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Pertussis Toxin by Inducing IL-6 Promotes the Generation of IL-17-Producing CD4 Cells

Xin Chen, O. M. Zack Howard, and Joost J. Oppenheim

Compelling evidence has now demonstrated that IL-17-producing CD4 cells (Th17) are a major contributor to autoimmune pathogenesis, whereas CD4+CD25+ regulatory cells (Treg) play a major role in suppression of autoimmunity. Differentiation of proinflammatory Th17 and immunosuppressive Treg from naive CD4 cells is reciprocally related and contingent upon the cytokine environment. We and others have reported that in vivo administration of pertussis toxin (PTx) reduces the number and function of mouse Treg. In this study, we have shown that supernatants from PTx-treated mouse splenic cells, which contained IL-6 and other proinflammatory cytokines, but not PTx itself, overcame the inhibition of proliferation seen in cocultures of Treg and CD4+CD25+ T effector cells. This stimulatory effect could be mimicked by individual inflammatory cytokines such as IL-1β, IL-6, and TNF-α. The combination of these cytokines synergistically stimulated the proliferation of CD4+CD25+ T effector cells despite the presence of Treg with a concomitant reduction in the percentage of FoxP3+ cells and generation of IL-17-expressing cells. PTx generated Th17 cells, while inhibiting the differentiation of FoxP3+ cells, from naive CD4 cells when cocultured with bone marrow-derived dendritic cells from wild-type mice, but not from IL-6−/− mice. In vivo treatment with PTx induced IL-17-secreting cells in wild-type mice, but not in IL-6−/− mice. Thus, in addition to inhibiting the development of Treg, the immunoadjuvant activity of PTx can be attributable to the generation of IL-6-dependent IL-17-producing CD4 cells. The Journal of Immunology, 2007, 178: 6123–6129.

Microbial infection has been proposed to initiate clinical manifestations of autoimmune disease in genetically predisposed individuals (1). A clear-cut example of microbial environment influencing autoimmunity is the observation that TCR-transgenic mice specifically reactive to myelin basic protein did not develop experimental autoimmune encephalomyelitis (EAE) when housed in a sterile facility. However, such mice developed EAE when housed in a conventional animal facility, or if they were administered pertussis toxin (PTx), which apparently can act as a surrogate for the environmental microbial effects (2, 3). PTx is a well-known microbial component with immunoadjuvant effects and the capacity to enhance the severity of EAE in permissive strains, and renders resistant strains susceptible to disease induction (4, 5). In addition to inducing CNS autoimmunity, PTx has been used widely to induce autoimmune diseases in other animal models, such as orchitis (6), uveitis (7), and inflammatory myopathy (8).

PTx is a major virulence factor of Bordetella pertussis, the agent that causes human whooping cough. PTx belongs to the A-B structure class of bacterial toxins (9). Its B subunit binds to a receptor on the cell surface, and the enzymatically active A subunit disrupts intracellular signalling by irreversible ADP ribosylation of the Gqi subclass of G protein (10). The mechanism by which PTx enhances autoimmunity appears rather complex and has not been completely understood. One prevailing proposal is that PTx breaks down the blood-brain barrier and thereby provides the primed T cells access to the target organ (11). However, this interpretation has recently come under scrutiny (12) due to the observation that PTx actually increased the expression of adhesion molecules, thereby initiating leukocyte infiltration into the brain (13). Accumulating evidence indicates that PTx induces the maturation of dendritic cells, which results in the expansion of T effector cells (14) and the differentiation of both Th1 and Th2 cells (12, 15). Activation of intracellular TLR4 signaling has also been implicated in PTx-mediated adjuvant activity (13, 16). Recently, we and others reported that in vivo treatment of PTx reduced the number of mouse FoxP3+ CD4+CD25+ T regulatory cells (Treg) (17, 18) and impaired the immunosuppressive functions of Treg (17).

Recent compelling evidence demonstrated that IL-17-producing T lymphocytes comprise a distinct lineage of proinflammatory Th cells, termed Th17 cells, that are major contributors to autoimmune disease (19). In contrast, Treg actively restrain the inflammatory response, suppress development of autoimmune diseases, and dampen a wide spectrum of immune responses (20, 21). Intriguingly, pathogenic Th17 and immunosuppressive Treg from naive CD4 cells are reciprocally induced, contingent upon the presence of either IL-6 or IL-2, respectively, in the presence of TGF-β (22–24). Based on evidence that PTx has the capacity to induce proinflammatory cytokines (25), while inhibiting IL-2 mRNA transcription induction by proinflammatory cytokines (26),
PTx promotes generation of Th17

**Materials and Methods**

**Mice and reagents**

Female wild-type (wt) C57BL/6 (Ly5.1 and Ly5.2) and BALB/c mice, 8–12 wk old, were provided by the Animal Production Area of the National Cancer Institute. The National Cancer Institute is accredited by the American Association for the Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (National Research Council, Washington, D.C.). IL-6 knockout mice were purchased from The Jackson Laboratory. Abs purchased from BD Pharmingen consisted of FITC anti-CD3 (145-2C11), FITC anti-CD4 (GK1.5), allophycocyanin anti-CD4 (L3T4), PE anti-CD25 (PC61), allophycocyanin anti-CD25 (PC61), PE anti-IL-4 (11B11), PE anti-IL-17 (TC11-18H10), PE anti-IFN-γ (XMG1.2), purified anti-CD3 (145-2C11), purified anti-CD16/CD32 (2.4G2), and anti-CD28 (35.71). PE anti-mouse/rat Foxxp3 staining set (FK1-16s) was purchased from ebioscience. Recombinant mouse cytokines (TNF, IL-1β, IL-6, and TNF levels were determined by multiplex mouse cytokine measurement. Data shown are representatives of three separate experiments with similar results. Error bars indicate SE derived from triplicate wells.

A theory that PTx may also promote the differentiation of Th17 cells, while concomitantly inhibiting Treg (17, 18).

In this study, we show that PTx-cultured supernatant (PTx-CS) that contained IL-6 and other proinflammatory cytokines, but not PTx itself, restored the proliferative response of cocultures containing CD4+CD25+ T effector cells (Teff) and Treg. Remarkably, the combination of IL-1β, IL-6, and TNF synergistically increased lymphocyte proliferation up to 400-fold in the cocultures and allowed Teff to escape from Treg-mediated inhibition, resulting in the generation of IL-17-producing cells, while reducing the proportion of Foxp3+ cells. Treatment with PTx both in vitro and in vivo promoted the generation of IL-17-producing CD4 cells in a TGF-β- and IL-6-dependent manner and suppressed the development of Foxp3+ CD4 cells. Our observation thus provides the first evidence that the immunoadjuvant activity of PTx may be based in part on the facilitation of IL-17 production. This property of PTx may also reflect a novel mechanism by which environmental infectious agents promote autoimmune diseases.

**PTx promotes generation of Th17**

**FIGURE 1.** Effect of PTx culture supernatant on proliferation of cocultures of Teff and Treg. A, PTx-CS was prepared by stimulating total splenocytes with indicated concentrations of PTx (0.1, 1, and 10 μg/ml) for 48 h. FACS-sorted CD4+CD25- cells (5 × 10⁴ cells/well), cultured alone or cocultured with CD4+CD25+ cells (2.5 × 10⁵ cells/well), were stimulated with APC and anti-CD3, in the presence of 50 μl of PTx-CS. The numbers under the column of PTx-CS are concentrations of PTx used to generate culture supernatant. B, In another set of experiments, PTx (1 μg/ml) or PTx-CS (stimulated with 1 μg/ml PTx) was added to cocultures of Teff and Treg (2:1). After 72-h incubation, cell proliferation was determined by [³H]thymidine incorporation assay. C, PTx stimulated production of proinflammatory cytokines and IFN-γ from splenocytes. Total splenocytes were cultured in 96-well plate (10³ cells/well) with medium alone or with 0.1–10 μg/ml PTX. After 48-h incubation, the supernatant was harvested, and IL-1β, IL-6, and TNF levels were determined by multiplex mouse cytokine measurement. Data shown are representatives of three separate experiments with similar results. Error bars indicate SE derived from triplicate wells.
plus 0.5 μg/ml soluble anti-CD3 Ab. CD4<sup>+</sup>CD25<sup>+</sup> T cells were added to the wells at desired ratio to CD4<sup>+</sup>CD25<sup>−</sup> cells. Cells were pulsed with 1 μCi of [<sup>3</sup>H]thymidine (Amersham Biosciences) per well for the last 6 h of culture period. In some experiments, the medium was supplemented with PTX-CS, PTX, IFN-γ, IL-1β, IL-6, or TNF-α, or their combinations. For CD4 cell activation, purified CD4 cells or naive CD4 cells (2.5 × 10<sup>4</sup> cells/well) were cultured with BMDC (1 × 10<sup>6</sup> cells/well) in a 24-well plate, stimulated with soluble anti-CD3 (0.5 μg/ml), in the absence or presence of PTX, for 3 days. In some experiments, anti-TGF-β (2 ng/ml) or anti-TNF (1–3) Ab (10 μg/ml) or IL-6 (20 ng/ml) was added to the cells. In some experiments, naïve CD4 cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) for 3 days.

**In vivo treatment with PTX**

The wt BALB/c, C57BL/6, or IL-6<sup>−/−</sup> mice used to evaluate the effect of PTX were injected intraperitoneally with 400 ng/mouse (in 0.2 ml of PBS) per day for 2 consecutive days. One week after treatment, the mice were sacrificed, lymphoid organs were removed aseptically, and a single cell suspension was prepared for the assays.

**Flow cytometry**

For blocking with anti-CD16/CD32 Ab, cells were incubated with appropriately diluted Abs. For CFSE-labeling assay, cells were labeled with 2 μM CFSE for 8 min at room temperature. For intracellular cytokine staining, the cells were stimulated with PMA (20 ng/ml; Sigma-Aldrich) and ionomycin (1 μM; Sigma-Aldrich) for 5 h in the presence of GolgiPlug (BD Pharmingen). The cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) and stained with relevant Abs. Samples were acquired on a FACSSort (BD Biosciences), and data analysis was conducted using CellQuest software (BD Biosciences).

**Statistical analysis**

Data were analyzed by two-tailed Student’s t test using GraphPad Prism 4.0 (GraphPad).

**Results**

**PTX-CS overcomes suppressed proliferation in cocultures of Teff and Treg**

We previously reported that in vivo treatment with PTX reduced the number and immunosuppressive activity of mouse Treg (17). However, treatment of Treg with PTX in vitro did not reduce FoxP3 expression and their immunosuppressive activity (17). This led us to hypothesize that PTX-elicited soluble factor(s) may be responsible for the in vivo effect of PTX. To test this idea, we generated PTX-CS by stimulating total splenocytes with various concentrations of PTX (0.1–10 μg/ml) for 48 h and then added the PTX-CS to cocultures of Teff and Treg. As shown in Fig. 1A, Teff potently suppressed the proliferation of Treg, and this inhibition could be reversed by addition of PTX-CS, in a dose-dependent manner. Contaminating PTX was not responsible for this effect because addition of PTX to the cells had only a minimal effect (Fig. 1B). Thus, soluble factor(s) contained in the PTX-CS, but not PTX itself, was responsible for the enhancement of proliferation of cocultures.

It was reported previously that proinflammatory cytokines had the capacity to abrogate inhibition of proliferation in cocultures of Treg and Teff (28, 29); we therefore identified the proinflammatory cytokines contained in PTX-CS. Consistent with previous reports (25, 30), we found that unfractonated mouse splenocytes treated with PTX produced proinflammatory cytokines, including IL-6, TNF-α, IL-1β, as well as IFN-γ in a dose-dependent manner. IL-6 appeared to be the major proinflammatory cytokine produced by PTX-treated splenocytes, because its concentration was much higher than that of TNF and IL-1β (Fig. 1C).

**Inflammatory cytokines synergize to overcome the inhibition of proliferation by Treg**

Next, we examined the effect of proinflammatory cytokines on the lymphoproliferative response in cocultures of Teff and Treg. Consistent with earlier reports, exogenous IL-1β (29) and IL-6 (28) enhanced proliferation in cocultures. Furthermore, TNF also shared this property with IL-1β and IL-6. In contrast, IFN-γ did not affect the proliferative response in cocultures. The combination of IL-1β, IL-6, and TNF cell dramatically synergized in reversing the inhibition of proliferation in the cocultures with up to 400-fold increase in proliferation over the effect of medium alone (p < 0.0001; Fig. 2, A and B). The combination of any two inflammatory cytokines merely had an additive effect, or in the case of IL-1β plus IL-6 a modest synergistic effect (Fig. 2, A and B). Thus, the participation of all of the three inflammatory cytokines was indispensable to yield the dramatic synergistic proliferative response in the cocultures.

To identify the subset of CD4 cells in the cocultures responsible for the proliferative response to the three cytokines, we added 5–10 ng/ml of each cytokine to Teff, Treg, or their cocultures, respectively, together with APC and anti-CD3. As shown in Fig. 3A, the combination of the three cytokines promoted maximal proliferation of Teff in a dose-dependent manner (p < 0.01). Consistent with previous observation, Treg were hyporesponsive to stimulation with APC and anti-CD3; however, the anergic status of Treg was overcome by the addition of the three cytokines in a dose-dependent manner (p < 0.05–0.01). Although the inhibition of proliferation in the cocultures was reversed by addition of the
mained by [3H]thymidine incorporation assay. Comparison of effects of cytokines with medium-only cultures (CD25+ and CD25−) respectively; *, p < 0.05; **, p < 0.01). B, CFSE-labeled Ly5.1 CD4+CD25− cells (5 × 10^5 cells/well) or CFSE-labeled Ly5.2 CD4+CD25+ cells (5 × 10^5 cells/well) were cultured alone or cocultured with APC and anti-CD3 in the presence of IL-1β, IL-6, and TNF (10 ng/ml each). After incubation for 72 h, proliferation was analyzed by gating on CD4+CD45.1+ or CD4+CD45.2+ cells to detect CD4+CD25− cells or by gating on CD4+CD45.1− cells to detect CD4+CD25+ cells. CFSE-labeled cells were marked with *. The percentage of CFSE-diluted cells was shown on the histogram. C, CD4+CD25− cells (5 × 10^5 cells/well) were cultured with CD4+CD25+ cells at a ratio of 2:1, stimulated with APC and anti-CD3, with or without IL-1β, IL-6, and TNF (10 ng/ml each). Seventy-two hours later, aliquot of cells was harvested, and intracellular expression of FoxP3 was analyzed by FACS (gated on CD3+CD4+ population). B. The rest of cells were restimulated with PMA and ionomycin. Intracellular expression of IL-17 was analyzed by FACS (gated on CD3+CD4+ population, A). Numbers in quadrants represent the frequency of cells in each. The results shown are representatives of three separate experiments with similar results.

It was reported recently that IL-6 in concert with TGF-β (or its Treg source) induced naive CD4 cells to differentiate into Th17
intracellular expression of FoxP3 was analyzed by FACS (gated on producing cells was markedly inhibited by anti-TGF-β. PTx treatment promotes the generation of IL-17-producing CD4 cells in vitro. The wt C57BL/6 naive CD4 cells were cultured with BMDC derived from either wt C57BL/6 mice (A) or IL-6 knockout mice (B) with soluble anti-CD3 Ab (0.5 μg/ml), in the presence of medium alone, PTx (1 μg/ml) alone, TGF-β (2 ng/ml) alone, or PTx plus TGF-β. a. After 3 days, aliquot of cells was harvested, and intracellular expression of FoxP3 was analyzed by FACS (gated on CD3+CD4+ population). b. The rest of cells were restimulated with PMA and ionomycin. Intracellular expression of IL-17 was determined by FACS (gated on CD3+CD4+ population). Numbers in quadrants represent the frequency of cells in each. The data were representatives of three separate experiments with similar results.

FIGURE 5. Effect of PTx on the differentiation of FoxP3+ cells and IL-17-producing cells from naive CD4 cells. The wt C57BL/6 naive CD4 cells were cultured with BMDC derived from either wt C57BL/6 mice (A) or IL-6 knockout mice (B) with soluble anti-CD3 Ab (0.5 μg/ml), in the presence of medium alone, PTx (1 μg/ml) alone, TGF-β (2 ng/ml) alone, or PTx plus TGF-β. a. After 3 days, aliquot of cells was harvested, and intracellular expression of FoxP3 was analyzed by FACS (gated on CD3+CD4+ population). b. The rest of cells were restimulated with PMA and ionomycin. Intracellular expression of IL-17 was determined by FACS (gated on CD3+CD4+ population). Numbers in quadrants represent the frequency of cells in each. The data were representatives of three separate experiments with similar results.

PTx treatment promotes the generation of IL-17-producing CD4 cells in vitro

Our foregoing experiment suggested that PTx may facilitate the generation of IL-17-producing CD4 cells by inducing proinflammatory cytokines. To examine this hypothesis in vitro, purified CD4 cells were cultured with BMDC in the presence of anti-CD3, with or without PTx. As shown in Fig. 4A, after treatment with PTx for 3 days, a substantial proportion (8.9%) of these CD4 cells became IL-17-producing cells. PTx-induced generation of IL-17-producing cells was markedly inhibited by anti-TGF-β Ab. In contrast, only 0.8% of CD4 cells expressed IL-17 in the absence of PTx. Concomitantly, the proportion of FoxP3+ cells was reduced by ~50% by PTx treatment (Fig. 4B, p < 0.05), probably due to the loss of Treg, which has been shown to be more sensitive than Teff to PTx-induced cell death in vitro (18).

It has been reported that naive CD4 cells stimulated with TGF-β and IL-6 differentiated into Th17 cells (22–24). We therefore determined whether PTx could induce naive CD4 cells to produce IL-17. When naive CD4 cells were cocultured with BMDC from wt mice, TGF-β was able to induce the generation of FoxP3+ cells (9.3%), and this was reduced to 6.4% when PTx was added (Fig. 5Aa). In contrast with unfractonated CD4 cells, naive CD4 cells treated with PTx alone only yielded 1.2% of Th17 cells. In combination with TGF-β, PTx was able to induce 6.6% of naive CD4 cells to produce IL-17 (Fig. 5Ab). Presumably, exogenous TGF-β promoted differentiation of Th17 cells from naive CD4 cells and acted as a surrogate for TGF-β-expressing Treg present in unfractonated CD4 population.

PTx, rather than suppressing, actually enhanced the TGF-β-mediated generation of FoxP3+ cells from naive CD4 cells when they were cultured with BMDC derived from IL-6−/− mice (from 9.9 to 16.6%; Fig. 5Ba). Furthermore, the combination of TGF-β and PTx failed to induce Th17 cells when IL-6-deficient BMDC was used (Fig. 5Bb). Thus, in the absence of IL-6 signaling, naive CD4 cells developed into Treg, rather than producing Th17 cells, in response to PTx.
PTx treatment promotes the generation of IL-17-producing CD4 cells in vivo

To verify our in vitro observation in an in vivo setting, PTx was injected into wt BALB/c mice using the same regimen we used to induce EAE (31). One week after PTx treatment, LN cells and splenic cells were restimulated in vitro with PMA and ionomycin. An appreciable fraction of LN and splenic cells from PTx-treated mice expressed IL-17 (7.8 and 6.5% in the LN and splenic cells, respectively). By comparison, only 0.9 and 1.2% in the LN and splenic cells from control mice (injected with PBS) expressed IL-17 (Fig. 6A). Presumably, PTx may promote the generation of Th17 cells to a greater degree when coadministered with autantigen to induce experimental autoimmune disease. The generation of Th1 (IFN-γ-producing) cells and Th2 (IL-4-producing) cells from BALB/c mice was also examined. As shown in Fig. 6, B and C, the proportion of IFN-γ-producing splenic CD4 cells was markedly reduced in PTx-treated mice (6.3%), as compared with CD4 splenic cells from control mice (9.9%, p < 0.01). In contrast, the proportion of IL-4-producing cells was not significantly changed by PTx treatment (p > 0.05). Again, the proportion of FoxP3+ cells in CD3+CD4−subpopulation was reduced by ~50% (Fig. 6D). The capacity of PTx to induce Th17 cells in vivo was IL-6 dependent, because injection of PTx resulted in only 1.2% of IL-17-producing cells in IL-6−/− mouse LN cells and 6% in IL-6+/+ wt C57BL/6 mouse LN cells (Fig. 6F). Thus, in vivo treatment with PTx not only reduced the number of Treg, but also selectively enhanced the generation of IL-17-producing cells in an IL-6-dependent manner.

Discussion

In this study, we examined the effect of PTx on the generation of IL-17-producing CD4 cells in both in vitro as well as in vivo settings. Our findings support the conclusion that PTx, through proinflammatory cytokine pathway, facilitates the generation of IL-17-producing cells, which may contribute to its immunological adjuvant activity. This is compatible with the observation that administration of PTx stimulates the development of considerably more pathogenic Th17 cells in mice of immune-mediated ocular inflammation (7) than other TLR ligands, such as LPS, polyinosinic-polycytidylic acid, and, particularly, CpG oligodeoxynucleotide (G. Shi, unpublished observation). Furthermore, during the preparation of this manuscript, it was reported that immunization with whole cell pertussis vaccination in mice results in the generation of Th17 cells (32). This is probably due to Th17-promoting effect of PTx, as shown in our study.

Proinflammatory cytokines have been shown to possess immunological adjuvant-like effects. For example, proinflammatory cytokines have been reported to control the expansion and survival of effector CD4 T cells, presumably by providing a third signal in addition to the Ag-specific signal delivered by MHC-peptide complex and costimulatory molecules (33–35). Combination of IL-1β, IL-6, and TNF, or use of an adjuvant that induces these cytokines was reportedly able to overcome age-related defects in CD4 T cell response in vivo and in vitro (36). According to our observation, this combination of proinflammatory cytokines resulted in a robust proliferation of Treg despite the presence of Treg. Our finding proves a novel interpretation for the adjuvant-like action of proinflammatory cytokines based on the observation that this combination of proinflammatory cytokines actually also suppresses the proportion and function of Treg. Although the combination of proinflammatory cytokines may induce autoimmune responses, this combination may warrant further study as an immunotherapy in patients failing to respond to conventional treatment to cancer and AIDS.

It is now well established that Th17 plays an important role in the pathogenesis of EAE (37). PTx is indispensable to generate EAE in a genetically resistant mouse strain (38). It is therefore not surprising that PTx actually facilitates the generation of IL-17 production in response to a proinflammatory cytokine pathway. IL-6 is a crucial cytokine in the generation of Th17 cells (22–24), and IL-1 is able to enhance IL-23-mediated production of IL-17 (39) as well as IL-6-mediated differentiation of Th17 cells (24). IL-6 knockout mice are completely resistant to EAE induction despite the administration of PTx (22, 40). Mice deficient in IL-1 signals (IL-1 knockout or IL-1RI knockout) are also resistant to EAE induction in response to the adjuvant effects of PTx, whereas IL-1R antagonist knockout mice can develop EAE even without PTx administration (39, 41, 42). Thus, it appears that PTx uses the Th17 pathway as well as IL-1 and IL-6 to trigger autoimmune EAE pathogenesis.

Previous reports showed that PTx promoted Th1 differentiation (12, 15), and IFN-γ (as well as IL-4) was reported to inhibit the differentiation of Th17 cells (22–24). Indeed, we also observed that in vitro treatment with PTx resulted in the production of IFN-γ from splenic cells. However, because PTx is able to promote generation of Th17 cells in vitro and in vivo, the proinflammatory cytokines elicited by PTx appear to prevail over any coproduced Th1 (or Th2) cytokines. Furthermore, following in vitro restimulation with PMA and ionomycin, splenic cells isolated from PTx-treated BALB/c mice actually contained fewer IFN-γ-producing CD4+ cells than cells isolated from PBS-treated control mice (Fig. 6B). This discrepancy may be attributable to the reported dual actions of PTx (43) and the potential suppressive effects of IL-17 on the differentiation of Th1 cells.

In our experimental studies, PTx was able to induce IL-17-producing CD4 cells from unfractonated CD4 cells that were cocultured with BMDC. However, neutralization of TGF-β partially blocked the generation of IL-17-producing cells by PTx (Fig. 4). These data suggest that TGF-β plays an important role in generation of IL-17-producing cells in response to PTx, and that naturally occurring Treg might be a major source of TGF-β. This notion is supported by the observation that PTx had very limited effect on the generation of IL-17-producing cells from naive CD4 cells, which were cocultured with wt BMDC. In contrast, exogenous TGF-β was able to restore the capacity of PTx to induce a substantial population of IL-17-producing cells (Fig. 5A,b). Therefore, although reducing the number of Treg, PTx may need some Treg to provide TGF-β to initiate the generation of Th17 cells. Surprisingly, PTx promoted TGF-β-mediated differentiation of FoxP3+ Treg from naive CD4 cells that were cocultured with BMDC derived from IL-6 knockout mice (Fig. 5A,b). Presumably, BMDC from IL-6 knockout mice can be activated by PTx to produce cytokines such as IL-1 and TNF and consequently activate cocultured CD4 cells to produce IL-2. It is known that activated dendritic cells themselves (for review, see Ref. 44), cytokines such as IL-2 (for review, see Ref. 45), and IL-1 (27) are capable of expanding Treg. Furthermore, we previously observed that TNF also could selectively and directly expand Treg by interacting with TNFR2, which is preferentially expressed by Treg, in the absence of IL-6 (our unpublished observation). Therefore, PTx has the capacity to elicit an IL-6-independent Treg response in cocultures of naive CD4 cells and BMDC, whereas PTx favors induction of the Th17 pathway in the presence of IL-6.

Taken together, we have revealed the capacity of PTx to promote generation of IL-17-producing CD4 cells through a proinflammatory cytokine pathway. This action of PTx may certainly contribute to the immunological adjuvant effect of PTx. PTx is a
major virulence factor of *B. pertussis*, and infection by this pathogen was reported to be capable of being an initiation or developmental factor of autoimmune disease (46, 47). Thus, our findings may represent a novel mechanism by which this environmental microbial toxin can promote autoimmunity.

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**Disclosures**

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