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Attenuated *Bordetella pertussis* Vaccine Candidate BPZE1 Promotes Human Dendritic Cell CCL21-Induced Migration and Drives a Th1/Th17 Response

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New vaccines against pertussis are needed to evoke full protection and long-lasting immunological memory starting from the first administration in neonates—the major target of the life-threatening pertussis infection. A novel live attenuated *Bordetella pertussis* vaccine strain, BPZE1, has been developed by eliminating or detoxifying three important *B. pertussis* virulence factors: pertussis toxin, dermonecrotic toxin, and tracheal cytotoxin. We used a human preclinical ex vivo model based on monocyte-derived dendritic cells (MDDCs) to evaluate BPZE1 immunogenicity. We studied the effects of BPZE1 on MDDC functions, focusing on the impact of *Bordetella*-primed dendritic cells in the regulation of Th and suppressor T cells (Ts). BPZE1 is able to activate human MDDCs and to promote the production of a broad spectrum of proinflammatory and regulatory cytokines. Moreover, conversely to its parental wild-type counterpart BPSM, BPZE1-primed MDDCs very efficiently migrate in vitro in response to the lymphatic chemokine CCL21, due to the inactivation of pertussis toxin enzymatic activity. BPZE1-primed MDDCs drove a mixed Th1/Th17 polarization and also induced functional Ts. Experiments performed in a Transwell system showed that cell contact rather than the production of soluble factors was required for suppression activity. Overall, our findings support the potential of BPZE1 as a novel live attenuated pertussis vaccine, as BPZE1-challenged dendritic cells might migrate from the site of infection to the lymph nodes, prime Th cells, mount an adaptive immune response, and orchestrate Th1/Th17 and Ts responses. ***The Journal of Immunology*****, 2011, 186: 5388–5396.**

Despite the fact that efficacious vaccines against *Bordetella pertussis* are licensed and widely used, *B. pertussis* continues to circulate even in populations with high vaccine coverage and represents the second largest cause of vaccine-preventable death in children under 5 y of age (1). Approximately 40 million whooping cough cases and between 200,000 and 400,000 pertussis-linked deaths are recorded each year (2).

Pertussis may be particularly severe and even life-threatening in infants, especially in neonates, who are not sufficiently protected until the age of 6 mo with the currently available vaccines and vaccination schedules. Hence, new vaccines are needed to evoke full protection in the most vulnerable infants and long-lasting immunological memory starting from the first administration.

Recently, a novel live attenuated *B. pertussis* vaccine strain, BPZE1, has been developed by eliminating or detoxifying three important *B. pertussis* virulence factors (3). Pertussis toxin (PT) was genetically detoxified, resulting in inactivation of the toxin without altering its immunogenicity. The gene encoding dermonecrotic toxin (DNT) was deleted from the *B. pertussis* chromosome by homologous recombination. Tracheal cytotoxin (TCT), a major factor destroying ciliated cells of the respiratory epithelium, was eliminated by replacing the *B. pertussis ampG* gene with that of *Escherichia coli*.

In mice, BPZE1 was able to induce strong protective immunity after a single nasal administration. Protection against *B. pertussis* was comparable with that induced by two injections of acellular vaccine in adult mice and was significantly better than that induced by two administrations of acellular vaccine in infant mice (3). Moreover, histopathological and physiological studies in mice confirmed that *B. pertussis* BPZE1 is highly attenuated, although it is still able to transiently colonize the mouse respiratory tract and thereby induce strong protection upon a single intranasal administration (4). It has also been shown that BPZE1 is safe in neonatal and immunodeficient mouse models and induces strong Th1 responses in adult mice (5). It is currently undergoing a phase I safety trial in humans (http://www.child-innovac.org).

With the aim to evaluate BPZE1 immunogenicity in human preclinical models, in this study we used an ex vivo model based on monocyte-derived dendritic cells (MDDCs) challenged with BPZE1. Ex vivo-generated MDDCs represent a powerful tool to reproduce several aspects of the immune response, from pathogen recognition to Th cell polarization, and may provide valuable preclinical immunological data, in particular concerning the modulation of innate and acquired immune responses (6). The effector T cell repertoire includes Th1, Th2, and Th17 cells, involved in protection against intracellular pathogens, parasite and extracellular bacteria, respectively (7). Activation and expansion of these cells are regulated by suppressor T cells (Ts), which act through

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Abbreviations used in this article: DC, dendritic cell; DNT, dermonecrotic toxin; mDC, LPS-matured MDDC; MDDC, monocyte-derived DC; PT, pertussis toxin; TCT, tracheal cytotoxin; Treg, regulatory T cell; Ts, suppressor T cells.

the production of immunoregulatory cytokines or cell-to-cell contact (7, 8).

We have previously shown that human immature MDDCs upon *B. pertussis* stimulation undergo phenotypic maturation, acquire APC functions, and drive the expansion of Th1 and Th17 effector cells. In particular, our studies shed light on the contribution of several virulence factors, such as adenylate cyclase toxin (9–11), lipooligosaccharide (12, 13), and PT (14, 15).

We studied the immunomodulatory properties of BPZE1 and its parental wild-type counterpart, BPSM, on human MDDC functions, focusing on the impact of *Bordetella*-primed dendritic cells (DCs) in the regulation of Th and Ts systems. We demonstrated that BPZE1 maintains the capacity to activate MDDCs, which acquire APC activity, and to induce a broad spectrum of proinflammatory and regulatory cytokines, driving Th1 and Th17 responses as well as Ts. Remarkably, the attenuation present in the BPZE1 strain impairs a key immune evasion mechanism promoted by *B. pertussis*, the inhibition of CCR7-driven migration of DCs to the lymph nodes, where Ag presentation to naive Th cells occurs.

Overall, BPZE1 appears to be a good vaccine candidate because of its ability to promote migration of MDDCs to the lymph nodes and to foster APC functions, driving a Th1/Th17 response. The induction of this Th response is considered involved in protection, even if conclusive demonstration in pertussis is still not available (16–18).

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. All blood donors provided written informed consent for the collection of samples and subsequent analysis, and the blood samples were processed anonymously.

Reagents

Polymyxin B and brefeldin A were purchased from Sigma Chemicals (St. Louis, MO). Purified *E. coli* LPS was from Cayla-InvivoGen Europe (Toulouse, France). Human rGM-CSF and rIL-4 were from R&D Systems (Minneapolis, MN). rIL-2 was obtained from Roche (Basel, Switzerland).

Bacterial strains and growth conditions

The strains used in this study are all derived from *B. pertussis* Tohama I. BPSM (19), BPZE1 and BPQJ20, deficient in TCT release (3), have been described previously. BPRA (PT^-) has a deletion of the PT gene (20). BPSA175 (dPT) produces genetically inactivated PT and was constructed by inserting pPT-RE (21) into the *ptx* locus of *B. pertussis* BPRA by homologous recombination. *B. pertussis* BPSMDN was constructed by deleting the DNT gene in *B. pertussis* BPSM using the pJQmp200rpsL18 derivative described in Ref. 3.

All *Bordetella* strains were grown on charcoal agar plates supplemented with 10% sheep blood (Oxoid, Basingstoke, UK) for 48 h at 37°C. Bacteria were then collected and resuspended in 2 ml PBS, and the concentration was estimated by measuring the OD at 600 nm. The suspensions were adjusted to a final concentration of 10^9 CFU/ml. The bacterial concentration was then checked retrospectively by CFU evaluation of the final bacteria suspension.

Purification and culture of MDDCs

Human monocytes were purified as described (10, 11) from peripheral blood of healthy blood donors (courtesy of Dr. Girelli, "Centro Trasfusionale Policlinico Umberto I," University La Sapienza, Rome, Italy) and cultured in RPMI 1640 medium (Life Technologies Invitrogen, Paisley, U.K.), supplemented with heat-inactivated 10% LPS-screened FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, all from Hyclone Laboratories (South Logan, UT), and 0.05 mM 2-mercaptoethanol (Sigma) (hereafter defined as complete medium) in the presence of GM-CSF (25 ng/ml) and IL-4 (25 ng/ml). After 6 d, immature MDDCs were washed and analyzed by cytofluorometry for the expression of surface markers CD1a, CD14, CD83, and CD38. MDDCs were used in the experiments if >80% CD1a and <10% CD14.

MDDCs (10^6 cell/ml) were resuspended in complete medium without penicillin and streptomycin and treated with *B. pertussis* cells as described elsewhere (9). After 2 h, cells were extensively washed in the presence of polymyxin B (5 μg/ml) to kill extracellular bacteria and incubated at 37°C, 5% CO_2 for 48 h in complete medium.

After 48 h, the treated MDDCs were harvested for immunophenotypic analysis, and the supernatants were collected for cytokine measurement by ELISA.

Immunophenotypic analysis

Cells were washed and resuspended in PBS containing 3% FBS and 0.09% NaN_3, then incubated with a panel of fluorochrome-conjugated mAbs (obtained from BD Biosciences, San Jose, CA) specific for MDDC (anti-CD14, CD1a, CD80, CD83, and CD38) or specific for T cells (anti-CD3, anti-CD45RA, anti-CD25, and anti-FOXP3). Isotype-matched Abs were used as negative controls. Cells were analyzed with a FACScan or FACSCanto (BD Biosciences). Fluorescence data were reported as percentages of positive cells when the treatment induced the expression of the marker in cells that were negative; median fluorescence intensity was used when the treatment increased the expression of the marker in cells that were already positive.

Chemokine-driven chemotaxis

Chemotaxis experiments were performed as described elsewhere (22). Briefly, 600 μl complete medium containing 10 ng/ml CCL21 was added to the lower chamber of polycarbonate filters of 5-μm-pore size, 24-Transwell chambers (Costar, Corning Life Sciences, Lowell, MA). Then, 1.25×10^5 MDDCs (cell input) challenged with *B. pertussis* for 48 h were added to the upper chamber in 100 μl complete medium and incubated for 3 h. Migrated cells were counted by flow cytometry in a FACScan, acquiring events for 60 s using CellQuest software (BD Biosciences).

Isolation of T lymphocytes and MDDC–T cell allogeneic MLR

CD3 T cells were purified from PBMCs by negative sorting with magnetic beads (*Pan* T-cell Kit, Miltenyi Biotec, Auburn, CA). Purity of cell preparations was assessed by cytofluorometric staining.

MDDCs were cultured with *B. pertussis* strains for 48 h, washed extensively, and cultured in an MLR with T cells (5×10^5) at different MDDC/T cell ratios in 48-well cell culture plates for 6 d. Cell proliferation in MLR was measured by BrdU incorporation evaluated by direct immunofluorescence with an FITC-conjugated anti-BrdU mAb (BD Biosciences). Briefly, BrdU (BD Biosciences) was added to MDDC and T cell MLR at 3 μg/ml final concentration on day 3 and on day 5 of culture. Cells, collected on day 6, were fixed in 0.5% paraformaldehyde, permeabilized and stained for intracellular BrdU by direct immunofluorescence with an FITC-conjugated anti-BrdU mAb (BD Biosciences). Cells were examined by flow cytometry, and T cell proliferation was evaluated. Data were expressed as percentage of positive cells.

Polarization of T lymphocytes

$CD45RA^+$ naive T cells were purified from T cells by negative sorting with anti-CD45RO–conjugated magnetic beads (Miltenyi Biotec). MDDCs (0.5×10^5) were treated with *B. pertussis* for 48 h and then cocultured with $CD3^+$ or $CD3^+/CD45RA^+$ T cells (0.5×10^6) in 24-well plates (Costar, Corning Life Sciences). On day 6, IL-2 (50 U/ml) was added to the cultures. On days 6 and 12, supernatants were harvested for cytokine measurement. On day 12, cells were activated with PMA (40 ng/ml) and ionomycin (1 μg/ml) for 5 h in the presence of brefeldin A, a compound that blocks proteins in the endoplasmic reticulum, thus inhibiting cellular secretion. Cytokine production in T cells was measured by intracellular staining.

Analysis of Ts function

Purified naive T cells were exposed to allogeneic MDDCs treated with *B. pertussis* (MDDC/T cell ratio 1:10) (primary MLR) to generate Ts. Six days later, T cells were recovered and cultured at different numbers with syngeneic T cells (5×10^5) in the presence of allogeneic LPS-matured MDDCs (mDCs) (0.5×10^5) in 48-well cell culture plates (secondary MLR). In some experiments to determine TGF-β production, MDDCs and T cells were cocultured in serum-free medium using a substitute of bovine serum (BIT 9500; Stemcell Technologies, Vancouver, BC, Canada). In some experiments, the cultures were performed in the presence of blocking anti–IL-10 (10 μg/ml) and anti–TGF-β (10 μg/ml) Abs. Results were reported as proliferation index calculated as the percentage of BrdU incorporated by the MLR in the presence of Ts with respect to control MLR set as 100%.

To determine the cell-contact dependence of the regulatory response, syngeneic T cells (5×10^5) and allogeneic mDCs (0.5×10^5) were cultured in the bottom well of polycarbonate filters of 0.4-μm-pore size, 24-Transwell chambers (Costar, Corning Life Sciences), which does not allow cell passage in the lower chamber. The T cells (5×10^5) recovered from the primary MLR with allogeneic mDCs were added in the upper Transwell chamber. After 6 d, the proliferative response of the T cells in the lower chamber was measured by means of BrdU incorporation.

Determination of cytokine levels by ELISA

To measure cytokine production, MDDCs were cultured in the presence of the indicated stimuli in 14-ml round-bottom tubes (Falcon; Becton Dickinson, Lincoln Park, NJ) at 37˚C and 5% CO_2. Supernatants were collected after 48 h, and IL-10, IL-12p70, IL-1β, IL-6 (Quantikine; R&D Systems), and IL-23 (Bender MedSystem, Burlingame, CA) production was assessed by ELISA with a sensitivity of 1.0 pg/ml for IL-1β, 0.7 pg/ml for IL-6, 3.9 pg/ml for IL-10, 5.0 pg/ml for IL-12p70, and 20.0 pg/ml for IL-23. OD obtained was measured with a 3550-ultraviolet Microplate Reader (Bio-Rad, Philadelphia, PA) at 450 nm.

Cytokines in the supernatants from polarized T cells were assayed by ELISA specific for IFN-γ, IL-5 and IL-17, IL-10 and TGF-β (Quantikine; R&D Systems). The lower detection limits were 8.0 pg/ml for IFN-γ, 3.0 pg/ml for IL-5, 15.0 pg/ml for IL-17, and 4.6 pg/ml for TGF-β.

Intracellular cytokine staining

T lymphocytes were stained using fluorochrome-conjugated anti-CD3 mAb (BD Biosciences). Cells were then fixed and permeabilized using Cytofix-Cytoperm and Perm/Wash protocols (BD Biosciences) and stained with a predetermined optimal concentration of fluorochrome-conjugated anti-cytokine Abs (IFN-γ, IL-4, IL-17, FOXP3) or appropriate isotype controls. Cells were analyzed by flow cytometry in a FACScan or FACSCanto flow cytometer.

Statistical analysis

Statistical descriptive analyses were carried out using the SPSS statistical package (SPSS, Chicago, IL). Differences between mean values were assessed by two-tailed Student *t* test and were statistically significant for *p* values <0.05.

Results

Phenotypic maturation, viability, and cytokine production in MDDCs challenged with different doses of BPZE1

In a previous study, we set up an experimental protocol for MDDC challenge with virulent *B. pertussis* (9). We found that the bacteria were scarcely phagocytosed and rapidly killed intracellularly, and that bacterial contact rather than internalization induced the onset of the maturation program. Moreover, we found that a bacteria/cell ratio of 100:1 allowed for optimal MDDC maturation. The optimal BPZE1 dose to challenge MDDCs (i.e., the ratio that allows maximization of MDDC activation without affecting MDDC viability) was determined in dose-response experiments ranging from 20 to 500 bacteria per MDDC, and the induction of phenotypic maturation, cell viability, and cytokine production were assessed.

Phenotypic maturation was evaluated by monitoring the surface expression of the costimulatory molecule CD80 and the maturation markers CD83 and CD38. At all bacteria/cell ratios tested, both the vaccine strain BPZE1 and the virulent parent strain BPSM induced a significant upregulation of all markers compared with that in untreated MDDCs (Fig. 1).

MDDC viability was assessed by annexin V/propidium iodide staining to measure apoptosis and necrosis. In preliminary experiments, a time-course analysis of apoptosis induction was performed, showing that both BPZE1 and BPSM bacteria did not induce protection from apoptosis at 8 h and induced a similar protection at 24 and 48 h. Therefore, 24 h was chosen for the next experiments. An increase in the percentage of apoptotic and necrotic cells was induced by both bacterial strains when the 500:1 dose was used (Fig. 2). In contrast, incubation at 20:1 and 100:1

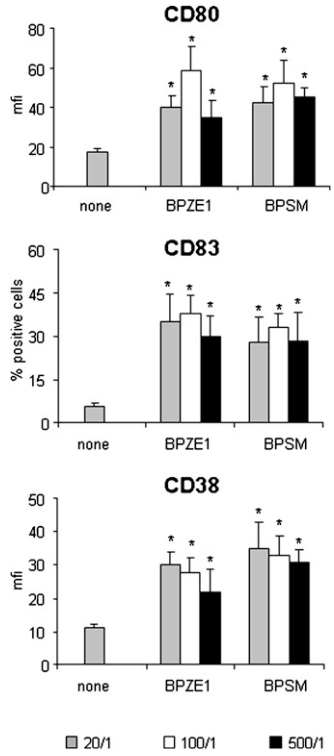

FIGURE 1. Induction of human MDDC maturation. MDDCs either untreated (none) or challenged with BPZE1 or BPSM at the indicated ratios for 48 h were analyzed for surface markers associated with a mature phenotype. Fluorescence data are reported as median fluorescence intensity (mfi) when treatment increased the expression of the marker in cells that were already positive (CD80, CD38); otherwise, percentage of positive cells is used (CD83). Mean expression ± SE of 11 independent experiments performed with MDDCs obtained from different donors is indicated. $*p < 0.05$ versus none.

ratios statistically significant promoted the resistance of MDDCs to apoptosis, spontaneously occurring when the cells were not stimulated (Fig. 2*A*), and both 20:1 and 100:1 ratios did not enhance necrosis (Fig. 2*B*).

To determine the impact of BPZE1 on the host response, we studied the induction of relevant cytokines produced by MDDCs. In preliminary experiments, MDDCs were challenged with two different ratios, 20:1 and 100:1, and the levels of IL-12p70, and IL-10 were measured (Table I). IL-10 was produced in a dose-dependent fashion, without statistically significant differences between BPZE1 and BPSM. For IL-12p70, we only recorded a very modest production by BPSM-treated MDDCs challenged with 100 bacteria per cell.

These data confirmed that optimal MDDC stimulation was achieved when 100 bacteria per cell were used, in accordance with the previously published data (9), and showed no differences between BPZE1 and BPSM. Thus, to maximize the challenge of *B. pertussis* on MDDCs, the 100:1 ratio was chosen.

Migratory ability of MDDCs treated with BPZE1

The migration of DCs to lymph nodes is pivotal to the establishment of an immune response. Upon maturation, DCs express the chemokine receptor CCR7, which enables migration in response to gradients of lymphatic chemokines, such as CCL19 or CCL21. Ex vivo chemotaxis experiments were undertaken to verify whether BPZE1-challenged MDDCs were able to perform lymphatic migration.

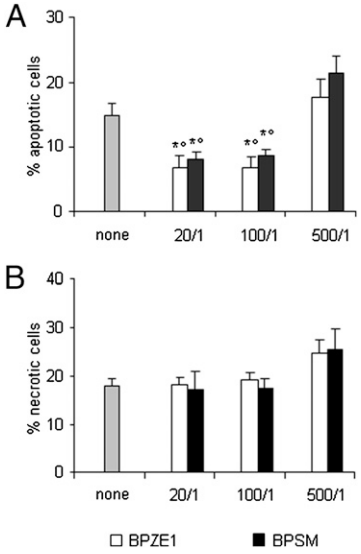

FIGURE 2. Resistance of MDDCs to spontaneous apoptosis. MDDCs were either untreated (none) or challenged with BPZE1 or BPSM at the indicated ratios for 24 h. Cells were stained with FITC-conjugated annexin V to assess apoptosis (*A*) and propidium iodide to assess necrosis (*B*) and analyzed by flow cytometry. Percentage of apoptotic or necrotic cells is shown. Mean expression ± SE of eight independent experiments performed with MDDCs obtained from different donors is indicated. *$p <$ 0.05 versus none, °$p < 0.05$ versus 500/1 ratio.

BPZE1-treated MDDCs efficiently sensed CCL21 gradients. In contrast, BPSM-treated MDDCs, although expressing the same cell-surface levels of CCR7 as those of BPZE1-treated MDDCs (data not shown), were completely blocked in their ability to migrate in response to CCL21 (Fig. 3).

It is well known that the enzymatic activity of PT specifically inhibits Gi/Go proteins and blocks cell migration induced by chemokines (23, 24). Furthermore, it has been shown that the B-oligomer of PT might inhibit chemokine receptor signaling (25). We have hypothesized that the PT expressed by the virulent BPSM strain may impair chemotaxis of MDDCs and that BPZE1-treated MDDCs were allowed to migrate due to the PT detoxification.

To test this hypothesis and rule out possible involvement of PT B-oligomer or other virulence factors lacking in BPZE1, chemotaxis experiments were performed in MDDCs challenged with *B. pertussis* mutants either lacking PT (BPRA) or producing genetically detoxified PT (BPSA175), deficient in TCT production (BPJQ20) or lacking DNT (BPSMDN). As expected, MDDCs incubated with either BPSA175 or BPRA migrated in response to the CCL21gradient, whereas neither BPJQ20-treated nor BPSMDN-treated cells were able to respond (Fig. 3).

Table I. IL-10 and IL-12p70 secretion by MDDCs challenged with different doses of BPZE1 and BPSM

Stimuli	IL-10 (pg/ml)	IL-12p70 (pg/ml)
None	34.6 ± 20.3	0.0 ± 0.0
BPZE1, 20:1	3027.6 ± 711.9*	0.0 ± 0.0
BPZE1, 100:1	5479.7 ± 367.9*	0.0 ± 0.0
BPSM, 20:1	4279.2 ± 154.7*	3.0 ± 2.7
BPSM, 100:1	5806.0 ± 225.5*	13.3 ± 11.9

MDDCs were treated with indicated stimuli for 48 h. IL-10 and IL-12p70 release in culture supernatants was assessed by ELISA. Values are expressed as mean ± SE from four independent experiments performed with MDDCs obtained from different donors and expressed as picograms per milliliter of cytokine released.

*$p < 0.05$ versus none.

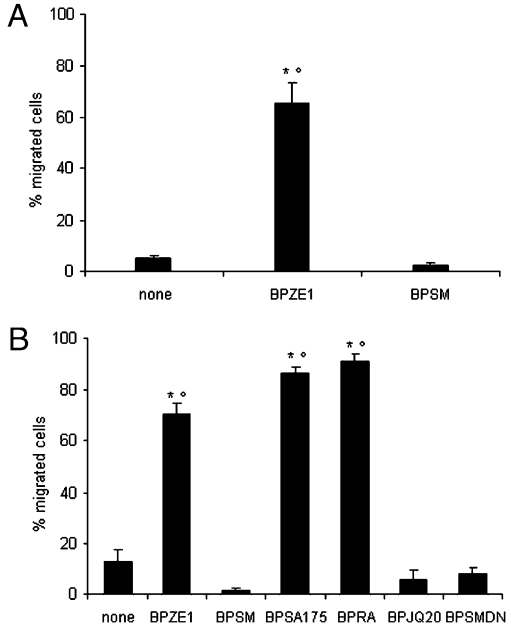

FIGURE 3. Lymphoid organ-derived CCL21-driven chemotaxis of MDDCs. *A*, MDDCs were either untreated (none) or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h. MDDCs were added to the upper chamber of a Transwell system, and CCL21-containing medium was placed in the lower chamber. After 3 h, cells migrated in the lower chamber were counted. Results are expressed as percentage of migrated cells with respect to the cell input (1.25×10^5 cells) and are the mean values ± SE of five independent experiments performed with MDDCs obtained from different donors. *B*, MDDCs were either untreated (none) or challenged with BPZE1, BPSM, BPSA175 (dPT), BPRA (PT^-), BPQJ20 (deficient in TCT release), or BPSMDN (DNT^-) at a 100:1 ratio for 48 h and chemotactic response assessed as described for *A*. Results are expressed as percentage of migrated cells with respect to the cell input (1.25×10^5 cells) and are the mean values ± SE of three independent experiments performed with MDDCs obtained from different donors. *$p <$ 0.05 versus none, °$p < 0.05$ versus BPSM.

Cytokine profile elicited in MDDCs challenged with BPZE1

Cytokine production is a key step in the regulation of the immune response exerted by DCs such as Th1-polarizing IL-12p70 and IL-10, involved in both Th2 and regulatory/Ts induction, and IL-23, IL-1β, and IL-6, involved in Th17 polarization (26). In our previous studies, we have shown that *B. pertussis* promotes the production by MDDCs of IL-23, IL-1β, and IL-10, whereas IL-12p70 is barely detectable (9–11). The cytokine profiles elicited by BPZE1 or BPSM were similar and characterized by high levels of IL-10, IL-1β, and IL-6 (Fig. 4). IL-23 was also produced by MDDCs treated with either strain, without any statistically significant difference. Concerning the IL-12p70 production, the mean values measured were at best modest and appeared to be influenced by a very high variation between the different MDDC donors. As shown in Fig. 4, BPZE1 induced IL-12p70 only in 4 of 15 independent experiments and BPSM only in 5 of 15 independent experiments performed with different donors. Statistically significant differences for IL-12p70 production with respect to untreated MDDCs were reached by BPZE1-challenged but not by BPSM-challenged MDDCs.

Ag presentation and polarization of Th lymphocytes by BPZE1-challenged MDDCs

We next investigated key functions of mature DCs, such as Ag presentation to T lymphocytes and polarization of the immune response. MDDCs stimulated with BPZE1 efficiently induced al-

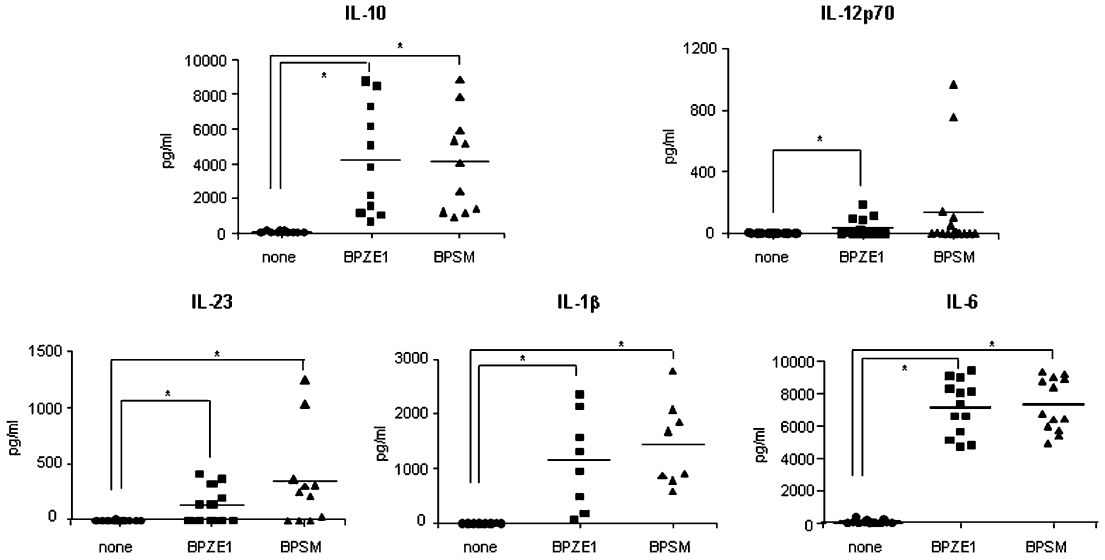

FIGURE 4. Analysis of cytokine secretion by MDDCs. MDDCs were either untreated (none) or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h. IL-10, IL-12p70, IL-23, IL-1β, and IL-6 release in culture media was assessed by ELISA. Values are expressed as mean ± SE from 15 (for IL-12p70), 13 (for IL-6), 11 (for IL-10 and IL-23), and 8 (for IL-1β) independent experiments performed with MDDCs obtained from different donors and expressed as picograms per milliliter of cytokine released. $*p < 0.05$ versus none.

logeneic T cell proliferation, with a maximum proliferation induced at a 10:1 T cell/MDDC ratio. Similar results were obtained with BPSM-treated MDDCs (Fig. 5).

Given the cytokine profile induced by BPZE1 in MDDCs, we analyzed their capacity to polarize purified T lymphocytes and, in particular, the possibility of Th1/Th17 induction, already described in human (11) and mouse models (16–18) and considered to contribute to protection induced by whole-cell pertussis vaccination (18). We assessed the capacity to induce polarization in naive CD45RA$^+$ T cells as well as in already primed CD3$^+$ T cells.

Fig. 6*A* and 6*B* shows that both BPZE1-treated and BPSM-treated MDDCs drove the expansion of IFN-γ– and IL-17–producing effector T cells both among the CD3$^+$ T cells (Fig. 6*A*) and the naive CD45RA$^+$ cells (Fig. 6*B*). A trend toward higher IL-17 production by BPSM- treated MDDC-driven T cell polarization was found, but the differences were not statistically different between the two bacterial strains. In contrast, the IL-5 production, typical of Th2 polarization, was decreased compared with that of the T cells cultured in the presence of untreated MDDCs.

These results were further confirmed by intracellular cytokine staining, showing a dramatic reduction of IL-4$^+$ cells accompanied by an increase of IFN-γ$^+$ and IL-17$^+$ cells when CD3$^+$ T cells were cocultured with MDDCs treated with either BPZE1 or BPSM (Fig. 6*C*). Remarkably, Th1/Th17 double positive cells accounted for the great majority of IL-17 effectors expanded. Similar results were obtained when CD45RA$^+$ T cells were used, although lower percentages of cytokine-producing cells were detected when intracellular staining of naive T cells was performed (data not shown).

Overall, BPZE1 and its virulent parent strain BPSM behave similarly as far as the capacity to strongly induce allogeneic T cell stimulation and polarization of Th cells are concerned, retaining the same properties already shown in our previous study using *B. pertussis* strain 18323 (11).

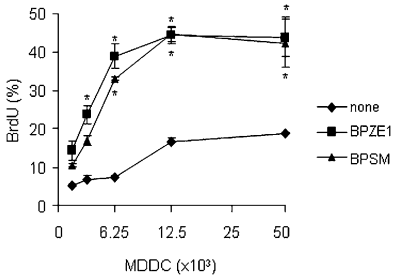

FIGURE 5. Ag presentation. MDDCs (5×10^5) either untreated (none) or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h were cocultured with allogeneic purified T cells (5×10^5) in different numbers [ranging from 50×10^3 (10:1) to 1.56×10^3 (1:320)] for 6 d. Proliferation was assessed by BrdU incorporation. Results are reported as percentage of positive cells (mean ± SE) of four independent experiments performed with MDDCs obtained from different donors. $*p < 0.05$ versus none.

Induction of functional Ts in vitro by BPZE1-challenged MDDCs

Previous studies in mice have shown that IL-10 produced in response to *B. pertussis* infection may in turn promote IL-10–producing Ts (27). Moreover, a reciprocal relationship between Th17 and Ts has been demonstrated (28–30). We therefore decided to investigate the possibility that BPZE1- or BPSM-challenged MDDCs drive the induction of regulatory/Ts activity.

To determine whether naive T cells exposed to *B. pertussis*-treated MDDCs become functional regulatory/suppressors, T cells were stimulated with mDCs in the presence of syngeneic naive T cells primed with BPZE1-MDDCs (BPZE1-Ts) or BPSM-MDDCs (BPSM-Ts). Both BPZE1-Ts and BPSM-Ts inhibited proliferation in the secondary MLR in a dose-dependent manner with statistical significance compared with that of T cells primed with immature MDDCs (Fig. 7*A*).

To rule out an inhibitory role of TGF-β present in the FCS added to the culture medium, we performed the same experiments with a serum-free medium. Also in this case, a marked reduction of T cell proliferation was induced by BPZE1-Ts and BPSM-Ts (data not shown), consistent with the opinion that suppression of proliferation was mediated directly by T cells.

Classical regulatory T cells (Tregs) are CD4$^+$/CD25$^+$/FOXP3$^+$ (7, 8). However, flow cytometry analysis showed that after 6 d of coculture, classical Tregs were not induced either in BPZE1-Ts or in BPSM-Ts (Fig. 7*B*).

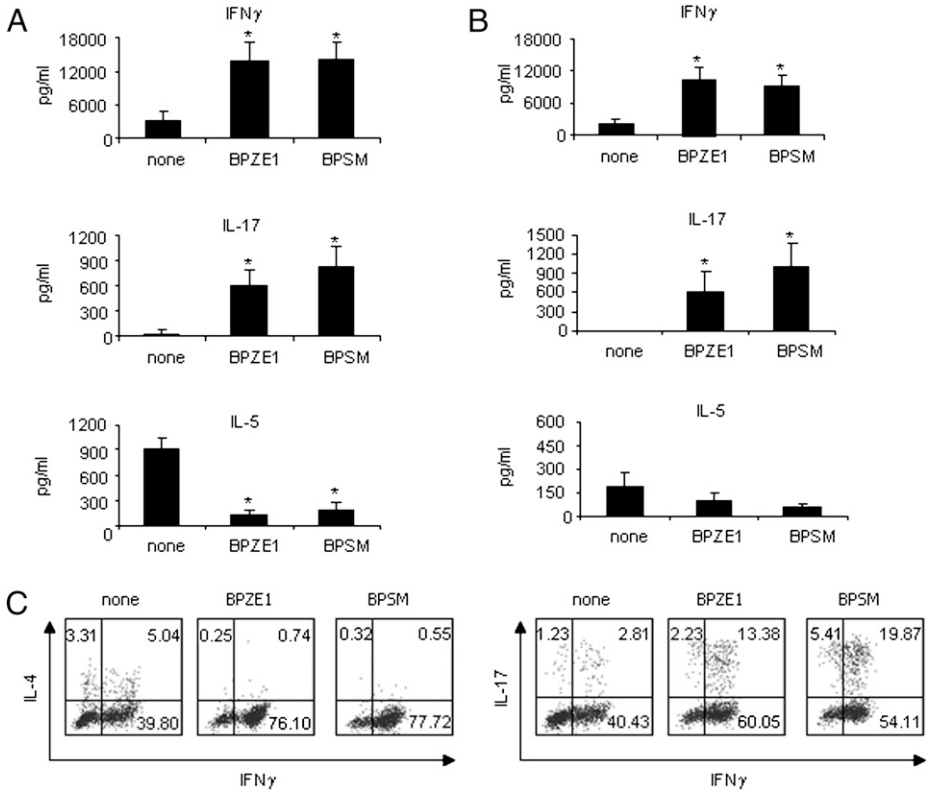

FIGURE 6. T lymphocyte polarization. *A* and *B*, MDDCs either untreated or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h were cocultured with purified allogeneic $CD3^+$ T cells (*A*) or $CD45RA^+$ naive T cells (*B*). On day 12, supernatants were collected, and secreted cytokines were measured by ELISA. Results are expressed as mean ± SE of five independent experiments performed with MDDCs obtained from different donors. *C*, MDDCs either untreated or treated as in *A* were cocultured with purified allogeneic $CD3^+$ T cells. On day 12, cells were stimulated for 5 h with PMA/ionomycin in the presence of brefeldin A for intracellular staining. Numbers in each quadrant indicate the percentage of positive cells. A representative experiment out of three performed with MDDCs obtained from different donors is shown. $*p < 0.05$ versus none.

The inhibitory effect on T cell proliferation might be mediated through soluble factors produced by Ts or cell contact (7). To correlate the regulatory/suppressor activity of the Ts with cytokine secretion, we further characterized BPZE1-Ts and BPSM-Ts. High levels of IFN-γ, IL-17, and IL-10 were induced by BPZE1-Ts and BPSM-Ts. IL-5 was barely detectable, whereas the levels of TGF-β were similar to those produced by T cells cocultured with untreated MDDCs (Fig. 7*C*). IL-10 and TGF-β play a crucial role among the soluble factors produced by Ts; thus, to evaluate the role of these cytokines, BPZE1-Ts or BPSM-Ts were added to a secondary MLR in the presence of both anti–IL-10 and anti–TGF-β neutralizing mAbs. As shown in Fig. 7*D*, the proliferation was still inhibited when neutralizing Abs were added, suggesting a minor role of these soluble factors in the Ts activity.

To demonstrate that the inhibitory effect on T cell proliferation was mediated by cell-to-cell contact and not through soluble factors, suppression experiments were performed in a Transwell system separating BPZE1-Ts or BPSM-Ts from syngeneic T cells by a polycarbonate membrane that allows exchange of soluble factors but excludes direct cell contact. The elimination of cell contact almost entirely restored the proliferation index, indicating that the inhibitory effect was mainly mediated by direct cell contacts (Fig. 7*D*).

Discussion

Studies in mice have shown that the live attenuated *B. pertussis* strain BPZE1 is a promising candidate as a new effective pertussis vaccine for young children (3–5, 16). Preclinical proof of concept has been established in murine models. However, evaluation of BPZE1 in a human experimental setting is important.

We used a well-established model of MDDC challenge with *B. pertussis* to analyze several aspects of BPZE1-driven immune responses in humans (9–11). In this study, we show that BPZE1, despite being strongly attenuated, maintains the capacity to promote MDDC maturation and, similar to BPSM, is able to protect cells from apoptosis. This is a crucial step in DC activation, because while enhancing the longevity of circulating DCs, it ensures a prolonged life span necessary to perform their functions (31).

A unique feature of mature DC is the ability to migrate toward the secondary lymph nodes where naive Th cells are encountered and activated. In this study, we add an important piece of information on the influence of *B. pertussis* on the modulation of the host's immune response and on its immune subversion capacity. Attenuated BPZE1 lacks the inhibitory effect that virulent *B. pertussis* exerts on the lymphatic migration of MDDCs. PT has been known to prevent chemokine receptor signaling through the enzymatic activity of the A subunit (23, 24). More recent studies revealed a novel mechanism by which PT may affect migration of T lymphocytes via the B subunit, which is mediated by interaction with the TCR (25). This process leads to desensitization of CXCR4; it occurs within a few minutes and it is reversible. Our data show that MDDCs challenged with *B. pertussis* mutants producing genetically detoxified PT (BPSA175) or lacking PT (BPRA) migrated in response to a CCL21 gradient. These results confirm that a crucial role in inhibiting cell migration is played by the enzymatically active A subunit, whereas the active B subunit, present in BPSA175, has a marginal role, if any, at least in MDDC lymphatic migration.

B. pertussis mutants deficient for DNT or TCT inhibited MDDC migration to the same extent as BPSM, suggesting that these two toxins do not influence migration in our settings.

These results imply that PT, in addition to its role in bacterial colonization (32) and inhibition of an early inflammatory response (33), mediates an immune evasion mechanism that strongly interferes with DC functions. More importantly, these findings highlight a crucial difference between BPZE1 and BPSM and suggest that one of the main advantages that the vaccine strain may have in vivo is its capacity to rapidly and efficiently activate

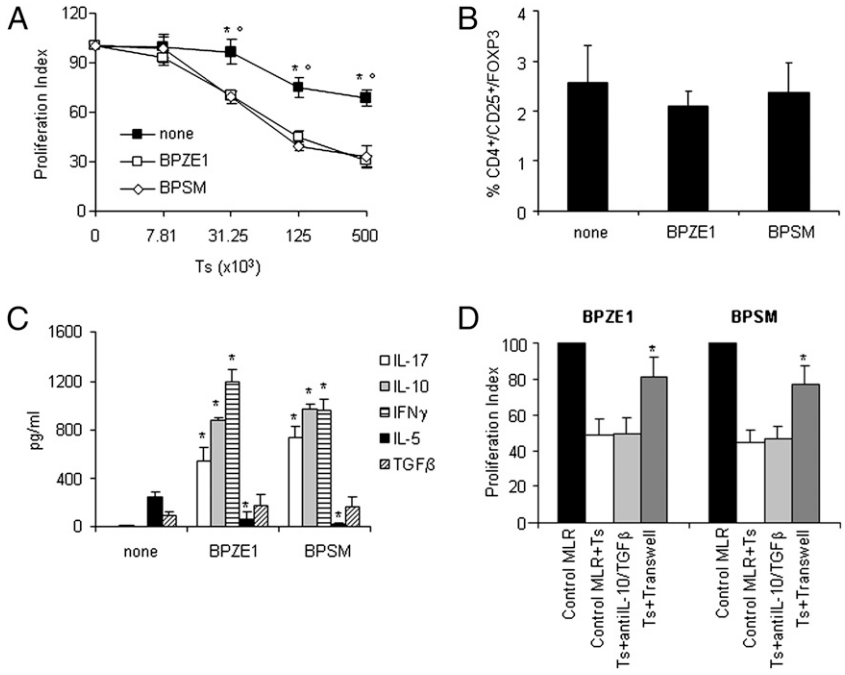

FIGURE 7. Characterization of Ts activity. *A*, MDDCs either untreated or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h were cocultured with purified allogeneic CD45RA$^+$ naive T cells at a 10:1 ratio to generate Ts. On day 6, Ts were collected and cocultured at different numbers, as indicated in the *x*-axis, with syngeneic T cells (5×10^5) in the presence of allogeneic mDCs (5×10^4). T cell proliferation was assessed by BrdU incorporation. Results are reported as proliferation index (mean ± SE) of six independent experiments performed with MDDCs and T cells obtained from different donors. *B*, MDDCs and naive T cells were cocultured as in *A*. On day 6, cells were collected and stained for intracellular FOXP3 and CD4 and CD25 cell surface expression. Results are reported as percentage of positive cells (mean % ± SE) of three independent experiments performed with MDDCs and T cells obtained from different donors. *C*, MDDCs and naive T cells were cocultured as in *A*. On day 6, supernatants were collected and cytokine production measured by ELISA. Results are reported as picograms per milliliter (mean ± SE) of three independent experiments performed with MDDCs and T cells obtained from different donors. *D*, Syngeneic T cells (5×10^5) were cocultured with allogeneic mDCs (5×10^4) (control MLR), added with Ts (5×10^5) (control MLR+Ts), in the presence of blocking anti–IL-10 and blocking anti–TGF-β mAbs (Ts+antiIL-10/TGFβ), or separated from Ts in a Transwell system (Ts+Transwell). The proliferative response was evaluated as in *A*. *$p < 0.05$ versus Ts.

the acquired immune response by allowing the migration of DCs to lymph nodes.

In our previous studies, we have shown that *B. pertussis* promotes in MDDCs the production of Th17-driving IL-23 and IL-1β and regulatory IL-10, whereas IL-12p70 is barely detectable (10, 11). BPZE1 and BPSM elicited a similar profile, with the exception of detectable levels of IL-12p70, which was produced by roughly one third of MDDC donors. This discrepancy could be ascribed to the different genetic background of the *B. pertussis* strains used in this study compared with that of the previous studies. BPSM and BPZE1 are both derivatives of the Tohama I strain, whereas in our previous studies we used the BP18323 strain, which differs from typical *B. pertussis* strains in many aspects (34).

Attenuation of the bacterium did not preclude the ability of BPZE1-challenged MDDCs to perform Ag-presenting activity and induce the expansion of polarized Th1 and Th17 effectors, in keeping with the results of our previous studies (9–11). Remarkably, IFN-γ was produced by T cells at high levels, independently of the amounts of IL-12p70 produced by polarizing MDDCs, confirming our previous finding that *B. pertussis*-driven Th1 immune responses may occur in the absence of IL-12p70 (10, 11).

Several studies have focused on the role of PT in modulating the host immune response, and evidence is accumulating on the role of PT in T cell polarization. We have recently shown that MDDCs stimulated with genetically detoxified PT induced a mixed Th1/Th17 response (15), and in another study PT-deficient *B. pertussis* was described as a poor inducer of both IFN-γ and IL-17 in mice (17). The findings presented here demonstrate that detoxification of PT in BPZE1 results in two interesting properties: it abrogates toxicity and the inhibitory effects on chemotaxis while the immunomodulatory properties are retained.

Notably, the induction of both IFN-γ and IL-17 by *B. pertussis* in T cells has been shown in mice (16–18) and humans (11), and these cytokines appear necessary to achieve protection in whole-cell vaccination in a mouse infection model (18). In our study, intracellular staining of polarized T cells highlights the presence of a double positive Th1/Th17 population. Th1/Th17 cells have recently been described (35). Although the host defense against extracellular bacteria is widely considered pertaining to the Th17 arm, some evidence indicates that efficient protection requires synergy between the Th1 and Th17 lineages (36, 37). Whether these double-positive cells represent a subset of cells in a transient stage of Th development or a new population derived from a distinct but unknown differentiation program remains unclear. The latter hypothesis is strengthened by our observation that Th1/Th17 cells are expanded by *B. pertussis*-challenged MDDCs independently of the use in polarization experiments of naive or already committed T cells and might suggest that, also in this model, these cells are involved in protection.

The fact that both BPZE1-primed and BPSM-primed MDDCs produced high levels of IL-10 argues for the induction of Ts. Our experiments show that BPZE1-treated or BPSM-treated MDDCs induce in vitro a population of T lymphocytes that are functionally able to suppress the proliferation of syngeneic T cells, and the suppressor activity was mediated, mainly by cell contact rather than by soluble factors. These cells were not classical CD4$^+$CD25$^+$

FoxP3$^+$ Tregs. Other phenotypes for Ts subsets have been described, such as IL-10–producing CD4$^+$FoxP3$^-$ type1 Tregs or CD8$^+$ Ts (8). Further studies are planned to better characterize the phenotype of BPZE1-Ts and BPSM-Ts. Remarkably, to our knowledge, the induction of Ts in a human preclinical model by *B. pertussis* is shown here for the first time.

These findings indicate that the interactions between *B. pertussis* and the human host have evolved a mechanism of suppression to limit T cell responses. Whether this represents an immune evasion mechanism for the bacterium or has a role in dampening exacerbated immune responses detrimental for the host still requires further investigation. However, it may be relevant in view of recent findings in mouse models where the administration of BPZE1 strongly reduces the cytokine storm induced by influenza A virus infection (38).

Because BPZE1 and BPSM showed the same capacity to promote Ts induction, bacterial products involved in this process are necessarily shared by the two strains. Studies are needed to unveil the role of other components, including filamentous hemagglutinin, adenylate cyclase toxin, and lipooligosaccharide, all described as inducers of DCs with a tolerogenic phenotype (10, 12, 13, 39, 40).

Our data demonstrate an equivalence of BPZE1 and BPSM in the modulation of DC function. It is not surprising that BPZE1 behaves similarly to BPSM, as the adenylate cyclase and the lipooligosaccharides that, together with PT, were demonstrated to have a direct effect on DC functions (41) are present in both strains at equal levels. PT is also present on BPZE1 but does not express its toxicity, due to the genetic abrogation of the enzymatic activity. The other genetic differences between BPZE1 and BPSM concern the DNT and TCT, but our data show that these two toxins do not appear to have a relevant effect on the DC functions that we analyzed.

In conclusion, our studies enable us to predict that in humans, the BPZE1 strain is a good vaccine candidate; indeed it strongly activated the maturation of DCs with full-blown activity, including the acquisition of a mature phenotype, resistance to apoptosis, and capacity to prime naive Th cells. Attenuation provides to BPZE1-challenged DCs the capacity to survive death signals and migrate from the site of infection to the lymph nodes. This allows priming of Th cells and mounting of the adaptive immune response. BPZE1-committed DCs have the ability to orchestrate a broad spectrum of protective, albeit proinflammatory, Th1/Th17 responses and Ts responses, which likely balance each other to restore local homeostasis.

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Disclosures

The authors have no financial conflicts of interest.

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