

Bordetella pertussis-Infected Human Monocyte-Derived Dendritic Cells Undergo Maturation and Induce Th1 Polarization and Interleukin-23 Expression

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Bordetella pertussis, the causative agent of whooping cough, is internalized by several cell types, including epithelial cells, monocytes, and neutrophils. Although its ability to survive intracellularly is still debated, it has been proven that cell-mediated immunity (CMI) plays a pivotal role in protection. In this study we aimed to clarify the interaction of *B. pertussis* with human monocyte-derived dendritic cells (MDDC), evaluating the ability of the bacterium to enter MDDC, to survive intracellularly, to interfere with the maturation process and functional activities, and to influence the host immune responses. The results obtained showed that *B. pertussis* had a low capability to be internalized by—and to survive in—MDDC. Upon contact with the bacteria, immature MDDC were induced to undergo phenotypic maturation and acquired antigen-presenting-cell functions. Despite the high levels of interleukin-10 (IL-10) and the barely detectable levels of IL-12 induced by *B. pertussis*, the bacterium induced maturation of MDDC and T helper 1 (Th1) polarized effector cells. Gene expression analysis of the IL-12 cytokine family clearly demonstrated that *B. pertussis* induced high levels of the p40 and p19 subunits of IL-23 yet failed to induce the expression of the p35 subunit of IL-12. Overall our findings show that *B. pertussis*, even if it survives only briefly in MDDC, promotes the synthesis of IL-23, a newly discovered Th1 polarizing cytokine. A Th1-oriented immune response is thus allowed, relevant in the induction of an adequate CMI response, and typical of protection induced by natural infection or vaccination with whole-cell vaccines.

The encounter between a microbe and cells of the innate immune system is a key event not only for the effectors of innate immunity, which trigger specific mechanisms to combat the invading pathogen, but also for the microbe, which activates its strategies to escape the immune response.

Dendritic cells (DC) represent a unique system of cells that link innate and acquired immunity. Immature DC are able to capture microbial antigens and directly phagocytose pathogens. These functions are shared with other natural effectors, such as monocytes and neutrophils, but DC upon encounter with microbial products undergo maturation, a profound rearrangement of gene expression that ultimately leads to mature DC. Mature DC are able to migrate to the lymph nodes to stimulate T helper (Th) lymphocytes and drive their functions in close association with the type of maturational stimulus received (6, 7, 33, 42).

Bordetella pertussis is the causative agent of whooping cough, a respiratory disease affecting infants and children that represents an important cause of morbidity and mortality in many parts of the world (46). The mechanism underlying protection from *B. pertussis* infection is still a matter of debate (28). One of the earliest immune responses elicited by the bacterium is the recruitment in the upper respiratory mucosa of phagocytic

cells, which may internalize the bacteria and thereafter kill them by a respiratory burst (8, 28).

A murine respiratory infection model suggested that phagocytic cells can play an important role in clearing the infection (21); thus, a critical issue in immunity against *B. pertussis* is represented by the possibility that an intracellular phase represents a way for *B. pertussis* to escape immune surveillance. However, studies addressing the abilities of monocytes and neutrophils to phagocytose *B. pertussis* have suggested that evasion of phagocytes, rather than intracellular survival, allows bacterial cells to escape the effector cells of innate immunity (38, 45).

In this study we aimed to clarify the interaction of *B. pertussis* and monocyte-derived DC (MDDC). In particular, we intended to evaluate the ability of human MDDC to phagocytose *B. pertussis* and the capacity of bacterial cells to survive intracellularly. Since many experimental observations led to the conclusion that humoral and cell-mediated immunity (CMI) play complementary roles in a protective immune response (2, 10, 28, 29, 30, 37), a key point in the present study was the assessment of the ability of *B. pertussis* to influence MDDC functions. In particular, we aimed to evaluate whether infected MDDC undergo phenotypic and functional maturation, and for which type of Th cells polarization is induced by infected MDDC.

MATERIALS AND METHODS

Reagents. Lipopolysaccharide (LPS) from *Escherichia coli*, cytochalasin D, polymyxin B, brefeldin A, phytohemagglutinin (PHA), phorbol 12-myristate 13-

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acetate (PMA), and ionomycin were obtained from Sigma Chemical Co. (St. Louis, Mo.). A monoclonal antibody (MAb) against interleukin-10 (IL-10), clone 23738.11, was from R&D Systems, Inc. (Minneapolis, Minn.). Human recombinant granulocyte-macrophage colony stimulating factor (hrGM-CSF) and hrIL-4 were obtained from Novartis Pharma AG (Basel, Switzerland). hrIL-2 was obtained from Roche (Basel, Switzerland).

Bacterial strains and growth conditions. *B. pertussis* strain 18323 (ATCC 97-97) was inoculated onto charcoal agar plates supplemented with 10% sheep blood (Oxoid, Basingstoke, United Kingdom) and grown at 37°C for 3 to 4 days. Bacteria were then collected and resuspended in 10 ml of phosphate-buffered saline (PBS). The bacterial concentration was estimated by measuring the optical density at 600 nm, and the suspension was adjusted to a final concentration of 10^9 CFU/ml. The bacterial suspension was used to infect MDCC. For an accurate measurement of the multiplicity of infection (MOI), the *B. pertussis* suspension was serially diluted onto charcoal agar plates and CFU were counted up to 5 days of culture.

Purification and culture of MDCC. Human monocytes were purified from peripheral blood of healthy donors as described elsewhere (3). CD14⁺ cells were cultured at 5×10^5 /ml in RPMI 1640 (ICN-Flow, Aurora, Ohio) supplemented with LPS-screened (*Limulus* amoebocyte lysate, <1 ng/ml) 10% heat-inactivated (HI) fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 25 mM HEPES, 100 U of penicillin/ml, and 100 µg of streptomycin/ml (all from HyClone Laboratories, Logan, Utah), and 0.05 mM 2-mercaptoethanol (Sigma) (referred to below as complete medium), at 37°C under 5% CO₂, in the presence of 1,000 U of hrIL-4/ml and 50 ng of hrGM-CSF/ml (3). After 6 days, immature MDCC (iMDCC) were washed and analyzed by cytofluorometric analysis for CD1a and CD14 expression.

Sytox green labeling and phagocytosis assay. Sytox green (Molecular Probes, Eugene, Oreg.) staining of *B. pertussis* and the subsequent phagocytosis assay were performed as described in reference 14. Briefly, bacteria (10^9 CFU) were centrifuged at $8,800 \times g$ for 3 min, resuspended in 1 ml of 70% isopropyl alcohol, and incubated 1 h for permeabilization. The bacterial suspension was washed twice in 0.9% NaCl and then stained with 5 µM Sytox green dye for 5 min at room temperature. Bacteria were pelleted, resuspended in complete medium, and incubated for 2 h at 37°C under 5% CO₂ with effector cells (i.e., monocytes or iMDCC [10^6]) at a bacterium-to-effector cell ratio of 100:1 or 20:1. After incubation, cells were washed and resuspended in PBS–2% paraformaldehyde, and cytofluorometric analysis was performed. Control experiments were performed in the presence of cytochalasin D, an inhibitor of phagocytosis, to evaluate membrane-bound, noningested fluorescent bacteria.

MDCC infection and maturation. iMDCC (10^6 /ml) were resuspended in complete medium without penicillin and streptomycin (referred to below as Bp medium) and then infected with *B. pertussis* cells at bacterium-to-cell ratios of 20:1 and 100:1. iMDCC and bacteria were centrifuged for 2 min at $640 \times g$ to facilitate contact (38) and then incubated for 2 h at 37°C under 5% CO₂. Cells were then extensively washed and incubated for 3 h in Bp medium in the presence of polymyxin B (20 µg/ml) to kill adherent extracellular bacteria. Polymyxin B is unable to penetrate the eukaryotic cell membrane and kills adherent extracellular, but not internalized, bacteria (38). MDCC were then washed and incubated at 37°C under 5% CO₂ in Bp medium supplemented with polymyxin B (20 µg/ml), and aliquots were lysed by addition of sterile water, at 0, 1, 17, and 24 h from the end of the infection. Control infections were performed in the presence of cytochalasin D, to evaluate the background represented by colonies grown from membrane-bound bacteria surviving after polymyxin B treatment.

To evaluate the induction of MDCC maturation and functions, infections were performed in the absence of polymyxin B in order to avoid killing the bacteria. After 24 h, infected MDCC were harvested for immunophenotypic analysis and cytokine measurement by immunoenzymatic assay. iMDCC (10^6 /ml) were also treated for 24 h with HI (1 h at 96°C) *B. pertussis* at a 100:1 bacterium-to-cell ratio, and then immunophenotypic analysis and MDCC functions were assessed. *E. coli* LPS (100 ng/ml) was used as a reference method to induce MDCC maturation. Conditioned media (CM) from *B. pertussis*-infected MDCC, *E. coli* LPS-treated MDCC, or untreated MDCC were harvested at the 24-h time point and used for polarization experiments (3).

Isolation of T lymphocytes and MDCC-T-cell cocultures. To measure allogeneic proliferation, T cells were purified from peripheral blood mononuclear cells by negative sorting with magnetic beads (Pan T-cell kit; Miltenyi Biotec, Auburn, Calif.). The purity of cell preparations was assessed by cytofluorometric staining (3).

iMDCC were cultured with live or HI *B. pertussis* for 24 h, washed extensively, and cultured at different numbers in round-bottom 96-well plates (Costar, Cambridge, Mass.) with 5×10^4 T cells per well. At day 5 of culture, plates were

pulsed with [³H]thymidine (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) at 0.5 µCi, and [³H]thymidine incorporation was measured as reported in reference 3.

Polarization of naïve T lymphocytes. To evaluate T-lymphocyte polarization, experiments were performed using naïve CD45RA⁺ T cells, purified from T cells by negative sorting with anti-CD45RO-conjugated magnetic beads (Miltenyi Biotec). In some experiments cord blood lymphocytes were also used.

Naïve T cells (0.5×10^6) were cultured in complete medium in 24-well plates (Costar) with *B. pertussis*-treated MDCC (0.5×10^5) or, to evaluate the polarization ability of CM, in the presence of PHA (1.5 µg/ml) and MDCC CM (50% vol/vol), obtained as described above.

On day 5, IL-2 (50 U/ml) was added to the cultures. On day 12, supernatants were harvested for cytokine measurement. T lymphocytes were then activated with PMA (40 ng/ml) and ionomycin (1 µg/ml) for 5 h in the presence of brefeldin A, and cytokine production was measured by intracellular staining.

Immunophenotypic analysis. Cells were washed, resuspended in PBS containing 3% fetal bovine serum and 0.09% NaN₃, and then incubated with a panel of fluorochrome-conjugated MAbs (obtained from BD Biosciences, San Jose, Calif.) specific for MDCC (anti-CD14, anti-CD1a, anti-CD80, anti-CD83, and anti-HLA-DR) or specific for T cells (anti-CD3 and anti-CD45RA). Isotype-matched antibodies were used as a negative control. Cells were analyzed with a FACScan (BD Biosciences). Fluorescence data are reported as the percent positive cells when treatment induces expression of the marker in cells that were negative; data are reported as median fluorescence intensity (MFI) when treatment increases expression of the marker in cells that were already positive.

Cytokine measurement by ELISA or cytometric bead array (CBA) assay. To measure cytokine production, MDCC were cultured in the presence of the indicated stimuli in 0.5 ml in 5-ml tubes (Falcon; Becton Dickinson, Lincoln Park, N.J.) at 37°C under 5% CO₂. Supernatants were collected after 24 h, and IL-10 and IL-12 p70 production was assessed by an enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Inc.) with a sensitivity of 3.9 pg/ml for IL-10 and 5 pg/ml for IL-12. Optical density was measured with a Bio-Rad (Philadelphia, Pa.) 3550-UV microplate reader at 450 nm. In some experiments, cells were incubated in the presence of a neutralizing anti-IL-10 MAb (10 µg/ml).

Cytokines in the supernatants of polarized naïve T cells were assayed by using a human Th1/Th2 II CBA (BD Biosciences). Six bead populations with distinct fluorescence intensities coated with capture antibodies specific for IL-2, IL-4, IL-5, IL-10, tumor necrosis factor alpha, and gamma interferon (IFN-γ) proteins were used. Samples were incubated with human cytokine capture beads and stained with phycoerythrin detection reagent. After a 3-h incubation at room temperature, samples were washed and analyzed by FACScan. Analysis of data was performed using the appropriate software provided by BD Biosciences.

Intracellular staining. iMDCC and T cells were subjected to intracellular staining after polarization experiments. Intracellular staining was performed in the presence of brefeldin A (10 µg/ml), a compound that blocks proteins in the endoplasmic reticulum, thus inhibiting cellular secretion and preventing the binding of secreted cytokines to the cell surface. T lymphocytes or MDCC were stained for cell surface antigens by using a pretitrated, fluorochrome-conjugated anti-CD3 or anti-CD1a MAb. Cells were then fixed and permeabilized as described by BD Biosciences (Cytofix-Cytoperm and Perm/Wash protocols) and were stained either with a predetermined optimal concentration of a fluorochrome-conjugated anti-cytokine antibody (anti-IFN-γ, anti-IL-4, anti-IL-12p40/p70; BD Biosciences) or with an appropriate isotype control. After a 30-min incubation at 4°C, cells were washed and subjected to cytofluorometric analysis.

mRNA cytokine expression by TaqMan real-time RT-PCR analysis. To measure cytokine mRNA expression, TaqMan real-time reverse transcriptase PCR (RT-PCR) analysis was used (Applied Biosystems, Foster City, Calif.). Total RNA was extracted from MDCC at different time points, and reverse transcription was carried out as previously described (3). TaqMan assays were performed according to the manufacturer's instructions with an ABI 7700 thermocycler (Applied Biosystems). PCR was performed, amplifying the target cDNA (p40, p35, and p19 transcripts), with β-actin cDNA as an endogenous control. A probe, labeled at the 5' end with a fluorescent reporter and at the 3' end with a quencher, annealing the amplicon, was added to the PCR mixture. During PCR the 5'–3' nuclease activity of TaqMan polymerase cleaves the probe, resulting in displacement of the quencher from the reporter, which releases a fluorescent signal. Specific primers and probes were obtained from Applied Biosystems. Data were analyzed with the PE Relative Quantification software of Applied Biosystems. Specific mRNA transcript levels were expressed as fold increase over the basal condition.

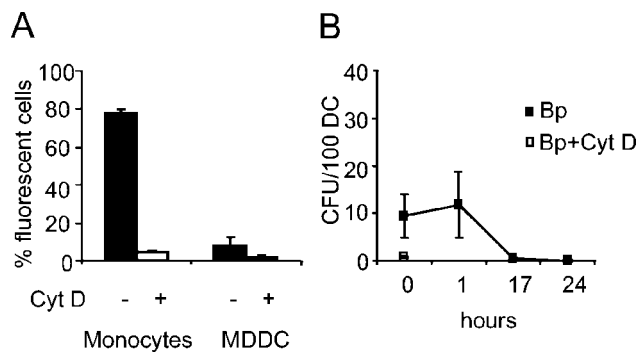


FIG. 1. (A) Internalization of *B. pertussis* by monocytes and MDDC. Sytox green-stained bacteria were incubated with monocytes or MDDC from the same donor. The samples were analyzed by flow cytometry to assess bacterial internalization. Black bars, percentage of fluorescent phagocytic cells; open bars, results of control experiments with the phagocytosis inhibitor cytochalasin D. Results are means $\pm$ SEs from three independent experiments. (B) Intracellular survival of phagocytosed bacteria. Black squares, counts of viable intracellular bacteria recovered at the indicated time points after MDDC infection; open squares, results of control experiments with the phagocytosis inhibitor cytochalasin D. Results are means $\pm$ SEs from 13 independent experiments.

Statistical analyses. Statistical descriptive analyses were carried out using the SPSS Inc. (Chicago, Ill.) statistical package. Differences between mean values were assessed by a two-tailed Student *t* test and were statistically significant at *P* values of <0.05 .

RESULTS

***B. pertussis* is phagocytosed by MDDC but does not survive intracellularly.** *B. pertussis* has been reported to be phagocytosed by THP-1 monocytic cells and by monocytes (20, 38, 41); thus, the ability of MDDC to phagocytose *B. pertussis* was assessed in parallel with that of monocytes obtained from the same donor. The phagocytic activities of the two different cell types were evaluated by assessing the internalization of Sytox green-labeled *B. pertussis*. The internalization of *B. pertussis* cells by MDDC was strikingly less efficient than that by monocytes (Fig. 1A); indeed, the vast majority of monocytes phagocytosed the fluorescent bacteria (mean $\pm$ standard error [SE], 78.0% $\pm$ 1.9%), compared to only a small fraction of MDDC (mean $\pm$ SE, 6.6% $\pm$ 5.4%).

Since internalization of permeabilized dead bacteria may not reflect the phagocytosis of live bacteria, in order to better characterize the ability of *B. pertussis* to survive in MDDC, infection experiments were performed. The results showed

that the optimal bacterium-to-cell ratio was 100:1. At this MOI, phagocytosis was maximized without affecting MDDC viability (data not shown).

A small fraction of bacteria were phagocytosed by MDDC within the 5 h of infection; indeed, only 8.8 $\pm$ 4.1 (mean $\pm$ SE) living intracellular bacteria were recovered from 100 infected MDDC (Fig. 1B). This datum is in agreement with the results shown in Fig. 1A.

To determine the intracellular survival of phagocytosed bacteria, counts of living bacteria were carried out at different time points. As shown in Fig. 1B, bacterial counts dropped rapidly; 17 h after the infection, no living intracellular bacteria were found.

MDDC treated with living and inactivated *B. pertussis* acquire mature phenotype and functions. The ability of iMDDC to undergo phenotypic maturation when infected with *B. pertussis* was addressed. Table 1 summarizes the surface expression of the maturation markers CD83, CD80, and major histocompatibility complex (MHC) class II (HLA-DR) after infection with living bacteria or after incubation with HI bacteria. The latter were used to assess whether killed bacteria retain the ability to trigger MDDC maturation and functions.

B. pertussis infection induced increased expression of the maturation markers, comparable to that induced by *E. coli* LPS, used as a control maturation stimulus. Remarkably, HI *B. pertussis* also induced maturation of MDDC, triggering up-regulation of phenotypic markers to levels similar to those obtained with living bacteria. No significant differences in the expression of maturation markers induced by *B. pertussis* preparations and that induced by LPS were observed. All stimuli were able to significantly up-regulate CD80 and CD83 expression relative to that in untreated MDDC. HLA-DR expression was increased by all treatments, but the differences in expression between stimulated and untreated MDDC did not reach statistical significance (Table 1).

Cytokine production is a key step in the regulation of the immune response exerted by MDDC. Thus, to determine the impact of *B. pertussis* infection on the host response, it was important to study the induction of two relevant immunoregulatory cytokines produced by MDDC: IL-12 and IL-10. To better compare the results of the different experiments, considering the high donor variability presented by the different MDDC tested, the data are reported as percentages of cytokines secreted relative to secretion after LPS stimulation (taken as 100%).

While LPS-treated MDDC secreted high levels of IL-12, *B. pertussis*-infected MDDC were practically unable to induce any

TABLE 1. *B. pertussis*-infected MDDC are phenotypically mature^a

Stimulus ^b	Mean expression $\pm$ SE of the indicated surface marker (isotype-matched control antibody)		
	CD80 (MFI)	CD83 (% positive cells)	HLA-DR (MFI)
<i>B. pertussis</i> (<i>n</i> = 12)	44.29 $\pm$ 7.74 ^c (2.65 $\pm$ 0.22)	48.43 $\pm$ 5.89 ^c (0.05 $\pm$ 0.02)	156.75 $\pm$ 26.75 (2.26 $\pm$ 0.27)
HI <i>B. pertussis</i> (<i>n</i> = 6)	51.08 $\pm$ 11.32 ^c (2.50 $\pm$ 0.30)	43.67 $\pm$ 12.62 ^c (0.10 $\pm$ 0.03)	150.61 $\pm$ 40.46 (2.60 $\pm$ 0.27)
LPS (<i>n</i> = 18)	38.09 $\pm$ 2.7 ^c (2.48 $\pm$ 0.24)	55.18 $\pm$ 4.35 ^c (0.06 $\pm$ 0.01)	179.91 $\pm$ 22.77 (2.30 $\pm$ 0.25)
None (<i>n</i> = 18)	14.85 $\pm$ 1.64 (2.45 $\pm$ 0.26)	3.78 $\pm$ 0.85 (0.06 $\pm$ 0.02)	114.16 $\pm$ 29.19 (2.72 $\pm$ 0.24)

^a iMDDC were treated with the indicated stimuli and analyzed for surface markers.

^b *n*, number of experiments.

^c *P* < 0.001 by Student's *t* test, in each column, for comparison between stimulated and unstimulated (none) DC cultures.

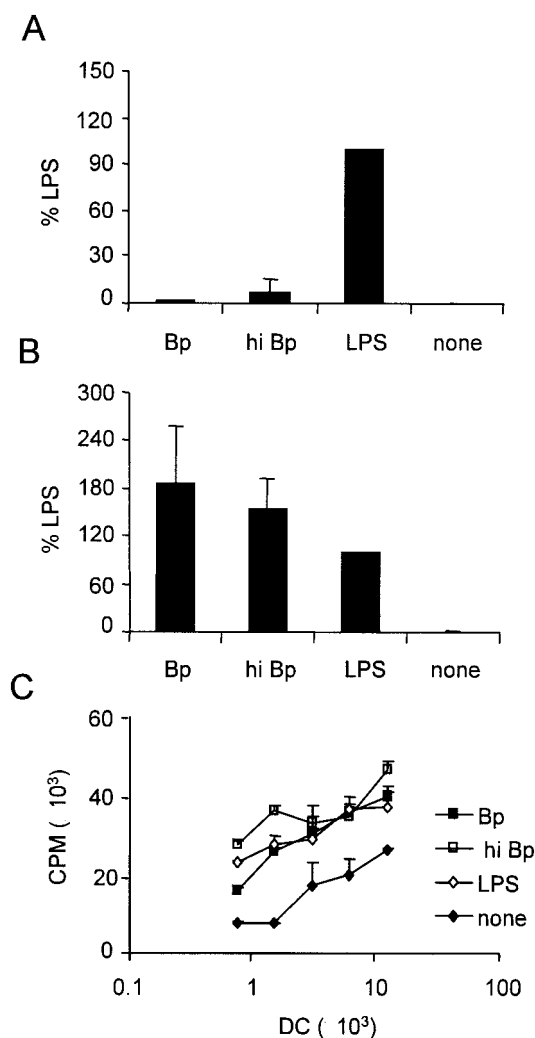


FIG. 2. Cytokine production and allostimulatory activity of MDDC treated with *B. pertussis*. (A and B) IL-12 (A) and IL-10 (B) production by MDDC after incubation with live (Bp) or HI (hi Bp) bacteria. Values are expressed as means $\pm$ SEs and are reported as percentages of cytokine production levels by cells treated with *E. coli* LPS, taken as 100% (in response to LPS, IL-12 production was 286.9 ± 60.78 pg/ml [$n = 15$] and IL-10 production was 546.3 ± 147.6 pg/ml [$n = 15$]). (C) MDDC treated with Bp, hi Bp, or LPS, or left untreated (none), were cocultured, at different numbers, with allogeneic purified T cells. Proliferation was assessed by [³H]thymidine incorporation. Results are reported as mean counts per minute (CPM) $\pm$ SE from three independent experiments.

IL-12 secretion (Fig. 2A). In contrast, *B. pertussis*-infected MDDC released high levels of IL-10, comparable to that induced by LPS (Fig. 2B). The differences in levels of secreted IL-12 between *B. pertussis*- and LPS-treated MDDC were statistically significant ($P < 0.001$). The results obtained with HI *B. pertussis* were similar to those obtained with living bacteria (Fig. 2A and B), and no statistically significant differences between live and HI bacteria were found.

Critical aspects of DC functions *in vivo* are antigen presentation and T-cell activation. Hence experiments were performed to evaluate the capacity of MDDC, following maturation in the presence of *B. pertussis*, to stimulate alloreactive T

cells. Figure 2C shows that MDDC matured in the presence of live or HI *B. pertussis* were able to increase allogeneic T-cell proliferation to the same levels as *E. coli* LPS, while unstimulated MDDC showed a low degree of allostimulatory activity.

Overall, these data show that *B. pertussis* is able to promote the transition from an immature to a mature stage of MDDC, including antigen-presenting-cell (APC) functions. Remarkably, matured MDDC are characterized by induction of elevated levels of IL-10 while they secrete barely detectable levels of IL-12.

***B. pertussis*-treated MDDC express IL-23 mRNA and drive a Th1-oriented polarization.** To further evaluate the ability of *B. pertussis*-treated MDDC to influence the specific antipertussis immunity, we performed a set of experiments assessing their capacity to drive polarization of autologous CD45RA⁺ T cells.

HI *B. pertussis* treatment enhances the Th1 polarization ability of MDDC. Indeed, higher levels of IFN- γ were produced by polarized T cells induced by HI *B. pertussis*-treated MDDC than by those induced by LPS-treated MDDC. Furthermore, the levels of IL-5 and IL-10 induced by HI *B. pertussis*-treated MDDC were lower than those induced by LPS (Fig. 3A).

These data were confirmed at the intracellular level. As shown in Fig. 3B, the percentage of IFN- γ -secreting cells was higher (38.9 versus 28.7%) in naïve T cells cocultured with HI *B. pertussis*-treated MDDC than in those cocultured with LPS-treated MDDC, while the percentage of IL-4-producing naïve T cells was decreased (4.9 versus 8.8%). Cord blood lymphocytes were also used to assess the polarizing ability of *B. pertussis*-treated MDDC; in this case also, a marked Th1 polarization was observed (data not shown).

Since polarization experiments require long-term cultures, use of MDDC infected with living bacteria was not possible because of the scarce amounts of T lymphocytes recovered at the end of the culture. We therefore performed polarization experiments on CD45RA⁺ naïve T lymphocytes by using CM obtained from infected MDDC (Bp-CM) and, as controls, CM from either untreated or *E. coli* LPS-treated MDDC. Table 2 summarizes data from IFN- γ and IL-4 intracytoplasmic staining of naïve T lymphocytes. The results were comparable to those obtained with HI *B. pertussis*-treated MDDC; indeed, the percentage of IFN- γ -producing cells was remarkably enhanced when Bp-CM were used.

Thus, taken together, these results underline a clear Th1 orientation of the immune response induced by *B. pertussis*.

In order to test the possibility that endogenous IL-10 was responsible for the inhibition of IL-12 production by *B. pertussis*-treated MDDC, experiments were performed in the presence of a neutralizing anti-IL-10 MAb. As shown in Fig. 3C, the presence of the anti IL-10 MAb markedly enhanced IL-12 production by LPS-treated MDDC, while *B. pertussis*-treated MDDC were still unable to release appreciable levels of IL-12.

Another possible explanation for the lack of IL-12 accumulation in culture supernatants is that *B. pertussis* may inhibit the synthesis and release of the IL-12 p40 subunit. To verify this hypothesis, MDDC were stained intracellularly with a MAb (IL-12 p40/p70) which reacts with the IL-12 p40 monomer and the IL-12 p70 heterodimer (composed of the p35 and p40 subunits) but not with the IL-12 p35 monomer. When protein secretion was blocked by brefeldin A, both live and HI *B. pertussis*-treated MDDC were positively stained by the intra-

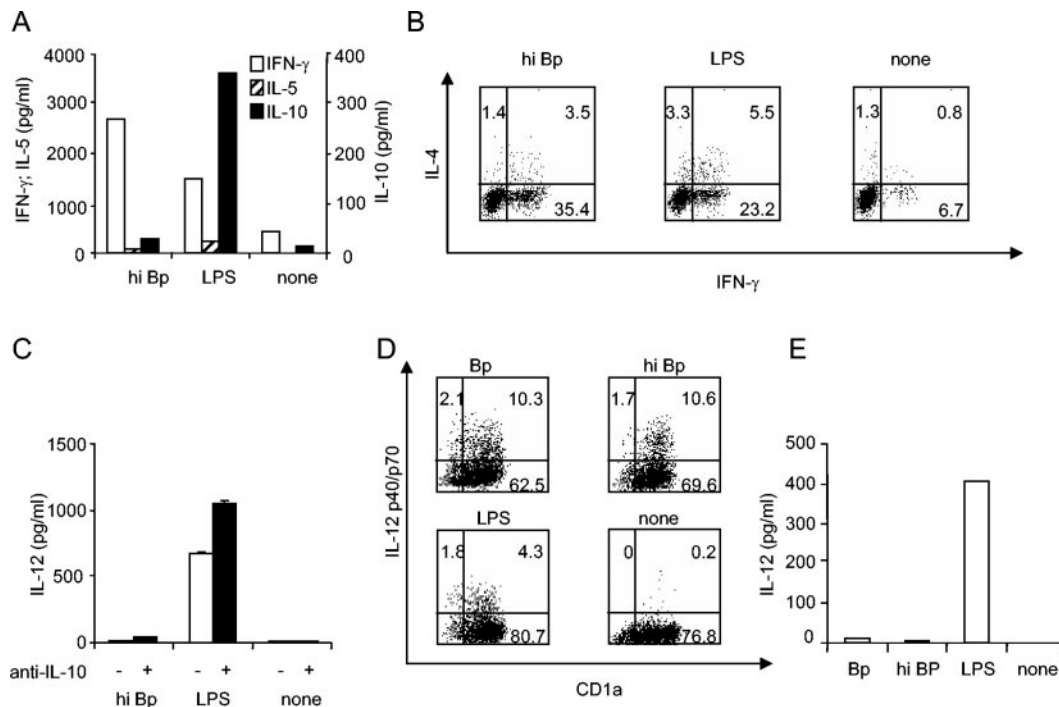


FIG. 3. *B. pertussis*-treated MDDC drive Th1 polarization of naïve T lymphocytes but do not produce a bioactive IL-12 p70 dimer. (A) MDDC either treated with HI *B. pertussis* (hi Bp) or *E. coli* LPS or left untreated (none) were cocultured with purified CD45 RA⁺ T cells as described in Materials and Methods. On day 12, supernatants were collected and secreted cytokines were measured by a CBA assay. Results of one representative experiment of three performed are shown. (B) T cells were washed and restimulated with PMA-ionomycin for IFN- γ and IL-4 intracellular staining. Numbers in each quadrant indicate the percentage of positive cells. Results of one representative experiment of three performed are shown. (C) IL-12 production by MDDC either treated with hi Bp or LPS or left untreated, in the absence or presence of an anti-IL-10 MAb (10 μ g/ml), as indicated. Results are means $\pm$ SEs from three independent experiments. (D) MDDC either treated with Bp, hi Bp, or LPS or left untreated were stained for intracellular IL-12. Numbers in each quadrant indicate the percentage of IL-12 p40/p70- and CD1a-positive cells. (E) IL-12 p70 release in cell culture supernatants from the experiment for which results are shown in panel D. Results of one representative experiment out of three performed for hi Bp and LPS and two performed for Bp are shown in panels D and E.

cellular MAb to an even greater extent than *E. coli* LPS-treated MDDC (Fig. 3D). When protein secretion was not blocked, both LPS- and *B. pertussis*-treated MDDC were negative for IL-12 p40/p70 intracellular staining (data not shown). Since IL-12 p70 was found in LPS-treated MDDC supernatants and was not detected in live or HI *B. pertussis*-treated MDDC supernatants (Fig. 3E), it was reasonable to assume that *B. pertussis* induces the expression and secretion of the IL-12 p40 subunit but not that of the IL-12 p70 dimer.

Several studies have shown that IL-12 p40 can assemble not only into heterodimeric IL-12 p70 but also into homodimeric

p40 or into the recently discovered Th1-promoting cytokine IL-23, which is composed of the p19-p40 dimer (31). Thus, the expression of p19, p35, and p40 by *B. pertussis*-treated MDDC was assessed by quantitative RT-PCR at different time points. As shown in Fig. 4, live (Fig. 4A) and HI (Fig. 4B) *B. pertussis* induced considerable levels of p40 and p19 mRNA transcription, similar to that obtained upon *E. coli* LPS-induced MDDC maturation, even though, in the case of live bacteria, decreased values and delayed kinetics for p40 mRNA expression were observed. However, the data from IL-12 p40 intracellular staining (Fig. 3D) indicate that live bacteria are able to induce the same protein levels as HI *B. pertussis*, suggesting that the differences in p40 mRNA expression observed here may depend on donor-to-donor variability. Conversely, p35 mRNA transcription, high in LPS-treated MDDC, is almost completely absent in both live and HI *B. pertussis*-treated MDDC.

These results suggest that *B. pertussis* is unable to induce the IL-12 p35 subunit, while it selectively induces production of the p40 and p19 monomers to form IL-23, a cytokine promoting Th1 polarized effector cells.

DISCUSSION

No data are available at the moment regarding infection of human DC by *B. pertussis*, their maturation, and their conse-

TABLE 2. *B. pertussis* CM induce Th1 polarization of naïve T lymphocytes^a

Stimulus	% of cells positive ^b for:	
	IFN- γ	IL-4
Bp-CM	48.11 $\pm$ 0.26	1.22 $\pm$ 0.14
LPS-CM	38.73 $\pm$ 4.19	0.38 $\pm$ 0.32
None-CM	18.61 $\pm$ 3.56	1.02 $\pm$ 0.41

^a Naïve T lymphocytes were cultured in the presence of PHA and diluted (50%, vol/vol) with CM obtained from MDDC cultured for 48 h with the indicated stimuli. On day 5, rIL-2 was added. On day 12, T cells were washed and restimulated with PMA-ionomycin in the presence of brefeldin A for IFN- γ and IL-4 intracellular staining.

^b Values are means $\pm$ SEs from three independent experiments.

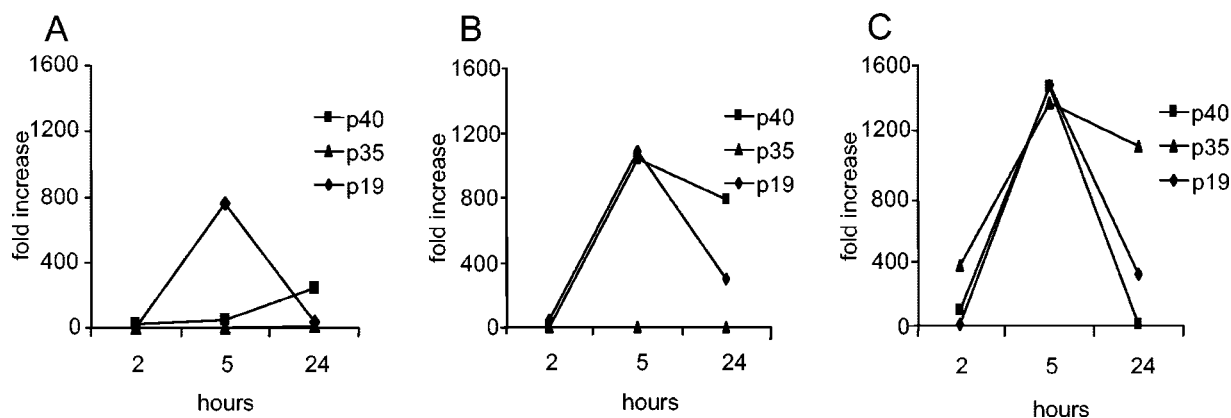


FIG. 4. *B. pertussis*-treated MDDC expressed p40 and p19 but did not express p35 monomers: MDDC were cultured in the presence of *B. pertussis* (A), HI *B. pertussis* (B), or *E. coli* LPS (C). At the indicated time point, a Taqman real-time quantitative RT-PCR for p35, p40, and p19 gene expression was performed. mRNA transcript levels were expressed as fold increase over those in unstimulated MDDC at 2 h. Results of one representative experiment of four performed for HI *B. pertussis* and LPS and two performed for *B. pertussis* are shown.

quent orchestration of the host immune response. Recent studies have shown that neutrophils and monocytes internalize *B. pertussis*, even if the cells are infected by a low number of bacteria, and efficiently kill intracellular bacteria, suggesting that professional phagocytes contribute to bacterial clearance (38, 45).

In a murine model of *Bordetella bronchiseptica* infection, DC counteract the infection; they are rapidly recruited in the upper respiratory tract and subsequently delivered to the lymph nodes draining the respiratory mucosa (16). In addition, Guzman and colleagues found that *B. bronchiseptica* efficiently invades mouse spleen DC and replicates intracellularly (18). These results are quite different from the data obtained in our study, confirming the intrinsic differences between *B. pertussis* and *B. bronchiseptica* (18, 39). Indeed, human MDDC are characterized by a low capacity to internalize *B. pertussis* compared to that of monocytes. In addition, *B. pertussis* fails to survive inside MDDC. Overall, even if we did not measure true phagocytosis by using live bacteria expressing green fluorescent protein (45), our results seem to confirm that the ability of *B. pertussis* to escape the effector cells of innate immunity is due more to its ability to evade phagocytosis than to intracellular survival (38, 45).

Phenotypic analysis of *B. pertussis*-treated MDDC reveals that maturation is induced even if only a low number of bacteria are internalized; thus, exposure to *B. pertussis* triggers maturation simply by contact with bacterial cells or bacterial products. Notably, no significant differences were observed between the abilities of living and HI bacteria to induce MDDC phenotypic maturation, suggesting that the process is triggered mainly by epitopes from *B. pertussis* that are also expressed in killed bacteria. Several studies concerning the influence of *B. pertussis* on cytokine production by APC have been carried out in both murine and human systems. In mice, *B. pertussis* infection and vaccination with whole-cell vaccines have been reported to induce IL-12 production by macrophages (25, 32). Moreover, murine DC have been shown to secrete IL-10 in addition to IL-12 and inflammatory cytokines upon encountering living or inactivated *B. pertussis* and have

been characterized as promoting the induction of T-regulatory (Tr) clones (22).

B. pertussis virulence factors such as filamentous hemagglutinin (FHA) and adenylate cyclase toxin (ACTX) have been shown in mice to drive the immune response toward a Tr type 1 (Tr1) and a mixed Th2–Tr1 response. In particular, both FHA and ACTX inhibited IL-12 production and stimulated IL-10 production by DC (27, 35).

In humans, induction of Th1 responses after *B. pertussis* infection or vaccination with whole-cell vaccines has been reported (2, 37), and we have previously shown that pertussis toxin (PTX) synergizes with LPS, promoting an MDDC-driven Th1 polarization mediated by elevated IL-12 production (3). These data were substantially confirmed by Tonon and colleagues, who showed that PTX induces DC maturation and IL-12 production (43). In addition, in an experimental model of autoimmune uveitis, PTX induced high levels of IL-12 and promoted a Th1 polarized response (23).

Recently reported data showed that human monocytes produce high levels of IL-10 after *B. pertussis* infection, but IL-12 production was not addressed (40). The same authors showed that *B. pertussis* infection of human peripheral blood monocytes determines a diminished surface expression of HLA-DR, reflecting intracellular redistribution (40). The authors state that PTX is responsible for suppression of HLA-DR expression and sequestration of peptide-MHC complexes. This seems not to be the case in the MDDC system. Indeed, in previous work we have shown that both native and detoxified PTX treatment of MDDC increased HLA-DR expression (3). Furthermore, in the present study, HLA-DR was up-regulated on the surfaces of MDDC upon *B. pertussis* infection (Table 1). However, we did not analyze the intracellular distribution of HLA-DR. *B. pertussis*-infected MDDC, nevertheless, enhance the proliferation of allogeneic T cells, giving evidence of efficient antigen presentation.

The production of high levels of IL-10 but only barely detectable levels of IL-12 is conceivable with a typical cytokine pattern of a Th2–Tr-oriented immune response (27, 35). We were surprised, therefore, to find that both live and HI *B.*

pertussis-treated MDCC promote a Th1 polarization of naïve T cells. To better understand the mechanisms underlying this polarization pattern, we decided to address the issue of IL-10 involvement in greater depth, since endogenous IL-10 has been shown to suppress IL-12 expression, acting at the level of transcription of both the p35 and p40 subunits (1, 12). Neutralization of endogenous IL-10 in our cultures with a specific MAb does not restore the production of IL-12, suggesting that in this case IL-10 is not responsible for IL-12 inhibition. In addition, intracellular staining indicates that only the IL-12 p40 subunit is produced and secreted by live and HI *B. pertussis*-treated MDCC, while mRNA transcription analysis showed that both live and HI *B. pertussis*-treated MDCC are unable to induce IL-12 p35 mRNA. All this indicates the inability of *B. pertussis*-treated MDCC to induce IL-12. Nonetheless, IL-12 p40 has been shown to be assembled in a heterodimeric complex with p19 to form the newly described Th1-promoting cytokine IL-23 (31); thus, the Th1 polarization of the immune response could be explained by the induction by *B. pertussis* of the IL-23 cytokine in MDCC. Quantitative RT-PCR results showed that p19 was indeed expressed at high levels by *B. pertussis*-treated MDCC.

IL-23 has been reported by Oppmann and colleagues to preferentially activate proliferation and IFN- γ production in CD45RO⁺ memory T cells but to be less active in the activation of CD45RA⁺ naïve T cells (31). In this study, we found that naïve T cells polarized by *B. pertussis*- or *E. coli* LPS-treated MDCC produced IL-23 alone or both IL-23 and IL-12, respectively, and we did not find great differences when an enriched population of CD45RO⁺ T cells was used (data not shown). However, the experimental settings were considerably different, since Oppmann and colleagues treated T-cell blasts with exogenous recombinant IL-23, while we tested the polarizing activity of endogenous IL-23 produced by *B. pertussis*-treated MDCC. It is also possible to hypothesize that, beside a direct role, IL-23 acts indirectly by inducing the production of other Th1-polarizing cytokines; indeed, IL-23 has been reported to induce IL-12 and IFN- γ production by murine DC (9).

The finding that IL-23 rather than IL-12 is produced by *B. pertussis*-treated MDCC confirms the pivotal role of IL-23 in counteracting invading pathogens (11, 13, 19).

Different mechanisms could be hypothesized in the inhibition of IL-12 production, a phenomenon that appears to be due to surface contact with the bacteria (external signaling) rather than to internalization (internal signaling). For example, selective inhibition of IL-12 p35 expression has already been described (15, 24). IFN regulatory factor 1 (IRF-1)-deficient macrophages have a selective impairment in the synthesis of IL-12 p35 mRNA but not of the p40 gene (15). Furthermore, differential regulation of IL-12 p35 occurs via a calcium-dependent but Erk-independent mechanism, which is likely to involve NF- κ B signaling (24).

One potential candidate for *B. pertussis*-mediated IL-12 inhibition in human MDCC is the complement 3 receptor (CR3). CR3 mediates the binding of several pathogens including *B. pertussis* (17, 34), and the binding of specific antibodies to CR3 suppresses IL-12 secretion by human monocytes via the inhibition of IFN- γ -induced tyrosine phosphorylation (26).

Furthermore, a recent study demonstrated the existence of

two subtypes of human macrophages (M ϕ -1 and M ϕ -2) active in immunity to mycobacteria (44). After mycobacterial stimulation, M ϕ -1, like *B. pertussis*-treated MDCC, do not secrete IL-12 but are able to drive Th1 polarization via secretion of IL-23. In contrast, M ϕ -2 produce neither IL-12 nor IL-23 but predominantly IL-10. Overall, it is possible to hypothesize that different stimuli trigger the induction of different Th1-polarizing cytokines. Alternatively, it is possible to imagine the existence of different subpopulations of MDCC which are primed by distinct stimuli and are able to produce different Th1-polarizing cytokines.

In addition, LPS from *B. pertussis* could play a role in the regulation of IL-12 cytokine family expression. In fact, purified LPS from *B. pertussis* is not able to induce the expression of either IL-12 p35 or IL-12 p40, and consequently the secretion of IL-12, in MDCC, but when it was used in association with PTX, a synergistic increase in the release of IL-12 was observed. Studies are now in progress to better define this regulation pathway (G. Fedele et al., unpublished data). The elevated levels of IFN- γ induced by PTX in MDCC may also play a role (3).

The present study provides new hints about the DC-mediated orchestration of immunity. The redundancy of the IL-12 family of dimeric cytokines is efficiently exploited by human DC to favor the onset of a Th1 immune response to *B. pertussis*. CMI has been shown to play a crucial role in anti-*B. pertussis* defense; in particular, evidence has accumulated indicating that type 1 immunity is protective both in unvaccinated, repeatedly exposed adults (4) and in *B. pertussis*-infected or convalescent children (2, 5, 37). Furthermore, the induction of IL-23, which is involved mainly in the activation of the T memory response, could explain the persistence of CMI despite the rapid decline in antibody response (4, 10, 36).

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