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Research Article

Bordetella pertussis outer membrane vesicles as virulence factor vehicles that influence bacterial interaction with macrophages

Bruno Blancá[1,†], Jimena Alvarez Hayes[1,†], Kristin Surmann[2], Valdez Hugo[1], Christian Hentschker[2], Yanina Lamberti[1], Uwe Völker[2], María Eugenia Rodriguez[1,*]

[1]CINDEFI (UNLP CONICET La Plata), School of Sciences, La Plata National University, La Plata, Argentina
[2]Department of Functional Genomics, Interfaculty Institute for Genetics and Functional Genomics, University Medicine Greifswald, Greifswald, Germany
*__Corresponding author__: CINDEFI, Facultad de Ciencias Exactas, Universidad Nacional de La Plata. Calles 47 y 115, La Plata, Argentina. Tel:/Fax: +54 221 5718039; E-mail: mer@quimica.unlp.edu.ar
One sentence summary: *Bordetella pertussis* outer membrane vesicles dampen macrophage bactericidal activity.
†These authors contributed equally to this work.
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ABSTRACT

Gram-negative pathogenic bacteria constitutively shed outer membrane vesicles (OMVs) which play a significant role in the host–pathogen interaction, eventually determining the outcome of the infection. We previously found that *Bordetella pertussis*, the etiological agent of whooping cough, survives the innate interaction with human macrophages remaining alive inside these immune cells. Adenylate cyclase (CyaA), one of the main toxins of this pathogen, was found involved in the modulation of the macrophage defense response, eventually promoting bacterial survival within the cells. We here investigated whether *B. pertussis* OMVs, loaded with most of the bacterial toxins and CyaA among them, modulate the macrophage response to the bacterial infection. We observed that the pre-incubation of macrophages with OMVs led to a decreased macrophage defense response to the encounter with the bacteria, in a CyaA dependent way. Our results suggest that CyaA delivered by *B. pertussis* OMVs dampens macrophages protective function by decreasing phagocytosis and the bactericidal capability of these host cells. By increasing the chances of bacterial survival to the innate encounter with the macrophages, *B. pertussis* OMVs might play a relevant role in the course of infection, promoting bacterial persistence within the host and eventually, shaping the whole infection process.

Keywords: *Bordetella pertussis*, outer membrane vesicles, adenylate cyclase toxin, macrophage response, bacterial intracellular survival

Introduction

Outer membrane vesicles (OMVs) are 10–300 nm diameter spherical bilayers released from the Gram-negative bacterial cell envelope that mainly contain periplasmic and outer membrane components. They are broadly considered a bacterial secretion system involved in host–pathogen interaction (Bonnington and Kuehn 2014). OMVs from pathogenic bacteria normally carry virulence factors, such as adhesins and toxins that are delivered to the host cells during infection (Kuehn and Kesty 2005) at local and distal sites, eventually compromising the host defense response. The vesicular membrane protects the virulence factors and any other luminal cargo from extracellular proteases and facilitates their penetration into the host cells. By delivering multiple virulence determinants as a package directly into the host cell OMVs play relevant roles during the infectious process. Accordingly, OMVs have been found implicated in promoting bacterial pathogenesis by stabilizing toxins (Horstman and Kuehn 2000, Kesty *et al.* 2004), altering bacterial adhesion to host cells (Metruccio *et al.* 2016), and/or regulating the host immune response (Kaparakis-Liaskos and Ferrero 2015), among others.

Bordetella pertussis is the etiological agent of whooping cough or pertussis, a re-emergent infectious disease. *Bordetella pertussis* is a strictly human pathogen and its continuous circulation within the population suggests the existence of a niche of persistence inside the host. We previously found that *B. pertussis* is able to survive the innate encounter with host immune cells such as neutrophils and macrophages (Rodriguez *et al.* 2001, Lamberti *et al.* 2010). Upon phagocytosis a significant number of bacteria avoids phago-lysosomal fusion, remains alive in phagosomes with early endosomal characteristics and eventually replicates within the macrophage (Lamberti *et al.* 2010). Importantly, *B. pertussis* was found capable of modulating the macrophage bactericidal response, ultimately enabling the bacterial intracellular survival (Valdez *et al.* 2016). *Bordetella pertussis* toxins were found involved in this modulation (Valdez *et al.* 2016) with adenylate cyclase (CyaA) toxin being one of the main effectors.

Bordetella pertussis is a Gram-negative bacterium that releases OMVs both *in vitro* (Hozbor *et al.* 1999) and *in vivo* (Donato *et al.* 2012). Among other virulence determinants, CyaA toxin was found present in *B. pertussis* OMVs (Hozbor *et al.* 1999, Donato *et al.* 2012). Importantly, it was demonstrated that CyaA delivered by OMVs causes an increase in the intracellular cAMP levels of murine macrophages after internalization, indicating that OMVs deliver active CyaA toxin into cells (Donato *et al.* 2012). It is, therefore, likely that CyaA delivered by *B. pertussis* OMVs into the host cell might also induce cellular response modulation (Valdez *et al.*

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2016, Ahmad *et al.* 2019) or other effects that eventually shape the pathogen fate upon the encounter with the cells. To our knowledge, despite its potential significance in *B. pertussis* pathogenesis, the influence of *B. pertussis* OMVs on the outcome of the bacterial interaction with macrophages has not been investigated. In the present study, we aimed at evaluating the impact of *B. pertussis* OMVs on macrophage responses, their influence on bacterial intracellular persistence, and the eventual role of CyaA in these processes.

Materials and methods

Bacterial strains and growth conditions

Bordetella pertussis Tohama I strain CIP 81.32 (Bp) and an isogenic mutant deficient in CyaA production (BpΔCyaA) (Cerny *et al.* 2015), were used in this study. For selected experiments, CIP 81.32 was transformed with plasmid pCW505 (Weingart *et al.* 1999) (kindly supplied by Dr. Weiss, Cincinnati, OH, USA) which induces cytoplasmic expression of green fluorescent protein (Bp-GFP) without affecting growth or antigen expression (Weingart *et al.* 1999). Bacteria were stored at −70°C and then grown on Bordet-Gengou (DIFCO Laboratories, USA) agar plates supplemented with 15% (v/v) defibrinated sheep blood (bBGA) at 35°C. After 3 days, the bacteria were sub-cultured in Stainer-Scholte (SS) (Stainer and Scholte 1970: 211–20) liquid medium at an initial cell density corresponding to an optical density at 650 nm (OD_{650}) of 0.2. SS cultures were maintained at 37°C with shaking (150 rpm) and grown until exponential phase for infection assays or subsequently subcultured into SS medium and grown until late stationary phase for outer membrane vesicles isolation.

Isolation of outer membrane vesicles

Outer membrane vesicles (OMVs) were obtained from Bp (OMVs) or BpΔCyaA (ΔCyaA-OMVs). They were isolated from culture supernatants after 48 h of growth. The bacterial suspension was centrifuged at 10 000 × *g* for 30 min. The supernatant was filtered through a 0.22 μm pore-size filter, and a sterility test was performed by culturing an aliquot on a BGA plate followed by incubation for 72 h at 36°C. The sterile supernatant was concentrated using 10 kDa Vivaflow 200 cross flow cassette (Sartorius, Epson, UK) at 4°C and subsequently centrifuged at 100 000 × *g* for 4 h at 4°C. Pellets containing OMVs were suspended in sterile PBS, followed by a final sterile filtration to exclude contamination and stored at −20°C. OMVs protein concentration was measured using a Bradford assay (Sigma-Aldrich Inc.) and the quality of OMVs preparations was confirmed by transmission electron microscopy (TEM). The average hydrodynamic diameter (Dh) and size distribution of the OMVs were measured by Dynamic Light Scattering (DLS) (scattering angle of $\theta = 173°$ to the incident beam, Zetasizer Nano-ZSP, Malvern Instruments, United Kingdom) at 25°C.

Transmission electron microscopy

The OMVs samples were suspended in 0.1 M ammonium acetate (pH 7.0). Droplets of these samples were placed on a grid coated with a collodion film. After removing the excess fluid with filter paper, the grids were stained with 1% (w/v) phosphotungstic acid pH 5.2 (with KOH). Observation was done with a JEM 1200 EX II (JEOL Ltd., Tokio, Japón) transmission electron microscope.

Antibodies

The following antibodies were used in this study: Cy3 or FITC conjugated goat F(ab')2 fragments of anti-rabbit IgG (Jackson ImmunoResearch, West Grove, USA), Alexa 647-conjugated goat F(ab')2 fragments of anti-mouse IgG (Jackson InmunoResearch, West Grove, PA), mouse monoclonal antibody against human lysosome-associated membrane protein 1 (LAMP1; BD Biosciences Pharmingen, San Diego, CA), polyclonal rabbit antibody against human flotillin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Alexa 594-conjugated anti-human CD11b mouse IgG1 (Biolegend, San diego, CA), PE-conjugated anti-human CD11b mouse IgG1 (Biolegend, San Diego, CA) and PE-conjugated mouse IgG1 isotype control antibody (Biolegend, San Diego, CA). Polyclonal rabbit anti-*B. pertussis* antiserum (Hellwig *et al.* 2001) and polyclonal mouse anti-*B. pertussis* antiserum were obtained as described elsewhere (Alvarez Hayes *et al.* 2011).

Cell culture

THP-1 monocyte cells (ATCC, Manassas, USA) were cultured as previously described (Lamberti *et al.* 2016) with minor modifications. Briefly, cells were cultured in RPMI 1640 (Grand Island, NY, USA) plus 10% (w/v) of fetal bovine serum (FBS; Gibco, NY, USA) at 37°C in a humidified 5% (v/v) CO_2 air atmosphere. The differentiation of THP-1 monocytes into macrophages was induced by adding 100 nM phorbol-12-myristate-13-acetate (PMA; SigmaAldrich, Saint Louis, USA) to RPMI 1640 plus 10% (w/v) of FBS for 24 h, followed by 24 h of culture in the absence of PMA (Lund *et al.* 2016: 64–70) prior infection. The viability of the cells was determined after PMA treatment with trypan blue. No significant cell death was observed.

Sample preparation for proteome analysis

OMVs were harvested as described above and suspended in 40 μL 5% (w/v) sodium dodecyl sulfate (SDS) dissolved in HPLC-grade water (J.T.Baker, Thermo Fisher Scientific, Pittsburgh, USA). Protein extraction was supported by 10 min shaking at 1400 rpm (Eppendorf, Germany) at room temperature and subsequent treatment in an ultrasonication bath (Sonorex, Berlin, Germany) for 3 min at 45 kHz and 60 W. Five μL of each sample were diluted in HPLC grade water to obtain a SDS concentration of 0.25% (v/v) and subsequently prepared using the SP3 (single pot solid-phase enhanced sample preparation) protocol with small adaptations as descripted before (Blankenburg *et al.* 2019: e1900192). In brief, the protein extracts were mixed with 8 μL beads [1:1 mixed beads, hydrophobic: Sera-Mag™ Speedbeads carboxylated-modified particles (Thermo Fisher Scientific, Schwert, Germany) and hydrophilic: Speedbead magnetic carboxylated modified particles (GE Healthcare, Chalfont Saint Giles United Kingdom)] and acetonitrile (ACN; Thermo Fisher Scientific, Waltham, USA) to a final concentration of 70% (v/v) ACN and allowed for binding for 18 min shaking at 1400 rpm (Eppendorf). Proteins bound to the beads were rinsed three times with 70% (v/v) ethanol and twice with ACN. After solvent removal, proteins were subjected to trypsin digestion using 50 ng trypsin (Promega Corporation, Madison, WI, USA) in 20 mmol L^{-1} aqueous ammonium hydrogen carbonate at 37°C for 17.5 h. Trypsin reaction was stopped and peptide binding on the beads supported by ACN in a final concentration of 95% (v/v). After solvent removal, peptides were eluted from the beads in 2% (v/v) aqueous dimethyl sulfoxide solution supported during 3 min incubation in the ultrasonication bath. After preparation with the SP3 protocol, the eluted peptides were rinsed one more time with C18 ZipTip columns according to manufacturer's instructions (Merk Millipore, Darmstadt, Germany) to improve the sample quality and remove high amounts of salts present in the buffers. After solvent removal by lyophiliza-

tion, samples were reconstituted in 20 μL buffer containing 2% ACN and 0.1% acetic acid (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in HPLC-grade water for subsequent mass spectrometry data acquisition.

NanoLC-MS/MS data acquisition and analysis

Peptides were separated using an UltiMate 3000 nano-LC device (Thermo Fisher Scientific) by combining a pre-column (Acclaim PepMap; Thermo Fisher Scientific) and an analytical column (Accucore; Thermo Fisher Scientific). A binary gradient of buffer A [0.1% (v/v) acetic acid in HPLC-grade water] and buffer B [0.1% (v/v) acetic acid in ACN] was used at a flow rate of 300 nL min^{-1}. After ionization with a TriVersa NanoMate source (Advion, UK), peptides were analysed using a Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific,) in data dependent acquisition (DDA) mode. Details are provided as supplementary data (Table S1). Obtained raw data were processed using MaxQuant version 1.5.3.30 (Cox and Mann 2008) and search against a Uniprot database limited to *B. pertussis* Tohama I entries (release 06/2018, 3296 protein sequences). Trypsin/P was set as proteolytic enzyme with two missed cleavages allowed and false discovery rate (FDR) for peptide to spectrum matches (PSM) and proteins was enabled. Proteins were only considered identified, if two or more unique peptides were found per protein. Protein intensities were median normalized over all replicates. Further details are provided as supplementary data (Table S2). Data are available as supplementary data (Table S3) and deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.* 2019) partner repository with the dataset identifier PXD033489. The proteins were functionally classified by the descriptive multifunctional classification (Multifun) of Serres and Riley as previously described (Alvarez Hayes *et al.* 2015).

Interaction of OMVs with THP-1 macrophages

To evaluate the OMVs' attachment to and internalization by macrophages, THP-1 in RPMI 1640 plus 10% (v/v) of FBS containing 100 nM of PMA were seeded onto glass coverslips in 24-well tissue culture plates (4×10^5 cells/well), for differentiation into macrophages. After 24 h, the monolayers were washed twice with sterile phosphate-buffered saline (PBS) and incubated with RPMI 1640 plus 10% (v/v) of FBS for other 24 h prior incubation with or without 10 μg $well^{-1}$ of OMVs for 4 h at 37°C. To facilitate interaction between vesicles and cells, the plates were centrifuged for 5 min at 640 × *g*. Attachment and phagocytosis of the OMVs were analyzed by mean of confocal and transmission electron microscopy. For confocal microscopy analysis, after incubation with or without OMVs, the cells were washed and fixed with 4% (v/v) paraformaldehyde (PFA) for 15 min. The cells were then incubated with polyclonal rabbit anti-*B. pertussis* antibodies for 30 min at 4°C, followed by incubation with FITC-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG antibodies for 30 min at 4°C. To differentiate surface attached from internalized OMVs, the cells were permeabilized by incubation with PBS containing 0.1% (w/v) of saponin (Sigma) and 0.2% (w/v) of BSA for 30 min. The intracellular and extracellular OMVs were then stained with polyclonal rabbit anti-*B. pertussis* antibodies in the presence of 0.1% (w/v) of saponin and 0.2% (w/v) of BSA for another 30 min. After three washing steps, cells were incubated with Cy3-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA for 30 min at room temperature, washed, mounted on microscope slides, and analyzed by confocal microscopy (Leica TCS SP5, Wetzlar, Germany). For transmission electron microscopy, after incubation with or without OMVs the cells were fixed for 2 h at 4°C in glutaraldehyde 2% (v/v) and post-fixed in 1% OsO4 for 1 h at 4°C. Samples were then serially dehydrated in ethanol and embedded in epoxy resin. Ultrathin serial sections (90 nm) were counterstained with uranyl acetate and lead citrate. and observed with a JEM 1200 EX II (JEOL Ltd., Tokio, Japón) transmission electron microscope.

To evaluate the colocalization of OMVs with flotillin, 4×10^5 THP-1 cells differentiated into macrophages were incubated with 10 μg of OMVs for 30 min at 37°C were washed and further incubated with polyclonal rabbit antibodies against human flotillin-1 and mouse anti-*B. pertussis* antiserum for 30 min at 4°C. After three washing steps, the cells were incubated with Cy3-conjugated F(ab')2 fragments of goat anti-rabbit IgG plus Alexa 647-conjugated goat F(ab')2 fragments of anti-mouse IgG for another 30 min at 4°C. To evaluate colocalization of OMVs with CR3, 4×10^5 THP-1 cells differentiated into macrophages were incubated with 10 μg of OMVs for 30 min at 37°C, washed, and further incubated with Alexa 594-conjugated anti-human CD11b mouse IgG1, plus polyclonal rabbit anti-*B. pertussis* antiserum for 30 min at 4°C. The cells were washed and further incubated with FITC-conjugated F(ab')2 fragments of goat anti-rabbit IgG for 30 min at 4°C. In order to avoid nonspecific binding of the antibodies, all incubations were carried out in the presence of 25% (v/v) of heat-inactivated human serum. Finally, the cells were analyzed using confocal microscopy (Leica TCS SP5, Wetzlar, Germany).

THP-1 infection assays and phagocytosis quantification

For infection experiments, THP-1 in RPMI 1640 plus 10% (v/v) of FBS containing 100 nM of PMA were seeded onto glass coverslips in 24-well tissue culture plates (4×10^5 cells $well^{-1}$), for differentiation into macrophages. After 24 h, the monolayers were washed twice with sterile phosphate-buffered saline (PBS) and incubated with RPMI 1640 plus 10% (v/v) of FBS for other 24 h prior incubation with or without 10 μg $well^{-1}$ of OMVs or ΔCyaA-OMVs for 4 h at 37°C. To facilitate interaction between vesicles and cells, the plates were centrifuged for 5 min at 640 × *g*. The cells were then washed twice and infected with *B. pertussis* in RPMI plus 0.2% (w/v) bovine serum albumin (BSA, Sigma) at a multiplicity of infection (MOI) of 100 bacteria per cell. To facilitate bacterial interaction with the eukaryotic cells, plates were centrifuged for 5 min at 640 × *g*. After 2 h of incubation at 37°C with 5% CO_2, non-adherent bacteria were removed by three washing steps. Next, the cells were either fixed for immune staining or further incubated with fresh RPMI medium plus 10% (v/v) FBS and 100 μg mL^{-1} polymyxin B (Sigma), an antibiotic that cannot penetrate mammalian cells (Lee *et al.* 1990), to kill extracellular bacteria. After 1 h incubation at 37°C, cells were washed three times with phosphate-buffered saline (PBS) and either harvested for intracellular bacterial survival determination 3 h post infection, or further incubated with fresh RPMI medium plus 10% (v/v) FBS and 5 μg mL^{-1} polymyxin B for another 48 h and harvested for intracellular bacterial survival determination at 48 h post-infection. In control experiments the number of colony forming units (CFUs) in the culture supernatants was examined and no viable bacteria were detected at any time post-infection after polymyxin B (100 μg mL^{-1}) treatment. To assess bacterial intracellular survival, THP-1 cells from each sample were lysed with sterile water, and serial dilutions of the lysates were rapidly plated onto bBGA plates to enumerate CFUs of recovered *B. pertussis*. Viable intracellular bacteria were expressed as the number of viable bacteria per well.

In parallel, the number of extracellular and intracellular bacteria 2 h after infection was determined by double staining and immunofluorescence as previously described (Lamberti *et al.* 2013). Briefly, surface-bound bacteria were detected by incubation with polyclonal rabbit anti-*B. pertussis* antiserum for 30 min at 4°C, followed by incubation with FITC-conjugated F(ab')2 fragments of goat anti-rabbit IgG for 30 min at 4°C. To determine the number of intracellular bacteria, cells were permeabilized by incubation with PBS containing 0.1% (w/v) saponin (Sigma) and 0.2% (w/v) BSA for 30 min, followed by incubation for 30 min with rabbit anti-*B. pertussis* antiserum in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA. After three washing steps, cells were incubated with Cy3-conjugated goat F(ab')2 fragments of anti-rabbit immunoglobulin for 30 min in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA. After washing, the cells were mounted on microscope slides and analyzed by fluorescence microscopy using a DMLB microscope coupled to a DC100 camera (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland). The number of extracellular (red and green, seen as yellow) and intracellular (red only) bacteria was evaluated by examination of at least 50 eukaryotic cells.

For competition assays between bacteria and OMVs for macrophage surface receptors, 4×10^5 THP-1 cells differentiated into macrophages were incubated with RPMI plus 10% (v/v) FBS in the presence or the absence of 1 μg mL^{-1} of cytochalasin D for 30 min, and further incubated with or without 10 μg of OMVs. After 4 h at 37°C the cells were incubated with Bp-GFP (MOI:100) in RPMI plus 0.2% BSA for 1 h at 37°C in the presence of 1 μg mL^{-1} cytochalasin D to inhibit phagocytosis. In selected experiments, 4×10^5 THP-1 cells differentiated into macrophages were incubated with or without 10 μg of OMV for 4 h at 37°C to promote OMV phagocytosis before incubation with RPMI plus FBS supplemented with 1 μg mL^{-1} cytochalasin D for 30 min at 37°C. The cells were then infected with Bp-GFP (MOI: 100) suspended in RPMI in the presence of 0.2% BSA and 1 μg mL^{-1}l cytochalasin D for 1 h at 37°C. The cell samples were analyzed by flow cytometry (Becton Dickinson, Lincoln Park, NJ). Ten thousand cells were analyzed per sample. The green fluorescence associated to the cells was used to evaluate cell surface-bound bacteria.

Cell surface complement receptor 3 (CR3) analysis

The surface-level of CR3 was evaluated in 4×10^5 THP-1 monocytes differentiated into macrophages incubated with or without 10 μg of OMVs or ΔCyaA-OMVs for 4 h at 37°C. The cells were then washed, fixed with 4% (w/v) PFA, and incubated for 30 min at 4°C with PE-conjugated anti-human CD11b mouse IgG1 for 30 min at 4°C. In order to avoid nonspecific binding of antibodies, all incubations were performed in the presence of 25% (v/v) heat inactivated human serum and isotype controls were performed in parallel. The expression of CR3 on the surface was evaluated by flow cytometry (Becton Dickinson, Lincoln Park, NJ).

Bacterial intracellular trafficking

Colocalization of intracellular bacteria with intracellular markers was performed as described before (Lamberti *et al.* 2010) with minor modifications. Briefly, infected THP-1 macrophages were pre-incubated or not with of OMVs or ΔCyaA-OMVs as described above fixed and permeabilized by incubation with PBS containing 0.1% (w/v) saponin (Sigma) and 0.2% (w/v) BSA for 30 min at 25°C. Bacterial colocalization with lysosomes was investigated by incubation with mouse anti-human LAMP-1 antibodies plus rabbit anti-*B. pertussis* antiserum for 30 min at 25°C in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA. After three washes, the cells were incubated with Alexa 647-conjugated $F(ab)_2$ fragments of anti-mouse antibodies (Jackson InmunoResearch West Grove, PA) plus Cy3-conjugated goat $F(ab)_2$ fragments of anti-rabbit immunoglobulin for another 30 min at 25°C. To avoid cytophilic binding of antibodies to FcγR, all incubations were done in the presence of 25% (v/v) heat-inactivated human serum. Microscopy analyses were performed using a confocal laser scanning microscope (Leica TCS SP5, Wetzlar, Germany). The percentage of phagosomes containing bacteria colocalizing with LAMP-1 was calculated by analyzing at least 50 cells per sample.

Quantitative real-time PCR

THP-1 cell monolayers differentiated into macrophages in 6-well plates (2.5×10^6 cell well^{-1}) were incubated or not with 62.5 μg well^{-1} of OMVs or ΔCyaA-OMVs for 4 h at 37°C. Monolayers were then washed twice and infected with *B. pertussis* in RPMI plus 0,2% (w/v) BSA at an MOI of 100 bacteria per cell. To facilitate bacterial interaction with the eukaryotic cells, the plates were centrifuged for 5 min at 640 × g. After 2 h of incubation at 37°C with 5% CO_2, non-adherent bacteria were removed by three washes and fresh RPMI medium plus 10% (v/v) FBS and 100 μg mL^{-1} polymyxin B was added to kill extracellular bacteria. After 1 h incubation at 37°C, cells were washed three times with phosphate-buffered saline (PBS) and then total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, USA) according to the manufacturer's instructions, including DNase I treatment (Promega, Madison, USA). RNA quality was assessed by agarose gel electrophoresis and photometrically by NanoDrop2000 (Thermo Scientific). A 260/280 ratio of around 2.0 was considered adequate. The cDNA synthesis was performed with M-MLV reverse transcriptase (Promega, Madison, WI) following the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA and 10 μM of random hexamers (Qiagen, Valencia, CA, USA) were used. A control reaction without M-MLV reverse transcriptase was included to confirm the absence of genomic DNA in the RNA samples. Quantitative real-time PCR (RT-qPCR) analyzes were performed on a qPCR Mx3000P system (Stratagene, USA) with SYBR Green-PCR Master Mix (Roche, Mannheim, Germany) and 0.5 μM of each forward and reverse primers (Invitrogen, Carlsbad, CA). Primer sequences are listed in Table 1. The PCR cycle comprised a 10 min pre-incubation at 95°C, followed by 40 cycles of a two-step PCR consisting of a denaturation step at 94°C for 15 sec and a combined annealing and extension phase at 60°C for 60 sec. The resulting amplicons were examined by melting curves and agarose gel electrophoresis. The relative expression level of each gene was calculated using the LinRegPCR program as described in (Ruijter *et al.* 2009). The β2-microglobulin gene (B2M) gene was used as a housekeeping marker for normalization, and uninfected cells served as a reference.

Statistical analysis

The Student's t-test (95% confidence level) or analysis of variance (ANOVA) was used for statistical evaluation of the data. The significance of the differences between the mean values of the data analyzed by ANOVA was determined with the least-significant-difference test at a 95% confidence level. The results are shown as the means ± the standard deviations (SDs).

Table 1. Primers used for real-time polymerase chain reaction

Gene	Protein name	Sense	Antisense
Antimicrobial peptides			
GNLY	Granulysin	5′-CGCAGCATTGGAAACACTT-3′	5′-GACCAAAACACAGGAGCTGG-3′
Enzymatic defense			
CTSB	Cathepsin B	5′-GGCCCCCTGCATCTATCG -3′	5′-AGGTCTCCCGCTGTTCCACTG-3′
CTSD	Cathepsin D	5′-CCCGCATCTCCGTCAACAA-3′	5′-GCCTTGCGGGTGACATTCAG-3′
CTSS	Cathepsin S	5′-TCTCTCAGTGCCCAGAACCT-3′	5′-GCCACAGCTTCTTTCAGGAC-3′
Housekeeping			
B2M	ß2-Microglubulin	5′-TCTCTGCTGGATGACGTGAG -3′	5′-TAGCTGTGCTCGCGCTACT-3′

Results

Isolation and characterization of B. pertussis outer membrane vesicles

Outer membrane vesicles (OMVs) purified from the stationary phase of *B. pertussis* culture supernatant were analyzed by negative staining TEM. Fig. 1 shows small spherical vesicles with a uniform size distribution. The absence of bacterial debris confirmed their purity (Fig. 1A). The diameter of the isolated OMVs ranged from 10 to 240 nm diameters (mean: 92.8 nm) (Fig. 1B). The proteomic analysis of these OMVs by nanoLC-MS/MS identified 348 proteins present in the vesicles, which represent 9% of all *B. pertussis* coding sequences (Parkhill *et al.* 2003). The detailed list of all identified proteins and their respective functional category is showed in Table S3. Fig. 1C shows the proportions of proteins belonging to each functional category. Most of the identified proteins belong to the categories 'hypothetical and conserved hypothetical' (30.5%), 'metabolism' (21.8%), 'transport' (16%) or 'cell processes' (8.2%). Importantly, most of the known *B. pertussis* virulence factors were identified in OMVs by proteome analysis (Table S3), including adenylate cyclase toxin (CyaA) and pertussis toxin (PT) (subunits, PtxA, PtxB, PtxC and PtxD). Additionally, several proteins involved in bacterial oxidative stress resistance, such as SodB, KatA, AhpC, and AhpD (Table S3) were identified. We also found some proteins, many of them exclusively secreted via OMVs (Luu *et al.* 2018a,b, Luu *et al.* 2020), that share homology with proteins involved in pathogenesis and persistence inside the host in other pathogens. For example, a significant number of lipoproteins was identified in the OMVs proteome (Table S3). Bacterial lipoproteins are known immune modulatory agents (Kovacs-Simon *et al.* 2011). Other identified proteins, potentially associated with virulence and intracellular survival, are OmpA (Confer and Ayalew 2013) and ClpB (Tripathi *et al.* 2020) (Table S3). Our results suggest that, besides known bacterial virulence factors, *B. pertussis* OMVs are loaded with different proteins that might influence the bacterial infection outcome.

OMVs attach to and are internalized by THP-1 cells

In order to evaluate the OMVs interaction with macrophages, THP-1 cells differentiated into macrophages were incubated with purified vesicles for 4 h at 37ºC, washed, fixed, and analyzed for OMVs attachment and internalization. TEM (Fig. 2A) analysis and two-color confocal microscopy (Fig. 2A) showed that *B. pertussis* OMVs were attached to and phagocytosed by THP-1 macrophages. According to our proteomic analysis, FHA, the main *B. pertussis* adhesin, is present in the OMVs obtained in this study (Table S3). We, therefore, investigated whether the OMVs and the whole bacteria share docking molecules on the macrophage surface. To that end, we analyzed the colocalization of OMVs with the complement receptor 3 (CR3, CD11b/CD18) and the membrane cholesterol-rich domains, so-called lipid rafts. These are two of the main targets of *B. pertussis* FHA on macrophage surfaces, and central macrophage receptors for the whole bacteria (Relman *et al.* 1990, Lamberti *et al.* 2008). Confocal microscopy of THP-1 cells incubated with OMVs for 30 min at 37ºC showed some *B. pertussis* OMVs colocalizing with the lipid raft marker, flotillin, and CR3 (Fig. 2B) suggesting that the OMVs and the whole bacteria might share these two important docking sites on macrophage surface.

OMVs decrease macrophage uptake of *B. pertussis*

Taking into account that OMVs and the whole bacteria might compete for the same receptors on the cell surface, we next investigated whether the presence of OMVs interfered with the macrophage uptake of *B. pertussis*. To that end, THP-1 cells differentiated into macrophages were incubated with OMVs or medium alone for 4 h at 37ºC prior to incubation with *B. pertussis*. Bacterial attachment and phagocytosis were analyzed using two-color microscopy to discriminate between extracellular and intracellular bacteria. As shown in Fig. 3A, the presence of OMVs determined a significant drop in bacterial attachment to the macrophage but did not affect bacterial phagocytosis (Fig. 3A). In order to gain a better insight into the effect of the OMVs on bacterial attachment, THP-1 cells were incubated with or without OMVs for 4 h followed by the incubation with GFP-expressing *B. pertussis* in the presence of cytochalasin D, an inhibitor of microfilaments polymerization that prevents phagocytosis. The level of bacteria attached to THP-1 cells was analyzed by flow cytometry. Figure 3C shows that pre-incubation of macrophages with OMVs caused a significant decrease in the number of bacteria attached to the cells (OMVs + CD + Bp). We then investigated whether this decrease in bacterial attachment was due to a competition for the docking molecules on the macrophage surface, with OMVs still attached to the cell at the moment of bacterial interaction. To that end, THP-1 macrophages were incubated with or without OMVs in the presence of cytochalasin D. Since the phagocytosis inhibitor was added before the addition of the OMVs and remained present during the experiment, OMVs internalization was prevented and all attached OMVs remained on the cell surface. Surprisingly, under these experimental conditions OMVs attached to the surface of THP-1 macrophages did not interfere with bacterial attachment (CD + OMVs + Bp) (Fig. 3B). These results indicate that the decrease in bacterial attachment depicted in Fig. 3A cannot be ascribed to a competition for the same receptors on the surface of the macrophages. They further suggest that OMVs have to be internalized in order to affect *B. pertussis* attachment. Ahmad *et al.* recently showed that *B. pertussis* CyaA induces macrophages dedifferentiation into monocytes (Ahmad *et al.* 2019). They proved that this dedifferentiation process led to a decrease in the level

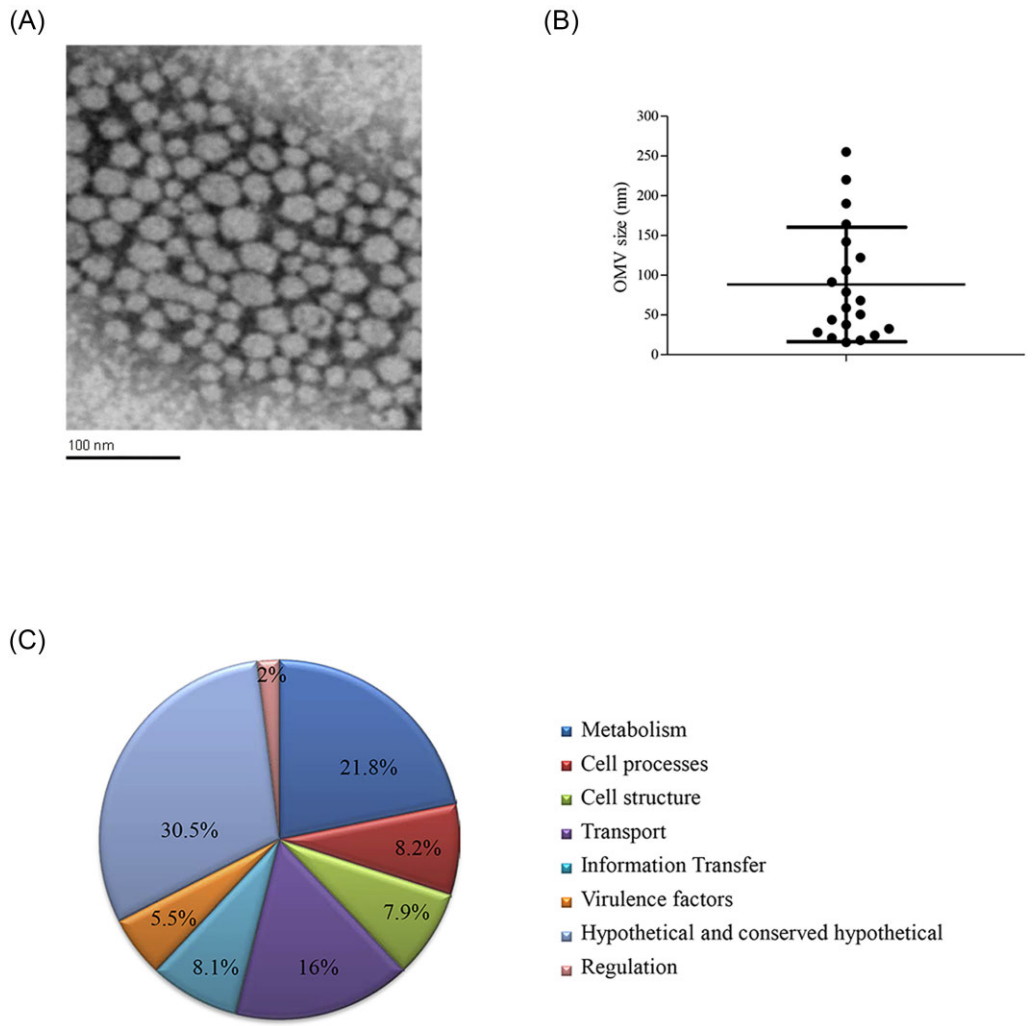

Figure 1. Analysis of *B. pertussis* outer membrane vesicles (OMVs). **(A)** Electron micrograph of the OMVs used in this study. **(B)** Size distribution of OMVs as determined by a Zetasizer-Nano-ZSP (Malvern, UK). Horizontal lines represent mean and standard deviation values. **(C)** Classification of the identified proteins in *B. pertussis* OMVs proteome according to their cellular function.

of cell surface molecules, CR3 among them. Since our proteomic analysis showed that *B. pertussis* OMVs are loaded with this toxin (Table S1), we next investigated the surface expression of CR3 in THP-1 cells differentiated into macrophages incubated with or without OMVs for 4 h at 37°C. Fig. 4A shows that CR3 surface level of THP-1 macrophages incubated with *B. pertussis* OMVs was significantly lower than that observed in macrophages control (incubated with medium alone) as determined by flow cytometry. To evaluate the involvement of CyaA in this decrease, the same experiment was performed with OMVs obtained from a *B. pertussis* mutant strain lacking CyaA (ΔCyaA-OMVs). Figure 4A shows that the CR3 surface level of THP-1 macrophages incubated with ΔCyaA-OMVs was not different from the level exhibited by control macrophages, indicating a role of the CyaA present in OMVs in the drop of THP-1 CR3 surface expression. The observed drop in CR3 surface level induced by CyaA associated to OMVs might explain the decreased bacterial attachment observed in OMVs treated macrophages. In order to further investigate this issue, THP-1 cells were incubated with OMVs obtained from the wild type bacteria (OMVs) or ΔCyaA-OMVs during 4 h at 37°C prior bacterial infection. Infection experiments of THP-1 macrophages incubated with medium alone were used as control. Fig. 4B shows that bacterial attachment to macrophages pre-incubated with OMVs was significantly lower than the bacterial attachment to macrophages pre-incubated with ΔCyaA-OMVs. No significant difference was observed in the number of bacteria attached to macrophages incubated with ΔCyaA-OMVs or medium alone (Fig. 4C). These results confirmed that CyaA present in *B. pertussis* OMVs is involved in the observed decrease in bacterial uptake.

B. pertussis OMVs modulate the macrophage response to the infection

Since previous studies have shown that *B. pertussis* CyaA modulates the macrophage defense response (Valdez *et al.* 2016) we here investigated the effect CyaA delivered by OMVs on the macrophage response to the innate encounter with the bacteria. To that end, THP-1 cells were incubated with OMVs, ΔCyaA-OMVs, or medium alone 4 h at 37°C prior incubation with *B. pertussis* for another 3 h. The transcriptional profile of a number of selected genes encoding for proteins involved in host antimicrobial response, such as the cathepsins-proteases *CTSB, CTSG, CTSD,* and the granulysin *GNLY* (Rivera-Marrero *et al.* 2004), was investigated by qRT-PCR three hours after infection. Cathepsins and granulysins are a family of proteases and hydrophobic peptides located almost entirely in lysosomes involved in pathogen

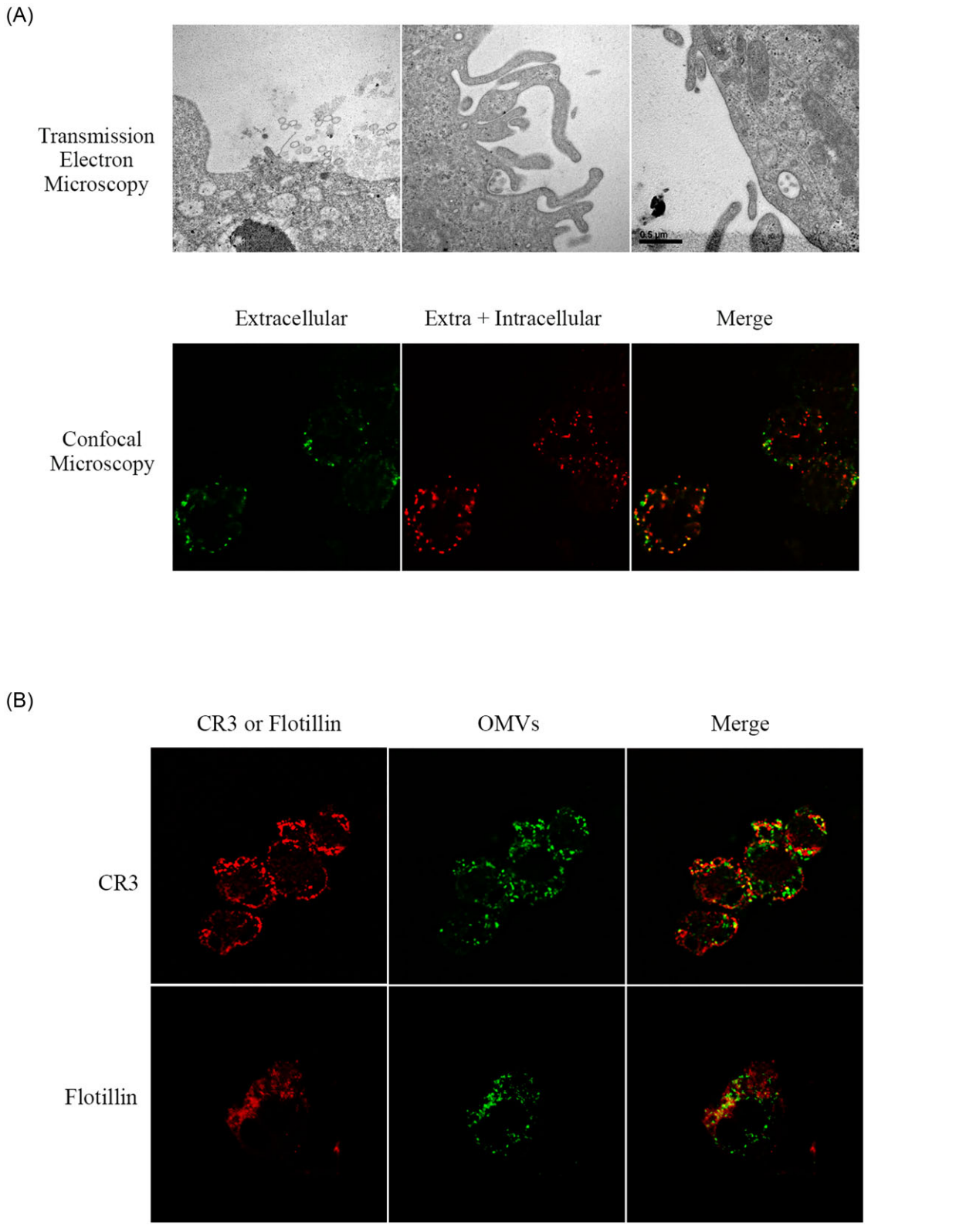

Figure 2. OMVs interaction with macrophages. THP-1 cells differentiated into macrophages were incubated with *B. pertussis* OMVs for 4 h at 37°C. **(A)** After incubation, the cells were washed, fixed and embedded in epoxy resin, stained with uranyl acetate and lead citrate, and analyzed by electron microscopy; or subjected to double staining to discriminate between surface attached OMVs (green and red) and internalized OMVs (red) by confocal microscopy. **(B)** OMVs colocalization with the lipid raft marker, flotillin, and CR3 was analyzed by confocal microscopy. THP-1 cells were incubated with OMVs for 30 min at 37°C. After washing and fixation, CR3 or flotillin were stained in red (red) and OMVs were stained in green. Colocalization is seen as yellow areas. Representative panels of one out of three independent experiments are shown

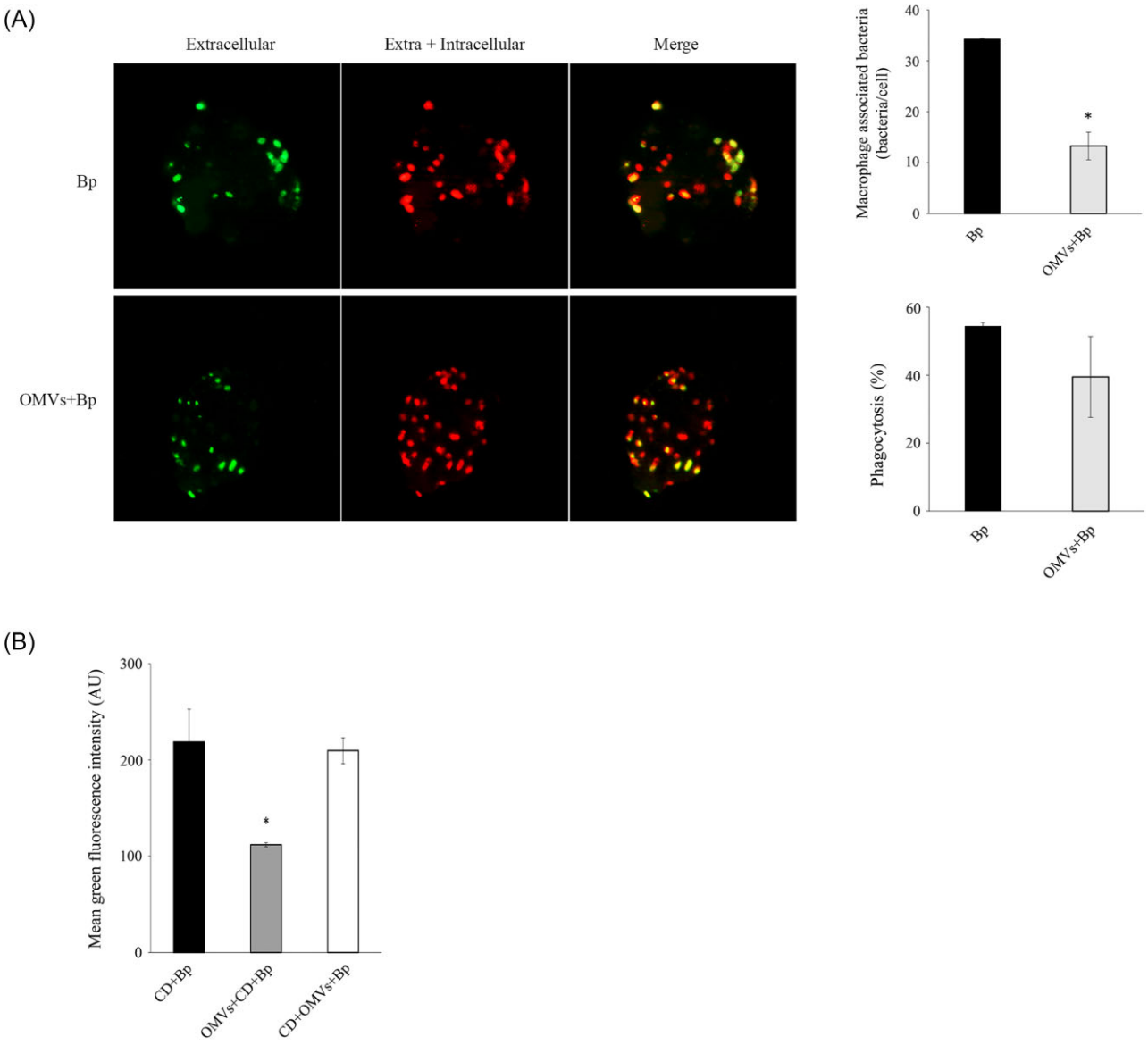

Figure 3. *Bordetella pertussis* OMVs induce a decrease in bacterial attachment to macrophages. THP-1 cells were incubated with or without OMVs for 4 h prior infection with bacteria (MOI 100) for other 2 h at 37°C. The cells were washed, fixed, and subjected to double staining to discriminate between surface attached (green and red) from internalized bacteria (red) by confocal microscopy. At least 50 cells were counted per slide. (A) The confocal image shows macrophage associated extracellular bacteria labeled both with green and red fluorescent dye and intracellular bacteria labeled only with red fluorescent dye. Fluorescence microscopy images representative of one out of three independent experiments are shown. The bars indicate the total number of bacteria (both extra and intracellular) per macrophage. The data represent the means ± SD of three independent experiments. The number of bacteria associated with macrophages pre-incubated with OMVs differs significantly from the number of bacteria associated with cells that were not pre-incubated with OMVs ($* P < 0.05$). Bacterial phagocytosis was expressed as the percentage of the macrophage associated bacteria that were found intracellular. The data represents the means ± SD of three independent experiments. (B) THP-1 cells, pre-incubated or not with OMVs for 4 h at 37°C, were further incubated with cytochalasin D for 30 min prior infection with GFP-Bp for other 2 h at 37°C in the presence of cytochalasin D (OMVs + CD + Bp and CD + Bp, respectively). In selected experiments, THP-1 cells were incubated with OMVs in the presence of cytochalasin D for 4 h at 37°C and further incubated with GFP-Bp for other 2 h at 37°C in the presence of cytochalasin D (CD + OMVs + Bp). The cells were fixed and analyzed by flow cytometry. The data represent the means ± SD of three independent experiments. AU: arbitrary units. The mean fluorescence of macrophages that were incubated with OMVs prior cytochalasin D treatment was significantly different from the mean fluorescence of macrophages that were incubated with cytochalasin D from the beginning of the experiment ($* P < 0.05$).

killing, antigen processing, and cytokine expression regulation (Hole *et al.* 2012, Pires *et al.* 2016). The expression of these genes was previously found affected by *B. pertussis* CyaA upon the macrophage encounter with the whole bacteria (Valdez *et al.* 2016). Figure 5 shows the transcriptional response of THP-1 cells under the different experimental conditions. As previously found (Valdez *et al.* 2016), *B. pertussis* infection of macrophages pre-incubated with medium alone induced an increase of *CTSB* and *CTSD* transcription and no significant changes in *CTSG* and *GNLY* expression early after infection (Fig. 5). Conversely, bacterial infection of OMVs treated macrophages induced a significant down-regulation of these genes upon infection as compared with infected macrophages pre-incubated with medium alone or ΔCyaA-OMVs (Fig. 5). These results suggest that OMVs modulate the macrophage response to the bacterial infection and that CyaA present in these vesicles plays a central role in the process. Fig-

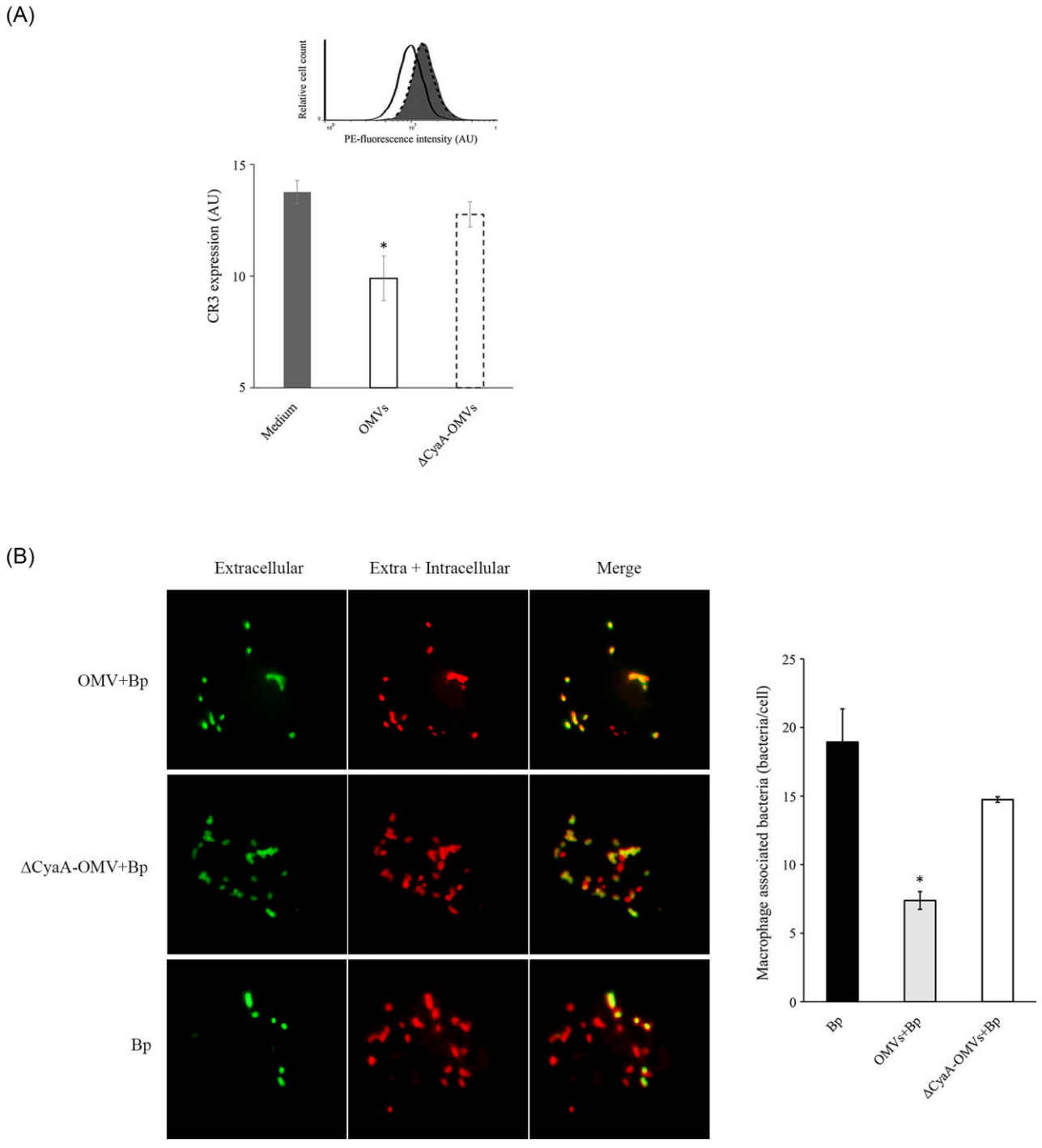

Figure 4. *B. pertussis* OMVs decrease macrophages CR3 surface expression and bacterial attachment to the cells in a CyaA dependent way. THP-1 cells were incubated with OMVs, ΔCyaA-OMVs, or medium alone for 4 h at 37°C, fixed, incubated with PE-conjugated anti-human CR3 antibodies and analyzed by flow cytometry. **(A)** Representative histograms of macrophages incubated with OMVs (bold line), ΔCyaA-OMVs (dashed bold line), and medium alone (filled in grey) are shown. The bars represent the mean red-fluorescence of macrophages incubated with OMVs (grey), ΔCyaA-OMVs (white), or medium alone (black) ± SD of three independent experiments. AU indicates arbitrary units. The mean red-fluorescence associated with OMVs-treated macrophages was significantly different from the mean red-fluorescence associated with the macrophages incubated with ΔCyaA-OMVs or medium alone. (∗$P < 0.05$). **(B)** THP-1 cells were incubated with OMVs, ΔCyaA-OMVs, or medium alone for 4 h at 37°C prior infection with bacteria (MOI 100) for other 2 h at 37°C. The cells were washed, fixed, and subjected to double staining to discriminate between surface attached (green and red) from internalized bacteria (red) by confocal microscopy. The bars indicate the number of bacteria (both intra and extracellular) associated to macrophages. The data represent the means ± SD of three independent experiments. The number of bacteria associated with macrophages pre-incubated with OMVs was significantly different from the number of bacteria associated with macrophages pre-incubated with ΔCyaA-OMVs or medium alone (∗$P < 0.05$). Fluorescence microscopy images representative of one out of three independent experiments are shown.

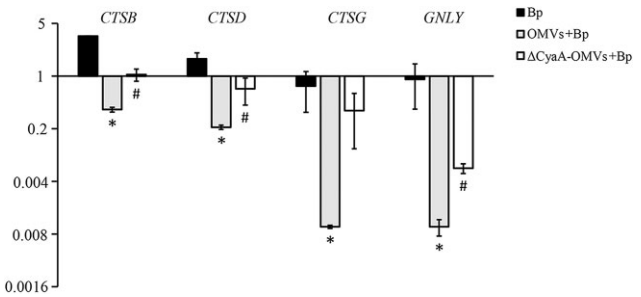

Figure 5. OMVs modulate the expression of genes involved in macrophage defensive response. THP-1 cells were incubated with OMVs, ΔCyaA-OMVs, or medium alone for 4 h prior infection with *B. pertussis* (MOI 100) for other 2 h at 37°C. The cells were then washed and further incubated with polymyxin B for 1 h to kill extracellular bacteria. RNA was extracted from infected and uninfected THP-1 cells and the expression level of the genes was quantitated by qRT-PCR. *B2M* was used as housekeeping gene for normalization, and uninfected cells served as reference. Relative quantification was performed using a LinReg algorithm with the data presented as the log2. Changes in gene expression were expressed as fold change in transcription level of a given gene in infected cells with respect to the uninfected cells. The data represent the mean relative gene expression levels ± SD of three independent experiments. The numbers that are < 1 denote down-regulation and those that are > 1 up-regulation. The asterisks (*) indicate statistically significant differences ($P < 0.05$) in the transcription level of a given gene in infected cells pre-incubated with OMVs (OMVs + Bp) with respect to the transcription level in infected cells pre-incubated with ΔCyaA-OMVs (ΔCyaA-OMVs + Bp) or medium alone (Bp). On the other hand, the number signs (#) indicate significant differences ($P < 0.05$) in the transcription level of a given gene in infected-cells pre-incubated with ΔCyaA-OMVs (ΔCyaA-OMVs + Bp) with respect to the transcription level in infected cells pre-incubated with medium alone (Bp)

ure 5 further shows that the transcription level of *CTSD*, *CTSB* and *GNLY* induced in macrophages treated with ΔCyaA-OMVs was not as affected as in macrophages treated with OMVs, but still significantly lower than those observed in macrophages pre-incubated with medium alone prior to bacterial infection suggesting that others factors present in OMVs contribute to the CyaA-induced down-regulation of these genes (Fig. 5).

Bordetella pertussis OMVs promote bacterial intramacrophage survival

The down-regulation of genes involved in the macrophage bactericidal response was expected to affect the bacterial intracellular survival. Therefore, we next investigated the bacterial intracellular trafficking in macrophages pre-incubated with OMVs, ΔCyaA-OMVs or medium alone. To that end, bacterial colocalization with the late endosomal/lysosomal marker LAMP-1 was investigated 3 h after infection. As shown in Fig. 6, the treatment of the macrophages with OMVs led to a significant decrease in bacterial colocalization with LAMP-1. The percentage of bacteria colocalizing with LAMP-1 in macrophages pre-incubated with ΔCyaA-OMVs was not significantly different from that observed in macrophages pre-incubated with medium alone supporting the relevance of CyaA present in the OMVs in the bacterial trafficking inside the cell (Fig. 6). Accordingly, the number of viable intracellular bacteria 48 h post infection was significantly higher in macrophages pre-incubated with OMVs as compared with macrophages pre-incubated with either ΔCyaA-OMVs or medium alone (Fig. 7). Intracellular survival in macrophages pre-incubated with ΔCyaA-OMVs was not significantly different from macrophages pre-incubated with medium alone (Fig. 7). Taken together, these results suggest that *B. pertussis* OMVs shape the intracellular fate of the bacteria leading to an increased intracellular survival in a CyaA-dependent way.

Discussion

Outer membrane vesicles constitute a bacterial secretion system through which pathogens eventually interact and intoxicate host cells without close contact with the whole bacterium. OMVs' delivery of virulence determinants, adhesion molecules, toxins, and immune modulatory compounds usually promotes host colonization while enhancing the pathogen survival inside the host (Ellis and Kuehn 2010, Chattopadhyay and Jaganandham 2015). Although *B. pertussis* OMVs have been widely characterized as vaccine components (Roberts *et al.* 2008, Raeven *et al.* 2015, Raeven *et al.* 2016), very little is known about their eventual role in bacterial pathogenesis. Donato *et al.* showed the presence of *B. pertussis* OMVs in the lung tissue of a fatal case of pertussis demonstrating that this pathogen sheds vesicles during infection (Donato *et al.* 2012). More recently, other studies (Gasperini *et al.* 2017) suggested that *B. pertussis* releases OMVs already during the initial phases of the upper respiratory tract colonization. Given their small size, or even by disrupting epithelial tight junctions (Chi *et al.* 2003, Hasan *et al.* 2018), *B. pertussis* OMVs might be able to migrate through host tissues and deliver virulence factors into different host cells. Accordingly, the delivery of active CyaA inside the cells trough *B. pertussis* OMVs was already demonstrated by Donato *et al.* (Donato *et al.* 2012). The presence of a variety of virulence factors packed in these OMVs and delivered at the site of infection might play a relevant role in the bacterial interaction with host, eventually shaping the infectious process. The studies published so far on *B. pertussis* OMVs interaction with the host cells were mainly focused on the changes induced by the OMVs in these target cells (Donato *et al.* 2012, Gasperini *et al.* 2017, Elizagaray *et al.* 2020). In the present study, we aimed at investigating the impact of OMVs on the outcome of *B. pertussis* interaction with macrophages since these cells are not only a potential niche of persistence for these bacteria (Lamberti *et al.* 2010, Lamberti *et al.* 2016, Valdez *et al.* 2016, Petráčková *et al.* 2020) but also the central players of the innate and adaptive immunity against invading pathogens.

Bordetella pertussis was historically considered an extracellular pathogen. However, we found that this bacterium is able to survive and even replicate inside human macrophages (Lamberti *et al.* 2010, Lamberti *et al.* 2016, Cafiero *et al.* 2018). We observed that, although a high percentage of phagocytosed bacteria is already killed soon after phagocytosis, a relevant number of intracellular bacteria are able to survive this initial killing by precluding phago-lysosomal fusion and remain alive inside the cells. Intracellular pathogens either obligate or facultative have evolved mechanisms that allow their survival within host cells (Bhavsar *et al.* 2007), which usually comprise the defense response modulation of the infected host cells (Jenner and Young 2005). In previous studies we showed that upon phagocytosis *B. pertussis* down-modulates the expression of genes encoding for proteins involved in the macrophage defense response and antigen presentation (Valdez *et al.* 2016), eventually leading to the bacterial intracellular survival within phagosomes that do not undergo lysosomal maturation (Lamberti *et al.* 2010). The present study showed that the exposure of macrophages to *B. pertussis* OMVs prior to infection potentiates the down-modulation of the macrophage bactericidal response, leading to a significant increase in the intracellular bacterial survival. Moreover, according to our results, macrophage priming with *B. pertussis* OMVs increases the odds of bacterial sur-

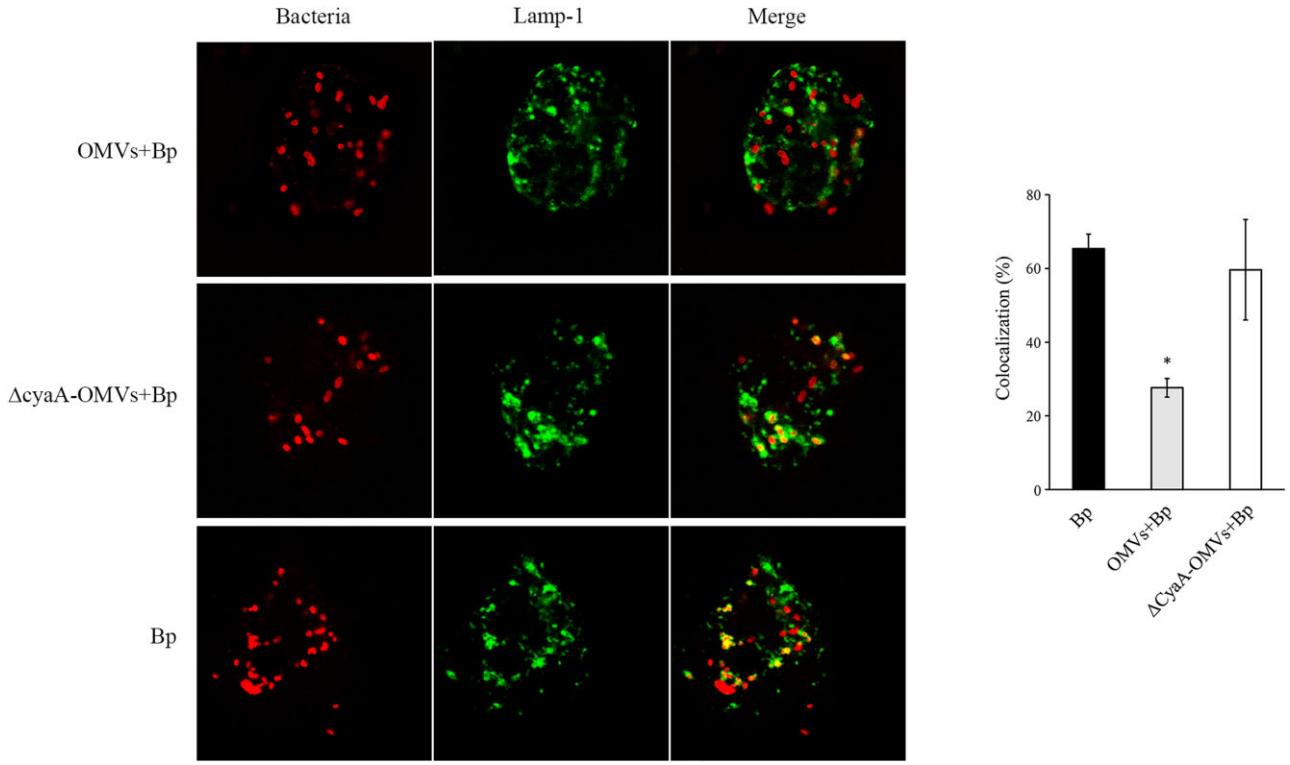

Figure 6. *Bordetella pertussis* OMVs decrease bacterial colocalization with LAMP-1. THP-1 cells were incubated with OMVs, ΔCyaA-OMVs, or medium alone for 4 h at 37°C prior infection with B. pertussis (MOI 100) for other 2 h at 37°C. Cells were then washed, incubated with polymyxin B for 1 h to kill extracellular bacteria, fixed, and permeabilized before incubation with antibodies against *B. pertussis* (red) and LAMP-1 (green). Colocalization of bacteria and LAMP-1 is reflected by the yellow areas. The bars indicate the percentage of LAMP-1-positive phagosomes. The means ± SD of three independent experiments are given. The percentage of bacteria colocalizing with LAMP-1 in macrophages pre-incubated with OMVs was significantly different from the percentage of bacteria colocalizing with LAMP-1 in macrophages pre-incubated with ΔCyaA-OMVs or medium alone (∗ $P < 0.05$). Fluorescence microscopy images representative of one out of three independent experiments are shown.

vival to the encounter with these cells not only by decreasing the intracellular killing but also by interfering with the innate bacterial uptake ability of these immune cells.

The present study was performed with spontaneously released outer membrane vesicles of *B. pertussis*. The proteomic analysis showed that these OMVs contain periplasmic proteins, outer membrane proteins, lipoproteins, and most of virulence determinants of *B. pertussis*, toxins and adhesins among them (Mattoo and Cherry 2005). The presence of adhesins in pathogenic bacteria OMVs grants their attachment to the host cells, their internalization and the eventual delivery of virulence determinants inside the cell (Shoberg and Thomas 1993, Demuth *et al.* 2003, Bauman and Kuehn 2009, Pollak *et al.* 2012). Consistent with the presence of FHA and other adhesins, such as fimbria, Prn, BrKa, Vag8, and SphB1 (Gasperini *et al.* 2018), in the spontaneously released OMVs we found these vesicles attached to and internalized by human macrophages. As expected, confocal studies showed OMVs colocalizing with CR3 and cholesterol-rich domains (lipidrafts), both of them known binding targets of FHA (Relman *et al.* 1990, Lamberti *et al.* 2008). According with these results OMVs and the whole bacteria might compete for the same docking sites on the macrophage cell surface. In our hands, however, although the presence of *B. pertussis* OMVs induced a significant decrease in the bacterial attachment to the macrophages, this decrease could not be ascribed to a competition between the whole bacteria and the OMVs for the same receptors on the surface of the cells. Attachment experiments run in the presence of cytochalasin D, a compound that would prevent OMVs phagocytosis, suggested that OMVs should be internalized to affect bacterial attachment. Although we cannot rule out that under different conditions (i.e. higher concentration of OMVs than that used in this study) there might be a competition for the same receptors as found for other pathogens (Shoberg and Thomas 1993). Under our experimental conditions the decrease in bacterial attachment upon OMVs exposure was found rather related to the macrophage response to CyaA delivered by *B. pertussis* OMVs. Ahmad *et al.* (Ahmad *et al.* 2019) had previously shown that macrophages exposure to *B. pertussis* CyaA induces their dedifferentiation into monocytes with the resulting reduction of macrophage markers expression, CR3 among others. The results of our study suggest a similar effect on macrophages exposed to CyaA delivered by the OMVs. Macrophages treated with *B. pertussis* OMVs showed a decreased level of surface CR3, which proved dependent on the presence of CyaA in these vesicles. The interaction of macrophages with OMVs derived from a *B. pertussis* mutant lacking the expression of CyaA affected neither the level of CR3 on macrophage surface nor the bacterial attachment to the cells. These results support a previous observation (Donato *et al.* 2012) indicating that *B. pertussis* OMVs deliver active CyaA into the host cells. The OMV delivery of active CyaA was further supported by the finding that CyaA associated to the OMVs shaped the macrophage response to the encounter with *B pertussis*. Previous studies had demonstrated that CyaA present in the infecting bacteria modulates the macrophage bactericidal response, eventually promoting *B. pertussis* intracellular survival (Valdez *et al.* 2016). Genes encoding for proteins involved in the macrophage defense response were

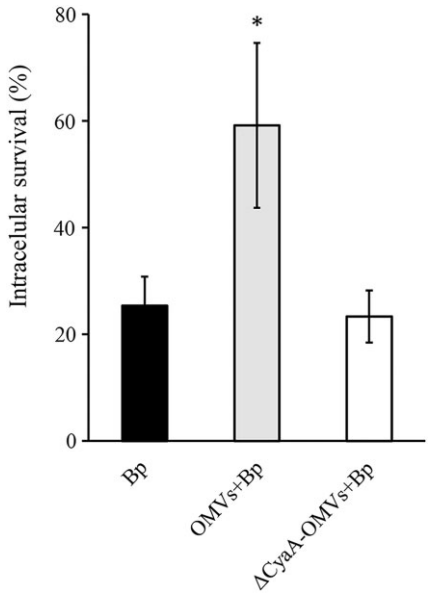

Figure 7. OMVs promote bacterial survival within macrophages. THP-1 cells were incubated with OMVs, ΔCyaA-OMVs, or medium alone for 4 h at 37ºC prior infection with *B. pertussis* (MOI 100) for 2 h at 37°C. The cells were washed extensively and further incubated with polymyxin B for 1 h to kill extracellular bacteria. Three and 48 h after infection, the cells were lysed and the number of CFUs of *B. pertussis* per cell was determined. Bacterial survival was expressed as the percentage of intracellular live bacteria at 3 h after infection that were still alive 48 h after phagocytosis. The data represents the means ± SD of three independent experiments. The percentage of viable intracellular bacteria per cell 48 h after infection in macrophages pre-incubated with OMVs was significantly different from the percentage of viable intracellular bacteria in macrophages pre-incubated with ΔCyaA-OMVs or medium alone. (∗$P < 0.05$)

found significantly down modulated as the intracellular infection progressed, and the CyaA present in the infecting bacteria proved to be involved in this effect (Valdez *et al.* 2016). The results of the present study showed the down regulation of genes encoding for antimicrobial proteins, namely cathepsins and granulysin, early after infection in macrophages exposed to OMVs but not in macrophages not exposed to OMVs. This effect proved mainly dependent on the presence of CyaA in the OMVs. Cathepsins are serine, aspartic, and cysteine peptidases that are involved in pathogen killing and antigen presentation, among other functions (Conus and Simon 2010). Granulysin is an antimicrobial protein involved in intracellular pathogens killing (Krensky 2000). Accordingly, we observed a decreased bacterial trafficking into the microbicidal pathway and a significant increased bacterial intracellular survival in OMVs primed macrophages as compared with macrophages not exposed to OMVs. Again, this impairment of the macrophage bactericidal capacity upon exposure to OMVs proved to be mainly dependent on CyaA. By decreasing the CR3 on macrophage surface, which would prevent bacterial uptake by these cell, together with the down-modulation of genes encoding proteins involved in bactericidal activity and antigen presentation (Shi *et al.* 1999, Zhang *et al.* 2000), OMVs will not only preclude bacterial killing but might also affect the induction of the adaptive immune responses, since macrophages are involved in CD4 + T-cells activation and expansion (Ben-Sasson *et al.* 2009).

Our data show a central role of CyaA in the immune modulatory effects of *B. pertussis* OMVs on macrophages. However, there might be other factors present in the OMVs that contribute to the cell bactericidal response modulation. Macrophages primed with OMVs lacking CyaA induced a down regulation of the cellular bactericidal response after bacterial phagocytosis which was not observed in untreated macrophages. Many other factors present in the OMVs might account for this effect. The proteomic analysis of the spontaneously released OMVs used in this study showed many proteins associated with immune modulatory effects, e.g. PT (Valdez *et al.* 2016), and lipoproteins (Kovacs-Simon *et al.* 2011), among other proteins, some of them solely secreted via OMVs (Luu *et al.* 2018a and b, Luu *et al.* 2020). Although further studies are needed to determine whether other OMVs components contribute to the macrophage response modulation, the present study shows that *B. pertussis* OMVs render macrophages less responsive to the infection, mainly through the delivery of CyaA into the cells.

Taken together our results support the hypothesis that OMVs are key virulence determinants for *B. pertussis*, eventually affecting the immunity against this pathogen.

Supplementary data

Supplementary data are available at *FEMSPD* online.

Acknowledgments

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Conflict of interest. The authors have no conflict of interest.

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