found that different *Brucella* strains adhere to and invade HUVEC and HMEC-1 cells, and that smooth strains undergo intracellular replication as shown by a 1000-fold increase of intracellular CFU in the first 48 h after infection. These data are in line with those obtained in other non-phagocytic cells, including epithelial cells and osteoblasts, in which smooth but not rough *Brucella* strains exhibited intracellular replication [35,36]. As in the case of these non-phagocytic cells, the initial magnitude of the infection in endothelial cells was modest, but the high rate of intracellular replication resulted in a greatly increased bacterial load at 48 h p.i. While a long lasting infection of endothelial cells by *Brucella* has not been demonstrated *in vivo*, the ability of smooth strains to survive and replicate intracellularly in endothelial cells (as shown in the present study) and the well known ability of *Brucella* to establish chronic infections in humans and animals suggest that such prolonged endothelial infection may occur in some cases. Interestingly, the three *Brucella* smooth species involved in most human cases of brucellosis, namely *B. abortus, B. suis* and *Brucella melitensis*, have been shown to produce vascular complications [13,17,37−39].

As mentioned above, a sustained infection may induce a long lasting proinflammatory response due to the continuous antigenic stimulus of immune system receptors. Studies performed in animal models, on pathology specimens, and *in vitro* indicate a central role of the proinflammatory response mounted by the infected endothelium in the development of infective endocarditis. It is estimated that this pathology can be initiated in intact endothelium by the adhesion and internalization of certain pathogens, which induce an important inflammatory response and also a procoagulant state that is essential for vegetation growth [40]. Notably, it has been found that the expression of proinflammatory chemokines is increased in valves from clinical cases of infective endocarditis [41]. In vitro studies and histological findings also suggest that both the proinflammatory and procoagulant state are further increased by the action of phagocytes recruited to the lesion in response to secreted and surface-expressed endothelial molecules upregulated during infection.

The proinflammatory response of the endothelium, including the enhanced transmigration of phagocytes, has been also proposed to be involved in vasculitis induced by bacteria that can invade endothelial cells, such as rickettsiae and *S. aureus* [42−44]. Therefore, it can be speculated that the inflammatory phenomena mounted by the *Brucella*-infected endothelium may have a role in the pathogenesis of endocarditis and vasculitis observed in some brucellosis cases.

As mentioned above, endothelial cells are activated not only by infection but also by the recognition of antigens from diverse types of pathogens. Bacterial antigens known to induce a proinflammatory response in human endothelial cells include LPS and OMPs (including lipoproteins), among others [30,31,45,46]. At variance with most Gram-negative bacteria *Brucella* possesses an atypical LPS that has been shown to be virtually devoid of proinflammatory activity in different cell types. In contrast, lipoproteins were found to be responsible for the inflammatory phenomena elicited by *Brucella* in those cell types [21−24]. In agreement with those previous studies, we found that the induction of proinflammatory cytokines and chemokines, and adhesion molecules in human endothelial cells is induced by *Brucella* lipoproteins (Omp19) and not by its LPS. Also in agreement with those reports, only the lipidated form of Omp19 induced these proinflammatory responses. This later observation also agrees with studies that showed that only the lipidated form of lipoproteins from *Borrelia burgdorferi* and *S. aureus* can induce proinflammatory responses in endothelial cells [30,46].

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The induction of adhesion molecules and chemokines in *Brucella*-infected or stimulated endothelial cells could be a direct effect of antigen recognition or could be mediated by the autocrine action of cytokines produced by the infected cells. This later situation seems unlikely in the present case, since the two main cytokines that activate the endothelium, namely IL-1β and TNF-α [47,48], were not detected in the supernatants of *Brucella*-infected endothelial cells (not shown). A similar situation has been reported for the production of IL-8 by endothelial cells in response to *B. burgdorferi*, which was also independent of IL-1β or TNF-α stimulation [49].

The repertoire of adhesion molecules induced by *Brucella* infection or by stimulation with *Brucella* antigens differed between HUVEC and HMEC-1 cells. While the three adhesion molecules here studied (CD54, CD106 and CD62E) were induced in HUVEC, only CD54 was induced in HMEC-1 cells. These results are in line with a previous report showing that HMEC-1 cells exhibit a reduced or null response of CD106 and CD62E to TNF-α stimulation as compared to HUVEC [50]. Interestingly, despite the restricted response of HMEC-1 cells to *Brucella* infection or antigen stimulation, these cells exhibited an enhanced capacity to mediate neutrophil transmigration. Therefore, this model of polarized endothelium was useful to demonstrate the functional consequence of endothelial activation by *Brucella* infection.

Our results showed that an enhanced migration of neutrophils from the apical side (luminal) to the basolateral side (abluminal) of the endothelium can be induced not only by the infection of the endothelium with *Brucella* from the apical side but also by its stimulation with bacteria from the basolateral side. The first situation may correspond to an intravascular route of infection, which may occur during the bacteriemic phase of brucellosis. The second situation, i.e. the interaction with the endothelium from the basolateral side, is likely to occur when the bacterium infects perivascular tissues. While this second scenario is common to many infectious processes and usually contributes to the resolution of the infection through the enhanced recruitment of phagocytes, the outcome may be different in the case of *Brucella* infection taking into account the potential of the bacterium to infect adjacent endothelial cells and its capacity to establish chronic infections. In the two scenarios (intravascular and perivascular origin of the infection), the sustained endothelial activation and proinflammatory response may lead to vascular complications.

In summary, the present study shows that smooth *Brucella* species invade and replicate within human endothelial cells, in which they induce a proinflammatory response characterized by the enhanced secretion of chemokines and the increased surface expression of adhesion molecules. These responses are functionally expressed as an increased ability of the infected endothelium to mediate the transmigration of neutrophils. Further studies are warranted to determine whether this proinflammatory response is involved in the vascular manifestations of human brucellosis.

Acknowledgements

We are grateful to Dr. Ignacio Moriyón (University of Navarra, Spain) for providing LPS from *B. abortus* and *E. coli*. We thank the staff of the Centro Nacional de Referencia del Sida, University of Buenos Aires, for their assistance with biosafety level 3 laboratory use. We also thank Dr. Roxana Toriano (University of Buenos Aires) for her help with transendothelial electrical resistance measurements.

This work was supported by grants 2006-0517, 2006-1335 and 2008-0764 from Agencia Nacional de Promoción Científica (ANPCYT), and grant UBACYT B015 and 20020090100083 from Universidad de Buenos Aires. M.C.F. is recipient of a fellowship from Consejo Nacional de Investigaciones Científicas (CONICET). M.V.D., P.B., C.A.F., G.H.G. and P.C.B. are members of the Research Career of CONICET.

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