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Original article

Brucella abortus activates human neutrophils

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Abstract

Human brucellosis is caused by infection with certain species of the genus *Brucella* and is characterized by bacterial persistence and inflammation of many host tissues. Neutrophils are one of the predominant cell types present in the infiltrate of these inflamed tissues, and due to their potential effect on the inflammatory response and tissue damage, direct activation of neutrophils by *Brucella abortus* might contribute to the pathology associated with human brucellosis. *B. abortus* expresses outer membrane lipoproteins (Omp) with inflammatory properties on a variety of cell types. This study examines the effect of *B. abortus* and its lipoproteins on neutrophil functions. *B. abortus* induced an increment in CD35 and CD11b expression and a decline in CD62L accompanied by IL-8 secretion, a response compatible with neutrophil activation. *B. abortus* lipoprotein Omp19 (L-Omp19), but not its unlipidated form, mimicked the changes associated with neutrophil activation induced by *B. abortus*. L-Omp19 primed neutrophils for oxidative burst as well as promoted neutrophil migration and prolonged neutrophil survival. Thus, *Brucella* lipoproteins possess pro-inflammatory properties that could contribute to the localize tissue injury and inflammation by direct activation of neutrophils. Data presented here, together with our previous results implicate *Brucella* lipoproteins in the pathogenesis of human brucellosis.

Keywords: *Brucella*; Brucellosis; Neutrophils; Lipoproteins

1. Introduction

Human brucellosis is a systemic febrile illness with a plethora of somatic complaints resulting from infection with *Brucella* species that are pathogenic to man: *Brucella melitensis*, *Brucella suis*, *Brucella abortus* and *Brucella canis* [1]. Inflammation is present in all stages of the disease and it is evident in all *Brucella* affected tissues, e.g. in the form of endocarditis, arthritis, meningitis, nephrithis, etc. Focal forms of brucellosis are present in approximately 30% of the patients being osteoarticular complications the most frequent (~70% of focal forms) [2], which may affect peripheral joints, sacroiliac joints or the spine [3]. As occurs with other tissue infections, persistence of the disease in the infected joint may result in tissue damage. Joint damage is often associated with the presence *of Brucella* organisms in these joints, as evidence by the recovery of bacterium from the synovial fluid of patients [4], demonstrating that the skeletal symptoms are caused by a true infection of the joint.

Synovial tissue characteristically reveals a cellular infiltrate mainly composed of monocytes and neutrophils [4,5]. It has also been reported the presence of neutrophilic infiltrates and bacteria in other sites of focalized disease [6,7]. Besides microbicidal function, neutrophil secrete a variety of mediators [8] capable of inducing an inflammatory response within the joint, and therefore neutrophil activation could support a range of pathological events, being the cells with the greatest ability to cause severe damage within diseased joints [8].

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Despite the diversity of signs and symptoms of brucellosis, the inflammatory trait of the disease; present both in the acute and chronic phases of human brucellosis; together with the detection of bacterium in the inflamed tissues, suggests that *Brucella* stimulates a robust inflammatory response at sites of localization. The bacterial constituents able to induce this response are still under investigation. Although having a low-activity LPS [9,10], *Brucella* possess lipoproteins with strong stimulatory effects on a variety of cells. In previous works, we have demonstrated that *B. abortus* lipoproteins are the molecules that confer the bacteria potent cytokine mediated inmunostimulatory properties in monocytes, macrophages and dendritic cells (DCs) [9,10], which could explain the correlation between tissue invasion and localized inflammation. Although some studies have been conducted in relation to the ability *of Brucella* to survive inside human neutrophils [11,12], the capacity of this bacterium to activate these cells endowing them with a potential role in tissue injury and inflammation has not been studied. Therefore, in the present study we evaluate if *B. abortus* and its lipoproteins may also affect the response of the other predominant cells present in cellular infiltrates of infected tissues, the neutrophils.

2. Materials and methods

2.1. Bacteria

B. abortus S2308 and *Escherichia coli* strain 11105 (ATCC) were cultured in tryptose–soy agar supplemented with yeast extract (Merck). Bacterial numbers were determined as described [9]. To obtain heat-killed *B. abortus* (HKBA), bacteria were washed five times for 10 min each in PBS and heat-killed by boiling for 20 min. Absence of *B. abortus* viability subsequent to heat-killing was verified by the absence of bacterial growth.

2.2. Lipoproteins and LPS

Lipidated Omp19 (L-Omp19) and unlipidated Omp19 (U-Omp19) were obtained as described [9]. Both recombinant proteins contained less than 0.25 endotoxin U/mg of protein as assessed by Limulus amebocyte assay (Associates of Cape Cod). *E. coli* O111k58H2 LPS were provided by I. Moriyon. The synthetic lipohexapeptide (tripalmitoyl-*S*-glyceryl-Cys-Ser-Lys4-OH [Pam_3Cys]) was purchased from Boehringer Mannheim.

2.3. Neutrophil isolation

Human neutrophils were isolated from venous blood by Ficoll-Paque (GE Healthcare) gradient followed by sedimentation of erythrocytes in 6% dextran and hypotonic lysis. Next, neutrophils were harvested, washed twice with phosphate buffer saline (PBS) and resuspended in RPMI 1640 supplemented with 5% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco-BRL Life Technologies) at a cell concentration of 1×10^6 cells/ml. 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.

2.4. Flow cytometry

Neutrophils (1×10^6 cells/ml) were incubated at 37 °C for different times with the indicated concentrations of live *B. abortus, E. coli,* HKBA, *E. coli* LPS, Pam3Cys, L-Omp19 or U-Omp19. PE-conjugated mAbs to CD11b, CD62L and CD35 were obtained from BD Pharmingen. After fixing and staining, cells were analyzed with a FACScan flow cytometer (Becton–Dickinson). Gating of neutrophils was based on light scatter properties. Data were processed using the CellQuest software (Becton–Dickinson).

2.5. Cytokine ELISA

IL-8 was quantified by ELISA (BD Pharmingen) according to manufacturer's instructions.

2.6. Assessment of oxidative burst

Purified neutrophils (1×10^6 cells/ml) were incubated for 30 min at 37 °C in the presence of the indicated priming stimulus. Dihydrorhodamine 123 (DHR, Invitrogen) (1 mM) was added and cells were incubated for other 15 min. PMA (5 ng/ml) was then added for 15 min to induce oxidative burst. Cells were immediately analyzed by flow cytometry. Events were acquired, gated for analysis of intact cells, and analyzed for emitted green fluorescence as previously described [13].

2.7. Apoptosis assays

Neutrophils (1×10^6 cells/ml) were incubated with the indicated stimulus for 6, 24, 48 or 72 h. Cells were washed and the percentage of apoptotic neutrophils was assessed by the Annexin V-FITC (AV; Sigma–Aldrich) assay and FACs analysis. AV and propidium iodide (PI) were added to cells according to manufacturers instructions. Cells that were AV^+/PI^- were in early apoptosis, and cells that were AV^+/PI^+ were in late apoptosis.

2.8. Chemotactic assay

Cell migration was quantified using 96-well microchemotaxis plates with 0.3 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 were placed in the lower wells of the chambers. Migration was scored by counting the number of cells that had reached the bottom well after 2 h incubation period. Migration toward *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1×10^{-7} M) (Sigma–Aldrich) served as a positive control.

2.9. Statistical analysis

Results were analyzed by one-way analysis of variance with Bonferroni post-test using GraphPad Prism version 4.00 for Windows (GraphPad Software).

3. Results

3.1. B. abortus induces neutrophil activation

Neutrophil activation includes a broad range of phenotypic and functional changes that occur in several stages [14]. Early events include modulation of surface receptors. CD62L (L-selectin) is highly expressed on resting neutrophils, mediates neutrophil–endothelial interaction before diapedesis, and is down-regulated by shedding during neutrophil activation and migration to extravascular sites. CD11b/CD 18 is an integrin that is located within the intracellular vesicles of resting neutrophils, which is transferred to the plasma membrane upon activation. CD35 (CR1) is stored in secretory vesicles and binds C3b and C4b. Neutrophil activation results in degranulation and increased cell surface expression of CD35 and CD11b as well as decreased expression of CD62L. Thus, to determine whether *Brucella* is able to induce human neutrophil activation, we first evaluated the expression of CD62L, CD11b and CD35 in neutrophils cultured with live *B. abortus*. Cells incubated with live *B. abortus* experienced a reduction in L-selectin expression as well as an increased expression of CD11b and CD35 in a dose- and time-dependent manner. These changes were comparable to those observed after incubation with live *E. coli,* used as a positive control for neutrophil activation (Fig. 1A and B).

Early neutrophil activation is followed by the induction of IL-8 production, a critical chemoattractant. The concentration of this cytokine was significantly ($P < 0.001$) increased in supernatants of cells incubated with live *B. abortus,* as well as in *E.* coli-incubated cells ($P < 0.01$) (Fig. 1C).

The response to *B. abortus* was not linked to bacterial viability, since heat-killed bacteria (HKBA) also led to the modulation of cell surface markers' expression and cytokine production in a dose-dependent manner in comparable amounts as those obtained with a known neutrophil activation stimulus such as *E. coli* LPS (Fig. 2).

The kinetics of surface molecule expression by neutrophils is also critical for the rapid neutrophil response. Activation of neutrophils by live bacteria and HKBA caused a rapid increase in CD11b and CD35 starting at 30 min and increasing thereafter, while CD62L down-modulation was evident as early as 15 min (Figs. 1B and 2B).

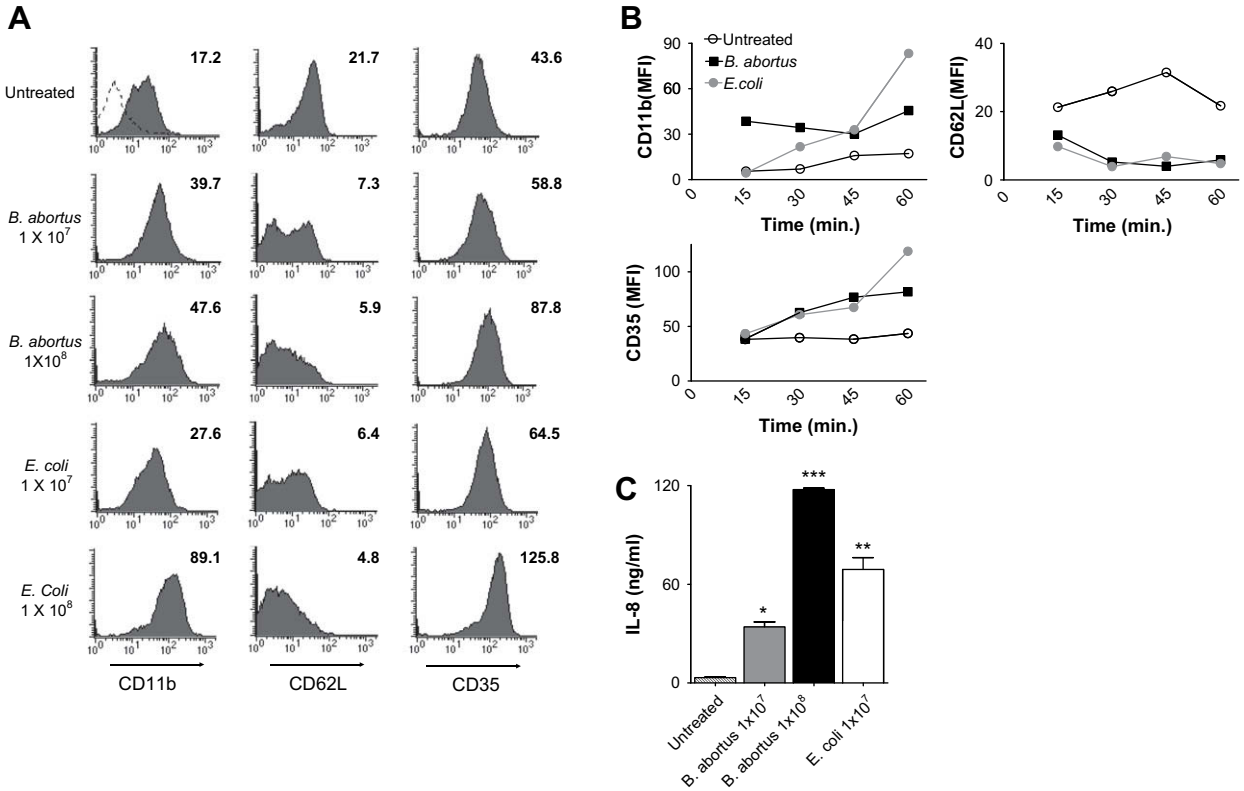

Fig. 1. *B. abortus* induces neutrophil activation. (A) CD11b, CD62L and CD35 expression on human neutrophils incubated at 37 °C for 60 min with medium alone (untreated), *B. abortus* or *E. coli* at a concentration of 1×10^7 and 1×10^8 bacteria/ml (dotted line, isotype control; shaded histograms, stimulated neutrophils). Histogram inserts show the Mean Fluorescence Intensity (MFI). (B) CD11b, CD62L and CD35 expression on human neutrophils incubated for 15, 30, 45 or 60 min with medium alone (untreated), *B. abortus* or *E. coli* (1×10^8 bacteria/ml). (A) and (B) data correspond to one representative of five independent experiments. (C) IL-8 concentration in the culture supernatants of neutrophils incubated for 24 h with the indicated stimulus quantified by ELISA. This experiment was performed five times in duplicate. ELISA results are expressed as the mean (ng/ml) ± SEM. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. untreated cells.

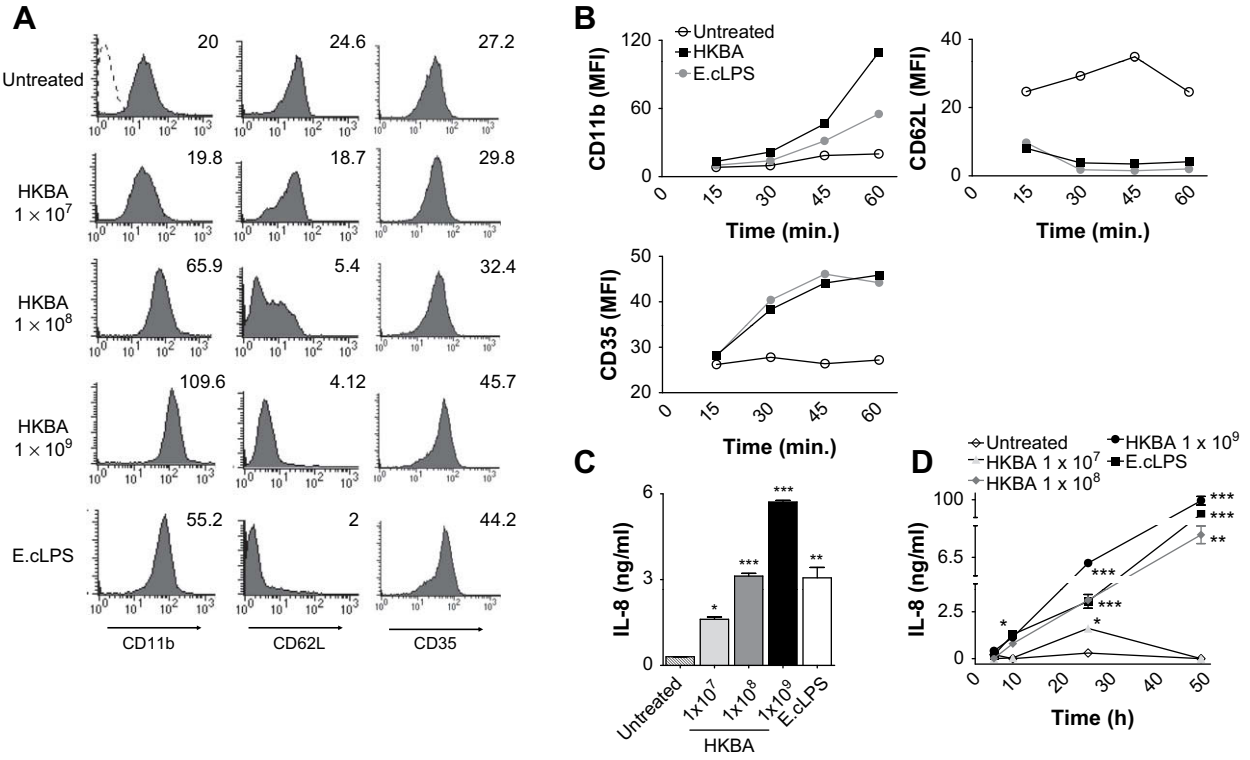

Fig. 2. HKBA induces neutrophil activation. (A) CD11b, CD62L and CD35 expression on human neutrophils incubated at 37 °C for 60 min with medium alone (untreated), HKBA (1×10^7, 1×10^8 and 1×10^9 bacteria/ml) or *E. coli* LPS (10 ng/ml) (dotted line, isotype control; shaded histograms, stimulated neutrophils). Histogram inserts show the Mean Fluorescence Intensity (MFI). (B) CD11b, CD62L and CD35 expression on human neutrophils incubated for 15, 30, 45 or 60 min with medium alone (untreated), HKBA (1×10^9 bacteria/ml) or *E. coli* LPS (10 ng/ml). (A) and (B) data correspond to one representative of five independent experiments. (C) IL-8 concentration in the culture supernatants of neutrophils incubated for 24 h with the indicated stimulus quantified by ELISA. (D) IL-8 concentration in the culture supernatants of neutrophils incubated for 4, 8, 24, or 48 h with the indicated stimulus quantified by ELISA. (C) and (D) were performed five times in duplicate. ELISA results are expressed as the mean (ng/ml) ± SEM. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. untreated cells.

As with the expression of cell surface markers, production of IL-8 in response to HKBA was also time-dependent (Fig. 2C and D). IL-8 concentration started to augment significantly at 8 h post-stimulation ($P < 0.05$) and continued to increase thereafter.

These results indicate that the exposure of neutrophils to *Brucella* stimulates L-selectin shedding, CD11b and CD35 up-regulation and IL-8 secretion; all responses consider characteristic of neutrophil activation; and these phenomena were also observed using dead bacteria, indicating that they can be achieved by a structural bacterial component.

3.2. B. abortus *lipoproteins mimic Brucella-induced neutrophil activation*

Although having a low-activity LPS, *Brucella* possess lipoproteins with potent stimulatory effects on a variety of cells. *Brucella* Omp19 lipoprotein has been found by our laboratory to induce the expression of IL-12 and other pro-inflammatory cytokines and up-regulation of cell surface markers in monocytes/macrophages and DC [9,10], but its impact on neutrophil activation has not been investigated. To test if *Brucella* lipoproteins could be one of the components able to induce the activation of neutrophils elicited by *B. abortus* we used Omp19 as a lipoprotein stimulant model. Lipidated Omp19 (L-Omp19) promoted a decrease in cell surface expression of CD62L and increased expression of CD11b and CD35 as well as IL-8 production in a dose- (Fig. 3A and C) and time-dependent fashion (Fig. 3B and D). Activation of neutrophils induced by L-Omp19 was abolished when the protein was devoid of the lipid moiety (U-Omp19). That the lipid portion of the protein was the one exerting the latter effects was further supported by the use of a synthetic lipohexapeptide (Pam3Cys) with an irrelevant peptide sequence, which showed a response nearly identical to that induced by L-Omp19 (Fig. 3). Kinetics of cytokine production and cell surface markers modulation in response to L-Omp19 was similar to that observed with HKBA (Fig. 3B and 3D). U-Omp19 was ineffective at all the times tested (Fig. 3D). Altogether, these results show that purified *Brucella* lipoproteins mimic live bacteria and HKBA-induced human neutrophil activation. Furthermore, as previously demonstrated for the activation of other cell types [9,10], the lipid moiety is also essential for the activity of this protein on neutrophils.

3.3. B. abortus *and Omp19 prime neutrophils for oxidative burst*

Further changes in neutrophil activation include the production of reactive oxygen species (ROS). ROS production is highly regulated in neutrophils, requiring a priming stimulation

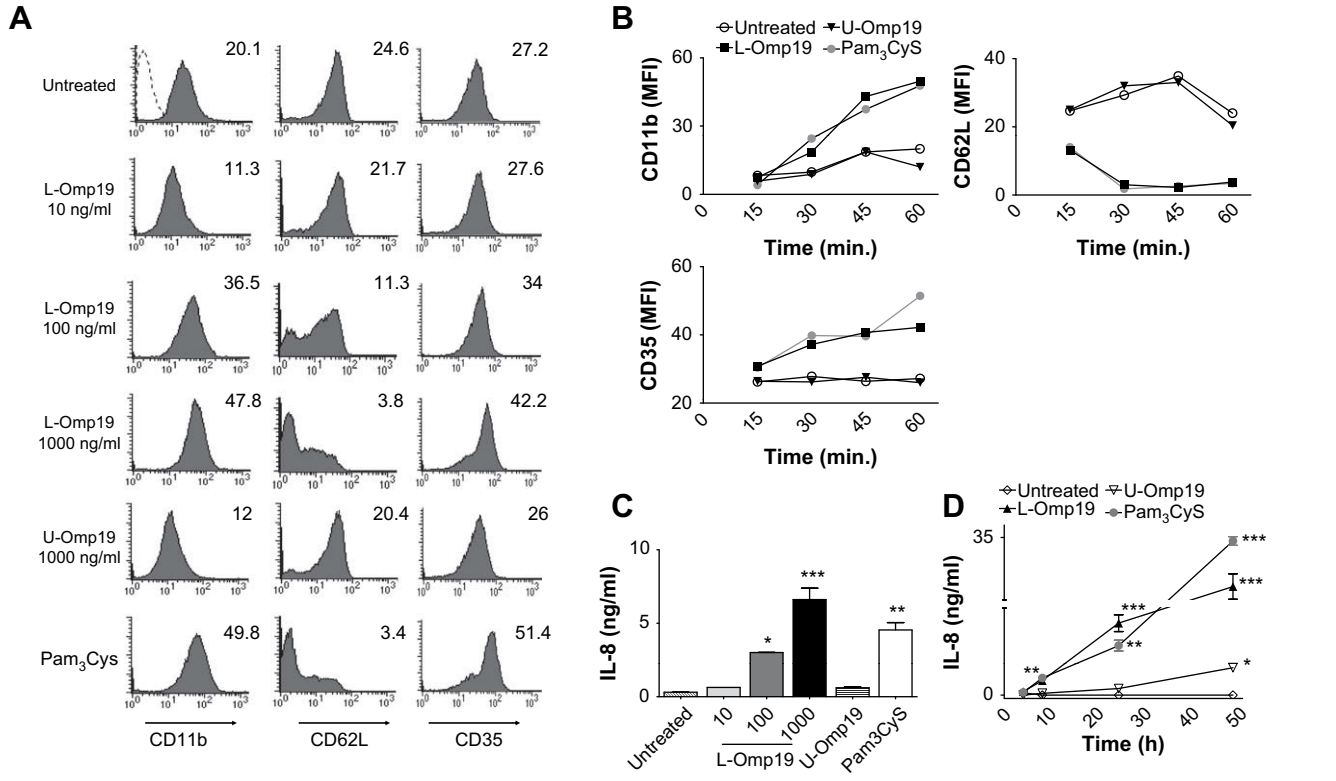

Fig. 3. Omp19 induces neutrophil activation. (A) CD11b, CD62L and CD35 expression on human neutrophils incubated at 37 °C for 60 min with medium alone (untreated), L-Omp19 (10, 100 and 1000 ng/ml); U-Omp19 (1000 ng/ml) or Pam_3Cys (100 ng/ml) (dotted line, isotype control; shaded histograms, stimulated neutrophils). Histogram inserts show the Mean Fluorescence Intensity (MFI). (B) CD11b, CD62L and CD35 expression on human neutrophils incubated for 15, 30, 45 or 60 min with medium alone (untreated), L-Omp19 (1000 ng/ml); U-Omp19 (1000 ng/ml) or Pam_3Cys (100 ng/ml). (A) and (B) data correspond to one representative of five independent experiments. (C) IL-8 concentration in the culture supernatants of neutrophils incubated for 24 h with the indicated stimulus quantified by ELISA. (D) IL-8 concentration in the culture supernatants of neutrophils incubated for 4, 8, 24, or 48 h with the indicated stimulus quantified by ELISA. (C) and (D) were performed five times in duplicate. ELISA results are expressed as the mean (ng/ml) ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. untreated cells.

for an appropriate detectable response [15]. Flow cytometry was used to detect oxidation of Dihydrorhodamine (DHR) as a measure of oxidative burst resulting from neutrophil activation. Stimuli concentration used in these set of experiments was selected on the basis that they presented the most evident effects on cell surface marker expression and cytokine production. HKBA and L-Omp19 both served as priming agents to enhance the subsequent oxidative burst of purified neutrophils exposed to PMA. After priming neutrophils with HKBA and L-Omp19, ROS production increased two and threefold, respectively (Fig. 4). The increased responsiveness to the subsequent oxidative burst of neutrophils exposed to PMA was similar to the control stimulus *E. coli* LPS. ROS production after priming with U-Omp19 remained unaltered, further supporting the role of the lipid moiety of L-Omp19 on its biological activity.

Thus, the ability of L-Omp19 to prime neutrophils for oxidative burst in response to PMA manifest that L-Omp19 is able to induce early and late neutrophil activation responses.

3.4. B. abortus *and Omp19 prolong neutrophil survival*

Bacterial wall products are known to delay the constitutive apoptotic rate of neutrophils as a consequence of cell activation [16]. Being *Brucella* lipoproteins capable of modulating cell surface receptors and increase reactive oxygen species production associated with an inflammatory neutrophil phenotype; and considering that joint damage in brucellosis is associated with the presence of the bacteria in the affected joints, we sought to analyze the effect *of Brucella* and its lipoproteins in neutrophil apoptosis. Cells were stimulated with *E. coli* LPS, different concentrations of HKBA, Pam_3-Cys, L-Omp19 or U-Omp19. Thereafter, cell surface exposure of phosphatidylserine by Annexin V-FITC (AV) was determined. Flow cytometric analysis showed that over a 72 h period up to 80% of isolated human neutrophils demonstrated evidence of apoptosis. The level of AV^+ cells significantly decreased ($P < 0.01$) in HKBA-stimulated neutrophils (Fig. 5). The higher the bacterial doses, the larger the differences in neutrophil apoptosis between untreated and HKBA-stimulated cells. In the presence of HKBA, the percentage of apoptotic cells still increased from 30% (at 6 h) to 65% (at 72 h), indicating that the pro-survival signal elicited by HKBA delay rather than prevented apoptosis. Similar delay in spontaneous apoptosis of human neutrophil was observed with L-Omp19, which in a concentration of 1000 ng/ml prolonged neutrophil survival. In contrast, U-Omp19 was incapable of inhibiting or delaying the progression of neutrophil apoptotic demise (Fig. 5).

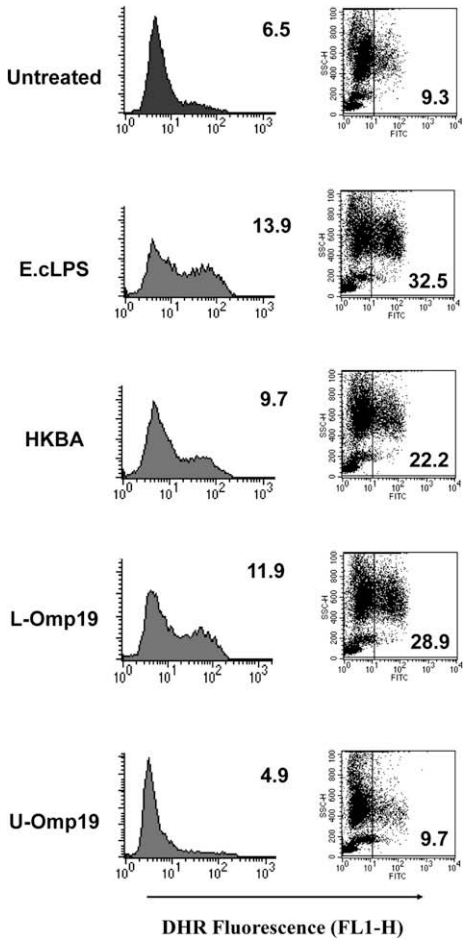

Fig. 4. HKBA and Omp19 prime neutrophil for oxidative burst. Neutrophils were incubated for 30 min at 37 °C with medium alone (untreated), *E. coli* LPS (10 ng/ml), HKBA (1×10^9 bacteria/ml), L-Omp19 (1000 ng/ml) or U-Omp19 (1000 ng/ml). DHR was then added and cells were incubated for other 15 min. Cells were then exposed to PMA for 15 min to induce oxidative burst. Cells were analyzed immediately by flow cytometry. Data correspond to one representative of five independent experiments. Histograms show the mean fluorescence intensity and dot blots show the percentage of FITC positive cells.

The present results provide evidence for a mechanism by which *Brucella* lipoproteins may contribute to inflammation by delaying neutrophil apoptosis and extending their functional lifespan.

3.5. B. abortus *and Omp19 induce neutrophil migration*

Neutrophils are relatively short-lived cells, undergoing death by apoptosis. Even when apoptosis is delayed, they survive in a functional state for only a few days. Thus, for neutrophils to contribute to the long-term process of focalized inflammation there must be further and continuous recruitment of new cells from the circulation. Infiltration of joints by immune cells requires the local production of chemoattractans. Considering that live *B. abortus*-, HKBA- and L-Omp19-activated neutrophils secreted one of the most powerful chemotactic substances; such as IL-8; and modulated important molecules that mediate the adhesion of neutrophils to the vascular endothelium (Figs. 1–3), additional experiments were conducted to evaluate whether HKBA and L-Omp19 was also able to induce neutrophil migration. To this purpose, purified neutrophils were placed in the top well of a microchemotaxis plate and the different stimulants were plated in the bottom well of the chamber. Migration was scored by counting the number of cells that reached the lower chamber after a 2 h incubation period. The number of migrating cells was significantly ($P < 0.01$) higher in wells containing HKBA (59 ± 4.6 cells/well) and L-Omp19 (100 ± 7.5 cells/well) than background levels (14 ± 4 cells/well), while U-Omp19 containing wells remained the same (10 ± 2 cells/well). Resembling L-Omp19 response, Pam_3Cys also augmented the number of migrating cells (50 ± 3 cells/well); and so did the controls fMLP (78 ± 13) and *E. coli* LPS (80 ± 10 cells/well) (Fig. 6A). In line with this, supernatants from HKBA-, L-Omp19-, Pam_3Cys- and *E. coli* LPS-treated neutrophils also induced neutrophil migration, while supernatants from U-Omp19-treated cells did not support neutrophil motion (Fig. 6B). This could indicate that IL-8 production derived from HKBA- and L-Omp19-stimulated cells may play an important local regulatory role in inflammation due to its chemoattractant function, recruiting more neutrophils to the site of infection.

4. Discussion

Human brucellosis originates from direct exposure to animal reservoirs or to unpasteurized dairy products derived from animals infected with *Brucella* zoonotic species (*B. melitensis*, *B. suis*, *B. abortus* and *B. canis*). The clinical manifestations may vary from subclinical disease, which may pass unnoticed, to a disease with general symptoms such as undulant fever, asthenia, myoarthralgias, weight loss, splenomegaly, hepatomegaly, lymphadenopaty, anorexia and malaise [17].

In previous works we have shown that *B. abortus* lipoproteins are one of the molecules responsible for the inflammation observed in brucellosis by direct activation of monocytes, macrophages and DC [9,10]. In this study, we present evidence of the ability of *B. abortus* and its lipoproteins to activate neutrophils, cells capable as well of inducing an inflammatory response and which are present in abundance in inflamed tissue infiltrates.

B. abortus induced an increment in CD35 and CD11b expression and a decline in CD62L accompanied by IL-8 secretion, a response compatible with neutrophil activation. Though the minimum concentration of dead bacteria required for modulation of marker's expression was higher than the one needed for live bacteria activity, HKBA was still able to induce activation. Although the reasons for enhanced live *B. abortus* stimulation are unknown to us, other bacteria have been reported to induce higher pro-inflammatory cytokines production in response to live organisms compared to dead bacteria or bacterial lysates [18]. Early contact *of Brucella* with phagocytic cells involves swimming on the cell surface

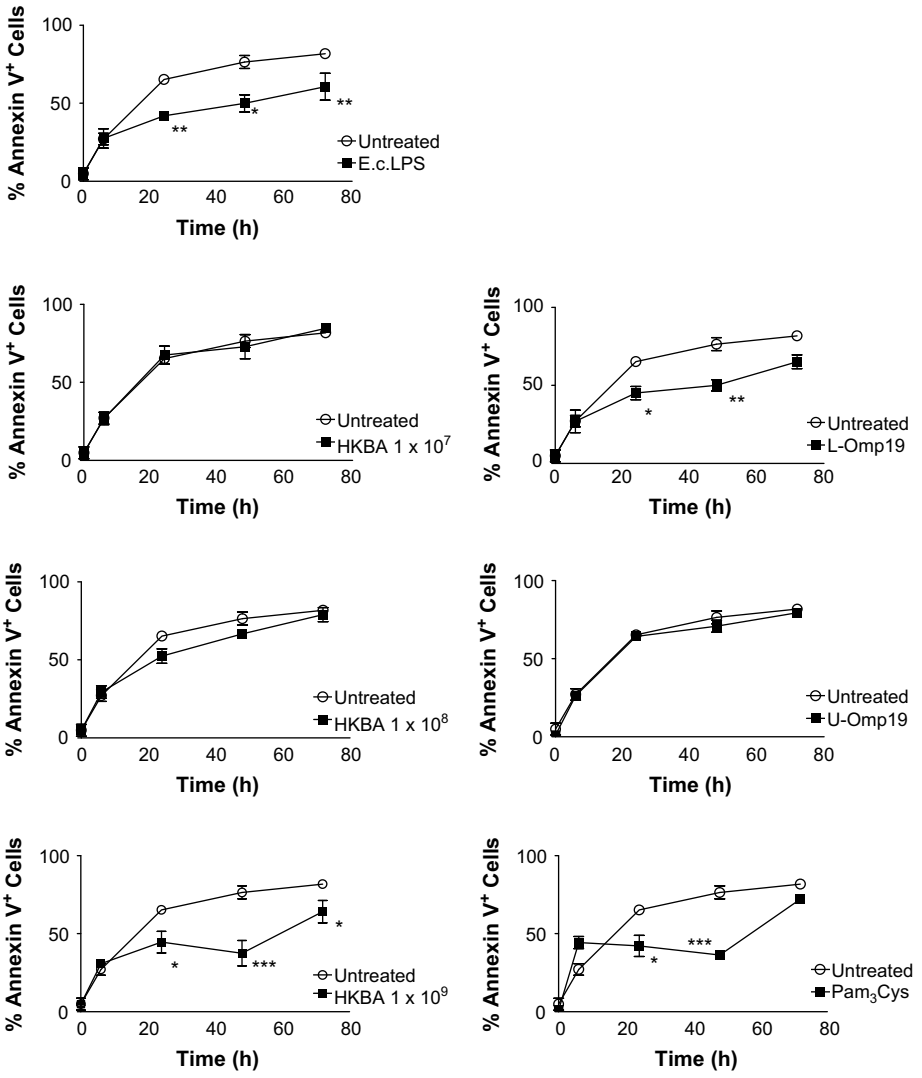

Fig. 5. HKBA and Omp19 prolong neutrophil survival. Neutrophils were incubated at 37 °C with medium alone (untreated), *E. coli* LPS (10 ng/ml), HKBA (1×10^7, 1×10^8, 1×10^9 bacteria/ml), Pam_3Cys (100 ng/ml), L-Omp19 (1000 ng/ml) or U-Omp19 (1000 ng/ml) for 6, 24, 48 or 72 h. Apoptosis was assessed by Annexin V protein binding assay. Results correspond to five independent experiments and are expressed as percentage of total Annexin V positive cells ± SEM. The proportion of necrotic cells was always less than 2%. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. untreated cells.

with generalized membrane ruffling [19]. During these early interactions bacteria are able to hijack lipid rafts [20], facilitating the innate recognition of bacterial components through TLRs confined within this rafts [21]. In addition, *Brucella* has been shown to form microcolonies during adherence to cell surface through interactions with sialic acid residues [22]. We can speculate that the improvement of any of these early cell interactions by live *B. abortus* may account for the observed differences between live and dead bacteria. For example, live bacteria could be more efficient in moving round from its initial membrane contact site due its swimming ability, resulting in a higher recruitment of TLRs to lipid rafts which may lead to enhanced signal recognition. Alternatively, enhanced cell activation may reflect different signaling events after internalization of bacteria. The fact that heat-killed *Brucella* is also able to induce neutrophil activation is proof of concept that at least one of the cell stimulatory ligands is a structural component of the bacteria; live bacteria could be more efficient in presenting this ligand or it could use additional stimulating mechanisms, nevertheless we cannot discriminate between these two possibilities.

B. abortus lipoprotein Omp19 mimics phenotypic and functional changes induced by HKBA associated with neutrophil activation. U-Omp19 was unable to induce any of these changes, confirming that the lipid moiety is required for neutrophil activation, as it has been reported with different cells types [9,10]. Even though it is difficult to determine the actual Omp19 concentration expressed by *Brucella* organism in vivo, in this study we used this protein as a model of *B. abortus* lipoprotein. Yet, considering that its bioactivity is conferred by the lipid moiety which is likely the same in all the lipoprotein molecules of this organism; and that *Brucella* genome contains at least 80 putative lipoprotein genes, many of which were shown to be expressed in the outer membrane of the bacterium [23,24], one can envision that the local concentration of *Brucella* lipoproteins in confined tissue

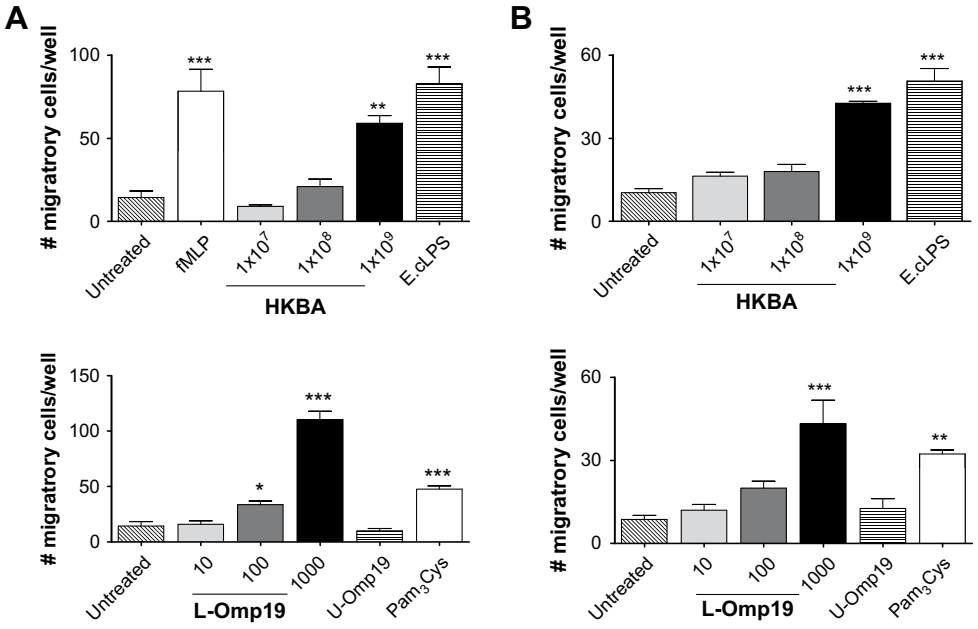

Fig. 6. HKBA and Omp19 induced neutrophil migration. Neutrophil migration induced by the indicated stimulus (A) or supernatants from cells incubated for 24 h with the indicated stimulus; (B) after a 2 h incubation period. Results represent the mean (# migratory cells/well) ± SEM of three independent experiments performed in triplicates. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. untreated cells.

spaces may be sufficient to exert their biological effects. In this context, we can hypothesize that any surface exposed *Brucella* lipoprotein may be relevant beyond in vitro assays and not one lipoprotein but rather a combination of them can cause the pro-inflammatory response. Furthermore, our results agree with the observed neutrophil activation exerted by lipoproteins from other bacteria [25,26]

Mature neutrophils undergo constitutive programmed cell death that renders them unresponsive to chemoattractans and allows their recognition and removal by scavenger macrophages, leading to the resolution of inflammation [27]. Neutrophil survival and apoptosis are, however, profoundly influenced by the inflammatory environment and suppression of neutrophil apoptosis ensue chronic inflammation [28]. Neutrophil accumulation at the inflammatory foci and survival have been implicated in the pathology of other inflammatory diseases such as arthritis, meningitis and peritonitis [27,29]. As previously observed for other bacterial lipoproteins [16], *B. abortus* lipoproteins mediated apoptotic delay. Hence, by delaying apoptosis *B. abortus* lipoproteins may further contribute to the pathogenesis of brucellosis, over-riding apoptotic neutrophils removal by phagocytic cells and limiting inflammation resolution. In this way, prolonged neutrophil survival may exacerbate leukocyte trafficking into inflamed tissues leading to tissue injury by continuous release of neutrophil cytotoxic granules content. Indeed, the augmented ROS production in response to either HKBA or Omp19 is compatible with reported increased ROS production by neutrophils from patients with bacteremia [30]. Furthermore, *B. abortus* and lipoprotein-stimulated cells as well as supernatants from these cells increased neutrophil migratory ability, supporting neutrophil recruitment to the inflammatory foci. This is in agreement with the increased neutrophil mobility found in patients with brucellosis [30].

Therefore, *Brucella* lipoproteins possess pro-inflammatory properties that could contribute to localize tissue injury and inflammation by direct activation of neutrophils. Cooperation between neutrophils and macrophages or direct macrophage activation by lipoproteins may also intensify neutrophil noxious outcome.

Collectively, data presented here, in conjunction with our previous results on monocytes, macrophages and DC, implicate *Brucella* lipoproteins in the pathogenesis of human brucellosis.

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