Pertussis Toxin Enhances Th1 Responses by Stimulation of Dendritic Cells

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Pertussis toxin (PTX) has been widely used as an adjuvant to induce Th1-mediated organ-specific autoimmune diseases in animal models. However, the cellular and molecular mechanisms remain to be defined. In this study, we showed that dendritic cells (DC) stimulated with PTX (PTX-DC) were able to substitute for PTX to promote experimental autoimmune uveitis (EAU). EAU induced by PTX-DC revealed a typical Th1 response, characterized by high uveitogenic retinal Ag interphotoreceptor retinoid-binding protein (IRBP)-specific IFN-γ and IL-12 production in the draining lymph nodes, as well as increased levels of anti-IRBP IgG2a and decreased levels of anti-IRBP IgG1 in the serum of IRBP-immunized mice. Furthermore, PTX-DC preferentially induced T cells to produce the Th1 cytokine, IFN-γ. After being stimulated with PTX, DC exhibited up-regulation of MHC class II, CD80, CD86, CD40, and DEC205. PTX-DC had also increased allostimulatory capacity and IL-12 and TNF-α production. Serum IL-12 was increased in naive mice that received PTX-DC i.p. In addition, PTX activated extracellular signal-regulated kinase in DC. Following the inhibition of extracellular signal-regulated kinase signaling, the maturation of PTX-DC was reduced. Subsequently, the ability of PTX-DC to promote IFN-γ production by T cells in vitro and to induce EAU in vivo was blocked. The results suggest that PTX might exert an adjuvant effect on DC to promote their maturation and the production of proinflammatory cytokines, thereby eliciting a Th1 response. The Journal of Immunology, 2003, 170: 1728–1736.

Pertussis toxin (PTX)3 is an exotoxin produced by Bordetella pertussis, known to have adjuvant properties that induce Th1 responses (1). PTX has been widely used to enhance Th1-mediated organ-specific autoimmune diseases, including experimental autoimmune encephalomyelitis, experimental autoimmune uveitis (EAU), and experimental autoimmune orchitis (2–7). PTX has also been reported to augment delayed-type hypersensitivity reactions (8). One study demonstrated that PTX is able to induce Th1 responses by inhibiting G protein signaling that negatively regulates IL-12 production (9). It is noted that the adjuvant effect of PTX is associated with its ability to induce production of proinflammatory cytokines and expression of the co-stimulatory molecules B7-1 and B7-2 on APCs, which affects T cell differentiation (10, 11). However, the cells targeted by PTX in vivo that promote Th1-mediated organ-specific autoimmune diseases, and the molecular mechanisms of this regulation are not clear.

Dendritic cells (DC) are the most potent APCs for priming naive T cells and the main source of IL-12 production (12–15). Upon contact with bacterial components or proinflammatory mediators, immature DC that have captured Ag in the periphery migrate to the T cell zone of lymphoid organs, where they present Ag in the context of MHC molecules. During migration from the periphery to the lymph nodes (LN), DC undergo a maturation process that results in morphological and functional changes. Expression of IL-12 by DC can be induced at the time of encounter with microbial compounds. This proinflammatory cytokine promotes cell-mediated response, stimulates IFN-γ secretion by NK and T cells, and favors the development of Th1 cells (16, 17).

Because DC are essential to generate and maintain an immune response, and because they play a crucial role in priming naive T cells and initiating autoimmunity (13, 18, 19), we hypothesize that the adjuvant effect of PTX results from a modulation of DC function. In this study, we show for the first time that DC stimulated with PTX (PTX-DC) are able to substitute for PTX to enhance Th1-mediated EAU in a syngeneic cell transfer model. Moreover, PTX-DC preferentially induced T cells to produce the Th1 cytokine, IFN-γ. Subsequent experiments demonstrated that the PTX-DC secreted high IL-12 in vitro and induced serum IL-12 production in vivo. The novel role of PTX-DC prompted us to assess PTX-mediated signaling and the correlation between PTX-DC maturation and its adjuvant effect on EAU induction.

Materials and Methods

Animals

Six- to 8-wk-old female B10.A and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were kept in a specific pathogen-free facility at the Chinese Academy of Sciences. Animal care and use were in compliance with institutional guidelines.

Medium and reagents

RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 5 × 10⁻⁵ M 2-ME was used for DC culture, fibroblast culture, and primary DC-T cell coculture, as well
as for secondary T cell stimulation culture. Interphotoreceptor retinoid-binding protein (IRBP) was a gift from R. Caspi, National Eye Institute, National Institutes of Health. Mouse rGM-CSF, BSA, α-methylmannopyranoside, PTX, and CFA were purchased from Sigma-Aldrich (St. Louis, MO). Mycobacterium tuberculosis strain H37RA and LPS (Escherichia coli O111:B4) were from Difco (Detroit, MI). Mitogen-activated protein extracellular signal-regulated kinase (ERK) kinase inhibitor PD98059 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). PD98059 was dissolved in DMSO.

**Generation of bone marrow-derived DC**

The method was modified from one initially described by Inaba et al. (20). Briefly, bone marrow cells were flushed from the femurs and tibias of B10.A mice and subsequently depleted of red cells with ammonium chloride. Cells were cultured at 2 × 10⁶ cells/well in 24-well plates in medium supplemented with 20 ng/ml murine rGM-CSF. Nonadherent cells were removed carefully, and fresh medium was added every 2 days. On day 8, nonadherent cells released spontaneously from proliferating cell clusters were collected. Surface phenotype analysis by flow cytometry consistently revealed a typical immature DC surface phenotype (CD11c⁺, CD80⁻, CD86⁻, I-Ak⁻) in 80–90% cells and microscopically revealed a dendritic, veiled morphology. As expected, no contaminating B cells, macrophages, and T cells were generated under these conditions, as determined by FACS analysis using mAbs specific for B220, F4/80, and CD3 (data not shown). PTX-DC were generated in the same way, but with the addition of 10 ng/ml PTX for the final 24 h of incubation. For in vitro chemotaxis analysis, 100 ng/ml LPS was added to DC cultures. DC were harvested, washed twice, and used in subsequent experiments. For immunization with DC in a syngenic cell transfer model, DC were resuspended in PBS and washed twice, and used in subsequent experiments. For immunization with PTX in a syngenic cell transfer model, DC were incubated with the following primary mAbs (conjugated or un-oupled): FITC-labeled anti-mouse I-Ak (clone 10-3.6; BD PharMingen), and rat anti-mouse DEC205/NLDC145 (a gift from P. Allavena, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy; 10% culture supernatant). If possible, cells were incubated with anti-CD16/CD32 (2.4G2) to reduce nonspecific binding of mAbs. Unconjugated rat mAbs were revealed by FITC-conjugated goat anti-rat IgG and IgM Ab (Southern Biotechnology, Birmingham, AL) as the second reagent. Isotype controls were conjugated or unconjugated rat IgG2a, and hamster IgG (all from BD PharMingen). All staining reactions were performed on ice. After each step, cells were washed twice with 1% FCS and 0.1% sodium

**Assay for IRBP-specific cytokine production by draining LN**

Cell suspensions were prepared from draining LN of mice that had been immunized with IRBP 21 days previously. The cells (5 × 10⁶ cells/ml) were cultured for 48 h with or without IRBP (30 μg/ml) in 24-well plates in complete RPMI 1640 medium containing 0.5% syngeneic normal mouse serum (24). The supernatants were collected after 48 h, and IRBP-specific IFN-γ, IL-4, and IL-12 secretion was measured by ELISA (R&D Systems, Minneapolis, MN). The sensitivity for IFN-γ, IL-4, and IL-12p70 was 10 pg/ml.

**Assay for serum levels of anti-IRBP IgG2a and IgG1**

Serum levels of anti-IRBP IgG2a and IgG1 subclasses were determined by ELISA in serum collected 21 days after immunization, as described previously (25). In brief, 96-well microtiter plates (Corning Costar) were coated with IRBP (1 μg/ml). After blocking the plates with BSA (Sigma-Aldrich), and an overnight incubation with diluted serum samples, the plates were developed using HRP-conjugated goat anti-IgG subclass-specific Abs (BD PharMingen, San Diego, CA). A series of serum dilutions were examined in the preliminary experiments. The OD values were positively correlated with the concentration of anti-IRBP Abs. In each assay, IgG subclass was measured in duplicate on a single plate. Data are mean ± SD of three separate experiments.

**IL-12 production in the serum of naive mice following DC administration**

DC or fibroblasts were resuspended in PBS, as mentioned above, and used to immunize mice in a syngeneic cell transfer model. naive B10.A mice were injected with fresh DC or fibroblasts (1 × 10⁵ cells/0.1 ml/mouse) via i.p. injection. The injection of PBS alone served as the negative control. Mice were sacrificed at various time points following injection, and the serum of individual mice was collected for IL-12 determinations.

**DC labeled with CFSE and in vivo migration assay**

DC were labeled with the tracking fluorochrome CFSE (Molecular Probes, Eugene, OR), as previously described (26). Briefly, either DC not stimulated with PTX (unstimulated DC), PTX-DC, or PTX-fibroblasts were incubated with CFSE at a final concentration of 5 μM in PBS for 10 min, and the labeling was terminated by the addition of FCS (10% of the total volume). DC were washed twice with RPMI 1640/10% FCS medium, washed once with PBS, and resuspended in PBS for i.p. injection. B10.A mice were injected with labeled DC or fibroblasts (1 × 10⁵ cells/0.1 ml/mouse, i.p.). Control mice received i.p. injection of PBS alone. After 48 h, the mice were sacrificed, and the inguinal LN was removed. Single cell suspensions of LN cells were prepared and were stained with CD11c–PE (BD PharMingen). CFSE⁻ and CD11c⁻ double-positive DC cells were detected by flow cytometry.

**Chemotaxis assay**

The chemotaxis analysis in vitro was performed, as described previously (27), with minor modifications. Recombinant macrophage-inflammatory protein (MIP)-3β (100 ng/ml; R&D Systems) was diluted in medium without FCS, and 500 μl was added to 24-well tissue culture plates (Corning Costar). Transwell culture inserts (50-μm pore size; Corning Costar) were placed in each well, and 80 μl of DC suspension (4.0 × 10⁵ cells/well) was added to the top chamber. After incubation at 37°C in 5% CO₂ for 3 h, the cells that had migrated to the bottom chamber were recovered and counted by light microscopy.

**Flow cytometry**

DC were incubated with the following primary mAbs (conjugated or unconjugated): FITC-labeled anti-mouse I-Ak (clone 10-3.6; BD PharMingen), FITC-labeled anti-mouse CD40 (clone 5C3; BD PharMingen), rat anti-mouse CD80 (clone 1G10; BD PharMingen), FITC-labeled anti-mouse CD86 (clone GL1; BD PharMingen), PE-labeled anti-mouse CD11c (clone HL3; BD PharMingen), and rat anti-mouse DEC205/NLDC145 (a gift from P. Allavena, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy; 10% culture supernatant). If possible, cells were incubated with anti-CD16/CD32 (2.4G2) to reduce nonspecific binding of mAbs. Unconjugated rat mAbs were revealed by FITC-conjugated goat anti-rat IgG and IgM Ab (Southern Biotechnology, Birmingham, AL) as the second reagent. Isotype controls were conjugated or unconjugated rat IgG2a, and hamster IgM (all from BD PharMingen). All staining reactions were performed on ice. After each step, cells were washed twice with 1% FCS and 0.1% sodium
azide in PBS. After the final incubation, cells were fixed with paraformaldehyde (1%). Cells were analyzed on a FACSCalibur cytometer using CellQuest software (BD Biosciences, San Jose, CA).

**Intracellular staining and ELISA**

For analysis of intracellular IFN-γ and IL-4 production, T cells from BALB/c mice were cocultured with allogeneic DC from B10.A mice (DC/T cell ratio of 1:20; 2 × 10^5 cells/well) in 96-well plates for 5 days. A suspension of splenic DC was cultured with an Ab cocktail composed of anti-I-A^d, CD45R0/B220 (BD Pharmingen), with a resulting purity of >98%. Primed cells were stained with anti-mouse anti-CD3e mAb (5 μg/ml; clone 145.2C11; BD Pharmingen) and anti-mouse CD28 mAb (3 μg/ml; clone 37.51; BD Pharmingen) for 6 h. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 4 h, and cells were collected, washed, fixed, saponin permeabilized (perm/fix solution; BD Pharmingen), and stained with FITC-labeled anti-IFN-γ and PE-labeled anti-IL-4 mAbs (BD Pharmingen), according to the manufacturer’s instructions. Appropriate fluorochrome-conjugated, isotype-matched, irrelevant mAbs were used as negative controls. Cells were analyzed on a FACSCalibur cytometer using CellQuest software (BD Biosciences).

For analysis of intracellular IL-12 and TNF-α production, on day 7, DC were incubated in the presence or absence of PTX for 6 h. Brefeldin A (10 μg/ml) was added for the last 4 h. Cells were stained as described above with PE-labeled anti-IL-12p40/p70 (clone C15.6; BD Pharmingen) or FITC-labeled anti-TNF-α (clone MP6-XT22; BD Pharmingen). Appropriate fluorochrome-conjugated, isotype-matched, irrelevant mAbs were used as negative controls.

**Mixed leukocyte reaction**

Unstimulated DC or PTX-DC from B10.A mice were exposed to 15 Gy of x-ray irradiation, and various dilutions from 3 × 10^7 to 1 × 10^8 cells/well were added to round-bottom 96-well microtiter plates. Allogeneic responder T cells from BALB/c mice were purifed using Dynabeads M-280 (Dynal) with the indirect method using an Ab cocktail composed of anti-I-A^d, CD45R0/B220 (BD Pharmingen), with a resulting purity of >98%, and added at a final concentration of 2 × 10^5 cells/well. The cultures were then incubated for 96 h, followed by an 18-h pulse with 0.5 μCi of [3H]thymidine/well. Results are expressed as mean cpm ± SD for triplicate wells.

**ERK phosphorylation**

DC at a concentration of 1 × 10^5 cells/ml were challenged with or without 10 ng/ml PTX in modified Eagle’s medium containing 0.1% BSA at 37°C for 15 min. The cells were then lysed in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2% SDS, and 1% 2-ME. Aliquots (50 μg of protein) of the whole cell extract were prepared and subjected to 10% SDS-PAGE and then electroblotted onto nitrocellulose membranes. The total amounts of ERK and phosphorylated ERK were detected by anti-total p44/p42 MAPK Abs and anti-phospho-p44/p42 MAPK Abs (New England Biolabs, Beverly, MA), respectively.

**Reproducibility and data presentation**

Experiments were repeated at least twice, and usually three or more times. Figures show data compiled from several experiments, or from a representative experiment, as specified. Results represent the mean ± SD, where applicable. Statistical significance of differences was analyzed using the independent Student’s t test. Values of p < 0.05 were considered significant. The results of statistical analyses are given in the figure legends. Statistical analysis of EAU scores was determined by Snedecor and Cochran’s (28) test for linear trend in proportions (nonparametric, frequency based). Each mouse (average of both eyes) was treated as one statistical event.

**Results**

**PTX-DC can substitute for PTX and play an adjuvant role in the induction of EAU**

We hypothesized that DC represent the cellular targets for PTX, and that DC could be manipulated by PTX to promote a characteristic Th1 response in vivo, characterized by high IFN-γ and IL-12p70 production, on day 7, DC were incubated in the presence or absence of PTX for 6 h. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 4 h, and cells were collected, washed, fixed, saponin permeabilized (perm/fix solution; BD Pharmingen), and stained with FITC-labeled anti-IFN-γ and PE-labeled anti-IL-12p40/p70 (clone C15.6; BD Pharmingen), according to the manufacturer’s instructions. Appropriate fluorochrome-conjugated, isotype-matched, irrelevant mAbs were used as negative controls. Cells were analyzed on a FACSCalibur cytometer using CellQuest software (BD Biosciences).

**Materials and Methods**

For the unstimulated DC control, the mice were injected i.p. with syngeneic DC that were cultured in the absence of PTX. For PTX-DC group, the mice were injected i.p. with syngeneic DC that were cultured in the presence of PTX for the final 24 h of incubation. For PTX group (EAU positive control), the mice were given PTX (0.5 μg/mouse) via i.p. injection. To exclude the possibility that PTX may be absorbed by DC in vitro and free PTX released following DC injection in vivo, the mice were injected i.p. with PTX as an additional adjuvant, B10.A mice fail to develop EAU (29). Therefore, the immunization with IRBP, CFA, and PTX is required to induce EAU (30). Preliminary experiments were performed to determine the numbers of DC that were needed to induce the disease. A total of 1 × 10^8 PTX-DC/mouse were optimal numbers for inducing EAU. The eyes from recipient mice were collected 21 days following immunization and cell transfer, and EAU severity was evaluated by histopathology. As expected, mice receiving unstimulated DC or PTX-fibroblasts did not develop EAU (Fig. 1, A and C), and EAU disease was restored in the mice receiving PTX-DC treatment (Fig. 1, B and C). Both incidence and severity of disease in PTX-DC group were comparable to the EAU-positive control mice (PTX group) (Fig. 1C). The results suggest that PTX-DC is capable of substituting for PTX’s adjuvant effect on EAU induction in vivo.

**Mice with EAU enhanced by PTX-DC have a typical Th1 response to IRBP**

Our previous work has shown that a dominant Th1-like response is required for EAU development in B10.A mice (29). Because the recipients developed EAU after PTX-DC treatment, we examined whether the recipients produced an IRBP-specific Th1 response. Twenty-one days after immunization with IRBP in CFA, draining LN cells were collected from mice that received different treatments. The cells were restimulated in vitro for 48 h in the absence or presence of IRBP. The supernatants were collected from mice that received PBS or unstimulated DC (Fig. 2, A and B). IL-4 was undetectable in cell culture supernatants in all groups (data not shown). This finding is consistent with our previous observation that a Th1-like response correlates with susceptibility to EAU in B10.A mice (29). To confirm the presence of a dominant Th1 response in vivo, we determined IRBP-specific IgG2a and IgG1 Abs in serum of individual mice 21 days after immunization. IFN-γ and IL-4 serve as switch factors for IgG2a and IgG1, respectively. Thus, an increased IgG2a/IgG1 ratio is evidence of a response shifting toward Th1. The data showed that in PTX-DC group, IgG2a levels were increased and IgG1 levels were decreased as compared with unstimulated DC group (Fig. 2, C and D), consistent with a Th1-dependent isotype switch. Taken together, these results suggest that PTX-DC induce a Th1 response in vivo, characterized by high IFN-γ and IL-12p70 secretion, increased levels of anti-IRBP IgG2a, and decreased levels of anti-IRBP IgG1.
PTX-DC acquired the functional features to induce Th1-like responses (IFN-γ) in vitro. Unstimulated DC or PTX-DC were cocultured with purified naive BALB/c T cells (DC-T cell ratio of 1:20) for 5 days. For intracellular cytokine staining, after 5-day coculture, IFN-γ, IL-4, and IL-12 levels were determined by ELISA. As shown in Fig. 3A, IFN-γ-producing T cells were significantly increased when allogeneic PTX-DC served as the stimulator cell compared with unstimulated DC. Both PTX-DC and unstimulated DC elicited few IL-4-producing T cells (Fig. 3A). To further confirm this observation, the supernatants were collected, and IFN-γ and IL-4 levels were determined by ELISA. As shown in Fig. 3B, PTX-DC induced higher IFN-γ secretion as compared with unstimulated DC. IL-4 was undetectable by ELISA (data not shown). These results clearly demonstrate that PTX-DC preferentially induce IFN-γ production by T cells in vitro.

PTX induces DC maturation

To efficiently prime naive T cells, immature DC must be stimulated and acquire a mature phenotype, as defined by the up-regulation of MHC class II, costimulatory molecules, and the secretion of cytokines, such as IL-12 and TNF-α (13, 31). The phenotype and cytokine secretion profiles of unstimulated DC and PTX-DC were analyzed by flow cytometry. The data showed that the expression of MHC class II, DEC205, CD80, CD86, and CD40 was all increased in PTX-DC (Fig. 4A). This implies that DC are activated by PTX. PTX-DC also secreted higher levels of IL-12 and TNF-α (Fig. 4, B and C). IL-6 and CCR7 mRNA expression were also up-regulated in PTX-DC (data not shown). To test their allostimulatory ability, unstimulated DC or PTX-DC were cocultured with naive BALB/c T cells in a standard 96-h primary MLR. PTX-DC had a higher ability to stimulate T cell proliferation as compared with unstimulated DC (Fig. 4D). Again, the stimulatory effect of PTX-DC on MLR was consistent with the ability of PTX to drive DC maturation.

Serum IL-12 secretion is increased in naive mice receiving PTX-DC injection

Because IL-12 is an initiator needed to switch on Th1 responses and because mature PTX-DC are able to produce IL-12 in vitro, we hypothesized that PTX-DC have a potential to alter the systemic environment in vivo and favor the development of a Th1 response. To address this hypothesis, serum IL-12 production was determined in naive mice that received unstimulated DC, PTX-DC, or PTX-fibroblasts. Injecting PBS served as the negative control. Serum samples were collected from the mice at 4 and 24 h after cell transfer. IL-12p70 protein was determined by ELISA. The data showed that mice receiving PTX-DC injection produced significantly higher levels of IL-12p70 than mice receiving unstimulated DC or PTX-fibroblasts. IL-12p70 peaked at 4 h and lasted at 24 h after PTX-DC transfer (Fig. 5). The results suggest that PTX-DC

FIGURE 1. B10.A mice develop EAU after immunization with IRBP plus PTX-DC, but not with unstimulated DC. B10.A mice were immunized with s.c. IRBP in CFA, and were then injected i.p. with freshly isolated syngeneic unstimulated DC or PTX-DC suspended in PBS, as described in Materials and Methods. Twenty-one days later, the eyes were processed for histopathology. A, Normal retina (R) with intact photoreceptors (+) and choroid (C) were observed in the eye of a B10.A mouse immunized with IRBP plus unstimulated DC (disease grade 0) (×400). B, In contrast, disorganized retina with retinal vasculitis (arrows) and thickening of the choroid are observed in the eye of a B10.A mouse immunized with IRBP plus PTX-DC (disease grade 2–3) (×400). C, EAU scores in mice receiving PTX-DC or PTX at all points differ significantly from mice receiving unstimulated DC or PTX-fibroblast controls (p < 0.05). Each symbol represents the pathology score for one mouse (average of both eyes). The average EAU score of each group is denoted by the horizontal bar. The results are a pool of two separate experiments.

FIGURE 2. IRBP-specific cytokine production by draining LN and serum concentrations of anti-IRBP IgG2a and IgG1 Abs in B10.A mice immunized with IRBP. B10.A mice were immunized with IRBP in CFA, and received an i.p. injection of PBS, unstimulated DC, or PTX-DC. Cells were prepared from draining LN of mice 21 days after immunization and were cultured for 48 h with or without (-) IRBP (30 μg/ml). The supernatants were collected, and IFN-γ, IL-4, and IL-12p70 were determined by ELISA. Sera were collected from IRBP-immunized individual mice, as mentioned above, and IRBP-specific IgG2a (1/400 dilution) and IgG1 (1/1600 dilution) were determined by ELISA. A, IFN-γ production. B, IL-12p70 production. IL-4 production is undetectable (data not shown). C, IRBP-specific IgG2a. D, IRBP-specific IgG1. Statistical signiﬁcant differences from control values are indicated by asterisks (*, p < 0.05; **, p < 0.01). Data are presented as the mean ± SD, n = 5, and are representative of two separate experiments. N.D., not detectable.

FIGURE 3. A, Purified naive BALB/c T cells (1×10⁶) were restimulated with plate-bound anti-CD3/anti-CD28 (1:20) for 5 days. For intracellular cytokine staining, after 5 days, IL-4 levels were determined by ELISA. As shown in Fig. 3A, IFN-γ-producing T cells were signiﬁcantly increased when allogeneic PTX-DC served as the stimulator cell as compared with unstimulated DC. IL-4 production by T cells cocultured with PTX-DC or PTX at all points differ signiﬁcantly from mice receiving unstimulated DC or PTX-fibroblast controls (p < 0.05). Each symbol represents the pathology score for one mouse (average of both eyes). The average EAU score of each group is denoted by the horizontal bar. The results are a pool of two separate experiments.
might increase systemic IL-12 production and in turn promote the development of Th1 responses in vivo.

**PTX-DC are capable of migration in vivo and in vitro**

To initiate specific T cell-mediated immune responses, the direct migration of DC to draining LN appears to be essential. Because PTX-DC are able to enhance Th1 responses and induce EAU in vivo, it is likely that PTX-DC have a potential to migrate after adoptive transfer in vivo. To confirm this, unstimulated DC, PTX-DC, and PTX-fibroblasts were labeled with the fluorescent cell tracker CFSE and injected i.p. into naive mice. The inguinal LN cells were harvested 48 h later. The migratory capacities of DC from the peritoneal cavity to the draining LN were evaluated by measuring CFSE-labeled DC in inguinal LN by flow cytometry. To exclude the possibility that fluorescent cells within the LN preparation were derived from host phagocytic cells that had ingested CFSE-labeled cells, the mice were injected i.p. with syngeneic CFSE-labeled PTX-fibroblasts as a control. The data showed that CFSE+ and CD11c+ double-positive cells were undetectable in inguinal LN from the mice injected with either PBS or PTX-fibroblasts (Fig. 6A). In contrast, these cells were detectable in inguinal LN of mice that had received either unstimulated DC or PTX-DC, although their percentages were low and there were no significant difference between two groups (Fig. 6A). To directly address whether PTX-DC can migrate, we designed the following experiment. Because CCR7 is highly expressed on the surface of mature DC and its ligand (MIP-3β)-triggered response plays critical roles in mature DC migration in mice (32), we measured the chemotaxis of PTX-DC in response to MIP-3β in vitro.

As shown in Fig. 6B, PTX-DC had a higher migratory capability in response to MIP-3β than that of unstimulated DC. In the same experimental system, LPS, a known DC maturation-enhancing factor...
maturation and IFN-ERK signaling pathway is involved in PTX-mediated DC activation in vitro and in vivo. Under our experimental conditions, PTX-DC were capable of migration and activation, the activation of the ERK signaling pathway was determined. When DC were exposed to PTX for 15 min, the level of phospho-p44/p42 MAPK was increased (Fig. 7A). The data indicated that ERK signaling pathway was activated in PTX-DC. To further confirm whether ERK signaling pathway has a regulatory role in the maturation of PTX-DC, PD98059, a highly specific inhibitor of the ERK signaling pathway (34), was used to treat PTX-DC. Preliminary experiments were performed to determine an optimal concentration of PD98059. The selected concentrations (50 or 100 μM) of PD98059 had a strong inhibition on ERK signaling, but had little influence on DC viability as determined by trypan blue dye exclusion (data not shown). When DC were pretreated with 50 μM PD98059 for 30 min before PTX was added, the up-regulation of CD86 expression was significantly diminished (Fig. 7B) and PTX-induced up-regulation of MHC class II exhibited minor changes (data not shown). When DC were pretreated with 50 or 100 μM PD98059 for 30 min before PTX stimulation, IL-12 and TNF-α production was reduced (Fig. 7C). These results revealed that ERK signaling pathway might be involved in the regulation of maturation and proinflammatory cytokine (IL-12 and TNF-α) production in PTX-DC. To address whether ERK signaling pathway is involved in the ability of PTX-DC to induce IFN-γ production by T cells, DC were pretreated with 50 μM PD98059 for 30 min before PTX stimulation, and then cocultured with naive allogeneic T cells. Intracellular IFN-γ and IL-4 in T cells induced by PTX-DC were analyzed by flow cytometry. It was found that once ERK signaling was inhibited in PTX-DC, the ability of PTX-DC to induce IFN-γ production by T cells was significantly eliminated (Fig. 7D). Taken together, these results demonstrated that ERK signaling pathway might play an important role in PTX-stimulated DC activation, contribute to DC maturation and proinflammatory cytokine secretion, and in turn promote IFN-γ induction by T cells in vitro.

**DC activation evoked by PTX is involved in EAU induction**

Because ERK signaling pathway might be involved in PTX-DC activation and function, then we asked whether PTX-stimulated DC activation was related to its adjuvant effect on EAU induction. To answer this question, we evaluated the induction of EAU conferred by injection of PTX-DC that had been pretreated with 50 μM PD98059 or DMSO (vehicle control) for 30 min before PTX stimulation (under this condition, the maturation and the cytokine secretion of PTX-DC were reduced). B10.A mice were immunized...
FIGURE 7. Inhibition of ERK signaling in PTX-DC reduces its maturation and cytokine production. A, PTX activates ERK kinases. DC were incubated with or without 10 ng/ml PTX for 15 min. The total amounts of ERK and phosphorylated ERK were detected by anti-total p44/p42 MAPK Abs and anti-phospho-p44/p42 MAPK Abs, respectively, by Western blot analysis. Lane 1, Unstimulated DC; lane 2, PTX-DC. DC were preincubated with the indicated concentrations of PD98059 for 30 min and then with 10 ng/ml PTX for 6 and 24 h. B, The up-regulation of CD86 by PTX is inhibited by 50 μM PD98059. After 24 h, cell surface expression of CD86 was determined by flow cytometry. Line 1, Isotype control; line 2, unstimulated DC; line 3, PTX-DC; and line 4, PTX-DC plus 50 μM PD98059. C, PD98059 inhibits PTX-induced up-regulation of IL-12 and TNF-α of DC. After 6 h, IL-12 and TNF-α production was determined by intracellular cytokine staining. D, PD98059 suppresses PTX-DC-induced IFN-γ production by T cells. After 24 h, DC were harvested, washed twice, and then cocultured with alloreactive T cells, as mentioned in Fig. 3A. Results are representative of three separate experiments.

with IRBP in CFA concurrently with the injection of PD98059-pretreated PTX-DC. EAU score was evaluated by histopathology 21 days following immunization. The results demonstrated that EAU was strikingly diminished in the mice receiving PD98059-pretreated PTX-DC (Fig. 8). The data suggest that the PTX-stimulated DC maturation and cytokine secretion might be involved in the adjuvant effect of PTX-DC on EAU induction.

Discussion
PTX serves as an adjuvant to induce Th1-mediated experimental autoimmune disease. Previous work indicates that PTX treatment strongly enhances Th1 responses to the immunizing Ag in parallel to enhancing disease scores (1, 30). One report confirms that PTX treatment strongly enhances Th1 responses and suggests that the activation of APCs and the induction of IL-12 are involved in these processes (9). However, it has been difficult to unambiguously characterize the cellular targets of PTX in vivo. In this study, we have shown that DC treated with PTX can substitute for PTX in the induction of Th1-mediated EAU. It was interesting to suggest that PTX may have a novel role in promoting DC maturation and secreting proinflammatory cytokines, which might be involved in EAU induction.

The enhancement of PTX on Th1 response was seen not only in EAU, but also in experimental autoimmune encephalomyelitis and orchitis. Therefore, this enhancement may be a general characteristic of the animal models in which PTX is used as an adjuvant to elicit a Th1-mediated autoimmune disease.

In this study, we focused on whether DC are target cells for PTX in promoting a Th1 response in vivo. We initially examined the possibility that PTX was able to stimulate DC and consequently favor the development of a Th1 response. This possibility was confirmed by in vivo and in vitro experiments, because PTX-DC were capable of enhancing EAU (Th1-mediated disease) in vivo and promoting IFN-γ production by T cells in vitro.

Because mature DC not only have the capacity to prime naive T cells, but also to provide different signals to induce different types of T cell response (35), we then asked whether the adjuvant role of PTX was correlated with its ability to drive DC maturation. The data reveal that PTX-DC are likely to be mature DC, because MHC class II, CD80, CD86, DEC205, and CD40 were highly expressed on the cell surface of PTX-DC. The cells also secreted high amounts of TNF-α and IL-12, the latter of which is critical in inducing Th1 responses (13, 31). Thus, PTX-DC provide stronger costimulatory signals and/or the proinflammatory cytokines needed for T cell activation and Th1 development. In humans, PTX has been proved to induce human monocyte-derived DC maturation and increase IL-12 production as well as to promote Th1 cell development in vitro (36, 37). In mice, Hofstetter et al. (38) have observed that PTX enhances the expression of MHC class II and costimulatory molecules on cells isolated from spleen or spinal cords. He et al. (9) have shown that inoculation of PTX in mice enhances IL-12 production by splenocytes in response to both microbial and nonmicrobial stimuli. All these experiments suggest that PTX has a potential to drive DC maturation.
Tarrant et al. (39) have reported that endogenous IL-12 is required to generate uveitogenic effector cells and the development of EAU. Yokoi et al. (40) have also observed that the anti-IL-12 treatment during the IRBP-priming phase prevents EAU induction. Our data showed that serum IL-12 was increased in naive mice that received PTX-DC injection. This finding implies the possibility that an increase in systemic IL-12 production might promote autoreactive T cell activation, favor Th1 development, and finally elicit EAU. The question raised by this new finding is that it is not clear for what cell types to produce IL-12 in vivo. It is most likely that IL-12 probably originates from donor PTX-DC because IL-12 secretion reached a peak 4 h after cell transfer. In our experimental model, it is possible that PTX-DC could initially secrete proinflammatory cytokines which might result in the activation of recipient APCs or NK cells, in turn to secrete IL-12 or IFN-γ, respectively, and drive T cell differentiation toward the Th1 pathway (41, 42).

In addition to secreting IL-12, PTX-DC may interact with host T cells. This communication between the cells might also contribute to the ability of PTX-DC to induce EAU. To address this possibility, the ability of PTX-DC to migrate was tested in vivo and in vitro. In vivo, both injected unstimulated DC and PTX-DC were detectable in draining inguinal LN, although the percentages were very low. The data suggest that PTX-DC are able to migrate from the peritoneal cavity into the draining inguinal LN, where autoreactive T cells can be activated and differentiate into Th1 cells. In vitro, we also found that PTX-DC could be stimulated to migrate in response to MIP-3β, suggesting that PTX-DC are likely to be mature DC that have the potential to migrate in vitro. Labeur et al. (43) have reported that murine bone marrow-derived DC appear to migrate very inefficiently into regional LN after s.c. injection, regardless of their maturation state; however, their Ag-presenting capacity in vivo correlates with their maturation stage. Eggert et al. (44) have demonstrated that bone marrow-derived DC are able to accumulate in the T cell areas of draining LN when DC are delivered by s.c. administration or i.p. injection.

In our experimental model, because PTX-DC were not pulsed with Ag in vitro, PTX-DC might affect the cytokine milieu in draining LN by secreting proinflammatory cytokines. This could favor the priming of newly Ag-exposed naive T cells to become Th1 cells or further induce activated Ag-specific T cells toward the Th1 phenotype in an Ag-independent manner (45, 46). In contrast, donor PTX-DC in draining LN might activate local DC and NK cells to secrete IL-12 and IFN-γ, respectively, further driving T cells to differentiate toward the Th1 pathway (41, 42). In addition, if autoantigen, IRBP would be presented by donor PTX-DC before naive T cells primed in vivo; PTX-DC could influence the Th1/Th2 balance as the major APCs involved in priming naive T cells in an Ag-dependent manner.

Eggert et al. (44) have presented evidence that after i.p. injection, most bone marrow-derived DC remain at the injection site. Labeur et al. (43) have reported that the vast majority of bone marrow-derived DC that remain at the site of injection exhibit equal in vitro survival, random migration in collagen gels, and allostimulatory capacity. In this study, we also observed that most injected PTX-DC did not reach draining LN. These data suggest that, in our experimental system, donor PTX-DC at the site of injection may also shift the Th1/Th2 balance through proinflammatory cytokine production without playing any direct effect on priming naive T cells, which are dependent on cell-cell contact in draining LN. This interpretation is consistent with the observation by Maldonado et al. (47), in which s.c. administration of CD8α+ DC combined with parenteral injection of IL-12 switches a Th2-type response to a Th1-type response. Smith and de St. Groth (48) have also reported that Ag-pulsed CD8α+ DC generate an immune response after s.c. injection without homing to draining LN.

Thus, there are three possibilities for PTX-DC in enhancing Th1 responses in vivo. First, PTX-DC might directly generate an immune response as APCs after homing to draining LN. Second, PTX-DC act as cytokine-producing accessory cells and indirectly deliver a proinflammatory message to T cells to enhance a Th1 response. Third, both the direct and indirect role of PTX-DC could be involved in its adjuvant effect.

PTX has the A-B architecture type of many bacterial toxins similar to cholera toxin and E. coli heat-labile toxin (49). Evidence shows that LPS and PTX bind to different domains on the same p73 receptor on murine splenocytes (50). Recent data imply that CD14 may be a binding site for PTX on myelomonocytic cells (51). These data suggest that PTX may share some endotoxin signal with LPS-mediated signaling (52). A prior report has shown that LPS activates ERK and regulates DC function (53). A recent study shows that PTX directly activates endothelial cell ERK kinases (33). We therefore propose that ERK kinases are key factors mediating the activation of DC by PTX. In this study, we found that PTX activated ERK signaling pathway. It is interesting to note that when ERK signaling was inhibited in PTX-DC, the expression of costimulatory molecules and the production of proinflammatory cytokines were reduced. Similarly, the ability of PTX-DC to promote IFN-γ production by T cells in vitro and its adjuvant effect on the EAU induction was blocked when the ERK signaling was inhibited. Bouchon et al. (54) have demonstrated that owing to selective activation of the ERK pathway, TREM-2/DAPI2 promotes a unique maturation program on human monocyte-derived DC. Thus, this DC activation pathway might be responsible for DC maturation and enhanced Ag presentation, which may result in T cell priming and differentiation in vivo.

In this study, we have shown DC activation evoked by PTX. PTX-DC acquired a feature of mature DC and secreted proinflammatory cytokines, which may be involved in its adjuvant effect in the EAU induction. Our data certainly do not exclude that pertussis toxin may be also affecting DC physiology by mechanisms that are not currently appreciated. In addition, our findings do not address the question as to whether PTX exerts effects on additional cell types that may contribute to its adjuvant activity in normal mice. NK cells or macrophages, which are the sources of IFN-γ and IL-12, respectively (55, 56), may also play a role in the enhancement of Th1-mediated EAU after PTX injection in vivo.

Overall, our results suggest that DC play a crucial role in the adjuvant effect of PTX on the induction of Th1 responses. The data show that this property of PTX is probably due to its ability to promote DC maturation and secrete proinflammatory cytokines. This mechanism might explain why microbial adjuvants or compounds, such as PTX, signal DC to undergo the coordinate maturation events that underlie efficient T cell priming and differentiation. Therefore, strategies that target DC activation and modulate DC function in vivo may have significant implications for vaccine design and the treatment of autoimmune disease.

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References


