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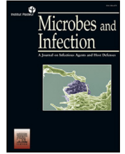

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Original article

Brucella abortus-infected B cells induce osteoclastogenesis

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Abstract

Brucella abortus is an intracellular bacterium that establishes lifelong infections in livestock and humans although the mechanisms of its chronicity are poorly understood. Activated B cells have long lifespan and *B. abortus* infection activates B cells. Our results indicate that the direct infection of B cells with *B. abortus* induced matrix metalloproteinase-9 (MMP-9), receptor activator for NF κB ligand (RANKL), tumor necrosis factor (TNF)-α and interleukin (IL)-6 secretion. In addition, supernatants from *B. abortus*-infected B cells induced bone marrow-derived monocytes to undergo osteoclastogenesis. Using osteoprotegerin, RANKL's decoy receptor, we determined that RANKL is involved in osteoclastogenesis induced by supernatants from *B. abortus*-infected B cells. The results presented here shed light on how the interactions of *B. abortus* with B cells may have a role in the pathogenesis of brucellar osteoarticular disease.
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Keywords: Osteoclastogenesis; *Brucella abortus*; B cells

1. Introduction

Human brucellosis is a zoonotic infection caused by different *Brucella* species [1]. Brucellosis is chiefly an inflammatory disease. Inflammation is present both in the acute and chronic phases of the disease. It can affect virtually any organ or system, causing focal forms that account for about 30% of the reported cases [2]. Osteoarticular involvement is the most common presentation of localized disease, which may affect peripheral joints, sacroiliac joints, or the spine [3–7]. The frequency of bone and joint involvement in brucellosis is mostly related to the *Brucella* species being most frequent in *B. melitensis*, followed by *B. suis* and *B. abortus* [8].

Since *Brucella* creates a niche inside macrophages that supports bacterial replication, it has been postulated that these cells are the major host cells responsible for chronic infection [9,10]. Although B cells have a low phagocytic capacity when compared to professional phagocytic cells [11], they internalize *Mycobacterium tuberculosis* [12], *Staphylococcus aureus* [11] and *Brucella abortus* [13] facilitating the establishment of infection. Activated B cells have long lifespan, and *B. abortus* infection activate B cells [13]. This suggests that these cells could be a survival niche for *Brucella* that contributes to the chronicity of infection [13]. Chronic brucellar lesions of bones and joints characteristically reveal, at the histological level, an inflammatory response with varying degrees of bone destruction and the presence of infiltrating lymphocytes [8]. While the primary function of B cells is to produce anti-microbial immunoglobulins against infecting pathogens; B cells may also contribute to the proinflammatory innate host response by secreting matrix metalloproteinases (MMP) and cytokines in response to innate immune receptor ligation [14,15].

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Mature B cells have the ability to migrate to the injured tissue and site of inflammation [16]. This process is mediated at least in part by the ability of B cells to secrete MMPs that degrades extracellular matrix [17,18]. However, excessive inflammation after infection may cause tissue damage due in part to increased levels of MMP activity. Locally increased levels of MMPs have been found in several osteoarticular diseases, including rheumatic conditions (rheumatoid arthritis, osteoarthritis, and spondyloarthritis) and in infectious arthritis such as that observed in Lyme disease [19–21].

During chronic inflammatory bone diseases, receptor activator for NF κB ligand (RANKL) and proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 have been important for disease progression and bone loss [22–26]. This cytokines could be secreted by activated B cells and contribute to the acceleration of bone resorption [27,28].

We have recently demonstrated that *Brucella* species can infect and survive within osteoblasts and osteocytes, and that this infection elicits the secretion of proinflammatory cytokines and chemokines. These lastly attract monocytes to the site of infection and contribute to the amplification of such response that might be involved in the osteoarticular manifestations of brucellosis [29–31]. Thus, it seems conceivable to speculate that infiltrating B cells, present in the inflammatory microenvironment generated by the bacterium, might activate and promote the generation of osteoclasts; the only cells capable to degrade bone. To investigate this hypothesis, we developed an *in vitro* model; in which murine splenic B cells that were infected with *B. abortus* were able to drive bone marrow-derived monocytes to undergo osteoclastogenesis in a RANKL-dependent fashion.

2. Materials and methods

2.1. Animals

Six to 8-week-old Balb/c mice were provided by University of La Plata (Argentina). Animals were housed in groups of five, under controlled temperature (22 °C_2 °C) and artificial light under a 12-h cycle period. Mice were kept under specific pathogen-free conditions in positive-pressure cabinets and provided with sterile food and water *ad libitum*. All animal procedures were performed according to the rules and standards for the use of laboratory animals of the National Institute of Health, USA. Animal experiments were approved by the Ethical Committees of the University of Buenos Aires CICUAL 3720/14.

2.2. Bacterial culture

B. abortus S2308 was grown overnight in 10 ml of tryptic soy broth with constant agitation at 37 °C. Bacteria were harvested by centrifugation for 15 min at 6000 × *g* at 4 °C and washed twice in 10 ml of phosphate-buffered saline (PBS). Bacterial numbers in the cultures were estimated by comparing the optical densities at 600 nm with a standard curve obtained in our laboratory. To prepare inocula, cultures were diluted in sterile PBS to the desired bacterial concentration on the basis of the optical density readings, but the precise concentrations of inocula were determined by plating cells onto tryptic soy agar. To obtain heat-killed *B. abortus* (HKBA), bacteria were washed five times for 10 min each in sterile PBS, heat-killed at 70 °C for 20 min, aliquoted, and stored at −70 °C until they were used. The total absence of *B. abortus* viability after heat killing was verified by the absence of bacterial growth on tryptose soy agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities located at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (Buenos Aires, Argentina).

2.3. Cells and media

All experiments were performed at 37 °C in a 5% CO_2 atmosphere in α-minimum essential medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Gibco-BRL, Life Technologies, Grand Island, NY), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (complete medium). B cells were obtained from murine spleens using a CD19 B cells-negative isolation kit (Miltenyi Biotech, Auburn, CA.), following the manufacturer's instructions. The purity of the isolated B cells were later confirmed by flow cytometry using anti-CD19 coupled to R-Phycoerythrin (PE) and anti B220 coupled to fluorescein isothiocyanate (FITC) antibodies and was more than 90%. The viability of cells was up to 95%, as measured by a trypan blue exclusion test.

The macrophage cell line J774.A1 was obtained from the ATCC (Manassas, VA) and was cultured as previously described [32].

2.4. Cellular infection

B cells (1×10^6 cells/well) were infected with *B. abortus* at a multiplicity of infection (MOI) of 100, 250, 500 and 1000. In some experiments results are only show at MOI of 100 and 1000 for clearer reading. Murine macrophages (J774.A1 cell line) were infected at a MOI of 100. After the bacterial suspension was dispensed, the plates were centrifuged for 10 min at 2000 rpm and then incubated for 2 h at 37 °C under a 5% CO_2 atmosphere. Cells were extensively washed with Dulbecco's modified Eagle's medium (DMEM) to remove extracellular bacteria and incubated in medium supplemented with 100 μg/ml of gentamicin and 50 μg/ml of streptomycin to kill extracellular bacteria. B cells or supernatants were harvested at 24 h post infection (p.i.) to determine matrix metalloproteinase (MMP) secretion, cytokine expression and to obtain culture supernatants to perform osteoclastogenesis assay. Supernatants from J774.A1 cells were harvested at 24 h p.i. to be used as conditioned medium.

2.5. Zymography

Gelatinase activity was assayed as described previously [33,34]. Briefly, a total of 20 μl of cell culture supernatants

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from infected B cells or from untreated controls was mixed with 5 µl of 5× loading buffer (0.25 M Tris [pH 6.8], 50% glycerol, 5% SDS, and bromophenol blue crystals) and loaded onto 10% SDS-PAGE gels containing 1 mg/ml gelatin (Sigma—Aldrich, Argentina SA). Following electrophoresis, gels were washed with a solution containing 50 mM Tris—HCl (pH 7.5) and 2.5% Triton X-100 (buffer A) for 30 min and with buffer A added with 5 mM $CaCl_2$ and 1 mM $ZnCl_2$ for 30 min and were later incubated with buffer A with additional 10 mM $CaCl_2$ and 200 mM NaCl for 48 h at 37 °C. Gelatin activity was visualized by the staining of the gels with 0.5% Coomassie blue.

2.6. Measurement of cytokine concentrations in culture supernatants

The secretion of interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α) in the supernatants was quantified with an ELISA kit from BD, and RANKL were quantified with ELISA kits from R&D Systems, Inc. (Minneapolis, MN).

2.7. RANKL expression on B cells

B cells were infected with *B. abortus* and, at 24 h post infection (p.i.) cells were washed and incubated with a fluorescein isothiocyanate (FITC)-labeled anti-mouse receptor activator for nuclear factor κB ligand (anti-RANKL) (BioLegend, San Diego, USA), or the appropriate isotype-matched control antibody for 30 min on ice. Cells were then washed and analyzed with a FACScan flow cytometer using CellQuest software (both from Becton—Dickinson, Franklin Lakes, NJ). The results were expressed as percentage of positive cells.

2.8. Osteoclast formation assay

Bone marrow-derived monocytes (BMM) were induced to undergo osteoclastogenesis as described previously [35]. Briefly, bone marrow cells from BALB/c mice were cultured in complete medium containing 5 ng/ml of murine recombinant M-CSF (R&D Systems, Minneapolis, MN, USA) for 12 h in 24-well plates. Nonadherent cells were harvested and cultured with 30 ng/ml M-CSF in 24-well plates for an additional 24 h. Nonadherent cells were washed out, and adherent cells were collected and used as BMM (5×10^4 cells/well), which were seeded onto glass coverslips in 24-well plates for 7 days and cultured in complete medium containing 30 ng/ml M-CSF and 0.2 ml of culture supernatants from B cells infected with *B. abortus*. As positive controls of osteoclast formation, BMM cultures received 50 ng/ml murine RANKL. On day 3, the culture medium and all reagents were replaced. To identify osteoclasts, cells were fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma—Aldrich, St. Louis, MO, USA). TRAP-positive, multinucleated (more than three nuclei) cells were defined as osteoclasts, and the number was determined by microscopic counts.

2.9. Neutralization experiments

Neutralization experiments were performed using 50 µg/ml of osteoprotegerin (OPG; R&D Systems), RANKL's decoy receptor. Supernatants from *Brucella*-infected B cells were preincubated with the decoy receptor for 1 h at 37 °C before being used to stimulate BMM.

2.10. Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by a *post hoc* Tukey test using GraphPad Prism, version 4.0, software. All data passed the normality test Shapiro—Wilk, except for data from Fig. 1F that were normalized before applying the statistical test. Data are represented as means ± standard errors of the means (SEM).

3. Results

3.1. B. abortus*-infected B cells secrete proinflammatory mediators*

During inflammatory bone diseases, MMP, RANKL and proinflammatory cytokines such as TNF-α, IL-1β and IL-6 have been involved in bone resorption [22,24,36]. Mature activated B cells have the ability to migrate to the injured tissue and site of inflammation [16] and secrete MMP and cytokines [14,15]. Then, B cells were infected with *B. abortus* and the ability to secrete MMP and cytokines was evaluated. *B. abortus* infection induced the secretion of MMP-9, TNF-α and IL-6 (Fig. 1A—D) but not IL-1β. In addition and taking into account that RANKL is the major cytokine involved in osteoclastogenesis, we investigated whether *B. abortus* infection would lead to membrane-bound and soluble RANKL expression from B cells. To this end, cell surface RANKL expression was evaluated by flow cytometry and soluble RANKL was determine in culture supernatants by ELISA. *B. abortus* infection induced membrane-bound RANKL expression on B cells (Fig. 1F). Quantitative analysis of RANKL expression in culture supernatants from *B. abortus*-infected B cells revealed that *B. abortus* induced the secretion of soluble RANKL (Fig. 1E). All cytokines were evaluated at 24 h post infection, the number of bacteria was 91,000 ± 5656.80 UFC/1×10^6 B cells for MOI 1000 and 13,500 ± 2121.30 UFC/1×10^6 B cells for MOI 100, indicating that only few B cells were infected. Taken together our results indicate that the infection of B cells by *B. abortus* induced inflammatory mediators, which could contribute to bone destruction.

3.2. B. abortus*-infected B cells induce osteoclastogenesis vía RANKL*

Osteoclasts play a major role in bone resorption. Osteoclastogenesis process involves soluble mediators from inflammatory cells, in conjunction with M-CSF [35]. To determine whether soluble factors produced by *B. abortus*-infected B cells could induce osteoclast formation. BMM were

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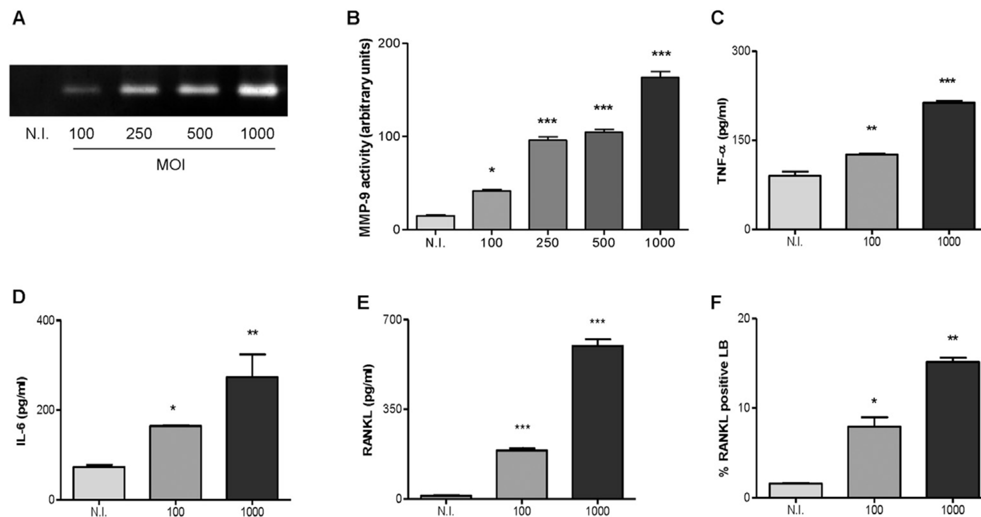

Fig. 1. *B. abortus* induces MMP-9 and cytokines secretion from B cells. B cells were infected with *B. abortus* at MOI 100 and 1000 and 24 h after infection MMP-9 production was determined by gelatin zymography (A). (B) Densitometric analysis of results from three independent experiments performed as described in panel A. ELISA determination of TNF-α (C), IL-6 (D) and RANKL (E) in culture supernatants of infected B cells. (F) Flow cytometry analysis of membrane bound RANKL positive B cells determined at 24 h p.i. Data are given as the means ± SEM of results from experiments performed in duplicate. Data shown are from a representative experiment of three performed. *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$, versus non-infected (N.I.) cells.

stimulated with M-CSF in conjunction with culture supernatants from B cells infected with *B. abortus*; and osteoclastogenesis was determined by the generation of multinucleated TRAP-expressing cells. RANKL was used as a positive control. Osteoclast-like cells were induced by culture supernatants from *B. abortus*-infected B cells but not by those of uninfected B cells (Fig. 2). These results indicate that *B. abortus*-infected B cells can promote functional osteoclast formation from BMM.

RANKL and proinflammatory cytokines are abundant in sites of inflammatory bone erosion [22–24]; and RANKL is a key molecule implicated in bone remodeling [37]. To assess the role of RANKL in osteoclastogenesis elicited by *B. abortus*, BMM cells were cultured with M-CSF and supernatants from *B. abortus*-infected B cells in the presence of osteoprotegerin (OPG), RANKL' decoy receptor, and osteoclastogenesis was evaluated by the generation of multinucleated TRAP-expressing cells. In contrast to what was seen in untreated cells, OPG treatment completely abrogated osteoclastogenesis induced by supernatants from *B. abortus*-infected B cells (Fig. 2). These results indicate that RANKL secreted by *B. abortus*-infected B cells is involved in the bone loss observed in osteoarticular brucellosis.

3.3. Heat-killed B. abortus *induce TNF-α and IL-6 secretion by not RANKL*

Next, to test whether viable bacteria were necessary to induce a proinflammatory response upon B cell infection, the ability of HKBA to induce the expression of membrane bound RANKL and the secretion of RANKL, TNF-α and IL-6 was also examined. The production of TNF-α and IL-6 was enhanced in culture supernatants from B cells that were stimulated with HKBA when compared with the unstimulated cells (Fig. 3). HKBA was unable to induce the expression of RANKL (Fig. 3C) and concomitant osteoclastogenesis (Fig. 3D and E).

These results indicate that although HKBA was able to induce TNF-α and IL-6, it failed to induce RANKL, suggesting that a structural component is not involved in osteoclastogenesis induction.

3.4. Macrophages do not induce an increase in RANKL expression on B cells during B. abortus *infection*

It has been described that *Brucella* virulence factors stimulate macrophages to induce B cells proliferation [38]. Then, experiments were conducted to determine whether macrophages could enhance B cell expression of RANKL during *B. abortus* infection. To this end B cells were stimulated with culture supernatants from *B. abortus*-infected macrophages or alternatively cocultures of macrophages and B cells were infected with *B. abortus*. Next, we analyzed membrane-bound RANKL expression on B cells, and the presence of RANKL in culture supernatants from B cells treated with culture supernatants from *B. abortus*-infected macrophages; and in supernatants from *B. abortus*-infected cocultures of B cells and macrophages. The evaluation of RANKL in culture supernatants was possible since we have previously demonstrated that macrophages do not secrete RANKL in response to *B. abortus* infection [29]. Supernatants from *B. abortus*-infected macrophages were unable to induce RANKL secretion by B cells (Fig. 4A). In addition, cocultures of macrophages and B cells could not induce an increase in RANKL secretion by B cells in the context of *B. abortus* infection (Fig. 4B). Taken together these results indicate that macrophages do not contribute to the induction of RANKL expression by B cells during *B. abortus* infection.

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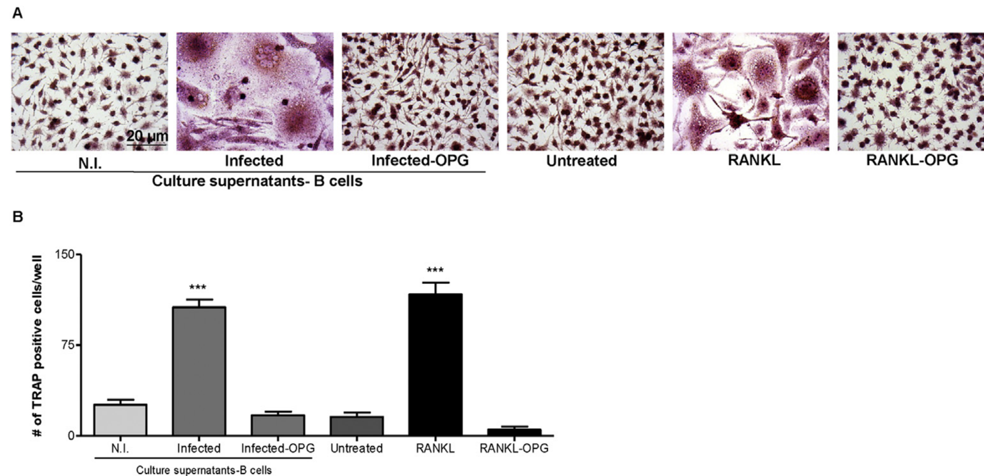

Fig. 2. Supernatants from *B. abortus*-infected B cells induce BMM-derived osteoclastogenesis. BMM cells were either untreated or stimulated with culture supernatants from *B. abortus*-infected B cells (added at a 1:2 proportion) or with culture supernatants from non-infected (N.I.) B cells (added at a 1:2 proportion) in conjunction with M-CSF. To inhibit RANKL, experiments were conducted in the presence of OPG. After 7 days, osteoclastogenesis was determined by the generation of multinucleated TRAP-positive cells. Representative digital images were taken by light microscopy (A), and multinucleated TRAP-positive cells were identified and counted (B). RANKL was used as a positive control. Data are given as the means ± SEM of results from experiments performed in duplicate. Data shown are from a representative experiment of three performed. ***, $P < 0.001$, versus cells treated with infected supernatants pre-incubated with OPG (Infected-OPG).

4. Discussion

It is well established that important regulatory interactions occurs between the cells in the immune and skeletal systems [39]. B cells are responsible for the generation and production of antibodies in the body, which allow an individual to develop specific responses and memory to an infection threat. In addition to this immune function, B cells have a close and multifaceted relationship with bone cells [40]. Because infiltration of B cells into the bones and joints is a hallmark

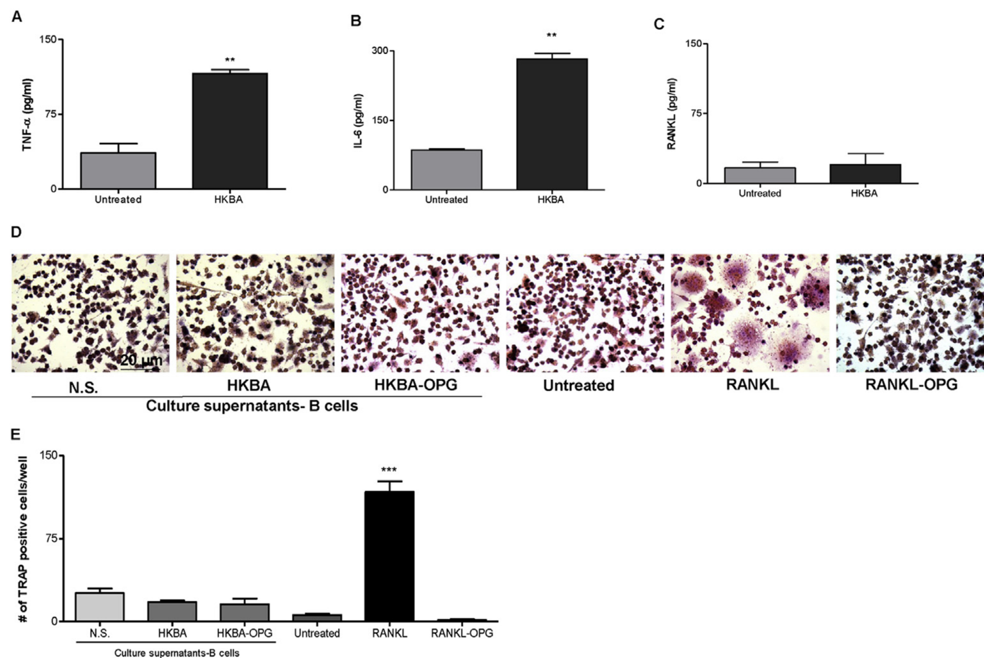

Fig. 3. HKBA induces TNF-α and IL-6 secretion. B cells were stimulated with HKBA (1×10^9 bacteria/ml). ELISA determination of TNF-α (A), IL-6 (B) and RANKL (C) was determined at 24 h p.i. in culture supernatants from stimulated B cells. (D) BMM cells were either untreated or stimulated with culture supernatants from HKBA-treated B cells (added at a 1:2 proportion) or with culture supernatants from non-stimulated (N.S.) B cells (added at a 1:2 proportion) in conjunction with M-CSF. To inhibit RANKL, experiments were conducted in the presence of OPG. After 7 days, osteoclastogenesis was determined by the generation of multinucleated TRAP-positive cells and representative digital images were taken by light microscopy. Multinucleated TRAP-positive cells were identified and counted (E). RANKL was used as a positive control. Data are given as the means ± SEM from at least three individual experiments. ***, $P < 0.001$; **$P < 0.01$ versus untreated cells.

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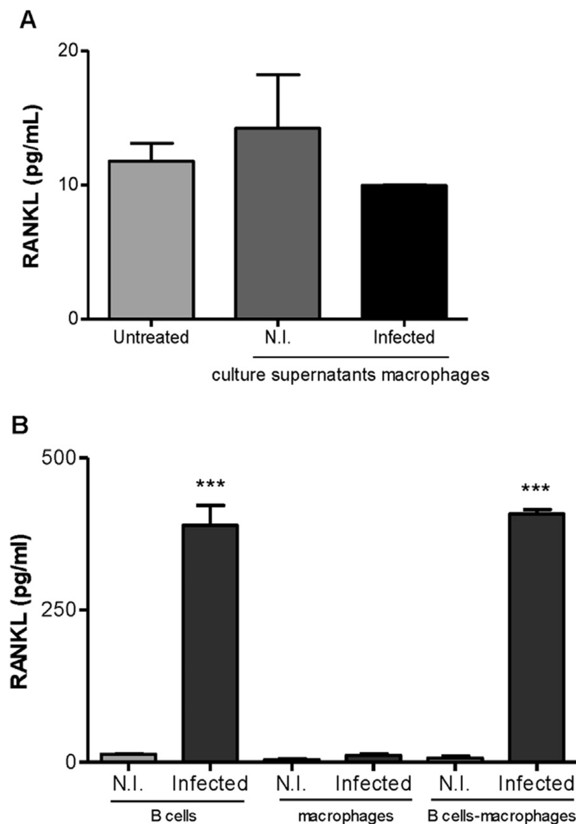

Fig. 4. Macrophages do not induce RANKL expression on B cells. B cells were stimulated with culture supernatants from *B. abortus*-infected B cells (added at a 1:2 proportion) or with culture supernatants from non-infected (N.I.) B cells (added at 1:2 proportion) and RANKL expression was measured in culture supernatant by ELISA at 24 h p.i. (A). (B) Cocultures of macrophages and B cells (at 1:10 proportion) were infected with *B. abortus* at MOI 100 and RANKL was determine by ELISA at 24 h p.i. Data are given as the means ± SEM from at least three individual experiments. ***, $P < 0.001$; versus N.I.

pathological finding of osteoarticular brucellosis, it is essential to address whether and how B cells are linked to enhanced bone resorption in this form of brucellosis.

In this paper, we studied the role played by B cells in bone damage caused by *B. abortus* infection. Our results indicate that *B. abortus* infection of B cells induced the expression of MMP-9, RANKL and proinflammatory cytokines. Increases in MMP levels provide to mature B cells the ability to migrate to the injured tissue and site of inflammation [16], but locally increased levels of MMP have been found in several osteoarticular diseases which suggest that MMP may be involved in the damage associate with this pathology [19−21]. Furthermore, activated B cells have been recently shown to enhance osteoclastogenesis [41]. RANKL is a homotrimeric molecule displayed on the membrane of osteoblasts that stimulates differentiation of osteoclasts and is a key molecule involved in bone resorption. Under pathological conditions, increased expression levels of this mediator lead to bone destruction [37]. In addition, proinflammatory cytokines could contribute to bone damage, inducing osteoclastogenesis [23−26,36]. Our results demonstrated that OPG (RANKL's decoy receptor) abrogates osteoclast formation. This indicates that RANKL is the key cytokine involved in osteoclastogenesis induced by *B. abortus*-infected B cells. The ability of *B. abortus* to induce RANKL in B cells is in line with its capacity to induce the expression of this molecule in other bone cells, including synoviocytes, osteoblasts and osteocytes [31,32,42].

B cells have the ability to respond to TLR agonist [43]. TNF-α and IL-6 production by B cells in response to *B. abortus* were not dependent on bacterial viability, as these cytokines were also induced by exposure to HKBA, suggesting that they were elicited by a structural bacterial component. However, HKBA was unable to induce RANKL expression and concomitant osteoclastogenesis by B cells. This indicated that B cell-induction requires the presence of viable bacteria.

Macrophages/monocytes are the main replication niche for *Brucella*. Macrophages can also mediate B cell proliferation during *Brucella* infection [38]. However, macrophages could not contribute to osteoclastogenesis induced by *B. abortus*-infected B cells, since they did not increase the levels of RANKL expression by B cells, either when both cells were cocultured or when supernatants from *B. abortus*-infected macrophages were used to stimulate B cells.

Based on the results obtained in the present study, we hypothesize that *B. abortus* may activate B cells directly inducing the expression of RANKL, contributing to the bone and joint destruction observed in patients with osteoarticular complication of brucellosis.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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