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ARTICLE

Human macrophage polarization shapes *B. pertussis* intracellular persistence

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Abstract

We previously demonstrated that *Bordetella pertussis*, the etiologic agent of whooping cough, is able to survive inside human macrophages. The aim of this study was to examine the influence of macrophage polarization in the development of *B. pertussis* intracellular infections. To this end, primary human monocytes were differentiated into M1, M2a, or M2c macrophages and further infected with *B. pertussis*. Infected M1 macrophages showed a proinflammatory response evidenced by the production of TNF-α, IL-12p70, and IL-6. Conversely, infection of M2a and M2c macrophages did not induce TNF-α, IL-12p70, nor IL-6 at any time postinfection but showed a significant increase of M2 markers, such as CD206, CD163, and CD209. Interestingly, anti-inflammatory cytokines, like IL-10 and TGF-β, were induced after infection in the 3 macrophage phenotypes. *B. pertussis* phagocytosis by M1 macrophages was lower than by M2 phenotypes, which may be ascribed to differences in the expression level of *B. pertussis* docking molecules on the surface of the different phenotypes. Intracellular bactericidal activity was found to be significantly higher in M1 than in M2a or M2c cells, but live bacteria were still detected within the 3 phenotypes at the late time points after infection. In summary, this study shows that intracellular *B. pertussis* is able to survive regardless of the macrophage activation program, but its intracellular survival proved higher in M2 compared with the M1 macrophages, being M2c the best candidate to develop into a niche of persistence for *B. pertussis*.

KEYWORDS
bacterial intracellular persistence, *Bordetella pertussis*, macrophage, macrophage polarization, whooping cough

Abbreviations: B2M, β—2-microglobulin; CD11b, CD11b molecule (Integrin, α M); CD163, CD163 molecule; CD18, CD18 molecule (Integrin, β 2); CD206, CD206 molecule (Mannose receptor C-type 1); CD209, CD209 molecule (Scavenger receptors); CD40, CD40 molecule; CD80, CD80 molecule; CD86, CD86 molecule; CR3, complement receptor 3 (CD11b/CD18); M1, M1 macrophage phenotype; M2, M2 macrophage phenotype; M2a, M2a macrophage phenotype; M2c, M2c macrophage phenotype; MOI, multiplicity of infection; MRC1, mannose receptor C-type 1; PFA, paraformaldehyde; Ptx, pertussis toxin.

1 | INTRODUCTION

Bordetella pertussis is a Gram-negative bacterium that causes whooping cough, a disease that showed an increasing impact in the public health worldwide. *B. pertussis* is a strictly human pathogen.[1] The introduction of pertussis vaccines led to a dramatic decrease in the morbidity and mortality but failed to control the bacterial circulation within the population.[2] There are currently 2 types of vaccines in use, the whole-cell vaccines that induce a mixed Th1/Th17 response and the acellular vaccines that mainly induce a Th2-polarized response.[3–5] Both types of vaccines induce a waning immunity that last only for a few years.[6] We have previously found that in the absence of opsonic antibodies, *B. pertussis* survives the encounter with the immune cells,[7–9] remaining alive within human macrophages and eventually replicating in phagosomes that do not undergo lysosomal maturation.[9] These results suggested that these cells may constitute a bacterial niche of persistence within the host. We further found that the 2 main toxins of *B. pertussis*, pertussis toxin (Ptx) and adenylate cyclase toxin, are expressed during the intracellular infection of macrophages[10] and are involved in the bactericidal cell response modulation that eventually leads to a less aggressive macrophage phenotype.[11]

Macrophages are a heterogeneous population of cells involved in several physiologic as well as pathologic processes. Macrophage plasticity allows them to adapt their phenotype and functions in response to different environmental inflammatory stimuli. Macrophage activation is broadly classified into 2 extreme phenotypes, proinflammatory or classical activation (M1) and anti-inflammatory or alternative activation (M2). The M1 macrophage is usually induced by bacterial components such as LPS and the Th1-cytokine IFN-γ that promote a proinflammatory cell response and a strong microbicidal capacity.[12,13] The M2 macrophage is induced in response to Th2 cytokines and can be further classified into different subsets based on their response to stimuli. Namely, the exposure to IL-4 and IL-13 induces M2a macrophage while the exposure to IL-10 and TGF-β induces M2c macrophage.[12] Unlike M1 macrophages, M2 macrophages are involved in immune regulation (in particular, M2a and M2c) and are poorly microbicidal. Due to their limited bactericidal activity and their nutrient-rich environment, M2 macrophages constitute a niche of persistence for several pathogens, favoring chronic infections.[14–16] Each macrophage phenotype is characterized by a particular morphology, chemokine and cytokine secretion profile, and superficial marker expression profile.[12,17] M1 macrophages are characterized by a high expression of costimulatory receptors like CD40, CD80, and CD86; the MHC antigen (HLA-DR), the chemotactic ligand CXCL10, the chemokine receptor CCR7; and a high production of TNF-α, IL-1β, IL-6, and IL-12. M2a macrophages show high expression of CD163, HLA-DR, mannose receptor (CD206), and scavenger receptor (CD209). They produce IL-10 and TGF-β. M2c macrophages are characterized by a high expression of CD163, CD206, and CD209 and a prominent production of IL-10, TGF-β, and CCL18.[13]

Intracellular bacterial pathogens that survive inside macrophages usually subvert the cell signaling eventually shaping the macrophage phenotype.[18,19] These pathogens have adapted to the macrophage plasticity and eventually modulate the classical (M1) or alternative (M2) activation skewing the polarization toward a state that increases their intracellular survival.[16] We previously found evidence indicating that *B. pertussis* might induce a M2-like phenotype in the infected macrophages.[11] Based on these findings, herein we investigated the ability of *B. pertussis* to survive inside M1 and M2 human macrophages, and how each activation program is modulated along the bacterial intracellular infection.

2 | METHODS

2.1 | Cell culture

Mononuclear cells from peripheral blood samples of 3 different healthy adult donors were isolated by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) gradient as previously described.[20] The mononuclear cell layer was washed and suspended in Macrophage-SFM™ (Invitrogen, Carlsbad, CA, USA) seeded in 6-well tissue culture plates (10^6 monocytes/well) and incubated for 2 h at 37°C in 5% CO_2. Nonadherent cells were then removed by gentle washing 3 times. Monocytes were cultured for 6 days in Macrophage-SFM™ supplemented with 10 ng/ml GM-CSF (ImmunoTools, Friesoythe, Germany) to obtain M1 macrophages or 10 ng/ml M-CSF (ImmunoTools) to obtain M2 macrophages. After 6 days, macrophages were treated for further 24 h with 10 ng/ml IFN-γ (Sigma–Aldrich, St. Louis, MO, USA) and 10 ng/ml LPS from *Escherichia coli* (Sigma–Aldrich) to obtain M1 macrophages, and either 20 ng/ml IL-4 (ImmunoTools) or 10 ng/ml IL-10 (ImmunoTools) to obtain M2a and M2c macrophages, respectively. Macrophages generated from human monocytes as described for M1 and M2 macrophages but without the addition of growth factors or cytokines were used as a control in quantitative real-time PCR studies performed to assess macrophage polarization.

The procedures involving human samples were in accordance with the ethical standards of the 1964 Helsinki declaration[21] and its later amendments and approved by the Institutional Review Board. All individuals provided written informed consent for the collection of samples.

2.2 | Bacterial strains and growth conditions

B. pertussis strain B213 (a streptomycin-resistant derivate of Tohama)[22] was transformed with plasmid pCW505[23] (kindly supplied by A. A. Weiss, Cincinnati, OH, USA), which induces cytoplasmic expression of GFP without affecting growth or antigen expression.[23] Bacteria was stored at −70°C and recovered by growth on Bordet-Gengou (BG) agar plates supplemented with 15% defibrinated sheep blood (Laboratorio Argentino, Caseros, Argentina) (bBGA) at 37°C and used in infection experiments. Animal handling and all procedures were in compliance with the Argentinean animal protection Law 14346.

2.3 | Macrophage infection and quantification of intracellular *B. pertussis*

Polarized macrophages (in 6-well tissue culture plates) were infected with GFP-*B. pertussis* suspended in Macrophage-SFM™ at a multiplicity of infection (MOI) of 100 bacteria per cell (10^8 bacteria/well). Bacterial concentration was estimated by OD_{650} and further quantified by CFUs counts. To facilitate bacterial interaction with macrophages, plates were centrifuged for 5 min at 640 × *g*. After 30 min of incubation at 37°C with 5% CO_2, nonadherent bacteria were removed by 3 washes. One sample of these cells were fixed with 4% (v/v) paraformaldehyde (PFA) for 10 min, washed with PBS, incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl, and stained to determine intra and extracellular bacteria as described below. Another sample of these cells was incubated for 1 h with fresh Macrophage-SFM™ medium containing 100 μg/ml polymyxin B (Sigma–Aldrich)—an antibiotic that cannot penetrate mammalian cells[24]—to kill the remaining extracellular bacteria, washed 3 times, and further incubated with Macrophage-SFM™ medium supplemented with correspondent stimulation for M1, M2a, or M2c profiles as described above, plus 5 4 μg/ml polymyxin B. Samples were taken at 3 and 48 h postinfection and washed prior fixation with 4% (v/v) PFA for 10 min. After washing with PBS, the cells were incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl. In all samples, macrophage surface-bound and phagocytosed bacteria were discriminated by a 2-step labeling procedure followed by fluorescence microscopy analysis as described before.[9] Briefly, surface-bound bacteria were stained with polyclonal rabbit anti-*B. pertussis* serum, obtained as described elsewhere,[25] for 30 min at 4°C, and then incubated with Cy3-conjugated goat $F(abt')_2$ fragments of anti-rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min at 4°C. In order to determine the number of intracellular bacteria, after 2 washing steps, the cells were permeabilized with PBS containing 0.1% (w/v) saponin (Sigma–Aldrich) and 0.2% (w/v) BSA (Sigma–Aldrich) for 30 min at 25°C, and further incubated with rabbit anti-*B. pertussis* serum in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA (30 min at 25°C). After 3 washing steps with PBS containing 0.1% (w/v) saponin and 0.2% (w/v) BSA, the cells were incubated with the FITC-conjugated $F(ab')_2$ fragments of goat anti-rabbit immunoglobulin (Jackson ImmunoResearch) in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA (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. To avoid cytophilic binding of antibodies to the FcR, all incubations were done in the presence of 25% (v/v) heat-inactivated human serum. After washing, the cells were mounted and analyzed by fluorescence microscopy using a DMLB microscope coupled to a DC100 camera (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland). The cell viability was analyzed by trypan blue dye exclusion at the different times after infection. No significant macrophage death was observed at any time post infection.

2.4 | Bacterial intracellular survival

Bacterial intracellular survival was determined as previously described[9] with minor modifications. Briefly, macrophages were infected with *B. pertussis* (MOI 100) suspended in Macrophage-SFM™ for 30 min at 37°C and nonadherent bacteria were removed by 3 washes. To kill extracellular bacteria, 100 μg/ml polymyxin B sulfate was added for 1 h and then the antibiotic concentration was decreased to 5 μg/ml. Control experiments indicated that at 5 μg/ml 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 EDTA) (Sigma-Aldrich and 4 mg/ml lidocaine (Sigma–Aldrich) to promote cell detachment. The number of viable macrophages was determined by trypan-blue dye exclusion. Next, the macrophages were lysed with 0.1% (w/v) saponin (Sigma–Aldrich) 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 this time point 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). Briefly, samples of 5×10^8 bacteria were incubated with the antibiotic for 1 h at 37°C and plated on bBGA. The subsequent incubation resulted in a 99.99% decrease in the CFUs. The viability of infected cells was determined at different times after infection with trypan blue. No significant cell death was observed in any of the different infection assays performed.

2.5 | Confocal microscopy analysis

Colocalization studies were performed as described previously[9] with minor modifications. Briefly, macrophages were infected with *B. pertussis* (MOI 100) at 37°C for 30 min, washed to remove nonattached bacteria. To evaluate colocalization of the macrophage mannose receptor and *B. pertussis*, the cells were incubated with mouse anti-human CD206 mAbs (Biolegend, San Diego, CA, USA) and rabbit anti-*B. pertussis* serum, for 30 min at 4°C, washed, and incubated with Cy3-conjugated goat F(ab)2 fragments of anti-rabbit antibodies (Southern Biotechnology, Birmingham, AL, USA) (shown in green in the Figure 4(C)), and anti-mouse Alexa Fluor® 647 (Jackson ImmunoResearch) for 30 min at 4°C. For trafficking studies, 48 h after infection the macrophages were incubated with or without 200 nM LysoTracker® Red DND-99 (Thermo Fisher Scientific, Waltham, MA, USA) (5 min at 37°C), washed, fixed with PFA and incubated with PBS containing 50 mM NH_4Cl for 10 min at room temperature. After

2 washing steps, the cells were incubated with PBS containing 0.1% (w/v) saponin and 0.2% (w/v) BSA, and further incubated with rabbit anti-*B. pertussis* serum in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA for 30 min at 25°C. After 3 washing steps, the cells were treated with FITC-conjugated goat F(ab')2 fragments of anti-rabbit immunoglobulin (Jackson ImmunoResearch) for 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. Analyses were performed using a confocal laser scanning microscope (Leica™ TCS SP5, Wetzlar, Germany).

2.6 | Cytokine determination

The amounts of TNF-α, IL- 12 (IL-12p70), IL-6, IL-10, IL-1β, and TGF-β in culture supernatants of uninfected or infected macrophages were determined by ELISA according to manufacturers' instructions kits (TNF-α, IL-12p70, and TGF-β from Ready-SET-Go!™ Kits from eBioscience (San Diego, CA, USA); IL-10, IL-1β, and IL-6 ELISA MAX™ Deluxe Kits from Biolegend. The detection limit was 8 pg/ml for IL-10 and IL-6; and 16 pg/ml for TNF-α, TGF-β, and IL-12p70; and 15.6 pg/ml for IL-1β.

2.7 | Flow cytometry analysis

Macrophages infected or not with *B. pertussis* were analyzed by flow cytometry to determine the surface-level expression of selected markers (FACScan, Becton Dickinson Immunocytometry System, San Jose, CA, USA). Briefly, 2×10^5 macrophages were fixed with 4% (w/v) PFA, and incubated for 30 min at 4°C with the following conjugated mouse anti-human mAb: PE-anti CD11b (Biolegend), APC-anti CD40 (Immunotools), PE-anti CD80 (Immunotools), PerCP-Cy™5.5-anti CD86 (BD Pharmingen, San Diego, CA, USA), FITC-anti HLADR (BD Pharmingen), PE-anti CD206 (Biolegend), PE-anti CD163 (Biolegend), and FITC-anti CD209 (R&D Systems, Minneapolis, MN, USA). Control experiments with the respective isotype control antibodies were run in parallel. The macrophage population was gated according to its Forward Scatter and Size Scatter properties.

2.8 | 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-column DNAase-I digestion (Qiagen™, Valencia, CA, USA) as previously reported.[26] RNA quality was assessed both by agarose gel electrophoresis and photometrically by means of NanoDrop 2000 (ThermoScientific™, Waltham, MA, USA). A 260/280 ratio of around 2.0 was considered adequate.

2.9 | cDNA synthesis

Synthesis of cDNA was performed with the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). For each cDNA synthesis, 40 ng of a given total RNA and 10 μM random primers (Qiagen, Germantown, MD, USA) were used as previously described.[26] Complementary DNA samples were stored at −20°C until the time of analyses by the PCR.

2.10 | Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) analyses were performed on an Mx3000P QPCR System (Stratagene, La Jolla, CA, 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.[27] Primers were purchased from Invitrogen (Invitrogen, Karlsruhe, Germany). The primers for targeted genes are listed in Table 1. PCR runs comprised a 10 min preincubation at 95°C, followed by 40 cycles of a 2-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.[28] The ß2 microglobulin gene (*B2M*) was used as a housekeeping marker for normalization. Untreated human macrophage (without the addition of growth factors or cytokines) served as a reference for macrophage polarization analysis while the respective uninfected phenotype served as a reference for the macrophage phenotype response to *B. pertussis* infection.

2.11 | Statistical analysis

The Student's *t*-test (95% confidence level) or 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 mean ± SD.

3 | RESULTS

3.1 | Phenotypic characterization of polarized macrophages

In order to obtain the different human macrophage phenotypes, monocytes from peripheral blood were incubated in serum-free media in the presence of GM-CSF + INF-γ/LPS, M-CSF + IL-4, or M-CSF + IL-10 to obtain M1, M2a, or M2c macrophage, respectively (Figure S1). Thereafter, macrophage polarization was confirmed by gene expression, surface markers, and cytokines/chemokines production. Figure 1(A)

TABLE 1 Primers used for real-time PCR

Gene	HGNC ID	Protein name	Sense	Antisense
CCR7	1608	C-C motif chemokine receptor 7	5′-CAGCCTTCCTGTGTGGTTTT-3′	5′-AAATGACAAGGAGAGCCACC-3′
CXCL10	10637	C-X-C motif chemokine ligand 10	5′-*GCTGATATGGGGATATAGGTTC*-3′	5′-*GCAGGTACAGCGTACAGTTC*-3′
MRC1	7228	mannose receptor C-type 1	5′-*TACCCCTGCTCCTGGTTTTT*-3′	5′-*CAGCGCTTGTGATCTTCATT*-3′
CCL18	10616	C-C motif chemokine ligand 18	5′-*TCCTTGTCCTCGTCTGCAC*-3′	5′-*GTGGAATCTGCCAGGAGGTA*-3′
IL10	5962	IL-10	5′-GTGATGCCCCAAGCTGAGA-3′	5′-CACGGCCTTGCTCTTGTTTT-3′
B2M	914	beta-2-microglobulin	5′-TCTCTGCTGGATGACGTGAG-3′	5′-TAGCTGTGCTCGCGCTACT-3′

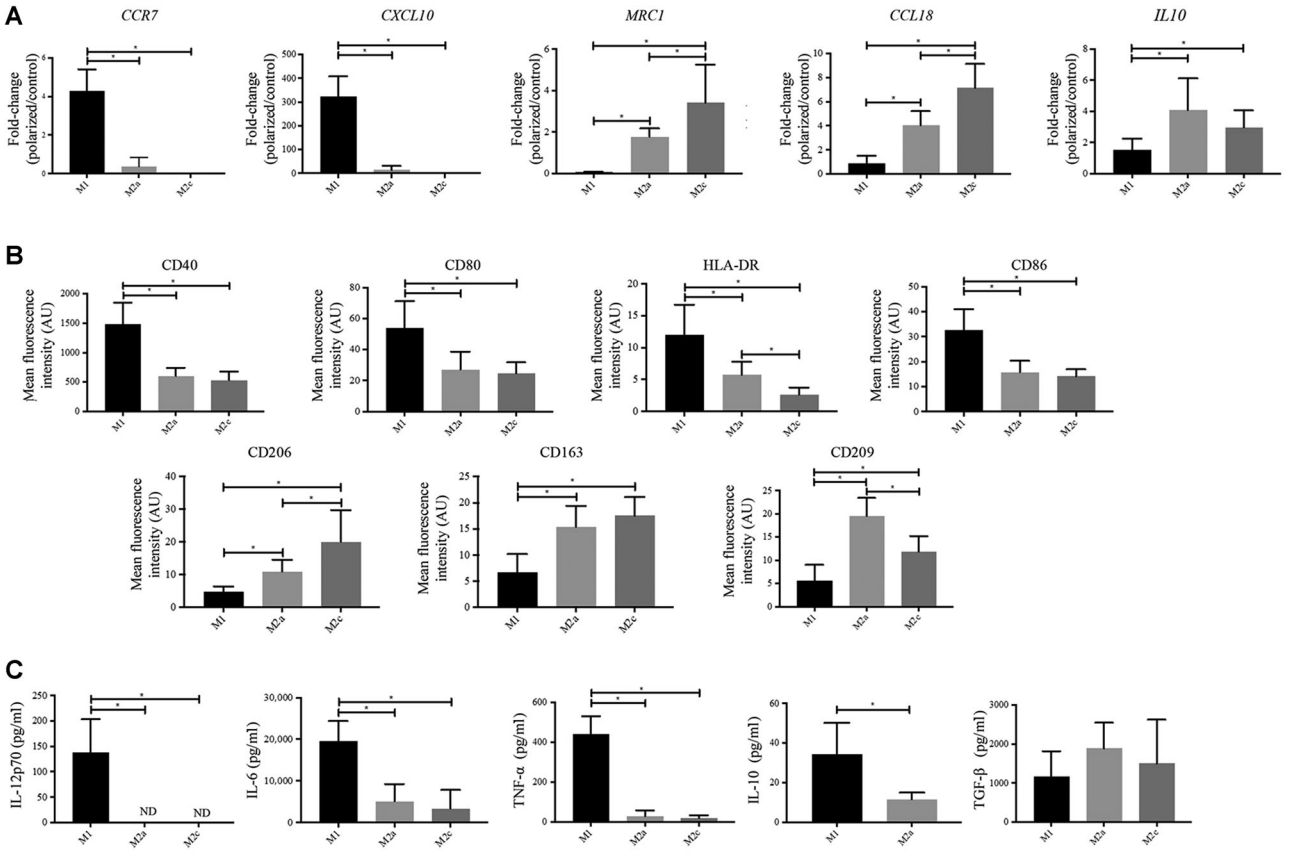

FIGURE 1 Characterization of polarized macrophages. Human monocytes were polarized into M1, M2a, or M2c macrophages. Panel (A) shows the expression of selected genes as determined by RT-qPCR. Changes in gene expression were expressed as fold change of mRNA level in polarized macrophages as compared with nonpolarized macrophages (control), *B2M* was used as a housekeeping gene for data normalization. Panel (B) shows the expression of surface markers as determined by flow cytometry. Panel (C) shows cytokine concentration in the culture supernatants of M1, M2a, and M2 macrophages 24 h after polarization as determined by ELISA (ND: not detected). The means ± SD of 3 independent experiments are given. $^*p < 0.05$

shows that, as expected,[13] the chemokine receptor *CCR7* and the chemotactic ligand *CXCL10*, that are selectively up-regulated in the M1 phenotype, were highly up-regulated in cells treated under M1 conditions. On the other hand, the transcription of mannose receptor *MRC1* and *CCL18*, that are selectively up-regulated in the M2 phenotype, were highly up-regulated in cells treated under M2 conditions. *IL-10* transcription was also significantly higher in M2 macrophages than in M1. The different polarization states were further confirmed by surface marker profiles, as determined by flow cytometry. Figure 1(B) shows that cells obtained under M1 conditions had a significantly higher level of CD40, CD80, CD86, and HLA-DR than cells obtained under M2 condition. On the other hand, as expected,[13] M2a and M2c phenotypes showed higher CD206, CD163, and CD209 than M1 cells. M2a cells, on the other hand, showed higher expression of cell surface HLA-DR and CD209 than M2c. Next, the cytokine production was quantified in the cell culture supernatant. Figure 1(C) shows that cells treated under M1 conditions released high amounts of proinflammatory cytokines such as IL-12p70, IL-6, and TNF-α, but

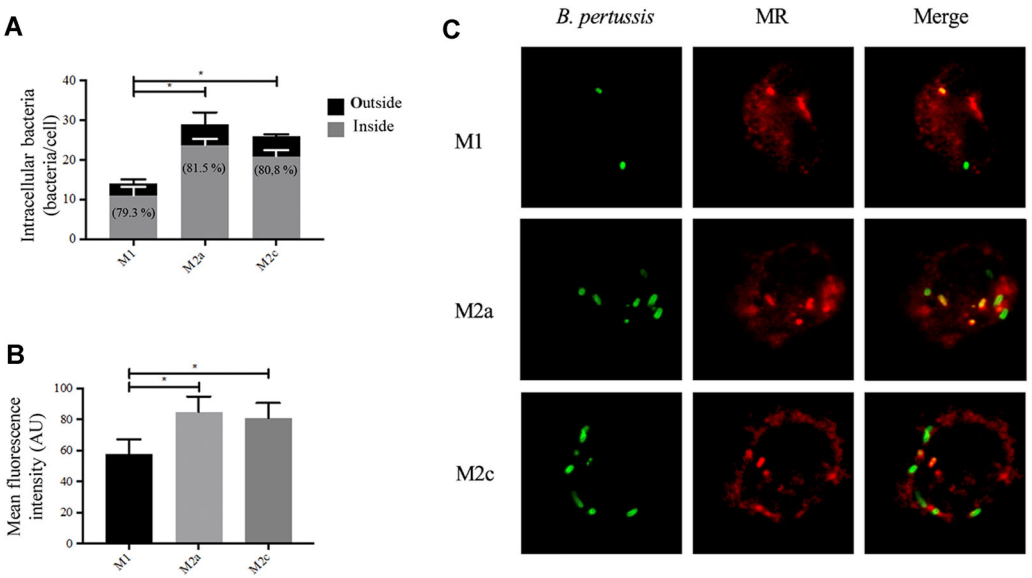

FIGURE 2 Macrophage phenotype and *B. pertussis* uptake. M1, M2a, M2c macrophages were infected with *B. pertussis* (MOI: 100). Thirty minutes after infection surface bound and intracellular bacteria were determined by double staining and fluorescence microscopy (A). Panel (B) shows CD11b surface expression on uninfected M1, M2a, and M2c as determined by PE-conjugated antibodies against CD11b and flow cytometry. Panel (C) shows *B. pertussis* colocalization with macrophage mannose receptor (MR) as determined by fluorescent staining and confocal microscopy. *B. pertussis* is seen in green and mannose receptor in red. Colocalization is indicated by the yellow areas. Representative images of 3 independent experiments are shown. Panels (A) and (B) show the means ± SD of 3 independent experiments. $^{*}p < 0.05$

also anti-inflammatory cytokines like TGF-β and IL-10. On the other hand, cells incubated under M2a or M2c conditions produced low amounts of IL-6, no detectable levels of either TNF-α or IL-12p70, and TGF-β level similar to M1 cells. Concerning IL-10 production, both M1 and M2a macrophages secreted this cytokine but it was produced at higher levels by the M1 phenotype (Figure 1(C)), which is likely related to the LPS stimulation used to generate this phenotype, whereas its production by M2a cells was not driven by a PAMPs. No relevant information could be obtained about IL-10 production by cells incubated under M2c condition, since this cytokine was already present in the culture medium to stimulate polarization. Altogether, these results show that the subsets of macrophages obtained in this study display the expected features of M1 and M2a and M2c macrophages.

3.2 | Phagocytosis of *B. pertussis* is dependent on docking molecules present in polarized macrophages

In order to evaluate the influence of macrophage polarization on *B. pertussis* uptake, M1, M2a, and M2c macrophages were incubated with bacteria for 30 min (MOI: 100), washed, and the number of intra and extracellular bacteria determined by double fluorescence staining. The number of bacteria phagocytosed by M1 macrophages (11.78 ± 1.21 bacteria/cell) was significantly lower than those phagocytosed by M2a (25.75 ± 2 b/c) or M2c (24.11 ± 2.2 b/c) macrophages (Figure 2(A)). However, around 80% of the bacteria associated with the macrophages were found inside the cells regardless the phenotype (Figure 2(A)), suggesting that the low bacterial uptake of M1 macrophages was primarily due to a deficient *B. pertussis* attachment to this phenotype. CD11b is part of the main *B. pertussis* docking molecule, the complement receptor 3 (CR3; CD11b / CD18), on macrophage surface.[29] Flow cytometry analysis of the surface expression of CD11b showed that M1 macrophages had a significant lower level of CD11b on their surface than M2a or M2c macrophages (Figure 2(B)). On the other hand, the mannose receptor (CD206 or MR) that showed a lower level of expression on M1 surface as compared with M2a and M2c macrophages (Figure 1(B)), has been recently proposed as a docking molecule for *B. pertussis* in mast cells.[30] We then investigated whether CD206 might also be a docking molecule for *B. pertussis* on macrophage surface. To this end, M1, M2a and M2c macrophages were incubated with *B. pertussis* for 30 min, CD206 and *B. pertussis* were further stained and analyzed by confocal microscopy. Figure 2(C) shows that in all evaluated macrophage phenotypes there were bacteria colocalizing with MR, suggesting that might *B. pertussis* target this receptor on the macrophage surface.

The lower expression of *B. pertussis* docking molecules on the surface of M1 macrophage as compared with M2 cells might explain the lower *B. pertussis* uptake of this phenotype.

3.3 | Changes in the macrophages phenotype upon *B. pertussis* phagocytosis

In order to investigate whether *B. pertussis* phagocytosis affects the activation program associated to each macrophage type, M1, M2a, and M2c macrophages were evaluated at 3 and 48 h after bacterial infection. Uninfected cells were used as controls.

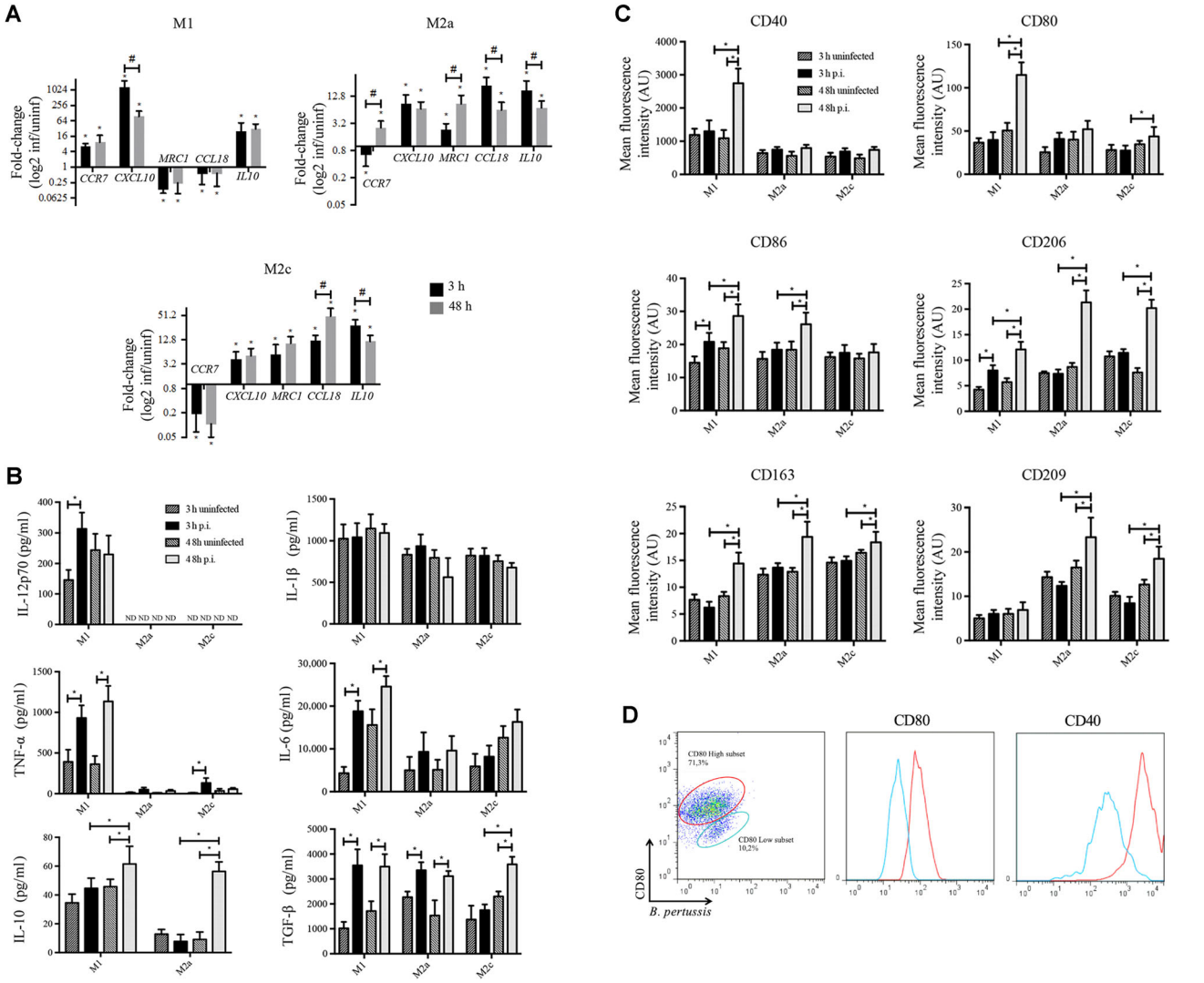

FIGURE 3 Influence of *B. pertussis* infection on macrophage polarization. M1, M2a, and M2c macrophages were infected or not with *B. pertussis* (MOI: 100). Samples were taken at 3 and 48 h postinfection. Panel (A) shows gene expression levels determined by RT-qPCR. *B2M* was used as a housekeeping gene for data normalization, and uninfected 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 log2. Changes in gene expression were expressed as fold change in transcription in infected cells with respect to the uninfected cells. The asterisks (∗) indicate statistically significant differences ($p < 0.05$) in the transcription level of a given gene between infected and uninfected cells. The numeral symbol (#) indicates statistically significant differences ($p < 0.05$) in the transcription level of given gene at different time points postinfection. Panel (B) shows the concentration of cytokines in the supernatants as determined by ELISA (ND: not detected). Panel (C) shows surface markers expression as determined by flow cytometry. Panel (D) shows a dot blot of CD80 versus *B. pertussis* infection of M1 macrophages 48 h after infection, and the overlapping histograms of CD80 and CD40 expression of the cells gated in the ellipses blue and red. Representative plots of 3 independent experiments are shown. Panels (A), (B), and (C) show the means ± SD of 3 independent experiments. $^{*}p < 0.05$

We evaluated the transcription profile of specific genes in each phenotype upon infection and found that the infection of M1 macrophages induced the up-regulation of the chemokine receptor *CCR7*, and the chemotactic ligand *CXCL10* while the transcription of alternative activation markers such as mannose receptor *MRC1* and *CCL18* was down-regulated upon infection (Figure 3(A)). On the other hand, the bacterial infection of M2a macrophages resulted in the up-regulation of *CCL18* and *IL-10* (Figure 3(A)). Although bacterial infection induced the expression of *CXCL10* in M2a cells, the up-regulation observed in this phenotype was significantly lower than in M1 macrophages ($p < 0.05$). The transcription of *MRC1* showed a moderate up-regulation at 3 h postinfection of M2a macrophages, but was more pronounced at 48 h (Figure 3(A)). The transcriptional response of M2c cells was similar to that observed in M2a except for *CCR7* expression, which was strongly down-regulated in M2c macrophages upon infection (Figure 3(A)). These results suggest that *B. pertussis* infection mainly induced the up-regulation of proinflammatory genes in M1 but also the expression of the anti-inflammatory cytokines, such as *IL-10*. On the other hand, bacterial infection of M2 macrophages induced mainly the up-regulation of anti-inflammatory genes and a

moderate induction of the proinflammatory mediators, particularly in M2a macrophages.

The analysis of cytokines released into the supernatant showed that *B. pertussis* infection of M1 macrophages induced the release of high amounts of proinflammatory cytokines, such as IL-12p70, IL-6, and TNF-α, as well as the anti-inflammatory cytokines TGF-β early after infection (Figure 3(B)). Interestingly, 48 h after infection the level of IL-6 and IL-12p70 in the supernatants of infected and uninfected M1 macrophages showed no significant differences, indicating that *B. pertussis* infection modulated the inflammatory response. Accordingly, the level of the anti-inflammatory cytokines, TGF-β and IL-10 was significantly higher in the supernatant of infected than in the supernatant of uninfected M1 macrophages 48 h after infection (Figure 3(B)). On the other hand, as shown in Figure 3(B), *B. pertussis* infection of M2a and M2c macrophages did not modify the level of proinflammatory cytokines, such as IL-6, IL-1β, or IL-12p70, in the culture supernatant, although the infection of M2c induced a transient production of TNF-α. Figure 3(B) further shows that *B. pertussis* infection induced the production of TGF-β soon after infection in M2a cells and late after infection in M2c cells. It also shows that IL-10 level was significantly higher in the supernatant of infected than in the supernatant of uninfected M2a cells 48 h postinfection. No relevant information on IL-10 production by infected M2c cells could be obtained since this cytokine was already present in the culture medium to stimulate polarization.

Figure 3(C) shows the level of specific markers on the surface of M1 and M2 cells over the time of infection. Three hours after infection M1 macrophages exhibited an increase of CD86 and CD206 surface expression. Forty-eight hours postinfection the expression level of CD40, CD80, and CD86 were significantly higher than those observed 3 h after infection. Interestingly, M1 surface expression of CD206 and CD163 were also found increased at late time points as compared with early times postinfection. On the other hand, the infection of M2a and M2c macrophages resulted in a significant increase of M2-associated markers, like CD206, CD163, CD209, at late times postinfection. In addition, CD86 expression was increased 48 h after infection exclusively in M2a cells. The analysis of CD40 and CD80 surface expression on M1 macrophages 48 h after *B. pertussis* infection revealed a heterogeneous cell population. Figure 3(D) shows that around 10% of the cells (all of them positive for *B. pertussis* infection) had lower expression of both CD80 and CD40 receptors as compared with the rest of the cells. This result suggests that *B. pertussis* infection may interfere with the M1-associated features likely hampering the maintenance of the microbicidal-associated activation program.

Altogether these results seem to suggest that *B. pertussis* infection of M2 macrophages, unlike M1 cells, does not induce a proinflammatory response. Particularly, none of the proinflammatory markers were found to be up-regulated in M2c cells after infection. On the other hand, they also suggest that *B. pertussis* might be able to interfere with the polarization of those M1 macrophages in which it manages to persist.

3.4 | Influence of macrophage polarization on the intracellular survival of *B. pertussis*

In order to evaluate the intracellular fate of *B. pertussis* in the different macrophage phenotypes, bacterial intracellular trafficking and intracellular bacterial viability were determined by confocal microscopy and CFU counting, respectively. Since the number of phagocytosed bacteria differed between the different macrophage phenotypes, we determined the percentage of the phagocytosed bacteria that were alive 3 h after infection in order to be able to properly compare the early bacterial survival inside different macrophage types. Figure 4(A) shows that the percentage of the phagocytosed bacteria that was still alive 3 h after infection in M1 macrophages was significantly lower than in M2a or M2c macrophages, indicating a higher ability of M1 to kill *B. pertussis* early after phagocytosis. By scoring the number of intracellular bacteria per macrophage over time of infection using fluorescence microscopy (Figure 4(B)), we found that 48 h after infection the percentage of macrophages containing less than 5 bacteria increased in M1 and M2a macrophages suggesting that bacteria were efficiently killed by some of the macrophages of these 2 phenotypes. On the other hand, the percentage of cells containing between 5 and 10 bacteria decreased over the time in all evaluated macrophage phenotypes, while the percentage of macrophages containing more than 10 bacteria increased in M2a and M2c macrophages. By means of confocal microscopy we observed that 48 h after infection, most of the intracellular bacteria within M2 macrophages were not colocalizing with lysosomes. Bacterial trafficking to lysosomes proved higher in M1 than in M2 macrophages. However, around 60% of the intracellular bacteria were found not colocalizing with lysosomes even in M1 macrophages (Figures 4(C) and 4(D)). These results suggest that some phagocytosed bacteria were able to avoid phagolysosomal fusion in the macrophages regardless their initial activation program. Accordingly, at 48 h after infection, we found viable bacteria in all the 3 phenotypes, although the number of intracellular bacteria that survived inside M1 cells was significantly lower than in M2 macrophages (Figure 4(E)).

4 | DISCUSSION

During a bacterial infection, several different environmental stimuli will affect the macrophage phenotype polarization which, in turns, will determine the result of the bacteria–immune cell interaction. The epithelium response to the pathogen, through the production of a variety of modulatory mediators, is a key player in this process. Due to the primary role of the macrophage in host innate immunity the evasion of macrophage bactericidal activity is particularly relevant for bacterial pathogens. The manipulation of macrophage polarization state is emerging as a key pathogenesis mechanism of intracellular bacteria. In this study, we used specific in vitro stimulation conditions to polarize primary human monocytes-derived macrophages into M1, M2a, or M2c profiles in order to investigate the interaction of *B. pertussis* with

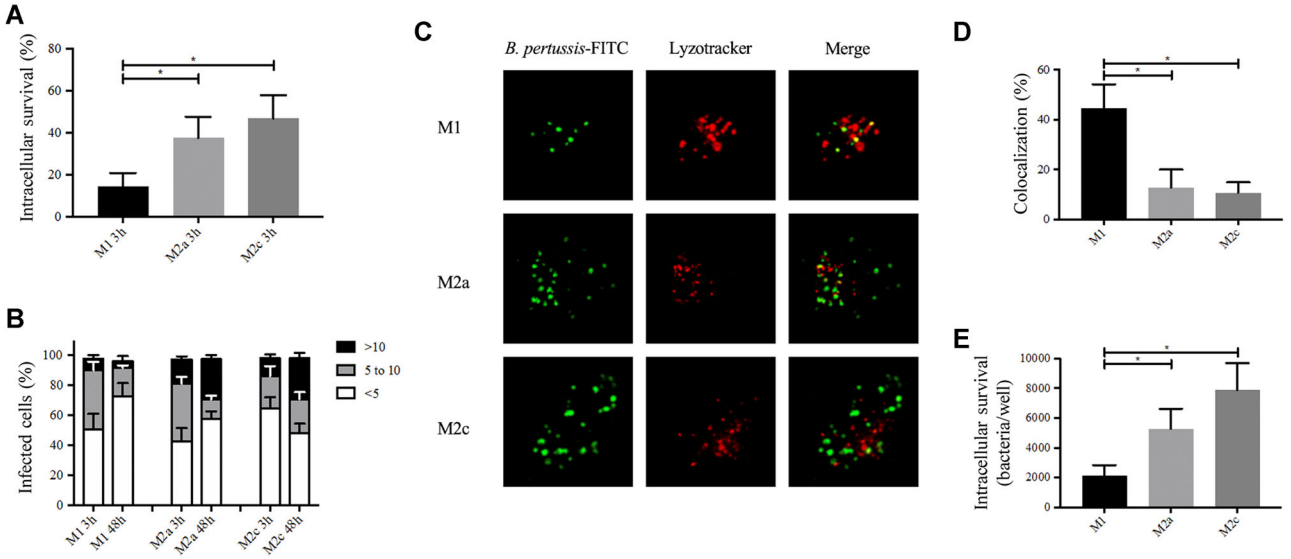

FIGURE 4 *B. pertussis* intracellular survival in polarized macrophages. M1, M2a, and M2c macrophages were infected with *B. pertussis* (MOI: 100). Three and 48 h after infection the number of viable intracellular bacteria was determined in each macrophage subtype. In parallel, the number of phagocytosed bacteria was determined by double staining and fluorescence microscopy. Panel (A) shows the intracellular survival calculated as the percentage of the bacteria phagocytosed by the different macrophage types that were still alive 3 h after infection. Panel (B) shows *B. pertussis* distribution at 3 and 48 h postinfection. Infected cells were scored as containing ≤5, 5–9, or ≥10 bacteria. Panel (C) shows the intracellular *B. pertussis* (green) and lysosomes (red) 48 h after infection. Colocalization is indicated by the yellow areas. Representative images of 3 independent experiments are shown. Panel (D) indicate the percentage of bacteria colocalizing with Lysotracker 48 h after infection. Panel (E) shows the intracellular survival of *B. pertussis* 48 h after infection. Panels (A), (B), (D), and (E) show the means ± SD of 3 independent experiments. $^{*}p < 0.05$

the different phenotypes and the possible implications for bacterial persistence.

This study showed that the M1 and M2 macrophage phenotypes differ in their ability to uptake *B. pertussis*, which seems primarily due to differences in bacterial attachment to each phenotype. The lower levels of CR3 and MR on M1 surface as compared to M2 macrophages might be involved in the different bacterial attachment to each phenotype. CR3 is a well-known docking molecule of *B. pertussis* on immune cell[29] while MR, being found able to interact with the GlcNAc (N-acetyl-D-glucosamine) of *B. pertussis* lipooligosaccharide, has been proposed as another receptor for this bacterium.[30] By means of confocal studies, we here showed the colocalization of MR with *B.* pertussis on the surface of the macrophages suggesting that this receptor might be a docking molecule for this pathogen also in these cells. Phagocytosis through CR3 was found to increase the odds of bacterial intracellular survival.[25,31] Likewise, bacterial–cell interaction through MR has been linked to nonbactericidal trafficking of the internalized bacteria.[32,33] Accordingly, we found intracellular *B. pertussis* alive 2 days after infection in all evaluated macrophage activation programs. As expected, we observed a higher bactericidal activity in M1 macrophages than in M2, as determined by the number of phagocytosed bacteria that were still alive 3 h after internalization. However, despite the early bactericidal response, M1 macrophages failed to completely eradicate intracellular *B. pertussis* and 2 days later a significant number of bacteria were still alive inside this phenotype. Accordingly, confocal studies showed intracellular bacteria not colocalizing with lysosomal markers at this time postinfection in M1, M2a, and M2c cells. Taken together, these results suggest that *B. pertussis* is able to avoid the bactericidal trafficking even if it is phagocytosed by M1 macrophages. As found before for other pathogens,[34,35] *B. pertussis* phagocytosis by M1 macrophages initially led to an increase of the markers associated to classically activated macrophages. The common response of macrophages against bacterial infections mainly involves the induction of an M1 program, thus preventing bacterial phagosome escape and stimulating intracellular killing of bacteria. This cellular activation upon bacterial phagocytosis explains the higher microbicidal capacity observed in M1 macrophages as compared with M2 cells. However, 48 h after infection M1 proinflammatory response seemed modulated by *B. pertussis* infection as anti-inflammatory cytokines, such as TGF-β and IL-10, were found induced. Interestingly, the phenotypic analysis of M1 macrophages 48 h after infection revealed the presence of 2 subpopulations of cells, one with a high level of CD80 surface expression and another one, containing around 10% of the cell population, displaying a low level of CD80 expression (Figure 3(D)). Interestingly, this latter population also exhibited a low level of CD40 surface expression as compared with the rest of the cells. Although not the whole population of cells was positive for *B. pertussis* infection, all the cells in this low-CD80/low CD40 group were loaded with *B. pertussis* (Figure 3(D)). The coexistence of noninfected and infected macrophages 48 h upon *B. pertussis* infection has been previously observed.[9,11] The presence of the cells positive for *B. pertussis* with reduced expression of the activation markers CD40 and CD80 suggests that *B. pertussis*, as other pathogens,[36,37] might be able to modulate the proinflammatory response and somehow interfere

with M1 polarization in those macrophages in which it manages to survive. CD40 is a relevant macrophage receptor that mediates the secretion of a vast array of cytokines, which are important in promoting and maintaining Th1 and a proinflammatory environment.[38] CD40 is also necessary for macrophage activation and the up-regulation of costimulatory molecules like CD80 and CD86 that play a critical role in antigen presentation and T-cell activation.[39] Our results suggest that *B. pertussis* intracellular infection of M1 might reduce the macrophage antigen-presenting capacity and interfere with the inflammatory amplification by decreasing CD40 and CD80 expression level. This study showed that *B. pertussis* is able to survive even in M1 macrophages. However, whether this intracellular survival means that this pathogen can find a niche of persistence inside M1 macrophages is still uncertain. The infection of M2a and M2c, on the other hand, did not induce an increase of the proinflammatory markers but rather induced the expression of anti-inflammatory markers, such as CD206, CD163, and CD209. Accordingly, the intracellular survival of *B. pertussis* in M2a and M2c cells was significantly higher than in M1. The encounter and phagocytosis of *B. pertussis* induced the release of TGF-β in M2a and M2c. Similar results were observed for the other anti-inflammatory cytokine, IL10. Both IL-10 and TGF-β have strong immunosuppressive effects and play a central role in the control of the immune response.[40] Increased IL-10 production in response to infection was previously found to be involved in intracellular persistence by contributing to bacterial–phagosome maturation arrest in human macrophages.[41,42] Moreover, by activating IL-10 expression, *B. pertussis* infection might further induce M2 polarization eventually promoting its own persistence.

Interestingly, according to the results of this study, the use of antibiotics to control the infection and the vaccination history of the infected individual might play a role in the outcome of *B. pertussis* interaction with the macrophages at the site of colonization. A limited number of antibiotics are available for *B. pertussis* control.[43] Among them, azithromycin is the agent of choice and the only macrolide recommended for neonates less than 1-month old.[44] Azithromycin was found to promote an anti-inflammatory environment not only by shifting macrophages from the inflammatory M1 toward the anti-inflammatory M2 phenotype but also by reducing the activation of other immune cells.[45,46] A recent study provided evidence that azithromycin induces the attenuation of the immune response against *B. pertussis* and reduces the activation status of macrophages and dendritic cells.[47] Together with these reports, our findings suggest that the use of azithromycin to control *B. pertussis* might promote the development of macrophage intracellular infections.

On the other hand, the reemergence of pertussis has reopened the discussion about the efficacy of the vaccines to prevent colonization and transmission. In particular, the introduction of acellular vaccines has been linked to the reemergence of pertussis. Previous studies have demonstrated that while *B. pertussis* natural infection or the whole cell vaccine induce a mixed Th1/Th17 response mediated by IFN-γ and IL-17, acellular vaccines mainly induce Th2-polarized response.[3,5] The results showed in this study support the importance of the Th1 cytokines to induce macrophage bactericidal activity against *B. pertussis*. Th2 cytokines, on the other hand, will induce M2 macrophage phenotypes, which are highly phagocytic but poorly bactericidal, increasing the odds for the development of intracellular infections and a niche of persistence for this pathogen. The results obtained in this study shed some new light into the influence that the inflammatory context might have on *B. pertussis*–macrophage interaction outcome and the bacterial persistence within the host. These findings might help to explain the well-known but still not completely understood need for Th1/Th17 response to clear *B. pertussis*, and the observed failure of acellular pertussis vaccines to protect against host colonization.[5] Our data highlight the influence of environment on the outcome of *B. pertussis* interaction with the macrophages, pointing at the M2 phenotype as the best suited for the intracellular persistence of this pathogen.

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AUTHORSHIP

H. A. V. and M. E. R. designed the research. H. A. V., L. B., J. P. G., J. A. H., and J. M. performed the experiments. H. A. V., M. E. R., L. B., J. P. G., J. A. H., J. M., M. L., and M. C. S. contributed to data analyses and interpretation. H. A. V. and M. E. R. wrote the manuscript. H. A. V., M. E. R., L. B., J. P. G., J. A. H., J. M., M. L., and M. C. S. critically reviewed and approved the final version of the manuscript.

DISCLOSURES

The authors declare no conflict of interest.

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