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Genetically Detoxified Pertussis Toxin Induces Th1/Th17 Immune Response through MAPKs and IL-10-Dependent Mechanisms

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Genetically detoxified pertussis toxin (dPT) maintains the protein structure and the immunological properties, but not the enzymatic activity. In search of an adjuvant able to direct polarization of T cells to induce/potentiate protective immune response to a variety of infectious disease, we investigated the role played by dPT on human dendritic cell-driven Th polarization and analyzed the intracellular signaling events. To reach these aims, we used a highly purified dPT preparation devoid of contamination and monocyte-derived dendritic cells, a well-characterized model to study ex vivo the polarization of the immune responses. First, we analyzed dPT-induced monocyte-derived dendritic cell maturation, longevity, and cytokine production and, in a second step, we analyzed TLR4/2 engagement by dPT, the connected signaling events, and their relevance to the skewing of Th cell polarization. These approaches allowed us to clarify some of the mechanisms that are responsible for dPT-driven regulation of T cell polarization. We demonstrated that dPT acts utilizing TLR4/TLR2 engagement, being the signaling induced by the former stronger. dPT, through a crucial role played by MAPK and IL-10, favors the expansion of the Th1/Th17 immunity. Indirect evidences indicated that dPT-induced Th17 expansion is counterregulated by the PI3K pathway. For its properties and being already used in humans as vaccine Ag in pertussis, dPT may represents a valid candidate adjuvant to foster immune protective response in vaccines against infectious diseases where Th1/Th17 are mediating host immunity. The Journal of Immunology, 2009, 183: 1892–1899.

Pertussis toxin, a key virulence factor of Bordetella pertussis, the causative agent of whooping cough, contains five different subunits that are arranged in a typical A-B structure. The A protomer contains the enzymatically active S1 subunit. The B oligomer consists of two dimers, S2–S4 and S3–S4, that are held together by an S5 subunit; it binds to cellular receptors and mediates the internalization of the S1 subunit (1, 2). Furthermore, the B oligomer possesses multiple glycan binding sites and it may bind to the glycan residues present in the receptor proteins and cause receptor cross-linking (3, 4). Several studies indicate that pertussis toxin possesses adjuvant properties able to potentiate local and systemic Ab responses, enhancing IgE, IgA, and IgG production (5, 6) and promoting Th1- and Th2-type responses to co-administered Ags (7, 8). Pertussis toxin also regulates IL-12p70 secretion in murine splenocytes (9) and human dendritic cells (DC) (10, 11). However, due to its intrinsic toxicity, the use of pertussis toxin as adjuvant in humans is not advisable. Studies performed so far have shown that a genetically detoxified pertussis toxin (dPT), which maintains an unchanged immunogenic protein structure but has lost enzymatic activity (12, 13), retains the immunological properties of the native protein (5, 7, 10, 11). dPT has been used to develop an acellular vaccine against whooping cough and its administration has been shown to be safe and protective in humans (14–16).

Of interest for pertussis toxin and dPT adjuvant properties is the action on APC and in particular on DC (17). The recognition of microbial products by DC occurs through pathogen recognition receptors, such as TLRs. Pathogen recognition triggers DC maturation and migration to the lymph nodes where they activate T cells and orchestrate the development of adaptive immunity (18, 19).

In this study, we focused our efforts on more deeply investigating the role played by dPT on human DC-driven Th differentiation and undertook an analysis of the intracellular signaling events that are involved. The original contribution of this study is the demonstration that dPT, through a crucial role played by MAPK and IL-10, favors the expansion of Th1/Th17 lineages, recently ascribed to be involved in mediating host immunity in several infectious pathologies (20, 21). A role of the PI3K pathway in the regulation of Th17 induced by dPT was also pointed out.

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Materials and Methods

Reagents

Ultrapure LPS from Escherichia coli 0111:B4 strain, synthetic bacterial lipopolysaccharide S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[RS]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysyl-X 3CF3COOH (Pan2CSK4), ERK1/2 inhibitor PD98059, p38 MAPK inhibitor SB203580, PI3K inhibitor LY294002, anti-human TLR4 mAb, and anti-human TLR2 mAb were purchased from Cayla-InvivoGen Europe. Human GM-CSF and rIL-4 were from R&D Systems. rIL-2 was obtained from Roche. Fluorochrome-conjugated anti-human CD1a, CD14, CD38, CD80, CD83, and CD86, and HLA-DR mAbs, purified anti-human IFN-inducible protein 10 (IP-10) mAb, and appropriated mouse isotype-matched mAbs were from BD Biosciences. Rabbit polyclonal IgG anti-phospho-STAT1 (Tyr701), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-PAK1/2 (Thr180/Tyr182), anti-phospho-JNK (Thr183/Tyr185), and anti-phospho-IkB-α (Ser27) were from Cell Signaling Technology. Mouse anti-β-tubulin and brefeldin A were from Sigma-Aldrich. Mouse anti-STAT1 was from Transduction Laboratories.

dPT purification

dPT was purified by a Bordetella pertussis mutant strain (PTX-9K/129G) which had lost enzymatic activity, according to the protocol developed in Novartis Laboratories starting from bulk cultures for acellular vaccine preparation (12). HPLC showed that no contaminating molecules were present in the preparation and the endotoxin content was below the detection limit of the Limulus amebocyte lysate assay (<0.6 endotoxin units/mg).

Cell lines

Human epithelial kidney (HEK) 293 cells stably transfected with human TLR4, MD2, and CD14 (HEK293/TLR4) or with human TLR2 (HEK293/TLR2) were purchased from InvivoGen. The HEK293/TLR2 clones were grown in D-MEM (Life Technologies) supplemented with heattreated 10% LPS-screened FCS (Limulus amebocyte lysate, <1 ng/ml; HyClone Laboratories) supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM l-glutamine (all from HyClone Laboratories), and Normocin (100 μg/ml; InvivoGen). HEK293/TLR4 culture medium was supplemented with 0.2 mM blasticidin (InvivoGen) and HygroGold (50 μg/ml; InvivoGen). HEK293/TLR2 culture medium was supplemented with 0.6 mM G418 sulfate (InvivoGen).

Stable transfection with NF-κB-inducible reporter plasmid

pNifty2-secreted alkaline phosphatase and TLR signaling assay

HEK293/TLR cells were transfected with a plasmid encoding secreted alkaline phosphatase (SEAP) (pNifty2-SEAP; InvivoGen) as previously described (22). HEK293/TLR/pNifty2-SEAP cells were either untreated or pretreated, using a predetermined optimal dose, with either neutralizing antibodies, 2 mM l-glutamine (all from HyClone Laboratories), or TLR2 and TLR4 signaling, respectively) were used as positive control. Supernatants were collected (22).

Cytokine measurement by ELISA

MDDC culture supernatants were collected and cytokines were assayed by ELISA specific for IFN-γ, IL-5, and IL-17 (Quantikine; R&D Systems), and IL-23 (Bender MedSystems). The lower detection limits were 1.0 pg/ml for IL-1β, 0.7 pg/ml for IL-6, 3.9 pg/ml for IL-10, 15.0 pg/ml for IL-12p40, 5.0 pg/ml for IL-12p70, and 20.0 pg/ml for IL-23. OD was read at 450 nm with a 3550-UV Microplate Reader (Bio-Rad).

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

IP-10 intracellular staining

MDDC were incubated with different stimuli. After 1 h, brefeldin A, a compound which blocks proteins in the endoplasmic reticulum, was added. On day 12, supernatants were harvested for cytokine measurement.

mRNA cytokine expression by TaqMan real-time RT-PCR analysis

To measure cytokine mRNA expression, TaqMan real-time RT-PCR analysis was used (Applied Biosystems). Total RNA was extracted from MDDC at different time points and reverse transcription was conducted as previously described (24). PCR was performed, amplifying the target cDNA transcripts and the β-actin cDNA as endogenous control. Specific primers and probes were obtained from Applied Biosystems (24). mRNA transcript levels were expressed as fold increase compared with basal condition.

Western blot analysis

MDDC were starved by culturing overnight in culture medium supplemented with 1% LPS-screened FCS. Cells were then stimulated and lysed at the indicated time points in radioimmunoprecipitation buffer as previously described (25). Proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare) and immunoreactive proteins were detected by incubating blots with anti-phosphorylated protein mAbs (22, 25).

Statistical analysis

All data were recorded on a computerized database. Results are reported as mean ± SEM. Statistical analyses were conducted using the SPSS 13.0 software. Differences between mean values were assessed by Student’s t-test. The statistical significance was set at p < 0.05.

Results

dPT induces MDDC maturation

In a previous study, we demonstrated that dPT induces human MDDC maturation (10); in this study, we confirmed that a new highly purified dPT preparation retained the same capacity up-regulating the CD80 and CD86 costimulatory molecules, CD83 maturation marker, and MHC class II molecules (HLA-DR) in a dose-dependent manner (Fig. 1A). In addition, dPT up-regulated the expression of CD38, a recently described maturation marker involved in IL-12p70 regulation (22, 23) (Fig. 1A). Heat inactivation of dPT abolished the capacity to induce DC maturation (data not shown), ruling out the possible involvement of a very low level of contaminating endotoxin.

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**FIGURE 1.** A, Induction of human MDDC maturation. MDDC were either untreated (none) or treated with dPT (1 and 5 μg/ml) for 48 h and analyzed for indicated surface markers associated with mature phenotype. Fluorescence data are reported as MFI when treatment increased the expression of the marker in cells that were already positive (CD80, CD86, CD38, HLA-DR); otherwise, the percentage of positive cells was used (CD83). Values are expressed as mean ± SE of 10 independent experiments performed with MDDC obtained from different donors, *p < 0.05 vs none; **p < 0.05 vs 1 μg/ml dPT. B, Protection from spontaneous apoptosis. MDDC were either untreated (none) or treated as in A. Cells were stained with annexin V to assess apoptosis and propidium iodide to assess cell death. Results are reported as percentage of apoptotic cells (annexin V positive) and dead cells (annexin V and propidium iodide positive). Positive control (PC) was E. coli LPS (0.1 μg/ml). Values are expressed as mean ± SE of three independent experiments performed with MDDC obtained from different donors, *p < 0.05 vs none.

dPT-matured MDDC are protected from spontaneous apoptosis

Pathogen-derived maturation stimuli confer resistance to environmental and intrinsic death signals in human DC, enhancing their longevity and potentiating their functions (26). We assessed the viability of MDDC treated with dPT. The cumulative values show that dPT-treated MDDC are protected from spontaneous apoptosis and from cell death, as shown in Fig. 1B.

dPT induces cytokine production in MDDC, and autocrine IL-10 has an inhibitory activity on proinflammatory cytokines

Mature DC acquire the capacity to produce key inflammatory and immunoregulatory cytokines (17–19). As shown in Fig. 2A, MDDC cultured in presence of dPT generated high levels of IL-12p70, as previously shown by our group (10). In this study, we focused our attention on IL-1β, IL-6, and IL-23 cytokines implicated in development and expansion of Th17 cells, a newly discovered subset of T cells. Th17 cells were first described in the autoimmunity literature (20), but growing evidence indicated that the Th17 lineage plays a significant role in mediating host immunity to a number of pathogens and in particular versus extracellular pulmonary pathogens (20, 21, 27–29).

Upon dPT treatment, MDDC produced high levels of IL-1β, IL-6, and IL-23. Cytokine induction by dPT was dose dependent (Fig. 2A). dPT was also able to induce a high level of IL-10 (Fig. 2A), endowed with anti-inflammatory effects and able to counter-regulate the induction of proinflammatory cytokines (30). Interestingly, in cultures where IL-10 activity was reduced (by 50%) by preincubation of MDDC with a neutralizing anti-IL-10 mAb, dPT enhanced the production of IL-12p70 (by 100%), IL-23 (by 170%), and IL-1β (by 160%) with respect to the activation exerted by dPT alone (Fig. 2B).

dPT induces DC activation via TLR4 and TLR2 engagement

TLR4 and TLR2 signal at the surface of several cells including DC (19, 31, 32). To verify whether TLR4 is a receptor for dPT, we used HEK293 cells expressing TLR4, MD2, and CD14. The results showed that TLR4/MD2/CD14-dependent signaling was triggered by dPT in a dose-dependent manner. Furthermore, the higher doses of dPT-induced TLR4 activation at similar levels compared with E. coli LPS, the positive control (Fig. 3A). To evaluate whether dPT was also able to activate TLR2, a similar experiment was performed using HEK293 cells expressing TLR2. dPT was able to trigger TLR2-dependent signal transduction. The activation induced was rather modest at the 1 μg/ml dPT dose, but reached the statistical significance vs the untreated control, and was more pronounced at the 5-μg/ml dPT dose, also reaching the statistical significance (Fig. 3A). To confirm the involvement of TLR2 and TLR4 in dPT-mediated signaling, we preincubated MDDC with neutralizing anti-TLR4 or anti-TLR2 mAbs. As shown in Fig. 3B, IL-12p70 and IL-10 were inhibited (by 90% for IL-12p70 and by 70% for IL-10 at the lower dPT dose) by the anti-TLR4
but not by the anti-TLR2 mAb, while IL-23 induction was inhibited by both anti-TLR mAbs at both dPT doses (by 50% at the lower dPT dose).

**Intracellular pathways involved in dPT-mediated MDDC activation**

The observation that dPT engaged either TLR4 and TLR2 forced us to focus on pathways described as master regulators of DC functions such as the MyD88-dependent pathway, activated by both TLRs, and the MyD88-independent pathway that is initiated only by TLR4 (33). The adaptor protein MyD88 plays a pivotal role in the activation of MAPK signaling components and the transcription factor NF-κB (33). The results, shown in Fig. 4A, indicated that phospho-ERK1/2 was induced by the 1 μg/ml dPT dose; however, at a higher dPT concentration, a more robust activation was evident. Similarly, in the case of phospho-p38 and phospho-JNK/SAPK, higher activation was noticed when the 5 μg/ml dPT dose was used. The phosphorylation of IκB-α is necessary to induce nuclear translocation of NF-κB and represents an indirect way of measuring its activation. We found that IκBα was rapidly phosphorylated upon exposure of MDDC to dPT. In this case, the 5 μg/ml dPT dose promoted earlier and higher phosphorylation levels (Fig. 4A).

**FIGURE 3.** TLR4 and TLR2 activation. A, Triggering of TLR4 and TLR2 in transfected HEK293 cells. HEK293/TLR4-p-Nifty2-SEAP and HEK293/TLR2-p-Nifty2-SEAP cells were either untreated or treated with dPT (1 and 5 μg/ml) for 16 h. Positive control (PC) for TLR4 stimulation was E. coli LPS (0.1 μg/ml) and positive control for TLR2 stimulation was Pam2CSK4 (0.1 μg/ml). SEAP activity in supernatants of cell cultures was measured. Data are reported as fold increase of SEAP activity over untreated values. Mean expression ± SE of 10 independent experiments is indicated. *p < 0.05 vs none; **p < 0.05 vs 1 μg/ml dPT. B, Neutralization of TLR4 and TLR2. MDDC were either untreated or treated as in A for 48 h either in the absence or presence of a neutralizing anti-TLR4 mAb (10 μg/ml) and anti-TLR2 mAb (10 μg/ml). Results of three independent experiments performed with MDDC obtained from different donors, measured by ELISA, are expressed as percentage of change of cytokines with respect to the corresponding stimulus in the absence of inhibitors. Mean expression ± SE of 10 independent experiments is indicated. *p < 0.05 vs none; **p < 0.05 vs 1 μg/ml dPT; IL-12p70 = 295 ± 87, IL-23 = 383 ± 66, and IL-10 = 220 ± 63 and for 5 μg/ml dPT: IL-12p70 = 457 ± 115, IL-23 = 416 ± 37, and IL-10 = 424 ± 57.

**FIGURE 4.** A, Analysis of TLR4/TLR2/MyD88-dependent pathway induction. MDDC were either untreated or treated with dPT (1 and 5 μg/ml). Phosphorylation of ERK1/2, p38, JNK/SAPK, and IκB-α was determined at the indicated time points by Western blot. A single gel was run and blotted to detect phosphorylated proteins and β-tubulin to normalize the results. Data are from one representative of four independent experiments performed with MDDC obtained from different donors. B, MDDC were treated as in A either in the absence or presence of p38 inhibitor (SB203580), ERK1/2 inhibitor (PD98059), or PI3K inhibitor (LY294002) for 48 h. Results of six independent experiments performed with MDDC obtained from different donors are expressed as the percentage of change of maturation markers (CD80, CD83, CD38) with respect to the corresponding stimulus in the absence of inhibitors. Mean ± SE of marker expression in MDDC not treated with inhibitors was for 1 μg/ml dPT: CD80 (MFI) = 41 ± 4, CD83 (%) = 33 ± 6, and CD38 (MFI) = 76 ± 20 and for 5 μg/ml dPT: CD80 (MFI) = 55 ± 8, CD83 (%) = 48 ± 8, and CD38 (MFI) = 107 ± 22. *p < 0.05 vs control, calculated from the raw data. C, MDDC were treated as in A and B. Results of six independent experiments performed with MDDC obtained from different donors, measured by ELISA, are expressed as the percentage of change of cytokines with respect to the corresponding stimulus in the absence of inhibitors. Mean ± SE of cytokine production (pg/ml) in MDDC not treated with inhibitors was for 1 μg/ml dPT: IL-12p70 = 326 ± 88, IL-23 = 201 ± 161, IL-1β = 140 ± 70, and IL-10 = 316 ± 92 and for 5 μg/ml dPT: IL-12p70 = 593 ± 118, IL-23 = 410 ± 150, IL-1β = 152 ± 65, and IL-10 = 728 ± 250. *p < 0.05 vs control, calculated from the raw data.
dPT induces MyD88-independent signaling

TLR4 MyD88-independent signaling is responsible for the activation of intracellular pathways that induce IFN-β and IFN-inducible genes, such as IFN regulatory factor 1 (IRF-1) or IP-10 (19, 36). To study this aspect in more depth, we analyzed the phosphorylation of the transcription factor STAT1, pivotal in the regulation of this pathway (36). Fig. 5A shows that dPT induced STAT1 phosphorylation. Accordingly, upon dPT stimulation, MDDC expressed consistent levels of IFN-β, IRF-1, and IP-10 (Fig. 5, B–D).

**FIGURE 5.** Analysis of TLR4-dependent MyD88-independent pathway induction. A, MDDC were either untreated or treated with dPT (1 and 5 µg/ml) for 2 h. Phosphorylation of STAT1 was determined by Western blot and total STAT1 was determined as control. Data are from one representative of four independent experiments performed with MDDC obtained from different donors. B, MDDC were treated as in A and total RNA was extracted at the indicated time points. Kinetics of mRNA expression for IFN-β was evaluated by real-time quantitative RT-PCR. mRNA transcript levels were expressed as fold increase over those in unstimulated (none) MDDC at 5 h. One representative experiment of three performed with MDDC obtained from different donors is shown. C, MDDC were treated as in A and processed as in B. One representative experiment of three performed with MDDC obtained from different donors is shown. D, MDDC were treated as in A for 1 h and brefeldin A was added. After further incubation for 5 h, intracellular staining for IP-10 was performed. Results are expressed as percentage of positive cells. Mean ± SE of three independent experiments performed with MDDC obtained from different donors is shown. *p < 0.05 vs none.

To further detail the dPT-induced MAPK intracellular signaling, the p38 inhibitor SB203580 and the ERK1/2 inhibitor PD98059 were introduced into the experiments. The results showed that the addition of the p38 inhibitor diminished the expression of all of the maturation markers analyzed and greatly reduced cytokine production (near to 100% inhibition in the case of IL-23) (Fig. 4, B and C). The addition of the ERK1/2 inhibitor caused a clear increase of CD80, a decrease of CD83 and CD38 expression, and an inhibition of IL-12p70 (by 70%) and IL-1β (by 40%) induced by dPT at both doses. IL-10 was inhibited when induced by dPT at 1 µg/ml (by 40%), whereas when induced by dPT at 5 µg/ml was not inhibited, probably due to the difficulty of inhibiting this kinase when strongly activated. The addition of the ERK1/2 inhibitor had a limited effect on IL-23 production.

Furthermore, we also investigated the role of PI3K using the specific inhibitor LY294002. PI3K promotes a negative regulation of TLR signaling in innate immune cells and can be considered an anti-inflammatory pathway (34). It is involved in a specific TLR2-mediated pathway that controls an IkB-α independent pathway to NF-κB activation (34, 35). The results indicated that the PI3K inhibitor induced a clear inhibition of CD38 expression and IL-10 production (by 50%). CD83 and CD80 expression and IL-12p70 production were minimally affected, whereas the PI3K inhibitor induced a strong increase of IL-23 (by 300%) and IL-1β (by 150%) production (Fig. 4, B and C).

In a previous study, we demonstrated that dPT promotes Th1 responses in human MDDC (10). In this study, we found that dPT drove, in MDDC, a cytokine profile that was compatible with the induction of Th17 lymphocytes, since it induced the production of IL-23, IL-6, and IL-1β (20, 27, 37). Thus, to clarify this point, polarization experiments were performed coculturing purified allogeneic T lymphocytes with MDDC stimulated with dPT and the levels of Th1 (IFN-γ), Th17 (IL-17), and Th2 (IL-5) cytokines were measured. The results obtained showed that MDDC treated with dPT induced statistically significantly higher levels of IFN-γ and IL-17 production by T cells compared with untreated MDDC (Fig. 6A). The production of IFN-γ and IL-17 by T cells was markedly enhanced when the highest dose of dPT was used. Worth of note, MDDC stimulated by dPT inhibited the expansion of Th2 cells producing IL-5 compared with untreated MDDC. Also in this case, a clear dose-dependent effect was observed (Fig. 6A).

We showed that dPT-induced IL-10 had an important autocrine role in modulating cytokine expression in MDDC (Fig. 2C) and therefore we decided to perform polarization experiments by treating MDDC with a blocking anti-IL-10 mAb before dPT stimulation and then coculturing activated MDDC with T cells. Reduction of IL-10 activity resulted in an inhibition of Th17- and Th2-polarizing capacity of dPT, with IL-17 and IL-5 secretion being reduced by 40 and 30%, respectively. Conversely, Th1-polarized T cells were strongly enhanced, being IFN-γ production increased by 80% (Fig. 6B).

To further understand the intracellular mechanisms underlying the dPT-driven polarizing ability of MDDC, we used MAPK and PI3K inhibitors in polarization experiments (Fig. 6C). When p38 or ERK1/2 inhibitors were added during the dPT-induced MDDC maturation, a clear and marked effect was observed on the Th balance induced in MDDC. Indeed, IFN-γ production by T cells was reduced by 70 and 50% in the presence of p38 or ERK1/2 inhibitors, respectively. Equally, IL-17 production by T cells was reduced by 70 and 40% in the presence of p38 or ERK1/2 inhibitors, respectively. IL-5 production by T cells was enhanced by 500 and 200% in the presence of p38 or ERK1/2 inhibitors, respectively. The inhibition of the PI3K pathway in MDDC favored the expansion of IL-17-producing cells (by 50%) having a limited effect on IFN-γ and IL-5 production (Fig. 6C).

**Discussion**

Despite the fact that adjuvants have been used to increase the immunogenicity of vaccines for >70 years, only a few have been licensed for human use. In particular, adjuvants able to direct polarization of T cells to induce and potentiate protective immune responses to a variety of infectious diseases are clearly needed. In this study, we propose dPT as a safe and effective adjuvant able to drive Th1/Th17 responses through the capacity to stimulate DC.

Other studies have referred to the contribution of Th1 and Th17 in mediating host immunity to a number of pathogens, including human fungal pathogens such as *Candida albicans* (37–40) and bacterial infections such as *Mycoplasma pneumoniae* (41), *Klebsiella pneumoniae* (42–44), *Mycobacterium tuberculosis* (45) and *B. pertussis* (Ref. 46 and G. Fedele, F. Spensieri, R. Palazzo,
of IL-10 was reduced in MDDC cultures using blocking anti-IL-10 mAb. Taking into consideration these data, dPT appears able to affect the balance between pro- and anti-inflammatory cytokines.

dPT was able to activate TLR4 in MDDC in accordance with other reports focused on fully enzyme-active pertussis toxin and on B oligomer (48). This is the first report showing that dPT also triggers TLR2. TLR2 engagement becomes more evident and strong with increasing dPT concentration, highlighting a peculiar situation in which a low dose of dPT efficiently triggers TLR4 signaling, while a high dose efficiently activates both TLR4 and TLR2. Blocking experiments allowed us to stress an essential role of TLR4 in dPT-induced-IL-12p70 and IL-10, while both the TLRs were involved in dPT-induced IL-23 secretion. The involvement of TLR4 and TLR4/TLR2 in the induction of IL-12p70/IL-10 and IL-23 production, respectively, was in agreement with data shown by other reports, mostly performed in murine models (49, 50). dPT engaged either TLR4 and TLR2 and was able to activate the MAPK signaling cascade, as testified by p38, ERK1/2, and JNK/SAPK phosphorylation. Of relevance, pERK1/2 induction was particularly strong when dPT was used at the higher dose, suggesting the need of both TLR2 and TLR4 receptors to fully activate this pathway. We demonstrated also that the increase of expression/secretion of all phenotypic markers and cytokines induced by dPT required the p38 pathway, while ERK1/2 activation was exclusively required in dPT-induced IL-12p70, IL-1β, and IL-10. At the moment, we have no explanations for the inhibitory role exerted by ERK1/2 in dPT-induced CDbO expression.

dPT was able to induce a MyD88-independent pathway leading to the synthesis of IFN-β, which in turn induced STAT1 phosphorylation and activated IRF-1 and IP-10. These results denoted relevant differences between the intracellular signaling activated by dPT and the B oligomer of pertussis toxin, both able to efficiently bind to the cell receptors and devoid of the enzymatic activity. Indeed the B oligomer was shown to trigger the MyD88-independent pathway only (48), dPT and pertussis toxin may activate a similar intracellular pathway due to the presence of the S1subunit per se, even enzymatic inactive, that probably maintains the conformational structure of the entire pertussis toxin.

To better define the mechanisms underlying the capacity of dPT to tuning Th cells development, we used the original approach of kinase activity inhibition in polarization experiments. This way allowed us to clarify that p38 MAPK activities have a pivotal role in inducing both the IL-12p70/IFN-γ and the IL-1β/IL-23/IL-17 axes. Indeed, we demonstrated that p38 inhibition strongly reduced Th17 and Th1 expansion, favoring the expression of Th2 profile. Furthermore, we found that ERK1/2 participates along with p38 in the activation of MDDC by dPT, but its activities seem to be, in general, less crucial for Th development.

PI3K/Akt axis is well known as a regulator of innate responses to microbial pathogens (34, 35). Our results demonstrated for the first time the key role played by PI3K in the regulation of DC-driven Th17 expansion. Indeed, blocking PI3K activity in dPT treated MDDC resulted in an up-regulation of proinflammatory cytokines, in particular IL-23 and IL-1β, and inhibition of IL-10. In accordance with the cytokine profile obtained in MDDC, PI3K inhibition increased IL-17 production in polarization experiments, thus pointing out an important inhibitory role of PI3K pathway in Th17 development.

IL-10 production has been linked to MAPK and PI3K activation (51, 52) and our data confirmed the involvement of these pathways in sustaining IL-10 production. Nonetheless our data unveil the pivotal role of IL-10 in the Th17/Th1 balance. Indeed, its partial neutralization provided a block of IL-17 production by Th cells, yet enhanced IFN-γ production. It is probable that IL-10 action is
mediated by its ability to fine tune regulatory cytokines expression by MDDC (IL-12p70, IL-1β, IL-23) and not by a direct action of IL-10 on Th17 cells. Indeed, preliminary experiment, where IL-10 was directly added to the polarization cultures, supported an indirect role of IL-10 on Th cell development.

An interesting consideration emerges from our data. Indeed, using a blocking anti-IL-10 mAb or PI3K inhibitor a similar IL-10 reduction (around 50%) in dPT-treated MDDC was reached but the Th polarization driven by the two MDDC preparations was different. A direct inhibition of IL-10 in dPT-treated MDDC induced a clear expansion of Th1 cells with a reduction of Th17 and Th2. Although, when IL-10 was reduced by inhibiting PI3K pathway a clear expansion of Th17 cell was observed. Recently a new subset of Th1/Th17 cells has been described (53). In this contest we could hypothesize that a reduction of anti-inflammatory IL-10 cytokine, induced by blocking mAb, may act on this subset by inducing a shift from Th1/Th17 to a Th1 subset, while the inhibition of PI3K anti-inflammatory pathway could induce a shift from Th1/Th17 subset to the expansion of Th17 subset. To verify this point further studies are needed, including intracellular staining experiments.

dPT behaves functionally similar to pertussis toxin, with the same adjuvant properties of the fully enzymatically active toxin and it is not surprising that our data are in keeping with that obtained in mice where pertussis toxin promotes the generation of Th17 cells (54).

We demonstrated also that dPT promotes protection of MDDC from spontaneous apoptosis. The capacity to increase DC longevity, thus prolonging the ability of DC to regulate the immune response, is an important aspect to consider when evaluating the properties of an adjuvant, and further studies are needed to characterize the potential of dPT to prolong longevity of specific DC subsets.

In conclusion, we were able to further expand the knowledge on dPT adjuvant properties focusing on the ability of dPT to influence Th development. In addition to Th1 (10), dPT is able to polarize the immune response toward the Th17 phenotype due to the activation of both TLR4 and TLR2. When used at a lower dose, dPT drives mainly TLR4 signaling, activating p38 and ERK1/2, and inducing appreciable levels of cytokines. In this situation T cell polarization is characterized by Th1 (IFN-γ) and Th17 (IL-17) expansion. When dPT is used at the higher dose, TLR4 triggering is stronger and TLR2 signaling is added. The release of IL-12p70, IL-10 and other cytokines is increased, as well as MAPK phosphorylation. As a result, dPT promotes a sharp Th1 and Th17 polarization, with a strong inhibition of Th2. However, it should be taken into consideration that dPT may promote cellular signaling not only via TLR4/TLR2 engagement, but also by cross-linking cell surface receptors through its multiple glycan binding sites (3, 4). Thus multiple pathways (involving both positive and negative regulatory loops) may be simultaneously engaged and perhaps down-regulated.

For its intrinsic properties, which we have largely depicted here, dPT represents a valid candidate adjuvant to foster immune protective response vs infectious diseases and in vaccines where the development of Th1/Th17 is needed. Recent evidences suggest that the Th17 response plays a role in vaccine-induced immunity and/or as target for therapeutic manipulation to treat a wide variety of pathogens. As elegantly illustrated by Khader et al. (45), although Th17 cells are not critical to primary response to *M. tuberculosis*, the Th17 response is clearly implicated in response to ESAT-6 peptide vaccination against murine tuberculosis. Th17 cytokine responses have been also implicated in vaccine-induced immunity against *B. pertussis* (46). Our data foster the adjuvant usage of dPT which has lost the undesired toxic activity of its enzymatic moiety, as already demonstrated by its safe use in clinical setting as vaccine Ag (12–16), and promote the dPT use as adjuvant in human trials. However, considering that dPT used in vaccines was treated with low concentration of Formaldehyde (0.06%), further studies are required, in particular to evaluate whether dPT can be used in humans without Formaldehyde inactivation, or, in alternative, whether this treatment affects the capacity of dPT to modulate DC activities here depicted.

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**Disclosures**

Detoxified pertussis toxin is a potential commercial product. P.C. and R.R. are employed by Novartis Vaccines. All other authors report no potential conflicts.

**References**
