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Brucella abortus Invasion of Synoviocytes Inhibits Apoptosis and Induces Bone Resorption through RANKL Expression

AQ: au **Romina Scian,**[a] **Paula Barrionuevo,**[a] **Ana María Rodriguez,**[a] **Paula Constanza Arriola Benitez,**[a] **Clara García Samartino,**[a] **Carlos Alberto Fossati,**[b] **Guillermo Hernán Giambartolomei,**[a] **María Victoria Delpino**[a]

Instituto de Inmunología, Genética y Metabolismo (INIGEM), Hospital de Clínicas "José de San Martín," Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina[a]; Instituto de Estudios de la Inmunidad Humoral (IDEHU), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina[b]

Arthritis is one of the most common complications of human active brucellosis, but its pathogenic mechanisms have not been completely elucidated. In this paper, we describe the role of synoviocytes in the pathogenesis of brucellar arthritis. Our results indicate that *Brucella abortus* infection inhibited synoviocyte apoptosis through the upregulation of antiapoptotic factors (cIAP-2, clusterin, livin, and P21/CIP/CDNK1A). In contrast, infection did not change the expression of proteins that have been involved in apoptosis induction such as Bad, Bax, cleaved procaspase 3, CytC, and TRAIL, among others; or their expression was reduced, as occurs in the case of P-p53(S15). In addition, *B. abortus* infection induced upregulation of adhesion molecules (CD54 and CD106), and the adhesion of monocytes and neutrophils to infected synoviocytes was significantly higher than to uninfected cells. Despite this increased adhesion, *B. abortus*-infected synoviocytes were able to inhibit apoptosis induced by supernatants from *B. abortus*-infected monocytes and neutrophils. Moreover, *B. abortus* infection increased soluble and membrane RANKL expression in synoviocytes that further induced monocytes to undergo osteoclastogenesis. The results presented here shed light on how the interactions of *B. abortus* with synovial fibroblasts may have an important role in the pathogenesis of brucellar arthritis.

Brucellae are Gram-negative intracellular pathogens that can survive and multiply within phagocytic cells (1, 2). Humans become infected by ingesting unpasteurized dairy products, being in direct contact with infected animals, or inhaling infectious aerosols (3). The distribution of this disease is worldwide, and areas of high endemicity include the Mediterranean, the Middle East, Latin America, and Asia (4–6).

Osteoarticular brucellosis is the most common presentation of the active disease in humans, affecting up to 85% of patients (7–9). The three most frequent forms of osteoarticular involvement are sacroileitis, spondylitis, and peripheral arthritis (7, 10–13). Arthritis is one of the most common presentations of localized disease in human brucellosis and may be caused by different *Brucella* species (7–9). Osteoarticular involvement may be observed in acute or chronic cases of human brucellosis (7–9) and may affect patients of any age (7–9, 14). Imaging studies have revealed cartilage loss and bone erosion in brucellar arthritis affecting different joints (12, 14). These lesions may eventually lead to permanent joint dysfunction. *Brucella* spp. are isolated from synovial fluid samples in about 50% of the cases (8, 11). The synovial membrane of the affected joint may present a lymphomononuclear infiltrate in the chronic phase of the disease but usually presents a polymorphonuclear infiltrate in acute cases (8, 11). Modulation of adhesion molecules by *B. abortus* might be central in this process.

Since *Brucella* spp. are intracellular pathogens, they may survive and multiply despite the hostile environment generated by the inflammatory immune response induced. Successful strategies for intracellular survival include a panoply of mechanisms, such as the ability to survive in membrane-bound vesicles (15–17), alteration of macrophage apoptosis (18, 19), and the inhibition of membrane expression of major histocompatibility complex (MHC) class II and I (20, 21), among others.

We have recently partially deciphered potential mechanisms that involved synoviocytes in bone damage caused by *Brucella*. We demonstrated that *Brucella* spp. can infect and survive within human synoviocytes and that this infection elicits the secretion of matrix metalloproteases (MMPs) that might be involved in the osteoarticular manifestations of brucellosis (22). Notwithstanding, at present it has not been investigated whether *Brucella*-infected synoviocytes are involved in the pathological increase in numbers of osteoclasts (cells involved in bone resorption) and whether *Brucella* infection alters synoviocyte survival. In addition, considering the relevance of macrophages and neutrophils as infiltrating cells in inflammatory tissues and taking into account that synoviocytes secrete monocyte chemoattractant protein 1 (MCP-1) and interleukin-8 (IL-8) in response to *Brucella* infection (22), we also decided to investigate the role of these cells as modulators of synoviocyte survival, which has been associated with osteoarticular damage (23).

In the present study, we demonstrated that *B. abortus* infection inhibited synoviocyte apoptosis. In addition, infection induced the upregulation of adhesion molecules (CD54 and CD106), which led to the adhesion of monocytes and neutrophils to synoviocytes. Despite this increased adhesion, *B. abortus*-infected synoviocytes were able to inhibit apoptosis induced by supernatants from *B. abortus*-infected monocytes and neutrophils. Moreover, *B. abortus* infection increased soluble and membrane RANKL expression in synoviocytes, which further induced monocytes to undergo osteoclastogenesis.

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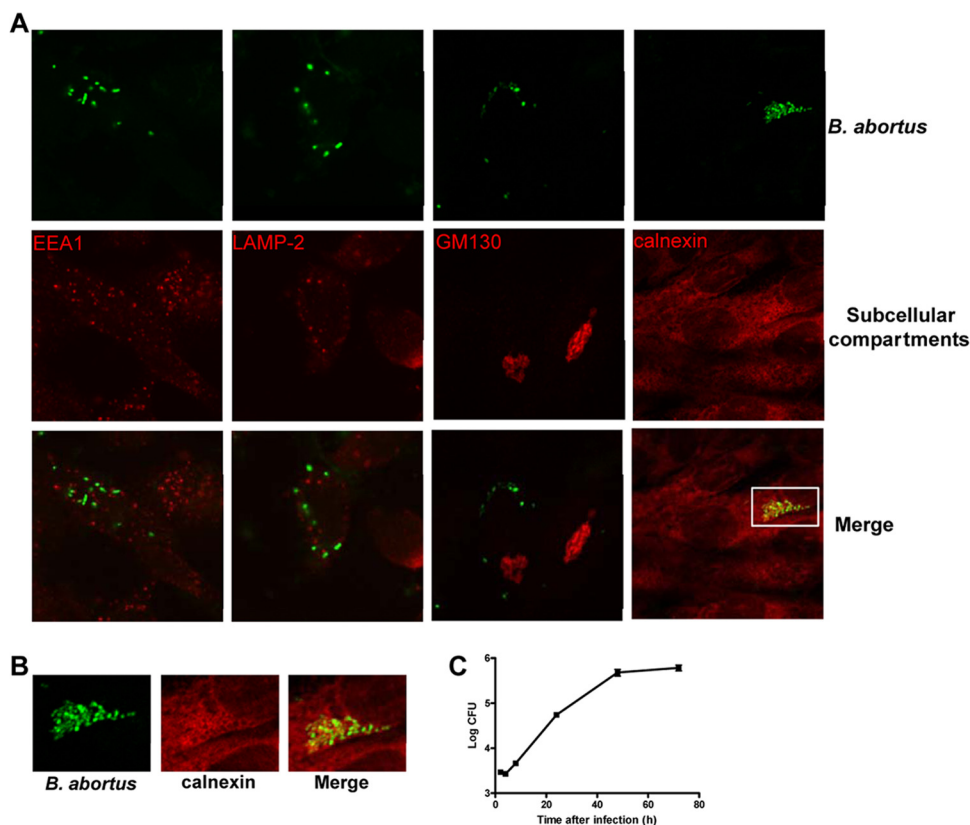
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FIG 1 *B. abortus* replicates within LAMP-positive vacuoles in human primary synoviocytes. (A) Confocal micrographs of human synoviocytes infected with GFP-expressing *B. abortus* at an MOI of 100 and stained at 24 h after infection with the subcellular localization markers EEA1, LAMP-2, GM130, and calnexin followed by Alexa 546-labeled secondary antibody (red). Results are representative of 3 independent experiments. (B) Magnification of the inset in panel A showing *B. abortus* enclosed within calnexin-positive vacuoles. (C) Infection and replication of synoviocytes. After infection with *B. abortus* (MOI, 100), cells were incubated with antibiotics to kill extracellular bacteria. Cells were lysed at different times p.i. and plated on agar to determine intracellular CFU. Values are means ± standard errors of the means (SEM) of triplicates from one experiment, which was repeated twice with similar results.

MATERIALS AND METHODS

Bacterial cultures. *Brucella abortus* S2308 was grown overnight in 10 ml of tryptic soy broth (Merck, Buenos Aires, Argentina) with constant agitation at 37°C. Bacteria were harvested by centrifugation for 15 min at $6,000 \times g$ at 4°C and washed twice in 10 ml of phosphate-buffered saline (PBS). The numbers of bacteria in stationary-phase cultures were determined by comparing the optical densities at 600 nm (OD_{600}) with a standard curve obtained in our laboratory. To obtain the standard curve, the spectrophotometer was calibrated using tryptic soy broth as the blank reference. A single colony of *B. abortus* was selected and inoculated into 1 ml of tryptic soy broth and then incubated at 37°C under agitation at 200 rpm for 16 h. Then, the bacterial solution was diluted in tryptic soy broth to an OD_{600} of 0.100 using the spectrophotometer reader. At each 30-min interval, an aliquot of the sample was used to determine the OD_{600}, and another aliquot was used to determine the CFU by plating cells onto tryptic soy agar. This procedure was followed during 48 h.

To prepare the 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. All live *Brucella* manipulations were performed in biosafety level 3 facilities located at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS).

Cell cultures. Primary synovial fibroblasts (synoviocytes) were isolated from synovial tissues obtained from patients undergoing total knee replacement surgery in accordance with the guidelines of the Ethical Committee of the INIGEM Institute. A written consent was obtained from all patients. The number of patients included in this study was 11. The tissue was finally minced and subjected to collagenase digestion at 1 mg/ml (Invitrogen, Carlsbad, CA) in Dulbecco's minimal essential medium (DMEM). Digested cells were resuspended in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (complete medium). When the cells were confluent, they were passaged by gentle trypsinization (0.25% trypsin in PBS) and were used between passages 4 and 8 (24).

Human neutrophils were isolated from venous blood from healthy human volunteers by a Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient, followed by the sedimentation of erythrocytes in 6% dextran and hypotonic lysis (25, 26). Neutrophils were harvested, washed twice with sterile PBS, and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 5% FBS, 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell viability was 98%, as determined by trypan blue exclusion. The purity of the final neutrophil preparation was 95%, as assessed by morphological examination after Giemsa staining and flow cytometry light scatter patterns as described earlier (27–32).

Human monocytes isolated from peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque using a monocyte-negative isolation kit (BD Biosciences, San Diego, CA). The human monocytic cell line THP-1 was cultured in a 5% CO_2 atmosphere at 37°C in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce THP-1 maturation, cells were cultured in the presence of 0.05 μM 1,25-dihydroxyvitamin D_3 (Calbiochem-Nova

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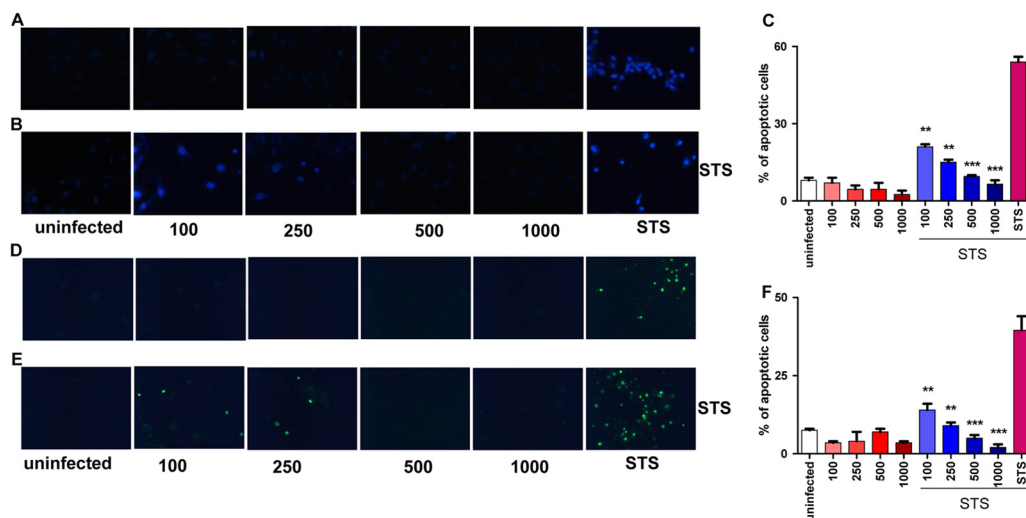*B. abortus* Inhibits Synoviocyte Apoptosis

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FIG 2 *B. abortus* inhibits STS-induced apoptosis of synoviocytes. Cells were infected or not at different MOI (100 to 1,000). After 24 h, some infected cells were treated with staurosporine (STS). Uninfected cells treated with STS were used as a positive control. Apoptosis was evaluated by the Hoechst 33342 and TUNEL techniques, using fluorescence microscopy. (A) Apoptosis of *B. abortus*-infected cells evaluated by Hoechst 33342. (B) Apoptosis of *B. abortus*-infected cells treated with STS 24 h after infection, by Hoechst 33342. (C) Quantitative analysis of experiments presented in panels A and B. (D) Apoptosis of *B. abortus*-infected cells, analyzed by TUNEL assay. (E) Apoptosis of *B. abortus*-infected cells treated with STS 24 h after infection, analyzed by TUNEL assay. (F) Quantitative analysis of experiments presented in panels D and E. Bars express the means ± SEM of duplicates. Data shown are from a representative experiment of five performed. **, $P < 0.01$; ***, $P < 0.001$ for STS-treated and uninfected cells.

Biochem International, La Jolla, CA) for 72 h, as described previously (33). All cells were seeded at 5×10^5 cells/well in 24-well plates.

Cellular infection. Synoviocytes were infected with *B. abortus* at different multiplicities of infection (MOI), and neutrophils and THP-1 cells were infected at an MOI of 100. After the bacterial suspension was dispensed, the plates were centrifuged for 10 min at 2,000 rpm and then incubated for 2 h at 37°C under a 5% CO_2 atmosphere. Cells were extensively washed with DMEM to remove extracellular bacteria and incubated in medium supplemented with FBS (10%), 100 μg/ml gentamicin, and 50 μg/ml streptomycin to kill extracellular bacteria. Synoviocytes were harvested at 24 h postinfection. Supernatants from THP-1 cells or neutrophils were harvested at 24 h postinfection (p.i.) to be used as conditioned medium.

CD54, CD106, CD62L, and RANKL expression on synoviocytes. Synoviocytes were infected at different MOI with *B. abortus*. As a positive control, some cells were stimulated with 200 mM phorbol 12–myristate 13–acetate (PMA). At the end of culture, cells were washed and incubated with a fluorescein isothiocyanate (FITC)-labeled anti-human CD54, anti-human CD106, and anti-human CD62L monoclonal antibody (BD Pharmingen, San Jose, CA), or FITC-labeled anti-human 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 mean fluorescence intensity (MFI). In addition, RANKL expression was assessed using a fluorescence plate reader (Victor3; PerkinElmer, Waltham, MA).

Adhesion of neutrophils and monocytes to synoviocytes. Synoviocytes grown in 96-well plates were infected with *B. abortus* S2308 at different MOI as described previously (34) or stimulated with PMA as a positive control for 24 h. Uninfected/untreated cells were used as a negative control. Then, synoviocytes were washed to eliminate residual bacteria or stimulant before the addition of 1×10^6 human neutrophils or monocytes (THP-1 cells or monocytes purified from venous blood) previously labeled with calcein acetoxymethyl ester fluorescent dye (BD Biosciences). After a 1-h incubation at 37°C, the nonadherent cells were washed away and plates were read in a fluorescence plate reader (Victor3; PerkinElmer) using 485/530-nm excitation/emission filters. Average percent adhesion was calculated according to

the following formula: (RFU after wash/RFU before wash) × 100, where RFU represents relative fluorescence units.

Apoptosis assays. Sinoviocytes were infected at different MOI and 24 h after infection were stimulated with 1 μM staurosporine (STS) or a 1/2 dilution of supernatants from *B. abortus*-infected monocytes or neutrophils at an MOI of 100. As a positive control, cells were treated with 4% paraformaldehyde (PFA) or STS. Twenty-four hours later, cells were washed, and the percentage of apoptotic cells was assessed by the annexin V-FITC (Sigma-Aldrich de Argentina S.A.) assay with fluorescence-activated cell sorter (FACS) analysis. The percentage of apoptotic cells was also assessed by fluorescence microscopy after the cells were labeled by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay or by staining with the Hoechst 33342 dye.

Osteoclast formation assay. THP-1 monocytes were induced to undergo osteoclastogenesis as described previously (35). Briefly, THP-1 cells were cultured with 30 ng/ml M-CSF in 24-well plates for 48 h. No adherent cells were washed out, and adherent cells were collected and seeded onto glass coverslips in 24-well plates for 6 days and cultured in complete medium containing 30 ng/ml of macrophage colony-stimulating factor (M-CSF; R&D Systems, Minneapolis, MN, USA) and 0.2 ml of supernatants from *B. abortus*-infected synoviocytes. As positive controls of osteoclast formation, THP-1 cultures received 50 ng/ml of human receptor activator for nuclear factor κB ligand (RANKL; R&D Systems). On day 3, the culture media and all reagents were replaced. To identify osteoclasts, cells were fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich). TRAP-positive, multinucleated (i.e., containing more than three nuclei) cells were defined as osteoclasts, and the number was determined by microscopic counts. In addition, vitronectin receptor (CD51) expression was determined using phycoerythrin (PE)-labeled anti-human CD51 (BD Biosciences). TRAP- or CD51-positive multinucleated cells were defined as osteoclasts, and their number was determined by microscopic counts.

Pit formation assay. THP-1 cells (2×10^4 cells/0.25-ml well), used as osteoclasts precursors, were plated on dentine disks (BD BioCoat Osteologic) in 96-well culture dishes and cultured in complete medium containing stimulated synoviocytes with 30 ng/ml of M-CSF for 6 days. Medium and all reagents were replaced every day to avoid acidification. After

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culture with cells, dentine disks were washed with 1 M NH$_4$OH to remove adherent cells. After rinsing with water, dentine disks were visualized by light microscopy to determine and enumerate resorption lacunae.

Neutralization experiments. Neutralization experiments were performed using 20 μg/ml of osteoprotegerin (OPG; R&D Systems), RANKL's decoy receptor. Supernatants from *Brucella*-infected synoviocytes were preincubated with the decoy receptor for 1 h at 37°C before being used to stimulate THP-1.

Antibody array. Determination of the relative levels of apoptosis-related proteins was determined using the human apoptosis array kit (R&D Systems) according to the manufacturer's instructions. Briefly, array membranes were incubated with 200 μg of lysates from *B. abortus*-infected synoviocytes or uninfected cells as a control, diluted in 250 μl of array buffer provided by the customer, and incubated during 18 h at 4°C. Then, membranes were washed with wash buffer and incubated with a detection antibody cocktail conjugated to peroxidase. Spots were visualized using Hyperfilm ECL (GE, Little Chalfont, United Kingdom) enhanced chemiluminescence.

Western blotting. Infected synoviocytes were lysed in ice-cold lysis buffer consisting of 20 mM HEPES (pH 8), 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 10% glycerol, 1 mM dithiothreitol, 400 mM KCl, 0.4% Triton X-100, 20 mM sodium β-glycerophosphate, and a protease inhibitor cocktail (Sigma-Aldrich). Lysates were incubated on ice for 10 min and cleared by centrifugation at 13,000 × *g* for 10 min. Protein concentrations were measured in the supernatants by the Bradford method (36), and equal amounts of proteins were loaded onto electrophoresis gels. After separation, proteins were transferred to a nitrocellulose membrane (GE Healthcare) and blocked for 1 h with 5% milk protein–0.1% Tween 20. Then, membranes were incubated with primary goat polyclonal IgG anti-human clusterin (R&D Systems) overnight at 4°C. After washing, the membrane was incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:2,000 dilution), for 1 h. Protein bands were visualized on Hyperfilm ECL (GE Healthcare) by chemiluminescence. Equal loading was checked by Ponceau S staining and by incubation of the blots with an anti-β-actin (clone C4; Santa Cruz Biotechnology).

ELISA. Clusterin was quantified by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). The protocol was performed according to the manufacturer's instructions.

RANKL was detected in culture supernatants using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by post hoc Tukey's test using GraphPad Prism 4.0 software. Data are represented as means ± standard deviations (SD).

RESULTS

***Brucella abortus* invades and multiplies in primary human synoviocytes.** We have demonstrated that *Brucella* spp. can infect and replicate within a human fibroblast-like synoviocytic cell line and that this infection may be involved in the osteoarticular manifestations of brucellosis (22). Yet the bacterium's capacity to infect and multiply in primary human synoviocytes has not been elucidated. Infection experiments showed that *B. abortus* is internalized by human primary synoviocytes *in vitro*. A follow-up of infected cultures revealed that *B. abortus* can replicate inside human primary synoviocytes (Fig. 1C) and that at 24 h postinfection intracellular CFU counts increased by about 3 log and then increased slightly during the next 24 h. Since in macrophages the establishment of a persistent infection relies on the ability of the bacterium to form a *Brucella*-containing vacuole (BCV), which traffics from the endocytic compartment to the endoplasmic reticulum (ER) (17, 37, 38), we decided to investigate the intracellular fate of the bacterium in human synoviocytes. By using green fluorescent protein (GFP)-expressing *B.*

TABLE 1 Protein array of pro- and antiapoptotic factors

Factor	Fold increase over uninfected cells
Antiapoptotic	
Bcl-2	1
Bcl-x	1
cIAP-1	1
cIAP-2	1.39
Claspin	1
Clusterin	1.33
HO-1/HMOX1/HSP32	1
HO-2/HMOX2	1
HSP27	1
HSP60	1
HSP70	1
Livin	1.54
PON2	1
P21/CIP/CDNK1A	1.16
Survivin	1
XIAP	1
Proapoptotic	
Bad	1
Bax	1
Procaspase 3	1
Cleaved	1
CytC	1
TRAIL R2/DR5	1
TRAIL R1/DR4	1
FADD	1
Fas/TNFRSF6	1
HTRA2/Omi	1
p27/Kip1	1
P-p53 (S15)	0.67
P-p53 (S46)	1
P-p53 (S392)	1
P-Rad17 (S635)	1
SMAC/Diablo	1
TNF RI/TNFRSF1A	0.40
HIF-1a	1

abortus and primary antibodies specific for subcellular compartments followed by Alexa 546-labeled (red) secondary antibody, we observed that after 24 h of infection *B. abortus* was enclosed within calnexin-positive vacuoles and did not colocalize with the early endosomal antigen 1 (EEA1), the lysosome-associated membrane protein 2 (LAMP-2), or the Golgi matrix protein GM130, indicating that the bacterium replicates in the endoplasmic reticulum of primary human synoviocytes (Fig. 1A and B).

***B. abortus* inhibits staurosporine-induced apoptosis of synoviocytes.** Because apoptotic and antiapoptotic effects have been observed in *Brucella* species infections (18, 19, 34, 39–41) and these phenomena were dependent on the cell type, we decided to investigate the effect of *B. abortus* infection on synoviocytes viability. For this, these cells were infected with *B. abortus* at different MOI, and after 24 h, cells were stained with annexin V-phosphatidylinositol (PI) and analyzed by flow cytometry to evaluate apoptosis. Paraformaldehyde (PFA; 4%) was used as a positive control. *B. abortus* infection did not induce synoviocyte apoptosis at any MOI tested. This result was confirmed by Hoechst 33342 staining (Fig. 2A and C) and TUNEL (Fig. 2D and F) assay. In contrast, *B.*
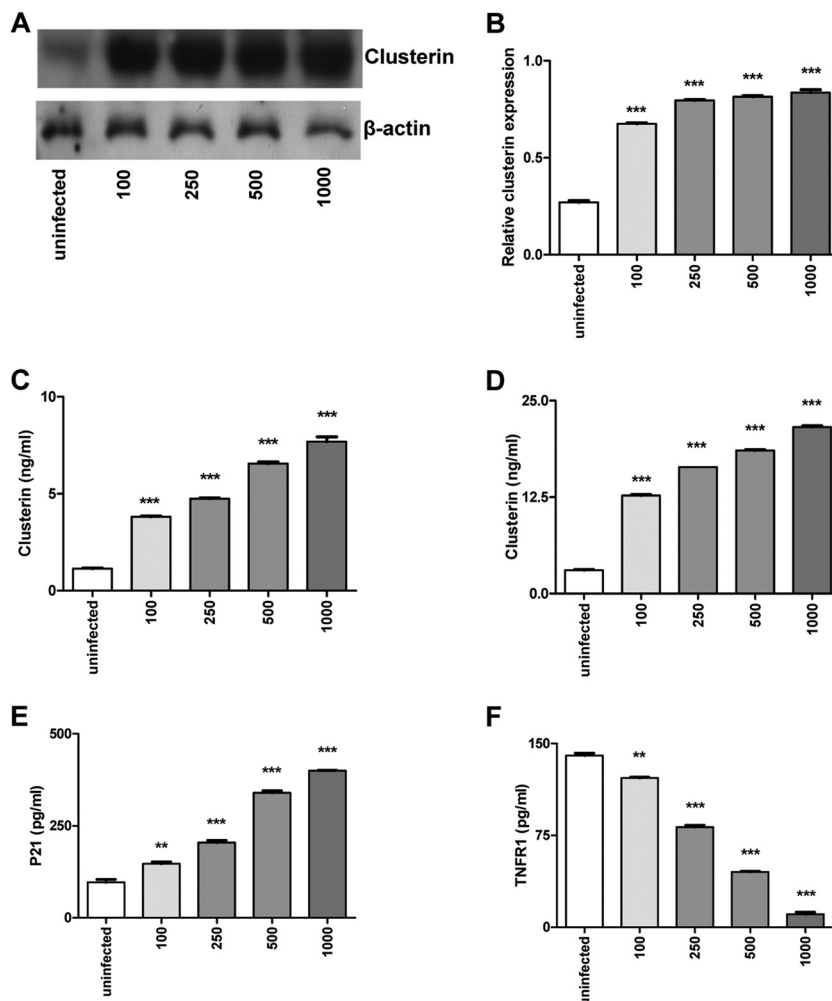
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B. abortus Inhibits Synoviocyte Apoptosis

FIG 3 *B. abortus* infection induces clusterin and p21 expression but inhibits TNFR1 expression. Synoviocytes were infected at MOI of 100 to 1,000. (A) Cell lysates obtained at 24 h postinfection were used to determine clusterin expression by Western immunoblotting. (B) Densitometric analysis of results from two independent experiments performed as described for panel A. Clusterin production by *B. abortus*-infected synoviocytes was measured at 24 h after infection with *B. abortus* by ELISA in cell lysates (C) and culture supernatants (D). (E and F) P21 (E) and TNFR1 (F) production by *B. abortus*-infected synoviocytes was assessed by ELISA in cell lysates. Bars express the means ± SEM of duplicates. Data shown are from a representative experiment of three performed. ***, $P < 0.001$ versus uninfected cells.

abortus infection was able to inhibit synoviocyte apoptosis induced by staurosporine (STS) in an MOI-dependent fashion as was determined by Hoechst 33342 staining (Fig. 2B and C) and corroborated by TUNEL (Fig. 2E and F). These results indicate that *B. abortus* infection prevents STS-induced synoviocyte apoptosis.

B. abortus infection of synoviocytes upregulates the expression of antiapoptotic factors. To further investigate the mechanism involved in the inhibition of synoviocyte apoptosis, differential expression of apoptotic and antiapoptotic proteins was determined in synovial fibroblast cells infected with *B. abortus*, and these results were compared to those obtained with uninfected cells. Protein array analysis revealed the upregulation of antiapoptotic factors such as cIAP-2 apoptotic suppressor (fold increase, 1.39), clusterin (fold increase, 1.33), livin (fold increase, 1.54), and P21/CIP/CDNK1A (fold increase, 1.16). In contrast, the expression of proteins that are involved in apoptosis induction [Bad, Bax, cleaved procaspase 3, CytC, TRAIL R1/DR4, FADD,

Fas/TNFRSF6, HTRA2/Omi, p27/Kip1, P-Rad17(S635), SMAC/Diablo, HIF-1a, and TRAIL R2/DR5] was not changed; or their expression was reduced, as in the case of P-p53(S15) (fold decrease, 1.48) and tumor necrosis factor (TNF) RI/TNFRSF1A (fold decrease, 2.44) (Table 1).

To further validate the protein array results, we corroborated the upregulation of two antiapoptotic factors, clusterin and p21; the downregulation of one of the apoptotic factors, TNFR1; and the unmodified regulation of two nonmodulated molecules including a proapoptotic factor, TRAIL R1DR4 and the antiapoptotic factor survivin. Clusterin expression was determined by Western blotting using the eukaryotic housekeeping β-actin protein as a reference for gel loadings. Upon infection of synoviocytes, *B. abortus* induced significant ($P < 0.001$) upregulation of the expression of clusterin when these cells were infected with MOI of 100 to 1,000 (Fig. 3A and B). Quantitative analysis of clusterin expression by ELISA in synovial fibroblast lysates and culture supernatants corroborated that *B. abortus* was able to induce the
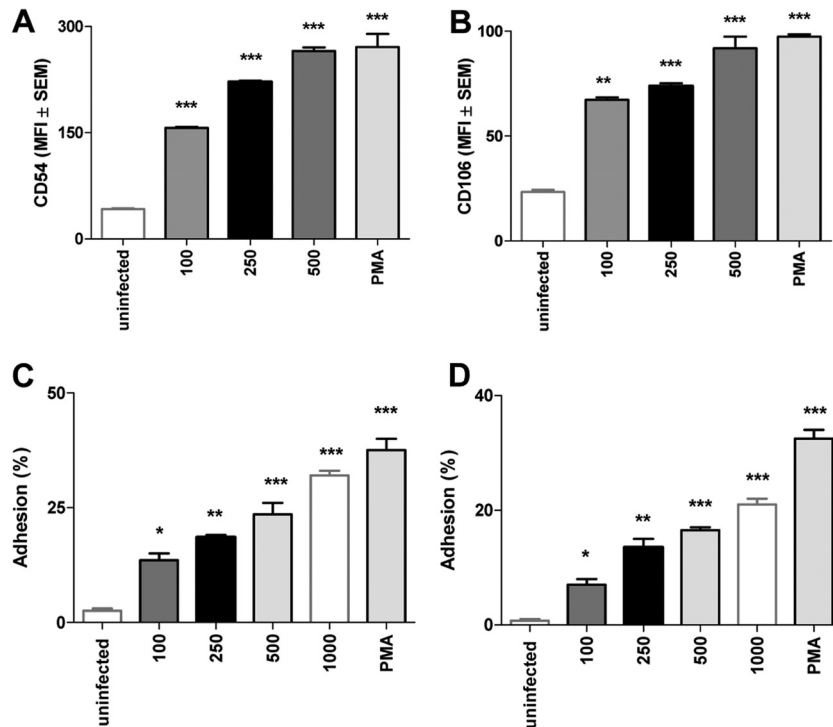
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FIG 4 Monocyte and neutrophil adhesion to *B. abortus*-infected synoviocytes. Flow cytometry analysis of CD54 (A) and CD106 (B) expression in *B. abortus*-infected synoviocytes and uninfected cells. Adhesion of calcein-labeled neutrophils (C) and purified human monocytes (D) to synoviocytes infected with *B. abortus* at different MOI, uninfected cells, or PMA-treated cells after coincubation for 1 h at 37°C. Nonadherent cells were washed away, and plates were read in a fluorescence plate reader. The percentage of adhesion was calculated as follows: (RFU after wash/RFU before wash) $\times$ 100. Data shown are from a representative experiment of five performed. *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$ versus uninfected cells (uninfected).

upregulation of clusterin expression with respect to the level of unstimulated cells in an MOI-dependent fashion (Fig. 3C and D). In addition, *B. abortus* infection induced the upregulation of p21 (Fig. 3E) and the downregulation of TNFR1 (Fig. 3F) in an MOI-dependent manner, as assessed by ELISA. Also, in concordance with the array, the expression of survivin and TRAIL R1DR4 did not change in response to *B. abortus* infection, as was determined by ELISA. Altogether, these results indicate that infection by *B. abortus* inhibits apoptosis of synoviocytes by upregulating the expression of antiapoptotic factors.

B. abortus infection upregulates CD54 and CD106 but not CD62L expression. Adhesion molecules are involved in cellular recruitment during immune responses, and intercellular adhesion molecule-1 (ICAM-1, CD54), vascular cell adhesion protein-1 (VCAM-1, CD106), or CD62L (L-Selectin) are expressed by several cell types, including synoviocytes (42, 43). As lympho-polymorphonuclear infiltrates are present in the synovia of infected patients (8, 11), we speculate that modulation of adhesion molecules in synoviocytes by *B. abortus* might be central in this process, and also this modulation of expression might lead to a harmful response induced by infiltrating cells. *B. abortus* infection upregulated the CD54 and CD106 expression on synoviocytes in an MOI-dependent fashion (Fig. 4A and B). In contrast, CD62L expression was not affected (not shown). PMA stimulation was used as a positive control. These results indicate that *B. abortus* infection may induce the upregulation adhesion molecules, and this upregulation could lead to an increase in the adhesion of cell from the innate immunity.

Neutrophils and monocytes adhere to B. abortus-infected synoviocytes. In previous studies, we demonstrated that *B. abortus*-infected synoviocytes secrete IL-8 and MCP-1, inducing neutrophil and monocyte migration (22). Cell-to-cell adherence is a mandatory step in the interaction between cells in the immune response, and neutrophilic and monocytic infiltrates have been found in histological studies on osteoarticular involvement in human brucellosis (42, 43). Since CD54 and CD106 expression was upregulated in *B. abortus*-infected synoviocytes, we investigated if neutrophils and monocytes can adhere to such cells. To this end, synoviocytes were infected at different MOI, and calcein-labeled uninfected neutrophils or monocytes (THP-1 cells or monocytes purified from human venous blood) were added to the culture. As shown in Fig. 4C and D, the percentage of adhered purified neutrophils and monocytes increased significantly when synoviocytes were infected with *B. abortus*, and this increase was MOI dependent. The same result was obtained with THP-1 cells (not shown). This indicates that *B. abortus*-infected synoviocytes may induce the adhesion of immune cells that could lead to a potential harmful response.

B. abortus infection inhibits apoptosis induced by supernatants from B. abortus-infected monocytes and neutrophils. Neutrophils and monocytes have been shown to induce apoptosis of cells from infiltrated tissues due to the release of MMPs, reactive oxygen species, and TNF-α (34, 44, 45). However, our results indicate that *B. abortus* infection inhibits apoptosis of synoviocytes induced by STS. Thus, experiments were conducted to determine whether *B. abortus* infection may also inhibit apoptosis
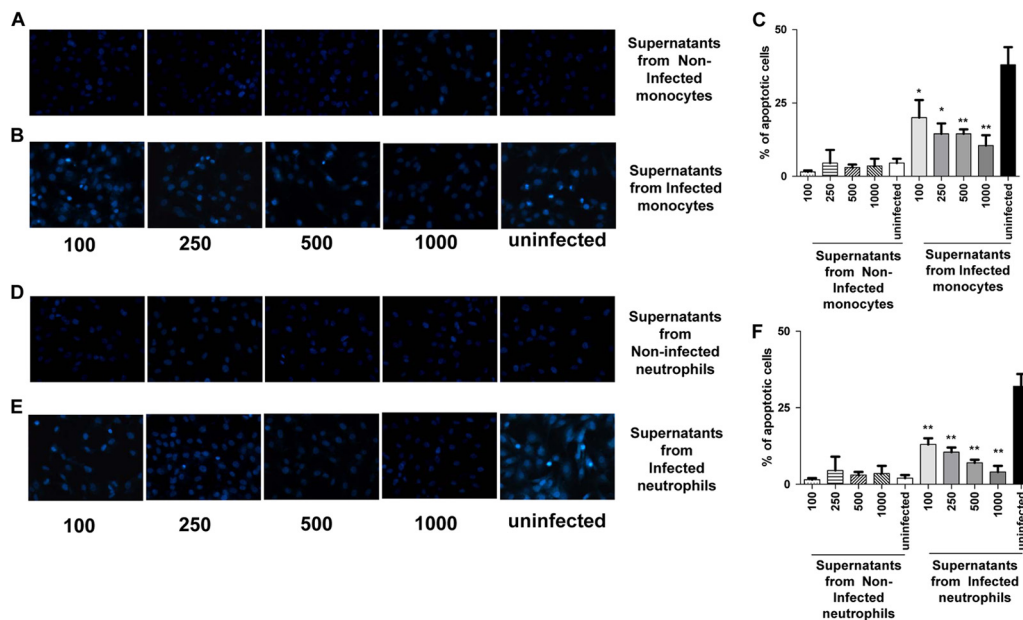
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B. abortus Inhibits Synoviocyte Apoptosis

FIG 5 Effects of *B. abortus*-infected monocytes and neutrophils on the viability of infected and uninfected synoviocytes. Fluorescence microscopy analysis of apoptosis using Hoechst 33342 techniques of *B. abortus*-infected synoviocytes treated with supernatants added at 1/2 proportion from noninfected purified human monocytes (A) and neutrophils (D) or with supernatants added at 1/2 proportion from *B. abortus*-infected purified human monocytes (B) and neutrophils (E). Data shown are from a representative experiment of five performed. *, $P < 0.1$; **, $P < 0.01$ versus uninfected cells (uninfected). Bars express the means ± SEM of triplicates. (C) Quantitative analysis of experiments presented in panels A and B. (F) Quantitative analysis of experiments presented in panels D and E.

induced by neutrophils' and monocytes' secreted factors. For this, supernatants from *B. abortus*-infected purified monocytes (or neutrophils) were used to stimulate synoviocytes previously infected with *B. abortus* for 24 h. Supernatants from monocytes and neutrophils infected with *B. abortus* induced apoptosis of uninfected synoviocytes. Yet upon infection of synoviocytes, apoptosis was inhibited in an MOI-dependent manner (Fig. 5A and B). These results indicate that supernatant from *B. abortus*-infected monocytes or neutrophils induces synoviocyte apoptosis but this phenomenon could be inhibited when synoviocytes were previously infected with *B. abortus*. Also, they indicate that despite the ability of *B. abortus*-infected synoviocytes to recruit and attach monocytes and neutrophils, infection protects them from the potential harm induced by these cells.

B. abortus-infected synoviocytes induce osteoclastogenesis. Osteoclasts play a key role in bone resorption and originate from the fusion of precursors belonging to the monocyte/macrophage lineage in the bone marrow (46, 47). This process may involve soluble mediators from inflammatory cells or membrane-bound and soluble RANKL in conjunction with M-CSF (48). To determine if factors secreted by *B. abortus*-infected synoviocytes could induce osteoclast formation from monocytic THP-1 cells, these cells were stimulated with M-CSF in conjunction with supernatants from *B. abortus*-infected synoviocytes, and *ex vivo* osteoclastogenesis was determined by the generation of multinucleated vitronectin receptor- and TRAP-expressing cells. RANKL was used as a positive control. Supernatants from *Brucella*-infected synoviocytes induced osteoclastogenesis. The magnitude of osteoclastogenesis induced was related directly to the MOI used to infect synovial fibroblasts (Fig. 6). Osteoclastogenesis was also induced when monocytic THP-1 cells were cocultured with synoviocytes in the presence of M-CSF (not shown).

B. abortus-infected synoviocytes induce osteoclastogenesis mediated by RANKL. RANKL and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been shown to be important in bone resorption (48, 49). We have previously demonstrated that synoviocytes are unable to produce detectable amounts of proinflammatory cytokines in response to *B. abortus* infection (22). Moreover, RANKL is upregulated in other bacterial osteoarthritis (50, 51), and we have detected RANKL (400 ± 22 pg/ml) in a synovial fluid from a patient with prepatellar bursitis caused by *B. abortus* (52). Therefore, we investigated whether *B. abortus* infection would lead to membrane-bound and soluble RANKL expression from synoviocytes. To this end, cell surface RANKL protein presence was evaluated by flow cytometry and fluorometry. *B. abortus* infection induced the upregulation of membrane-bound RANKL expression in an MOI-dependent fashion (Fig. 7A, B, and C). Quantitative analysis of RANKL expression on synoviocytes supernatants revealed that *B. abortus* was also able to induce the secretion of soluble RANKL expression as determined by ELISA (Fig. 7D). These results indicated that *B. abortus* induced the upregulation of RANKL, which could contribute to bone destruction through activation of osteoclastogenesis.

To assess the role of RANKL in osteoclastogenesis elicited by *B. abortus*, THP-1 cells were cultured with M-CSF and supernatants from *B. abortus*-infected synoviocytes in the presence of OPG, the 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 synovial fibroblasts (Fig. 8 A and B). These results indicate that RANKL secreted by *B. abortus*-infected synoviocytes could be involved in the bone loss observed in osteoarticular brucellosis.
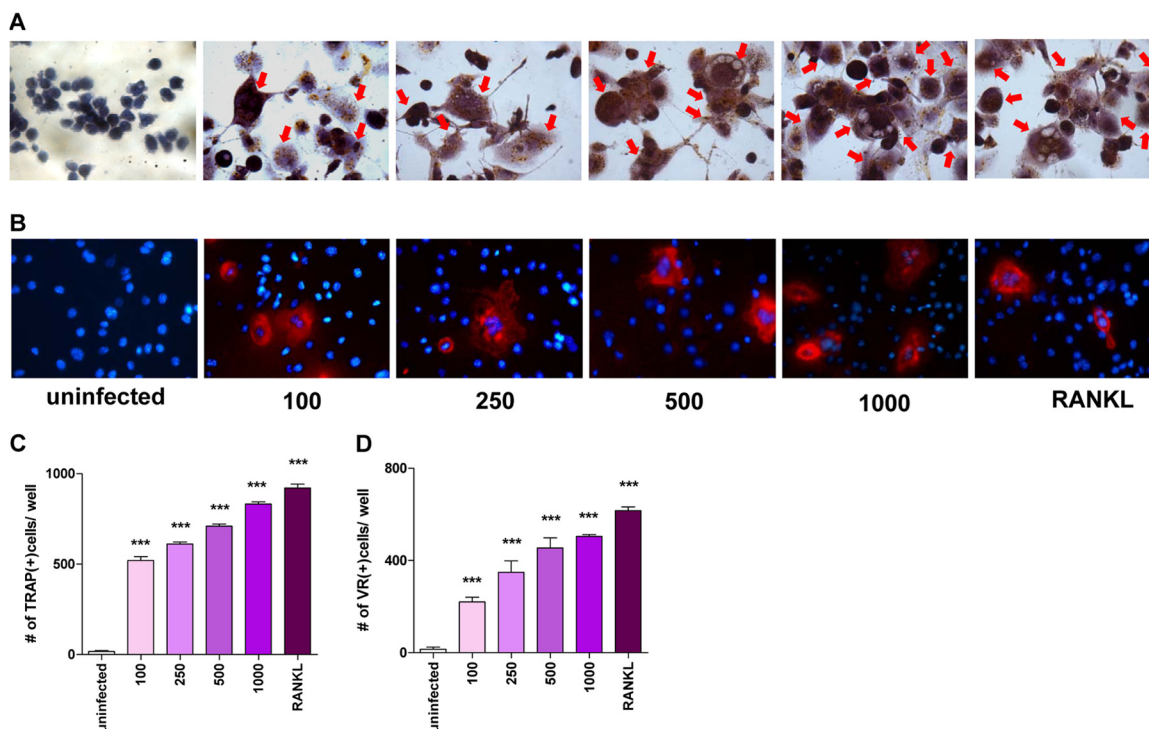
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FIG 6 Supernatants from *B. abortus*-infected synoviocytes induced monocyte-derived osteoclastogenesis. THP-1 cells were stimulated with supernatants from synoviocytes infected with *B. abortus* for 24 h at different multiplicities or uninfected in conjunction with M-CSF. RANKL was used as a positive control. After 5 days, osteoclastogenesis was determined by the generation of multinucleated TRAP-expressing (A and C) and vitronectin receptor (VR)-expressing (B and D) cells. Representative digital images were taken by light microscopy (A) or fluorescence microscopy (B), and multinucleated TRAP-positive (C) or vitronectin receptor-positive (D) cells were identified and counted. Bars express the means ± SEM of triplicates. Data shown are from a representative experiment of five performed. ***, $P < 0.001$ versus uninfected cells.

COLOR

***B. abortus*-infected synoviocytes induce functional osteoclast-like cells.** Our premise is that *B. abortus* infection might activate synoviocytes in a way that would promote the generation of osteoclasts, leading to bone loss. Thus, we assessed the functional activity of *B. abortus*-induced osteoclast-like cells by their ability to resorb dentine. THP-1 cells treated with supernatants from *B. abortus*-infected synoviocytes were able to induce a significant ($P < 0.001$) dentine resorption in an MOI-dependent manner. In contrast, supernatants from uninfected synoviocytes did not (Fig. 8C and D). Additionally, the resorption of dentine was blocked when the experiments were performed in the presence of OPG (Fig. 8C and D). Taken together, these results indicate that supernatants from *B. abortus*-infected synoviocytes could promote functional osteoclasts formation from human monocytes, leading to bone loss.

DISCUSSION

Osteoarticular brucellosis is the most common presentation of human active brucellosis disease (7–9, 11, 53). In this paper, we studied the role played by synoviocytes in this form of the disease, as they have been recognized as central mediators of joint damage in inflammatory arthritides of either infectious or noninfectious origins (54, 55).

Smooth *Brucella* species have developed several mechanisms to survive intracellularly, especially inside macrophages. We have demonstrated that *B. abortus* infects and replicates in human synoviocytes (22). In this study, we confirm and extend these results, demonstrating the bacterium's capacity to infect and multiply in

primary human synoviocytes. The ability of *B. abortus* to invade, survive, and replicate within synovial fibroblast is in line with its capacity to replicate in other nonphagocytic cells, including hepatocytes, astrocytes, and osteoblasts (34, 40, 56, 57). However, unlike what happens in all these cells, the infection did not induce synoviocyte apoptosis. Moreover, it inhibits the apoptosis induced by STS and by culture supernatants from *B. abortus*-infected neutrophils and monocytes. Thus, *B. abortus* could use these cells as an alternative replicative niche in the joints. Confocal imaging confirmed this speculation, indicating that, as occurred in macrophages, *B. abortus* replicates within calnexin-positive vacuoles in human primary synoviocytes.

Since the first report of macrophage apoptosis following infection with *Shigella flexneri* (58), the ability of parasites to alter the balance between pro- and antiapoptotic signals in phagocytes has generated considerable interest (59, 60). To analyze the strategy adopted by *B. abortus* to develop within synovial fibroblasts, we examined the modulation of apoptotic and antiapoptotic factors during *B. abortus* synovial fibroblast infection. In contrast with previous results from *B. abortus*-infected macrophages (18), the proapoptotic BCL-2 family members were not affected in *B. abortus*-infected synoviocytes. We observed an increased expression of inhibitor of apoptosis (IAP) family of proteins, clusterin, and the regulator of cell cycle p27 in *B. abortus*-infected synoviocytes relative to their expression in uninfected cells. In addition, apoptotic factors were down-modulated or not affected after *B. abortus* infection. The antiapoptotic signaling mediated by live *Brucella* oc-

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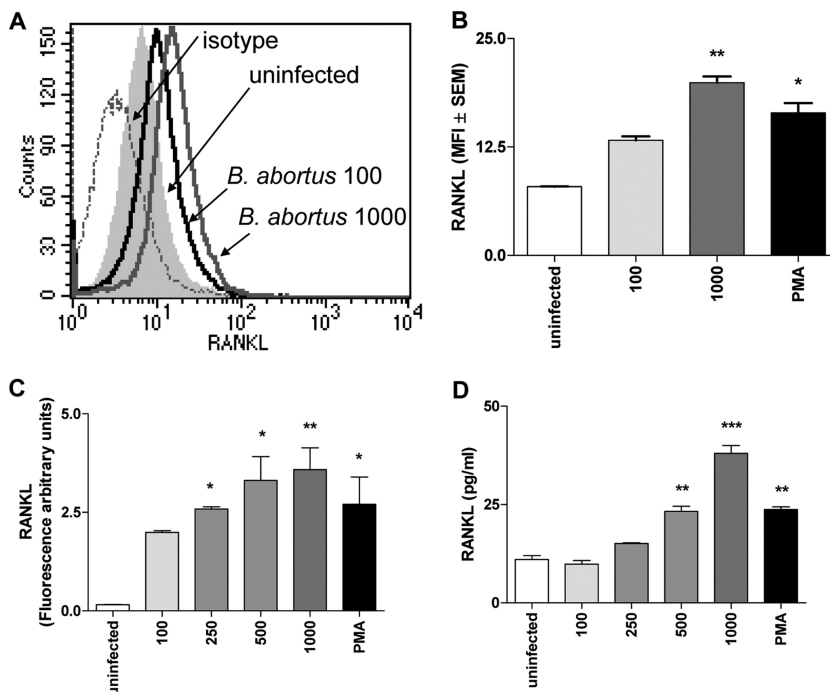
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FIG 7 RANKL expression induced by *B. abortus* infection in synoviocytes. (A) RANKL surface expression on synoviocytes infected with *B. abortus* at MOI of 100 and 1,000 or uninfected, measured by flow cytometry. The histograms show the results of one experiment representative of three independent experiments. (B) Bars represent the arithmetic means of three experiments, and the error bars indicate the standard errors of the means. MFI, mean fluorescence intensity. (C) RANKL expression in synoviocytes infected with *B. abortus* at MOI of 100 to 1,000 or uninfected, measured by fluorometry. (D) RANKL measured in supernatants from *B. abortus*-infected synoviocytes by ELISA. Data shown are from a representative experiment of five performed. *, $P < 0.1$; **, $P < 0.01$ versus uninfected cells.

curs downstream from phagocytosis and requires that brucellae counteract the stress induced by their internalization within phagosomes and reorient maturation of their phagosomes (16). Given that the intracellular fate of *Brucella* in human primary synoviocytes is similar to the one observed in its natural niche (the macrophage), the antiapoptotic phenotype observed in *B. abortus*-infected synoviocytes could be related to a strategy of this bacterium to survive in different host cells.
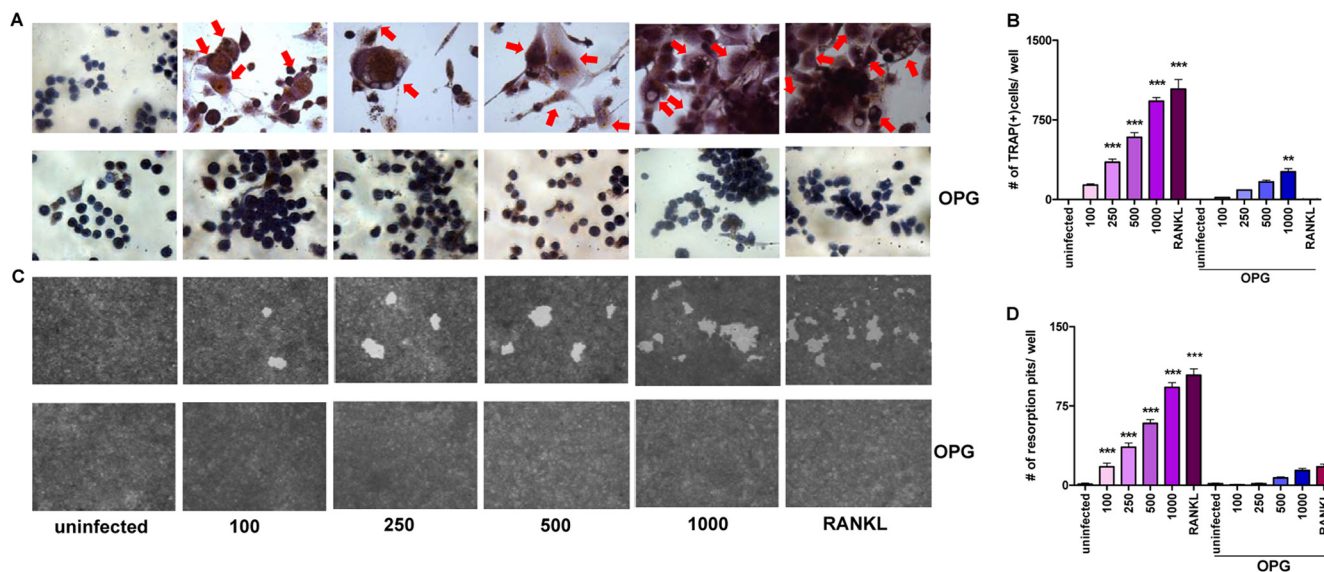

FIG 8 Supernatants from *B. abortus*-infected synoviocytes induced functional osteoclast-like cells via RANKL. Supernatants from *B. abortus*-infected or uninfected synoviocytes were preincubated or not with OPG. (A) Light microscopy of multinucleated TRAP-positive cells. (B) Quantitative analysis of the experiment shown in panel A. (C) The functional activity of osteoclast-like cells was determined by their ability to resorb dentine, determined by light microscopy. (D) Quantitative analysis of the experiment shown in panel C. Bars express the means ± SEM of duplicates. Data shown are from a representative experiment of five performed. ***, $P < 0.001$ versus cells stimulated with supernatants from uninfected synoviocytes.

To our knowledge, there are no reports on the ability of *Brucella* to infect human synoviocytes *in vivo*. This may be explained in part by ethical restrictions, since a biopsy of the affected articulation may be justified only in very select cases. Nevertheless, in the few published studies in which a culture of synovial membrane samples was performed, *Brucella* was isolated from such samples (61, 62). Importantly, cultures of synovial fluid were negative in these cases, suggesting that the bacterium was located intracellularly in the synovial membrane. In support of this hypothesis, foci of intracytoplasmic granular reactions were detected by immunofluorescence with anti-*Brucella* antibodies in the synovial surface of joints obtained from cattle experimentally infected with *B. abortus* S19 (63, 64). Interestingly, synoviocytes are located in the intimal layer of the synovium, which agrees with the "synovial surface" location described in that study.

Monocytes and neutrophils are typically seen as infiltrating cells in damaged joints infected with *Brucella* (8, 11, 14). We have previously demonstrated that *B. abortus*-infected synoviocytes secrete IL-8 and MCP-1, chemokines that attract neutrophils and monocytes, respectively (22). Therefore, synoviocytes are implicated, at least in part, in leukocyte recruitment to the infected joint. The upregulation of adhesion molecules by *B. abortus*-infected synoviocytes would facilitate the interaction of these cells with the infiltrating leukocytes, since these intracellular adhesion molecules play a central role in the interaction between cells in the immune responses (65). In agreement with this hypothesis, we demonstrated that infection of synoviocytes with *B. abortus* upregulated the surface expression of CD54 and CD106 and that, as a consequence, monocytes and neutrophils adhere to *B. abortus*-infected synoviocytes. This can potentially increase the ability of these cells to interact with infiltrating inflammatory cells, thereby propagating immunologically mediated inflammation such as occurs in rheumatoid synovitis (54). Besides, *B. abortus*-infected synoviocytes inhibited the apoptosis induced by supernatants from *B. abortus*-infected monocytes and neutrophils. These phenomena could contribute to the establishment of a successful chronic infection by *Brucella*.

RANKL is a newly discovered key mediator of osteoclast differentiation and bone resorption (66). Several investigations have revealed the involvement of RANKL in the bone destruction that occurs in rheumatoid arthritis and osteoarticular infectious disease (67, 68). It has been reported that resting synovial fibroblasts do not express RANKL. However, we found increased concentrations of RANKL in a synovial fluid from a patient with bursitis caused by *B. abortus* infection. In addition, RANKL expression was found in surface membrane and culture supernatants from synoviocytes infected *in vitro* with *B. abortus*. The marked change in RANKL expression between uninfected and infected cells suggests that under normal conditions synovial fibroblasts may be only weakly osteoclastogenic and that after infection they become potent stimulators of osteoclast formation. This is consistent with the fact that multinucleated TRAP-positive and vitronectin receptor-positive cells were observed only when osteoclasts precursors were treated with culture supernatants from *B. abortus*-infected synoviocytes. These multinucleated cells also resorbed dentine, indicating that they are differentiated functional osteoclasts. Moreover, our results demonstrated that OPG (RANKL's decoy receptor) abrogates osteoclast formation and dentine resorption, further supporting the contention that RANKL determines the osteoclastogenesis induced by *B. abortus* and underscoring the role of this factor in the bone loss reported in osteoarticular brucellosis. In previous results, in other models of *Brucella* osteoclastogenesis that involved the role of macrophages and T cells in bone resorption, we reported that TNF-α plays a key role in these processes (69). Although synovial fibroblasts secrete TNF-α and other inflammatory cytokines in response to infection by other bacteria (70), *B. abortus* does not induce significant levels of production of TNF-α and IL-1β in these cells.

Finally, the results presented here, together with our previous observations (22, 35, 41, 56, 57, 69), are shedding light on how the interactions of *B. abortus* with synovial fibroblasts may have an important role in the pathogenesis of brucellar arthritis by producing RANKL and consequent osteoclastogenesis and also by attracting and adhering phagocytes and neutrophils and inducing them to produce damage in the joint.

ACKNOWLEDGMENTS

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