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Research Article

RESEARCH ARTICLE

Bordetella pertussis modulates human macrophage defense gene expression

Hugo Alberto Valdez, Juan Marcos Oviedo, Juan Pablo Gorgojo, Yanina Lamberti and Maria Eugenia Rodriguez*

CINDEFI (UNLP CONICET La Plata), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900 La Plata, Buenos Aires, Argentina

*Corresponding author: CINDEFI, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 47 y 115, 1900 La Plata, Buenos Aires, Argentina. Tel/Fax: +54-221-4833794; E-mail: mer@quimica.unlp.edu.ar

One sentence summary: *Bordetella pertussis* manipulates host cell defense response creating a permissive intracellular environment for its survival.
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ABSTRACT

Bordetella pertussis, the etiological agent of whooping cough, still causes outbreaks. We recently found evidence that *B. pertussis* can survive and even replicate inside human macrophages, indicating that this host cell might serve as a niche for persistence. In this work, we examined the interaction of *B. pertussis* with a human monocyte cell line (THP-1) that differentiates into macrophages in culture in order to investigate the host cell response to the infection and the mechanisms that promote that intracellular survival. To that end, we investigated the expression profile of a selected number of genes involved in cellular bactericidal activity and the inflammatory response during the early and late phases of infection. The bactericidal and inflammatory response of infected macrophages was progressively downregulated, while the number of THP-1 cells heavily loaded with live bacteria increased over time postinfection. Two of the main toxins of *B. pertussis*, pertussis toxin (Ptx) and adenylate cyclase (CyaA), were found to be involved in manipulating the host cell response. Therefore, failure to express either toxin proved detrimental to the development of intracellular infections by those bacteria. Taken together, these results support the relevance of host defense gene manipulation to the outcome of the interaction between *B. pertussis* and macrophages.

Keywords: *Bordetella pertussis*; intracellular survival; adenylate cyclase; pertussis toxin; host cell defense response

INTRODUCTION

Whooping cough is a re-emerging disease caused by *Bordetella pertussis*. In spite of extensive vaccination coverage worldwide, this disease is still poorly controlled (Burns, Meade, and Messionnier 2014). Little is known about the forms and mechanisms of persistence of this pathogen. Our group has shown that the encounter of *B. pertussis* with human primary monocyte-derived macrophages leads to the intracellular survival of a significant number of bacteria that then seemed able to replicate inside the macrophages (Lamberti *et al*. 2010). These results suggest that *B. pertussis* might have an intracellular phase that contributes to bacterial persistence.

Macrophages have the ability to recognize bacterial products and initiate an immune response for removal of the microbes. In order to survive within the immune cell, pathogens need to be able to adapt to the intracellular environment and disrupt macrophage regulation by modulating microbicidal effector mechanisms (Thi, Lambertz and Reiner 2012). Recent studies on the intracellular proteome evolution of *B. pertussis* revealed that *B. pertussis* undergoes an adaptive response to that intracellular environment characterized by changes in proteins

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involved in metabolism, iron uptake and the stress response (Lamberti *et al*. 2016). Whether *B. pertussis* is able to modulate macrophage bactericidal activity, however, is still not fully understood.

The classical activation of macrophages leads to the acquisition of microbicidal competence and the inflammatory capacity that usually produces effective immunity (Benoit, Desnues and Mege 2008; Murray and Wynn 2011). The innate immune response of the macrophage is mediated by antimicrobial peptides, an oxidative response and various enzymatic activities that play a fundamental role in the defense against pathogens. Among the main antimicrobial peptides are the defensins and cathepsins located almost entirely in the lysosomes (Flannagan, Cosío and Grinstein 2009). The defensins are cationic and hydrophobic peptides, such as defensin-ß 1 and granulysin, whereas the cathepsins are a large family of proteases that play key roles in antigen processing and the regulation of cytokine expression (Kaiser and Diamond 2000; Colbert *et al*. 2009). Other proteins, like the acid phosphatases 2 and 6, play a major role inside the lysosome by cleaving different phosphomonoesters (Suter *et al*. 2001). After pathogen recognition, the macrophages usually kill the bacteria and help to develop an adaptive immunity through cytokine production and antigen presentation, which steps eventually activate T cells. Macrophage activation has been shown to be rapid and fully reversible. Recent studies have established that the macrophage phenotype is regulated by a suppressor of cytokine signaling (SOCS). Thus, macrophages with microbicidal capacity can be turned into immunomodulators with scarce or null microbicidal activity in a relatively short time by modulation of the SOCS1/SOCS3 balance (Benoit, Desnues and Mege 2008; Wilson 2014).

Intracellular pathogens have evolved to gain the ability to control host cell gene expression, which development eventually enables their survival and intracellular persistence. Such pathogens usually subvert macrophage signaling and effector mechanisms such as phagocytic capability, bactericidal capacity, or NF-κB and IFN-γ signaling, among others. These immune evasion strategies usually depend on bacterial effectors (Flannagan, Cosío and Grinstein 2009; Reddick and Alto 2014). *Bordetella pertussis* has two potent toxins, adenylate cyclase (CyaA) and pertussis toxin (Ptx), both of which have been reported to play a role in modulation or even suppression of host immune and inflammatory responses (Carbonetti 2011; de Gouw *et al*. 2011). Both toxins agents were found to be highly expressed in intracellular bacteria even after 2 days postinfection (Lamberti *et al*. 2016). In this study, we investigated whether the intracellular survival of *B. pertussis* depends on the modulation of the macrophage defense response along with the role of Ptx and CyaA in that process.

MATERIALS AND METHODS

Cell culture

THP-1 monocytes were obtained from ATCC (Manassas, USA) and 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 ng ml^{-1} phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, Saint Louis, USA) to RPMI 1640 plus 10% (w/v) of FBS according to Maess, Sendelbach and Lorkowski (2010). Cells were incubated in order to enable differentiation for 24 h before infection.

Bacterial strains and growth conditions

Bordetella pertussis strain B213 (a streptomycin-resistant derivative of Tohama, wtBp; King *et al*. 2001), *B. pertussis* strain BPM3183—a mutant deficient in CyaA production, BpCyaA(–) (Weiss *et al*. 1989)—and *B. pertussis* strain Bp357 (Weiss *et al*. 1983)—a mutant deficient in Ptx production, BpPtx(–)—were transformed with plasmid pCW505 (Weingart *et al*. 1999), kindly supplied by A. A. Weiss, Cincinnati, OH, which induces the cytoplasmic expression of green fluorescent protein (GFP) without affecting cellular growth or antigen expression (Weingart *et al*. 1999). The bacteria were stored at –70°C and recovered by growth on Bordet-Gengou (BG) agar plates supplemented with 15% (v/v) defibrinated sheep blood (bBGA) at 35°C for 3 days. Bacteria were subsequently plated on bBGA, cultured for 20 h at 35°C and used in infection experiments.

THP-1 infection and quantification of intracellular *Bordetella pertussis*

The number of intracellular bacteria per THP-1 cell was evaluated by double staining and fluorescence microscopy. THP-1 cells were infected with GFP-wtBp, GFP-BpCya(–) or GFP-BpPtx(–) suspended in RPMI 1640 plus 0.2% (w/v) bovine serum albumin (BSA, Sigma) at a multiplicity of infection (MOI) of 120 bacteria per cell. Bacterial concentration was estimated by OD_{650} and further quantified by counts of colony-forming units (CFUs). For experiments with heat-killed bacteria, the bacterial suspension was heated at 56°C for 30 min. To facilitate bacterial interaction with the eukaryotic cells, plates were centrifuged for 5 min at 640 × *g*. After 20 min 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. After incubation for 1 h at 37°C, cells were washed three times with phosphate-buffered saline (PBS) and then either harvested (3 h postinfection) and fixed with 4% (v/v) paraformaldehyde for 10 min or incubated with fresh complete RPMI medium plus 10% (v/v) FBS and 5 μg ml^{-1} polymyxin B for another 45 h. Samples were taken at 24 and 48 h postinfection and washed as above before fixation with 4% (v/v) paraformaldehyde for 10 min. After fixation, cells of all samples were washed once with PBS and incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl. Macrophage surface-bound and phagocytosed bacteria were discriminated by a two-step labeling procedure followed by fluorescence microscopy analysis. Surface-bound bacteria were evidenced by incubation with polyclonal rabbit anti-*B. pertussis* antiserum for 30 min at 4°C, followed by incubation with the Cy3-conjugated goat F(ab′)$_2$ fragments of anti-rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA) for 30 min at 4°C. In order to determine the number of intracellular bacteria, after two washes as above, the cells were permeabilized by incubation with PBS containing 0.1% (w/v) saponin (Sigma) and 0.2% (w/v) BSA for 30 min at 25°C, then further incubated with rabbit anti-*B. pertussis* antiserum in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA (30 min at 25°C). After three washes as above, the cells were incubated with the FITC-conjugated F(ab′)$_2$ fragments of goat anti-rabbit immunoglobulin for 30 min at 25°C. Labeling of the bacteria with FITC-conjugated antibodies was performed to minimize the loss of read-out sensitivity resulting from the quenching of GFP fluorescence after internalization. After washing with distillated water, the coverlips were mounted

on microscope slides and analyzed by confocal laser scanning microscopy (Leica TCS SP5, Wetzlar, Germany). The number of extracellular (red and green, seen as yellow) and intracellular (green) bacteria was evaluated by examination of at least 100 cells. All experiments were carried out in triplicate at least three times.

Bacterial intracellular survival

Bacterial intracellular survival was determined as previously described (Lamberti *et al*. 2010) with minor modifications. Briefly, THP-1 cells were infected with wtBp, BpCya(–) or BpPtx(–) suspended in RPMI 1640 plus 0.2% (w/v) BSA at an MOI of 120 bacteria per cell as described above. After 30 min of incubation at 37°C, non-adherent bacteria were removed by three washes. After the addition of 100 μg ml^{-1} polymyxin B sulfate for 1 h to kill the extracellular bacteria, the antibiotic concentration was decreased to 5 μg ml^{-1}. Control experiments had indicated that in the presence of 5 μg ml^{-1} polymyxin B *B. pertussis* was unable to replicate or remain alive in the culture medium. The number of CFUs in the culture supernatants was examined and no viable bacteria were detected at any time postinfection. At 3 and 48 h after infection, *B. pertussis* intracellular survival was determined as follows. After washing with PBS, the monolayers were incubated for 15 min at 37°C with PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 4 mg ml^{-1} lidocaine to promote THP-1-cell detachment. The number of viable eukaryotic cells was determined by trypan-blue dye exclusion. Next, the THP-1 cells were lysed with 0.1% (w/v) saponin in sterile water and serial dilutions rapidly plated onto bBGA plates to enumerate the CFUs. Control experiments to determine the number of phagocytosed bacteria at 3 h after infection were run in parallel to be used for calculating the percentage of phagocytosed bacteria that were still alive at those time points after phagocytosis. Bacterial phagocytosis was determined by fluorescence microscopy as described above. Control experiments were run in parallel to assess the efficacy of the bactericidal activity of polymyxin B (100 μg ml^{-1}). Stated in brief, samples of 5×10^8 bacteria were incubated with the antibiotic for 1 h at 37°C and plated on bBGA. Subsequent incubation resulted in a 99.9% decrease in the CFUs. No significant differences in bacterial sensitivity to the antibiotics were detected among the strains used in this study. The viability of infected THP-1 cells was determined at the different times after infection with trypan blue. No significant cell death was observed in any of the different infection assays performed in this study.

Confocal microscopical analysis

Colocalization studies were performed as described previously (Lamberti *et al*. 2010) with minor modifications. Briefly, THP-1 cells were infected with *B. pertussis* (MOI 120) at 37°C for 20 min, washed to remove non-attached bacteria and then further incubated with 100 μg ml^{-1} polymyxin B for 1 h at 37°C to kill extracellular non-phagocytosed bacteria. Next, the concentration of polymyxin B was reduced to 5 μg ml^{-1}. At 3 and 48 h after infection, macrophage samples were incubated with or without 200 nM Lysotracker DND-99 (5 min at 37°C) followed by fixation with paraformaldehyde. The cells incubated with the Lysotracker stain were washed twice with PBS, incubated for 10 min at room temperature with PBS containing 50 mM NH4Cl and finally subjected to fluorescence *in situ* hybridization (FISH) staining of the intracellular live bacteria present as described below. The samples of infected cells that were not incubated with the Lysotracker stain were washed twice with PBS and incubated with PBS containing 50 mM NH4Cl for 10 min at room temperature. After two washes, the cells were incubated for 30 min with PBS containing 0.1% (w/v) saponin and 0.2% (w/v) BSA. Next, the cells were incubated with mouse anti-human LAMP-1 (CD107a) polyclonal antibodies (Pharmingen, San Diego, CA) 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 Cy3-conjugated F(ab′)$_2$ fragments of goat anti-mouse antibodies (Southern Biotechnology, Birmingham, USA) plus FITC-conjugated goat F(ab′)$_2$ fragments of anti-rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA) 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. In addition, isotype controls were run in parallel. Microscopical analyses were performed through the use of a confocal laser scanning microscope (Leica TCS SP5, Wetzlar, Germany). The percentage of phagosomes containing bacteria that colocalized with a given marker was calculated by analyzing at least 20 cells per sample.

FISH staining of intracellular live bacteria

Samples of THP-1 cells infected with *B. pertussis*, probed with the Lysotracker stain and fixed with 4% (v/v) paraformaldehyde were examined for live intracellular bacteria by FISH staining. Hybridization with the following fluorescently labeled oligonucleotides was done as described previously (Gorgojo, Harvill and Rodríguez 2014) with minor modifications. The Alexa 488-conjugated DNA probes BET42a (5′-GCCTTCCCACTTCGTTT-3′) and EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) were designed for rRNA labeling of the gamma subclass of the proteobacteria and eubacteria, respectively (Manz *et al*. 1993). As a negative control, an Alexa 488-conjugated non-EUB338 DNA probe complementary to EUB338 was used. Bacterial detectability depends on the presence of enough ribosomes per cell, thus providing information on the viability of the bacteria based on the amount of ribosomes per cell. The BET42a and EUB338 probes were used together to produce a stronger signal. Both probes were added to a final concentration of 10 nM in the hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 7.4, 0.01% SDS and 35% [v/v] formamide). Next, the hybridization mixture was incubated for 1.5 h at 46°C, in a humid chamber. The cells were then incubated for 30 min with washing buffer (80 mM NaCl, 20 mM Tris/HCl, pH 7.4, 0.01% (w/v) sodium dodecyl sulfate and 5 mM EDTA, pH 8) at 48°C. In control experiments, the total number of intracellular bacteria (both live and dead) was determined by immune staining with polyclonal mouse anti-*B. pertussis* antiserum followed by Cy3-conjugated F(ab′)$_2$ fragments of goat anti-mouse antibodies as described above. Samples were mounted onto glass slides. The FISH labeling of free bacteria incubated with polymyxin B did not give any detectable signal.

RNA isolation

Total RNA was prepared from the cell lysates with the RNeasy Mini Kit according to the manufacturer's instructions, including the use of an *in columna* DNAase-I digestion (Qiagen, Valencia, CA, USA) as previously reported (Maess, Sendelbach, and Lorkowski 2010). RNA quality was assessed both by agarose gel electrophoresis and photometrically by means of NanoDrop

2000 (Thermo Scientific, Waltham, USA). A 260/280 ratio of around 2.0 was considered adequate.

cDNA synthesis

Synthesis of cDNA was performed with the Sensiscript RT Kit (Qiagen). For each cDNA synthesis, 40 ng of a given total RNA and 10 μM random primers (Qiagen) were used as previously described (Maess, Sendelbach and Lorkowski 2010). Complementary DNA samples were stored at –20°C until the time of analyses by the polymerase chain reaction (PCR).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) analyses were performed on an Mx3000P QPCR System (Stratagene, La Jolla, USA) with the SYBR Green PCR Master Mix (Roche, Mannheim, Germany) and 0.5 μM of each forward and reverse primer. Primer sequences were taken from qPrimerDepot database (Cui, Taub and Gardner 2007) and are listed in Table 1. Primers were purchased from Invitrogen (Karlsruhe, Germany). PCR runs comprised a 10 min preincubation at 95°C, followed by 40 cycles of a two-step PCR consisting of a denaturing phase at 94°C for 15 s and a combined annealing and extension phase at 60°C for 60 s. The resulting amplicons were examined by melting peaks and agarose gel electrophoresis. The PCR products were further characterized by nucleotide sequencing. The relative expression level of each gene was calculated by the threshold cycle ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001). The ß2 microglobulin gene (*B2M*) was used as a housekeeping marker for normalization, and uninfected cells served as a reference.

Cytokine determination

The amount of tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) in culture supernatants of either uninfected or infected THP-1 cells was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The level of TNF-α was determined through the use of the Human TNF-α ELISA kit (ImmunoTools, Friesoythe, Germany) and the level of IL-8 by means of the Human IL-8 ELISA kit (CLB, Amsterdam, the Netherlands).

LAMP-2, cathepsin S (CTSS) and cathepsin D (CTSD) determination

The cellular level of LAMP-2, CTSS or CTSD was determined by flow cytometry as previously described (Grützkau *et al.* 2004) with minor modifications. The steps stated in brief as follows: THP-1 cells infected with GFP-wtBp, GFP-BpCyaA(–) or

Table 1. Primers used for real-time PCR.

Gene	Protein name	Sense	Antisense
Antimicrobial peptides			
DFB1	Defensin, beta 1	5′-GGGCAGGCAGAATAGAGACA-3′	5′-TTTTGTCTGAGATGGCCTCA-3′
GNLY	Granulysin	5′-CGCAGCATTGGAAACACTT-3′	5′-GACCAAAACACAGGAGCTGG-3′
Oxidative defense			
CAT	Catalase	5′-ACGGGGCCCTACTGTAATAA-3′	5′-AGATGCAGCACTGGAAGGAG-3′
Enzymatic defense			
ACP2	Acid phosphatase 2	5′-ATACAGCCAAGGCCACAATC-3′	5′-GACTTCCTTCGCCTCACAGA-3′
ACP6	Acid phosphatase 6	5′-ATAGCTGGGGGTTCCACTCT-3′	5′-AAAATGGTGCAGGTCGTGTT-3′
CTSA	Cathepsin A	5′-GAGACGGCGGTACTGTAAGTTT-3′	5′-TCCACCTACCTCAACAACCC-3′
CTSB	Cathepsin B	5′-GGCCCCCTGCATCTATCG-3′	5′-AGGTCTCCCGCTGTTCCACTG-3′
CTSC	Cathepsin C	5′-CCTTGCTGCCCTCTTCTTTA-3′	5′-ATTCTGGCCATTTCACCATC-3′
CTSD	Cathepsin D	5′-CCCGCATCTCCGTCAACAA-3′	5′-GCCTTGCGGGTGACATTCAG-3′
CTSG	Cathepsin G	5′-AAGATACGCCATGTAGGGGC-3′	5′-TTCTGCTGGCCTTTCTCCTA-3′
CTSS	Cathepsin S	5′-TCTCTCAGTGCCCAGAACCT-3′	5′-GCCACAGCTTCTTTCAGGAC-3′
LYZ	Lysozyme	5′-ACAAGCTACAGCATCAGCGA-3′	5′-GTAATGATGGCAAAACCCCA-3′
Inflammatory response			
IL-8	Interleukin 8	5′-ATGACTTCCAAGCTGGCCG-3′	5′-CTCCACAACCCTCTGCACC-3′
IL-10	Interleukin 10	5′-GTGATGCCCCAAGCTGAGA-3′	5′-CACGGCCTTGCTCTTGTTTT-3′
TNF-α	Tumor necrosis factor-alfa	5′-AACCTCCTCTCTGCCATCAA-3′	5′-CCAAAGTAGACCTGCCCAGA-3′
SOCS1	Suppressor of cytokine signaling 1	5′-TTTTTCGCCCTTAGCGTGAA-3′	5′-GCCATCCAGGTGAAAGCG-3′
SOCS3	Suppressor of cytokine signaling 3	5′-GAAGATCCCCCTGGTGTTGA-3′	5′-TTCCGACAGAGATGCTGAAGA-3′
Lysosomal membrane protein			
LAMP-2	Lysosomal associated membrane protein 2	5′-CTCTGCGGGGTCATGGTG-3′	5′-CGCACAGCTCCCAGGACT-3′
Housekeeping			
B2M	ß2-Microglubulin	5′-TCTCTGCTGGATGACGTGAG-3′	5′-TAGCTGTGCTCGCGCTACT-3′

GFP-BpPtx(–) were incubated for 30 min with PBS containing 0.1% (w/v) saponin and 0.2% (w/v) BSA. Next, the cells were incubated with mouse anti-human LAMP-2 (CD107b; BD Pharmingen, San Diego, Santa Cruz, CA), mouse anti-human Cathepsin S (B-12; Santa Cruz biotechnology, CA), or mouse anti-human cathepsin D (Abcam Inc., Cambridge, UK) for 30 min at room temperature in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA. After three washes, the cells were incubated with phycoerythrin- (PE-) conjugated $F(ab')_2$ fragments of goat anti-rabbit antibodies, washed three times and analyzed by flow cytometry (FACScan, Becton Dickinson Immunocytometry System, San Jose, CA).

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).

RESULTS

Subcellular localization of bacteria phagocytosed by THP-1 cells

We had previously shown that the interaction of *Bordetella pertussis* with primary human monocyte-derived macrophages in the absence of opsonic antibodies leads to the intracellular survival of a significant number of phagocytosed bacteria. Trafficking studies have demonstrated that *B. pertussis* replicates inside cells in a non-acidic compartment (Lamberti *et al.* 2010). Primary monocyte-derived macrophages are difficult to use in functional studies where a large number of cells is required. We therefore sought a suitable cell line for studies on *B. pertussis* intracellular infection. Our findings indicated that the intracellular trafficking of *B. pertussis* in PMA-differentiated THP-1 cells was comparable to that observed in primary human cells. Two color confocal microscopy studies indicated that 48 h after infection most of the intracellular bacteria were not colocalizing with the lysosomal marker LAMP-1, suggesting that those bacteria had managed to avoid the degradative pathway by remaining in non-acidic compartments (Fig. 1A), such as found in primary human cells. In order to further investigate whether the bacteria that had prevented phagolysosomal maturation remained alive inside the cells, we used FISH staining for viable bacteria and the lysosomal marker Lysotracker. Colocalization studies demonstrated that 48 h after infection those bacteria that were not colocalizing with the lysosomal marker remained alive (Fig. 1B). Polymyxin B protection assays confirmed the intracellular survival of *B. pertussis* in PMA-differentiated THP-1 cells. At 48 h after infection, 35% of the bacteria internalized at 3 h after infection remained alive. The number of intracellular bacteria over the time of infection was assessed by double-staining microscopy. Figure 2A depicts the distribution of intracellular bacteria among the THP-1 cells expressed as the percentage of cells loaded with a certain number of bacteria at each time point. At 3 h after infection, most of the THP-1 cells contained 1 to 20 bacteria and no THP-1 cell contained more than 20. At 24 h, an increase in the number of cells containing none or a low number (i.e. 1–10) of bacteria was observed, suggesting that some of the THP-1 cells cleared the bacteria upon phagocytosis. By 48 h after infection, however, the percentage of THP-1 heavily loaded with bacteria (i.e. containing more than 20) had drastically increased. That this increase was not observed in THP-1 cells infected with inactivated bacteria (Fig. 2B) is of critical relevance. Since the extracellular medium is bactericidal, this result seemed consistent with intracellular

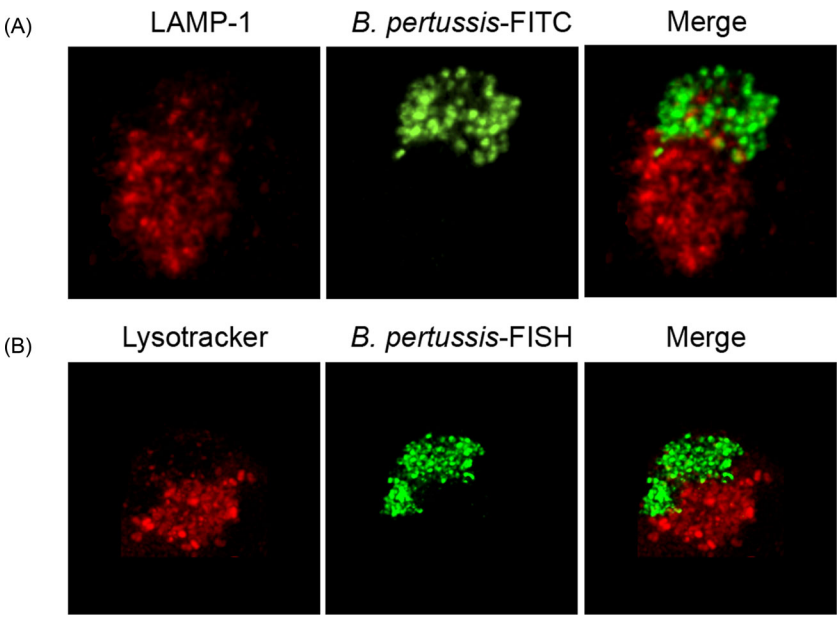

Figure 1. Intracellular trafficking of *B. pertussis* in THP-1 cells. THP-1 cells were incubated with *B. pertussis* (at an MOI of 120) for 20 min at 37°C, extensively washed and then incubated with polymyxin B to kill the extracellular bacteria (cf. Materials and Methods). Samples were taken at 48 h. The cells were either (**A**) fixed and permeabilized before incubation with antibodies against LAMP-1 (red) and antibodies against *B. pertussis* (green) or (**B**) incubated with the acidotrophic dye Lysotracker (red), fixed and stained for live *B. pertussis* bacteria by FISH (green), and then visualized by confocal microscopy. Colocalization is indicated by the yellow areas. The images are representative of those from three independent experiments.

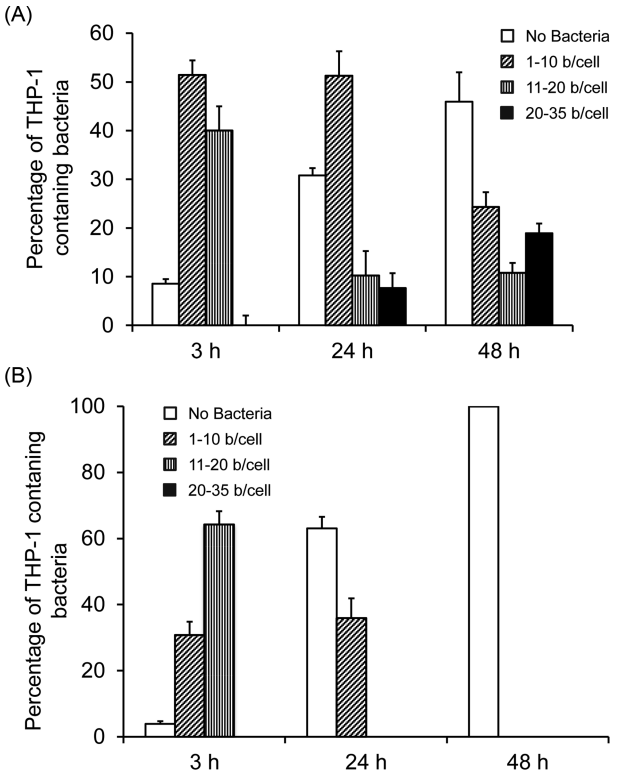

Figure 2. Quantification of bacterial loads in THP-1 cells over time. THP-1 cells were incubated with (**A**) live *B. pertussis* or (**B**) heat-killed *B. pertussis* (at an MOI of 120) for 20 min at 37°C, extensively washed and then incubated with polymyxin B to kill the extracellular bacteria. At 3, 24 and 48 h after infection, the cells were fixed and the number of bacteria per cell analyzed by double fluorescent staining and confocal microscopy. The number of intracellular bacteria was determined by analyzing 100 cells in each sample. The data represent the means ± SD of three independent experiments.

bacterial replication as found in primary human cells (Lamberti *et al.* 2010).

Host defense gene expression during *Bordetella pertussis* infection

Bacterial survival inside a cell usually comprises a dynamic interaction between the cell and the pathogen that eventually leads to transcriptional changes in the infected host cells. We therefore investigated the transcriptional response of THP-1 cells to intracellular infection. To this end, the level of expression of genes that encode antimicrobial peptides and proteins involved in enzymatic or oxidative defense mechanisms (Table 1) was investigated by qRT-PCR at 3, 24 and 48 h after infection. Uninfected THP-1 cells were analyzed in parallel as controls. Figure 3A depicts the gene expression over the time course of infection as compared with uninfected cells. Genes such as *ACP2* (acid phosphatase 2), *ACP6* (acid phosphatase 6) and *GNLY* (granulysin) were found to be downregulated during *B. pertussis* infection. The downregulation of these genes was initially observed at early time points after infection and remained as such during the whole time course investigated in this study. Conversely, the transcriptional level of *CAT* (catalase), *CTSB* (cathepsin B), *CTSD* (cathepsin D), *CTSG* (cathepsin G) and *CTSS* (cathepsin S) underwent an increase early after infection followed by a significant downmodulation at later time points after infection compared to the control group. The downregulation of the expression of *ACP2*, *ACP6*, *CAT*, *GNLY*, *CTSB*, *CTSD*, *CTSG* and *CTSS* was found to be dependent on bacterial viability since those changes in expression level were not observed in macrophages infected with heat-killed bacteria (Fig. 3B).

Involvement of CyaA and Ptx in *Bordetella pertussis* intracellular survival

Bordetella pertussis produces two potent toxins, CyaA and Ptx, with those having been previously found to be involved in host defense manipulation (Boldrick *et al.* 2002; Carbonetti 2011). CyaA induces an increase in the intracellular level of cAMP, a second messenger that modulates the activity of several cellular processes (Kambayashi, Wallin and Ljunggren 2001; McDonough and Rodriguez 2011). Ptx manipulates cellular responses through different mechanisms (Locht, Coutte and Mielcarek 2011). Proteomic analysis of intracellular *B. pertussis* indicated a high expression of both toxins even at 48 h after infection, suggesting that these virulence factors might be required for the intracellular persistence of the bacteria (Lamberti *et al.* 2016). In order to investigate the role of these toxins in *B. pertussis* intracellular survival, we examined bacterial viability in polymyxin B protection assays. Figure 4 reveals that the intracellular survival of the wild-type strain of *B. pertussis* was significantly higher than the intracellular survival of the two *B. pertussis* mutants BpCyaA(–) and BpPtx(–), defective in the expression of CyaA and Ptx, respectively. These results demonstrate that both those toxins are involved in bacterial intracellular persistence.

Contribution of CyaA and Ptx to the modulation of host defense genes

In order to investigate the eventual role of each toxic agent in host defense gene modulation, we examined the transcriptional response of THP-1 cells to the infection of the two *B. pertussis* mutants BpCyaA(–) and BpPtx(–). The transcriptional response of THP-1 cells either uninfected or infected with each deficient mutant or the wild-type strain of *B. pertussis* was accordingly determined in parallel. Figure 5 illustrates the cellular transcriptional response to BpCyaA(–) compared to that of uninfected cells. The expression of defense genes in cells infected with BpCyaA(–) was similar to that observed in cells infected with the wild-type strain (Fig. 3A) except for the transcription of *CTSB*, *CTSD* and *CTSS*. These genes were not downmodulated over the time of infection with BpCyaA(–), thus suggesting a role of CyaA in regulating the expression of those three genes. Figure 6 depicts the cellular response in gene expression to infection with BpPtx(–) compared to the expression in uninfected cells. This figure demonstrates that in the absence of Ptx production the downregulation of *ACP2*, *ACP6* and *CTSS* observed in cells infected with the wild-type strain at 48 h after infection (Fig. 3A) was abolished, whereas a significant increase in the expression level of those genes occurred. Similar results were obtained for the expression of *GNLY*, *CTSB*, *CTSD* and *CTSG*, but the change in the expression profile of those genes was observed as early as 24 h after infection. These data suggest a role of Ptx in the downmodulation of these host defense genes. The modulation of the expression of two of these cathepsins, CTSS and CTSD, was further investigated at protein level at 3, 24 and 48 h after infection (Fig. 7A and B). The results confirmed what was observed at transcriptional level. Figure 7 shows that the level of both cathepsins remained low in THP-1 cells infected with wtBp until 48 h after

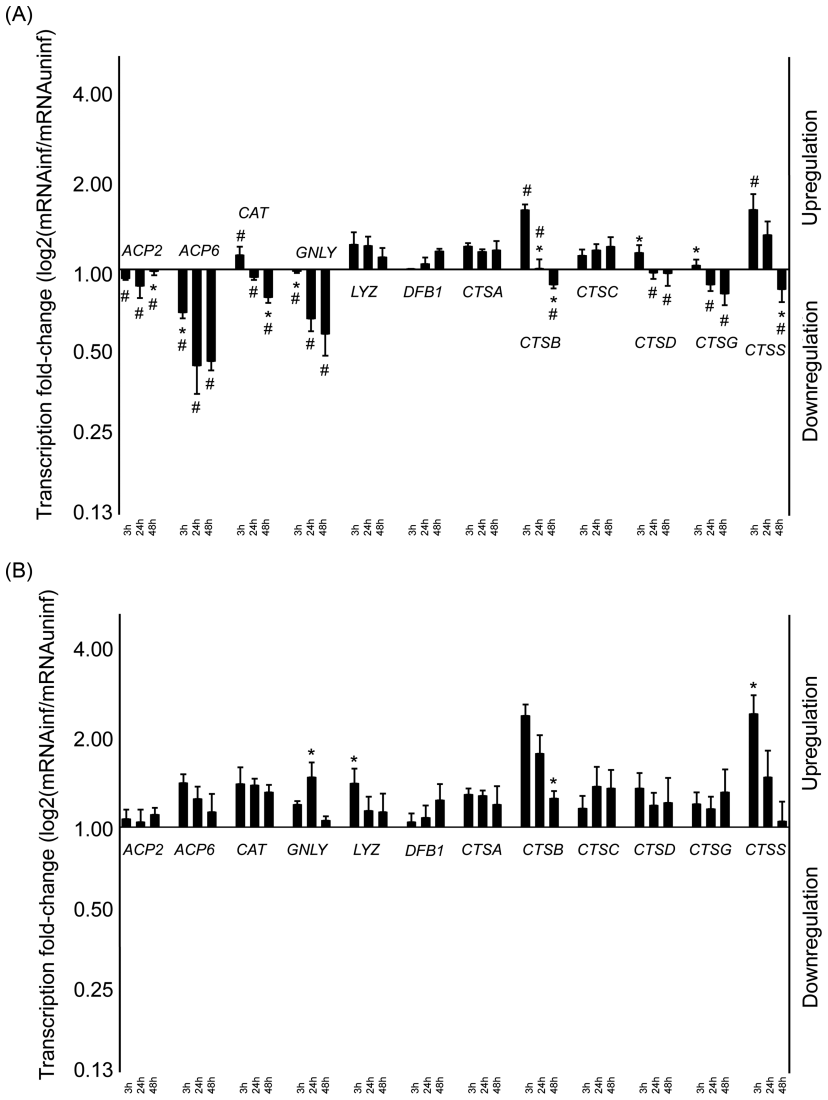

Figure 3. Host defense gene expression in THP-1 cells upon *B. pertussis* infection. THP-1 cells were incubated with (**A**) live *B. pertussis* or (**B**) heat-killed *B. pertussis* (at an MOI of 120) for 20 min at 37°C, washed extensively and further incubated with polymyxin B to kill the extracellular bacteria. RNA was extracted from infected and uninfected THP-1 cells at 3, 24 and 48 h after infection and the expression of defense genes quantitated by qRT-PCR. *B2M* was used as a housekeeping gene for data normalization, and untreated cells served as a reference. The relative gene expression level of each gene tested was calculated by the $2^{-\Delta\Delta CT}$ method with the data presented as the $\log_2$. Changes in gene expression were expressed as fold change in transcription in infected cells with respect to the uninfected cells. The data represent the mean relative gene expression levels ± SD ($n = 3$ independent experiments). Numbers that are <1 denote downregulation and those that are >1 upregulation. The asterisks (*) indicate statistically significant differences ($P \leq 0.05$) in the transcription level of a given gene at the different time points after infection. The number signs (#) indicate significant differences ($P \leq 0.05$) in the gene expression level in cells infected with live bacteria with respect to that in cells infected with heat-inactivated bacteria.

infection but not in THP-1 cells infected with either BpCyaA(–) or BpPtx(–).

LAMP-2 levels in infected THP-1 cells

We next investigated the transcriptional profile of one of the most abundant lysosomal membrane proteins, LAMP-2. The LAMPs are critical for phagosome maturation, through their involvement in lysosomal lumen acidification (Huynh *et al.* 2007). Figure 8A demonstrates that macrophage infection with wild-type *B. pertussis* or the two mutant strains BpCyaA(–) or BpPtx(–) increased the transcriptional level of *LAMP-2*. Nevertheless, at 48 h after infection the transcriptional level induced in cells infected with the wild-type strain was significantly lower than that observed in cells infected with either of the deficient mutants. Using flow cytometry, we determined the cellular level of LAMP-2 at 3 and 48 h after infection (Fig. 8B). The changes in the protein abundance over the time after phagocytosis in cells infected with each bacterial strain correlated with the changes in mRNA levels observed under the influence of each of those bacterial strains (Fig. 8A). At 48 h hours after infection, the level of LAMP-2 in cells infected with the wild-type *B. pertussis* was similar to the level observed in uninfected cells, but significantly lower than that resulting in cells infected with either of the deficient mutant strains.

Response of host inflammatory genes

In order to evaluate the inflammatory response of the infected cells, we determined the transcriptional response of the *IL-10*,

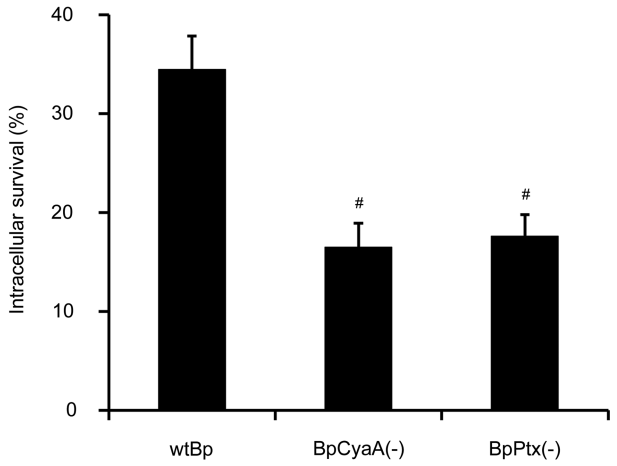

Figure 4. Role of CyaA and Ptx in bacterial survival within THP-1 cells. THP-1 cells were incubated with wild-type *B. pertussis* and the mutant strains BpCyaA(–) and BpPtx(–) at an MOI of 120 of each for 20 min at 37°C, washed extensively and further incubated with polymixin B to kill the extracellular bacteria. At different times after infection, cells were lysed and the number of CFUs of *B. pertussis* per cell was determined. Bacterial survival was expressed as the percentage of intracellular bacteria at 3 h after infection that were still alive 48 h after phagocytosis. The data represent the means ± SD of three independent experiments. The percentage of viable intracellular wtBp per cell 48 h after phagocytosis was significantly different from the percentage of viable intracellular BpCyaA(–) and BpPTx(–) at that same time point (#, $P \leq 0.05$).

TNF-α, *IL-8*, *SOCS1* and *SOCS3* in THP-1 cells infected with the wild-type *B. pertussis* and the BpCyaA(–) and BpPtx(–) mutant strains. Figure 9A indicates that 3 h after infection *IL-10* was highly induced in cells infected with any of the *B. pertussis* strains tested, though in cells infected with BpCyaA(–) the level was significantly lower. At 48 h after infection, *IL-10* was significantly downregulated in cells infected with any of the three strains, but the level of expression in cells infected with the wild type was significantly higher than in cells infected with either of the toxin-deficient mutant strains, thus suggesting that both Ptx and CyaA might be implicated in the induction of this anti-inflammatory cytokine. The analysis of *TNF-α* expression (Fig. 9B) demonstrated that although an early upregulation occurred in the cells infected with any of the strains tested, at later time points after infection the level of *TNF-α* transcription decreased significantly in the cells infected with the wtBp strain, but still remained high in those infected with either of the mutant strains, thus suggesting that both toxins might be involved in the regulation of this proinflammatory cytokine. Similar results were also observed with respect to the transcriptional level of the gene encoding another major proinflammatory cytokine, *IL-8* (Fig. 9C).

In order to investigate whether these changes in mRNA levels correlated with alterations in protein levels, we determined the amount of TNF-α and IL-8 in the culture media of cells infected with the wild-type *B. pertussis* and the BpCyaA(–) or BpPtx(–) mutant strains by ELISA (Fig. 10). Figure 10A shows that the level of TNF-α in the supernatant of cells infected with either BpCyaA(–) or wtBp correlated with the mRNA levels found over the course of infection. The level of TNF-α in the supernatant of cells infected with BpPtx(–) also correlated with the mRNA transcription at early time points, but not at 48 h after infection. At this later time point, the level of TNF-α in supernatants of cells infected with BpPtx(–) was lower than expected from the mRNA level, which discrepancy might indicate some form of posttranscriptional regulation. Figure 10B shows a significant decrease in the amount of IL-8 elaborated in the supernatants of cells infected with the wtBp 48 h after infection as compared with the amount present 24 h after infection. Both Ptx and CyaA would appear to be involved in this decrease since the amount of IL-8

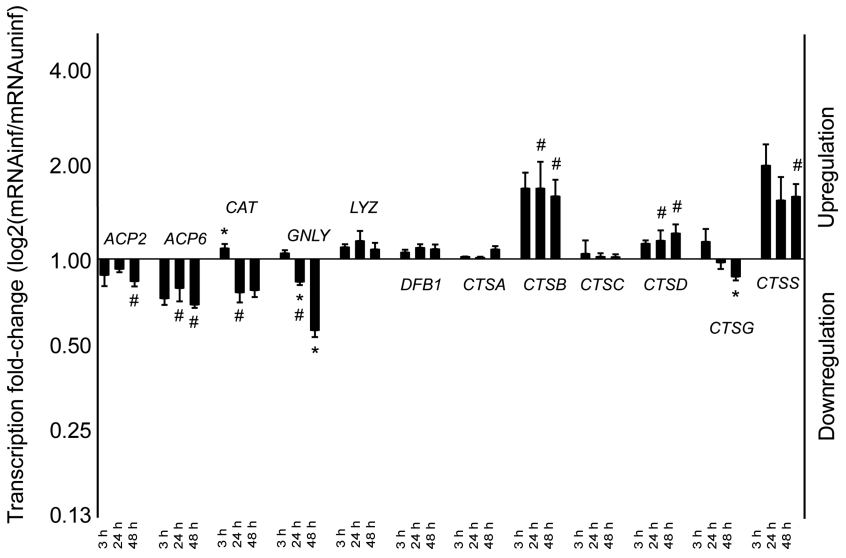

Figure 5. Role of CyaA in the expression of THP-1 cell defense genes upon *B. pertussis* infection. THP-1 cells were incubated with the *B. pertussis* strain deficient in adenylate cyclase production BpCyaA(–) (at an MOI of 120) for 20 min at 37°C, washed extensively and further incubated with polymyxin B to kill the extracellular bacteria. RNA was extracted from infected and uninfected THP-1 cells at 3, 24 and 48 h after infection and the expression of defense genes was quantitated by qRT-PCR. *B2M* was used as a housekeeping gene for normalization, and untreated cells served as a reference. The relative expression level of each gene tested was calculated by the $2^{-\Delta\Delta CT}$ method with the data presented as the $\log_2$. Changes in gene expression were expressed as fold change in transcription in infected cells with respect to the uninfected cells. The data represent the mean relative gene expression levels ± SD ($n = 3$ independent experiments). The numbers that are <1 denote downregulation and those that are >1 upregulation. The asterisks (*) indicate statistically significant differences ($P \leq 0.05$) in the transcription level of a given gene at the different time points after infection. The number signs (#) indicate significant differences ($P \leq 0.05$) in the gene expression level in cells infected with BpCyaA(–) with respect to that in cells infected with the wild-type *B. pertussis*.

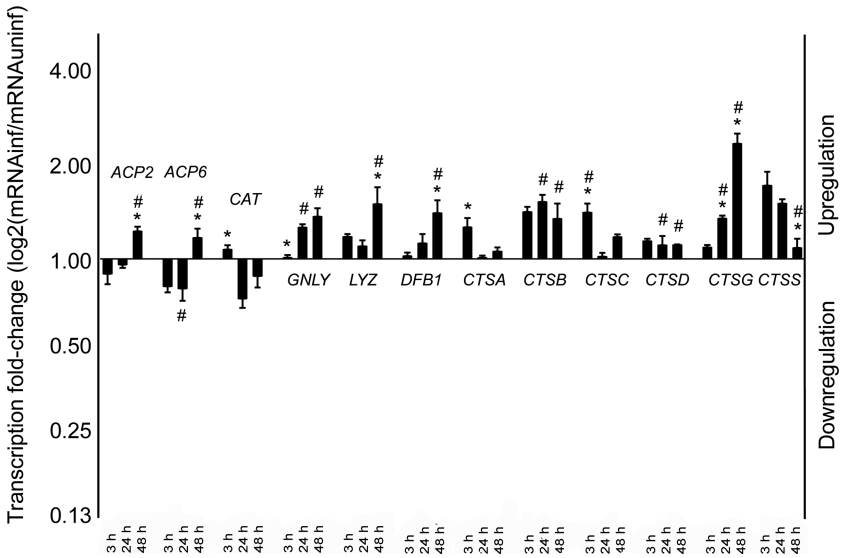

Figure 6. Role of Ptx in the modulation of THP-1 cell defense gene expression upon *B. pertussis* infection. THP-1 cells were incubated with the *B. pertussis* strain deficient in pertussis toxin production BpPtx(–) at an MOI of 120 for 20 min at 37°C, washed extensively and further incubated with polymyxin B to kill the extracellular bacteria. RNA was extracted from infected and uninfected THP-1 cells at 3, 24 and 48 h after infection and the expression of defense genes quantitated by qRT-PCR. *B2M* was used as a housekeeping gene for data normalization, and untreated cells served as a reference. The relative gene expression level of each gene tested was calculated by the $2^{-\Delta\Delta CT}$ method with the data presented as the $\log_2$. Changes in gene expression were expressed as fold change in transcription in infected cells with respect to the uninfected cells. The data represent the mean relative gene-expression levels ± SD ($n = 3$ independent experiments). Numbers that are <1 denote downregulation and those that are >1 upregulation. The asterisks (*) indicate statistically significant differences ($P \leq 0.05$) in the transcription level of a given gene at the different time points after infection. The number signs (#) indicate significant differences ($P \leq 0.05$) in the gene expression level in cells infected with BpPtx(–) with respect to that in cells infected with the wild-type *B. pertussis*.

found in the supernatants of cells infected with either of the deficient mutants was significantly higher than that found in the supernatant of cells infected with the wild-type strain.

Finally, we investigated the expression level of the genes encoding the suppressors of cytokine signaling 1 and 3 (*SOCS1*, *SOCS3*). Figure 11A indicates that 3 h after infection *SOCS3* was strongly induced in cells infected with any of the three strains tested although a significantly lower extent of transcription occurred in cells infected with BpCyaA(–) than in cells infected with either of the other two strains. At 48 h after infection, *SOCS3* proved to be strongly downregulated in cells infected with all three strains. In contrast, Fig. 11B demonstrates that the *SOCS1* expression level was not significantly affected by bacterial infection at 3 h after infection. At 48 h after infection, however, *SOCS1* became upregulated in cells infected with the wild-type *B. pertussis*. The *SOCS1* expression level was likewise upregulated in cells infected with BpPtx(–), but the level of expression was significantly lower than that observed in wtBp-infected cells. In contrast, the transcription of that suppressor gene remained weakly downregulated in cells infected with the BpCyaA(–) mutant at this late time point.

DISCUSSION

Bordetella pertussis has been historically considered an extracellular pathogen. Recent studies, however, have challenged this concept by demonstrating that this bacterium is able to survive and even replicate inside human macrophages (Lamberti *et al.* 2010). We previously demonstrated that although a high percentage of phagocytosed bacteria were killed soon after phagocytosis, a relevant number survived this initial bactericidal confrontation by following a non-bactericidal intracellular route and replicating inside phagosomes that, for their part, did not undergo lysosomal maturation by 48 h after infection (Lamberti *et al.* 2010). Macrophages seem to play a peculiar role in the host response to *B. pertussis* infection since those phagocytes, though being effector cells for bacterial killing, might nevertheless constitute a niche for persistence within the host. Intracellular pathogens have accordingly evolved mechanisms that allow their survival within host cells (Bhavsar, Guttman and Finlay 2007). These mechanisms usually involve transcriptional changes in the infected host cells that preclude bacterial clearance. Both facultative and obligate intracellular pathogens have the capacity to manipulate the host cell immune response at least to some extent (Jenner and Young 2005). In order to examine whether the intracellular survival and eventual duplication of *B. pertussis* inside macrophages involved a manipulation of the innate immune response of those host cells, we investigated the transcriptional response of human macrophage infected with this pathogen for up to 48 h after phagocytosis, using a qRT-PCR to study the transcriptional response of genes encoding proteins involved in the bactericidal mechanisms and inflammatory response. The most significant modulations in gene transcription observed were further confirmed at the protein level. Because of the large number of cells needed for this kind of study, we used the THP-1 cells instead of primary human monocyte-derived macrophages. Before employing this cell line, however, we confirmed that *B. pertussis* intracellular trafficking and the bacterial survival inside THP-1 cells resembled what was known to occur in primary human cells (Lamberti *et al.* 2010).

In this study, we observed that *B. pertussis* partially succeeded in reducing the cell bactericidal response early after phagocytosis since some of the genes involved in this activity were significantly downregulated at that time point (i.e. 3 h) with respect to levels in uninfected cells. In particular, *ACP2* and *ACP6*, encoding acid phosphatases with bactericidal activities, were downmodulated at that time after phagocytosis and remained so for 2 days after infection. In contrast, genes such as *CAT*, *CTSB*,

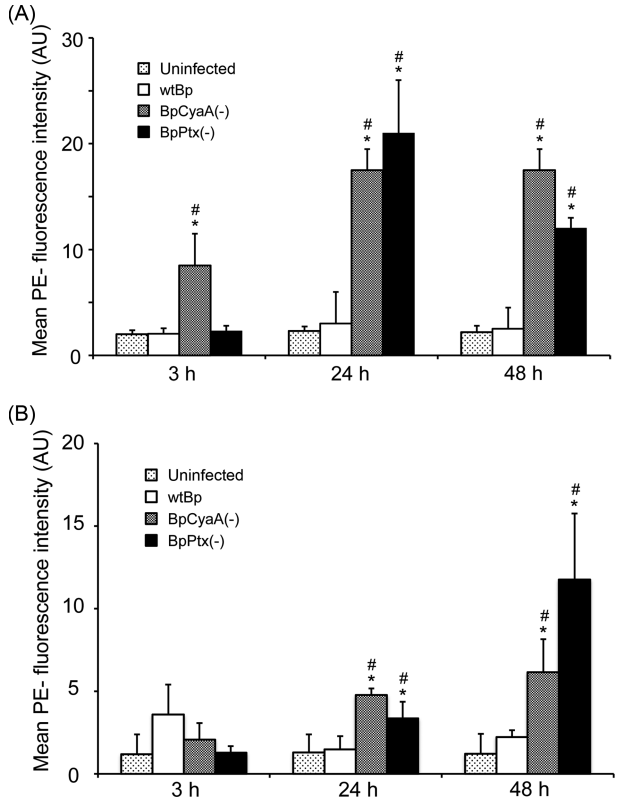

Figure 7. Cathepsin S and D abundance in *B. pertussis*-infected THP-1 cells: role of CyaA and Ptx. THP-1 cells were incubated with the wild-type *B. pertussis* (wtBp) or the mutant strains BpCyaA(–), or BpPtx(–) at an MOI of 120 for 20 min at 37°C, washed extensively and further incubated with polymyxin B to kill the extracellular bacteria. At 3, 24 and 48 h after infection, cells were fixed and incubated for 30 min with PBS containing 0.1% (w/v) saponin and 0.5% (w/v) BSA., The cells were next incubated for 1 h at 4°C with mouse anti-human CTSS (**A**) or CTSD (**B**) monoclonal antibodies. After three washes, the cells were incubated with the PE-conjugated F(ab)$_2$ fragment of goat anti-mouse antibody for 30 min and washed three times before analysis by flow cytometry. The data are the means ± SD of three independent experiments. PE, phycoerythrin; AU: arbitrary units. The asterisks (*) indicate statistically significant differences ($P \leq 0.05$) in the protein level in cells infected with the different Bp strains relative to that in uninfected cells. The number signs (#) indicate significant differences ($P \leq 0.05$) in the expression level in cells infected with the different *B. pertussis* mutant strains BpCyaA(–) or BpPtx(–) relative to that of the cells infected with the wild-type strain.

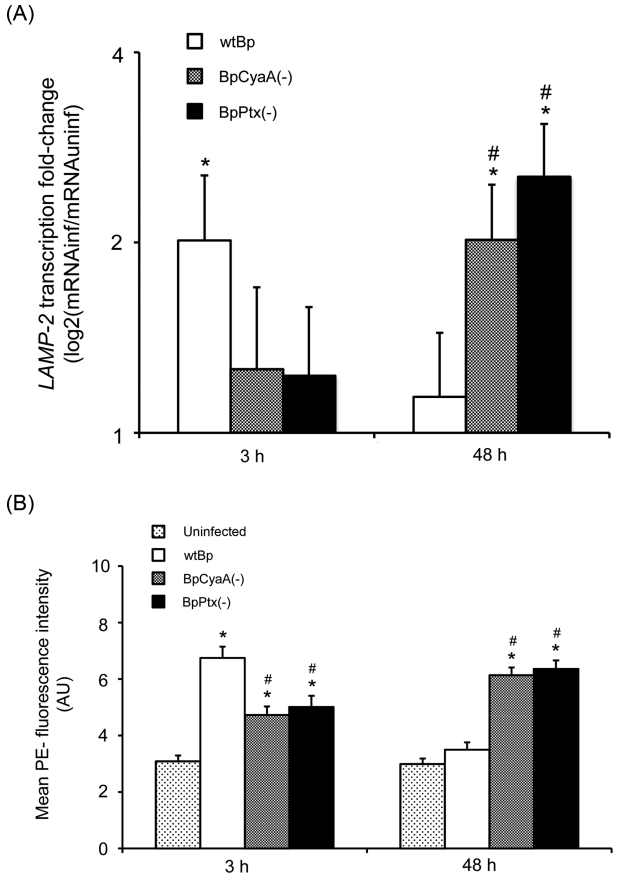

Figure 8. LAMP-2 in infected THP-1 cells: role of CyaA and Ptx. THP-1 cells were incubated with the wild-type *B. pertussis* (wtBp) or the mutant strains BpCyaA(–), or BpPtx(–) at an MOI of 120 for 20 min at 37°C, extensively washed and further incubated with polymyxin B to kill the extracellular bacteria. At 3 and 48 h after infection, cell samples were taken. (**A**) One aliquot was used for RNA extraction and quantification of the *LAMP-2* transcription level by qRT-PCR using *B2M* as a housekeeping gene for normalization, and untreated cells served as a reference. The relative expression level of each gene tested was calculated by the $2^{-\Delta\Delta CT}$ method with the data presented as the $\log_2$. The data represent the mean relative gene expression levels ± SD ($n = 3$ independent experiments). The numbers >1 indicate upregulation. (**B**) Another aliquot was used to determine the abundance of LAMP-2 protein by flow cytometry. The cells were fixed and permeabilized before incubation with mouse anti-human LAMP-2 monoclonal antibodies for 1 h at 4°C. After three washes, the cells were incubated with the PE-conjugated F(ab)$_2$ fragment of goat anti-mouse antibody for 30 min, washed and analyzed by flow cytometry. The data represent the means ± SD in arbitrary units from three independent experiments. PE, phycoerythrin; AU: arbitrary units. The asterisks (*) indicate statistically significant differences ($P \leq 0.05$) in the expression level in cells infected with the different *B. pertussis* strains relative to that in uninfected cells. The number signs (#) indicate significant differences ($P \leq 0.05$) in the expression level in cells infected with the different *B. pertussis* mutant strains BpCyaA(–) or BpPtx(–) relative to that of the cells infected with the wild-type strain.

CTSD, *CTSG* and *CTSS*—all encoding proteins involved in the cellular defense response—were found to be upregulated, likewise at an early time after infection. As the time after infection progressed, however, several of these early upregulated genes became downregulated—e.g. *CAT* encoding a protein that regulates antioxidant mechanisms in macrophages; *GNLY* encoding a granulysin involved in the killing of intracellular pathogens (Krensky 2000); and genes encoding cysteine proteases that are members of the cathepsin family such as *CTSB*, *CTSD*, *CTSG* and *CTSS*, all those being involved in the bactericidal activity of macrophages. For example, the downmodulation of *CTSG* had been found to be associated with an increase in the intracellular survival of *Mycobacterium tuberculosis* (Danelishvili *et al.* 2011). Similarly, the downregulation of *CTSD* was reported to be involved in promoting the intracellular persistence of other bacterial pathogens, such as *Streptococcus pneumoniae*, in macrophages (Bewley *et al.* 2011). In our experiments, the decrease in the bactericidal activity of macrophages infected with *B. pertussis* during the period after infection concurs with the increase observed in the number of cells heavily loaded with live bacteria.

Live intracellular *B. pertussis* bacteria reside within vacuoles that are part of the endosomal network (Lamberti *et al.* 2010). This intracellular location might lead to a sampling of bacterium-derived peptides and the presentation of those bacterial antigens to T cells. *Bordetella pertussis*, however, seems to have evolved strategies to avoid T-cell activation. Boldrick *et al.* (2002) previously demonstrated that the infection of mononuclear cells of peripheral blood by *B. pertussis* induced a

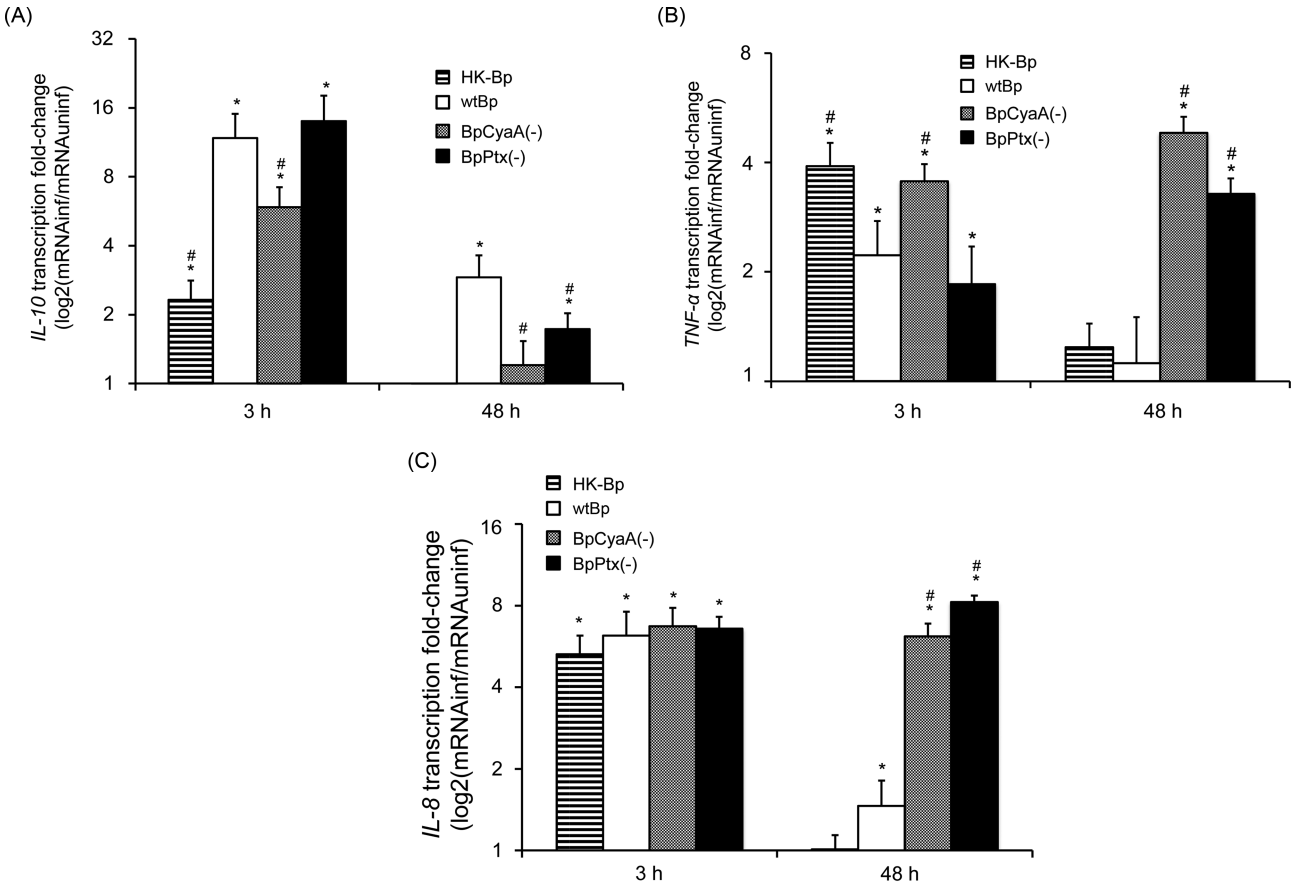

Figure 9. Inflammatory response of THP-1 cells to *B. pertussis* infection. Role of CyaA and Ptx. THP-1 cells were incubated with the heat-killed *B. pertussis* (HK-Bp) along with the live wild-type *B. pertussis* (wtBp) and the mutant strains BpCyaA(–) and BpPtx(–) at an MOI of 120 for 20 min at 37°C, washed extensively and further incubated with polymyxin B to kill the extracellular bacteria. At 3, 24 and 48 h after infection, cell samples were taken and the expression of (**A**) *IL-10* (**B**), *TNF-α* (**C**) and *IL-8* genes was quantitated by qRT-PCR. *B2M* was used as a housekeeping gene for normalization, and untreated cells served as a reference. The relative expression level of each gene tested was calculated by the $2^{-\Delta\Delta CT}$ method with the data presented as the $\log_2$. The data represent the mean relative gene-expression levels ± SD ($n = 3$ independent experiments). The asterisks (*) indicate statistically significant differences ($P \leq 0.05$) in the transcription level in cells infected with the different *B. pertussis* strains relative to that of uninfected cells. The number signs (#) indicate significant differences ($P \leq 0.05$) in the gene expression level in cells infected with the either of the two mutant strains, BpCyaA(–) and BpPtx(–), or the HK-Bp relative to that of the cells infected with the wtBp.

downregulation of genes encoding the lysosomal protease cathepsin B and the antigen presentation cofactor HLA-DM, both involved in antigen processing and presentation to T cells. In agreement with these findings, at 48 h after phagocytosis *CTSS*, *CTSB* and *CTSD*—all encoding proteins involved in antigen presentation (Shi *et al.* 1999; Zhang *et al.* 2000)—were found to be downregulated in the present work. This downmodulation of *CTSS* and *CTSD* was also confirmed at the protein level. These results—together with the observed downmodulation of genes encoding inflammatory cytokines such as *IL-8* and *TNF-α* during the period of infection, along with the high expression of immunosuppressive cytokines such as *IL-10*—strongly indicate that 2 days after infection those bacteria that have survived intracellularly in phagosomes by avoiding the degradative pathway have compromised the macrophage response in order to achieve a permissive environment in which they might persist. According to our results, this host cell modulation depends mainly on the two main toxins of *B. pertussis*, CyaA and Ptx. Pertussis toxin expression proved to be involved in the regulation of the expression of most of the genes implicated in the microbicidal activity that became accordingly downmodulated in infected cells, while the complementary downregulation of certain others required the additional expression of CyaA. Cells infected with *B. pertussis* lacking the expression of Ptx manifested a significant increase in the transcripts of *APC2*, *APC6*, *GNLY*, *LYS*, *DBF1* and *CTSG* at 48 h after infection in comparison with the cells infected with the wild-type strain. The transcriptional level of *CTSG* and *GNLY* in cells infected with the strain lacking Ptx expression was higher than that observed in cells infected with the wild-type *B. pertussis* by 24 h after infection. In contrast, the downmodulation of *CTSB*, *CTSD* and *CTSS* was found to require the expression of both Ptx and CyaA since the lack of expression of either of those toxins abolished the downmodulation observed in cells infected with the wild-type *B. pertussis* at the later time points after infection (i.e. 24 and 48 h). A significant downregulation of inflammatory cytokines, such as TNF-α and IL-8, occurred by 48 h after infection. This downmodulation, for its part, was likewise dependent on the expression of both Ptx and CyaA with respect to IL-8, but mainly on the expression of CyaA in the instance of TNF-α. Ptx seems to be involved in the downmodulation of TNF-α at only a short time after infection, an observation that is in agreement with the previous report (Boldrick *et al.* 2002). This finding might be partially explained by the well-documented effect of cAMP elevation on the inflammatory response since adenylate cyclase is the specific synthetase for cAMP. Ptx might be capable of downmodulating TNF-α

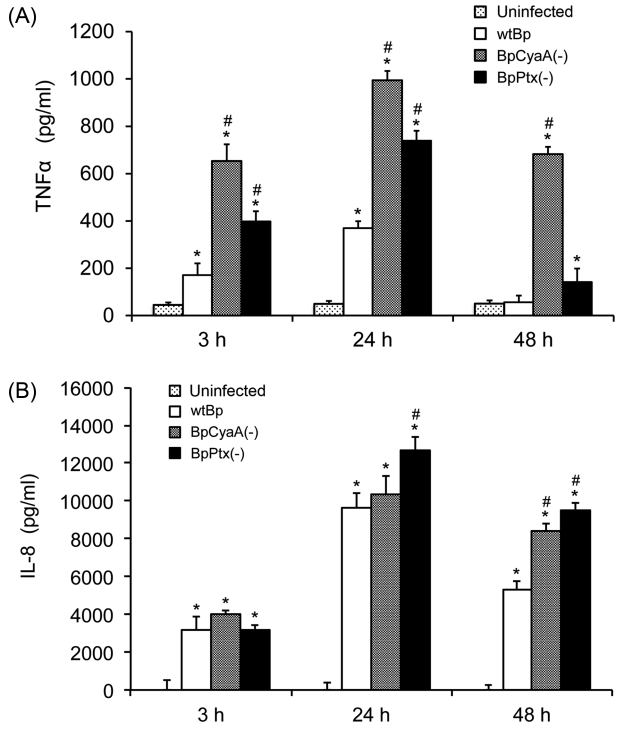

Figure 10. Time course of the secretion of proinflammatory cytokines upon *B. pertussis* infection of THP-1 cells: role of CyaA and Ptx. THP-1 cells were incubated with the wild-type *B. pertussis* (wtBp) and the mutant strains BpCyaA(–) and BpPtx(–) at an MOI of 120 for 20 min at 37°C, washed extensively and further incubated with polymixin B to kill the extracellular bacteria. At 3, 24 and 48 h after infection samples of cell culture supernatants were removed to determine the level of (**A**) TNF-α and (**B**) IL-8 by ELISA. The data represent the means of triplicate samples ± SD from three independent experiments. The asterisks (*) indicate statistically significant differences ($P \leq 0.05$) in the transcription level in cells infected with the different *B. pertussis* strains relative to that in uninfected cells. The number signs (#) indicate significant differences ($P \leq 0.05$) in the expression level in cells infected with the different *B. pertussis* mutant strains BpCyaA(–) or BpPtx(–) relative to that of the cells infected with the wild-type strain.

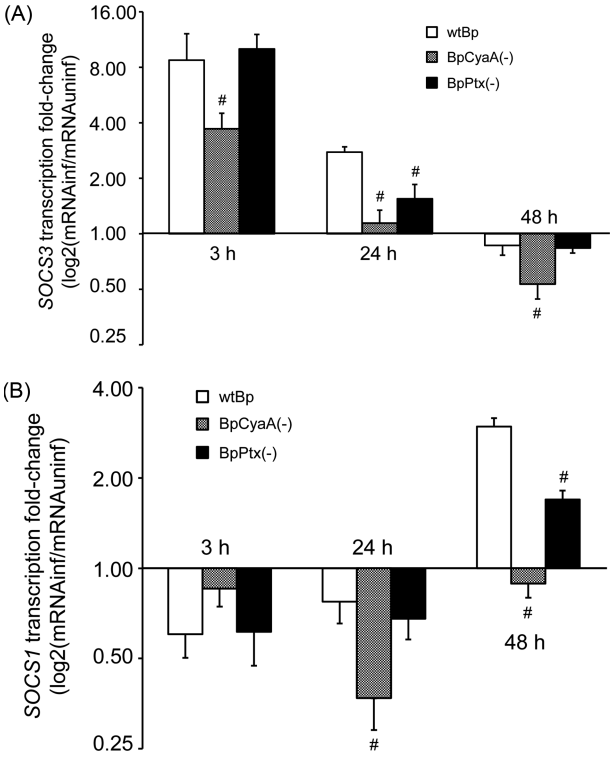

Figure 11. Expression of genes encoding SOCS in THP-1 cells upon *B. pertussis* infection: role of CyaA and Ptx. THP-1 cells were incubated with the wild-type *B. pertussis* (wtBp) or the mutant strains BpCyaA(–) or BpPtx(–) at an MOI of 120 for 20 min at 37°C, washed extensively and further incubated with polymixin B to kill the extracellular bacteria. At 3, 24 and 48 h after infection, cell samples were taken and the transcription of *SOCS3* (**A**) and *SOCS1* (**B**) genes was quantitated by qRT-PCR. *B2M* was used as a housekeeping gene for normalization, and untreated cells served as a reference. The relative expression level of each gene tested was calculated by the $2^{-\Delta\Delta CT}$ method with the data presented as the $\log_2$. The data represent the mean relative gene expression levels ± SD ($n = 3$ independent experiments). The number sign (#) indicates a significant difference ($P \leq 0.05$) between the gene expression level in cells infected with either mutant strain BpCyaA(–) or BpPtx(–) and that in cells infected with the wild strain.

production by other mechanisms (Mangmool and Kurose 2011). Our results are in agreement with previous findings indicating that both CyaA and Ptx are involved in the inhibition of cytokine production in other immune cells, such as dendritic cells (Bagley *et al.* 2002). *In vivo* studies have further demonstrated that Ptx inhibits chemokine upregulation in alveolar macrophages along with other cells in the lungs, eventually enabling a colonization of the respiratory tract (Andreasen and Carbonetti 2008).

The complex control of the inflammatory response in cells like macrophages critically depends on suppressors of cytokine signaling proteins (i.e. the SOCSs). A number of microorganisms, such as *Mycobacterium* spp., *Listeria monocytogenes* or *Chlamydia pneumoniae,* have developed sophisticated strategies to hijack the SOCS system in order to inhibit the immune defense signaling pathways (Delgado-Ortega *et al.* 2013). In the present work, we found *IL-10* to be highly upregulated at 3 h after infection in cells exposed to wild-type *B. pertussis*. In agreement with previous reports (Boyd *et al.* 2005), we also observed that CyaA is partially involved in this upregulation since cells infected with *B. pertussis* lacking the expression of CyaA exhibited a significantly lower *IL-10* upregulation at this same time point. An analysis of the transcriptional level of *SOCS1* and *SOCS3* provided a further insight into the macrophage response to infection by this pathogen. By 3 h after infection, a strong upregulation of *SOCS3* occurred in cells infected with the wild-type strain. CyaA would appear to be involved in this upregulation since cells infected with the BpCyaA(–) mutant evidenced a significantly lower upregulation of *SOCS3* at the same 3 h. *SOCS3* expression is strongly induced in the presence of *LPS*, *IL-6* or *IL-10* and is a key regulator of both the pro- and anti-inflammatory responses of macrophages (Delgado-Ortega *et al.* 2013). For example, in the presence of *IL-10*, *SOCS3* blocks signaling by *IL-6*, and the anti-inflammatory activity of *IL-10* prevails (Yasukawa *et al.* 2003). Accordingly, the upregulation of *IL-10* and *SOCS3* has been suggested to play a role in preventing the clearance of bacteria by macrophages (Weiss, Evanson and Souza 2005). In this investigation, we observed that *B. pertussis* infection induces the expression of both *SOCS3* and *IL-10* early after phagocytosis, thus suggesting that, like other pathogens, this bacterium is able to downmodulate the inflammatory response of the macrophage (Cyktor and Turner 2011). Conversely, *SOCS1* expression was not significantly modified at early time points after infection, though by 48 h after phagocytosis of the wild-type *B. pertussis* a marked upregulation of *SOCS1* and downregulation of *SOCS3* transcription were observed leading to a high *SOCS1*/*SOCS3* ratio. This result is particularly significant because the exclusive upregulation of *SOCS1* or a high *SOCS1*/*SOCS3* expression ratio is a characteristic of the M2 macrophage

phenotype (Wilson 2014) that is permissive for the intracellular survival of bacterial pathogens. Both Ptx and CyaA were found to be involved in this increase since the lack of either toxin resulted in a lower *SOCS1/SOCS3* expression ratio than seen with the wild strain. These results are in agreement with the observed necessity of both toxins for bacterial intracellular survival at later time points after infection. Both BpCyaA(–) and BpPtx(–) displayed a decreased ability to survive inside the cells. Evidence is accumulating that macrophage polarization plays a key role in infectious diseases (Benoit, Desnues and Mege 2008). While M1 macrophages develop a strong bactericidal activity, particularly against intracellular pathogens, M2 macrophages promote the growth of the same pathogens. Several bacterial pathogens—such as *Francisella tularensis*, *Coxiella burnetii* and *Tropheryma whipplei*—have been shown to induce an M2 polarization in macrophages that eventually enables the establishment of a niche of persistence within those cells (Desnues, Raoult and Mege 2005; Meghari *et al.* 2008; Shirey *et al.* 2008). Although further studies are needed to ascertain whether this circumstance obtains for *B. pertussis*, the pathogen seems to be able to manipulate the macrophage response by downmodulating the bactericidal and inflammatory responses so as to ultimately create a permissive intracellular environment. Although many bacterial factors might play a role in this process, the study reported here demonstrates that the two main *B. pertussis* toxins, CyaA and Ptx, are certainly involved. From our results, however, we cannot determine whether the observed effect was caused by the toxins released during the intracellular period or by those elaborated during the initial 30 min of incubation with the eukaryotic cells. Although this question constitutes an issue that deserves further investigation, the finding that both Ptx and CyaA are highly synthesized by intracellular *B. pertussis* 24 and 48 h after infection (Lamberti *et al.* 2016) would indicate that these toxic agents play a role during bacterial intracellular residence.

To our knowledge, the present work constitutes the first study in which the time course of the macrophage response to *B. pertussis* infection has been investigated. Our data provide a biological basis for the documented survival of this bacterium inside macrophages and shed new light on *B. pertussis* pathogenesis.

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