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Pertussis Toxin Reduces the Number of Splenic Foxp3⁺ Regulatory T Cells¹

Cécile Cassan,* Eliane Piaggio,* Jacques P. Zappulla,* Lennart T. Mars,* Nicolas Couturier,* Florence Bucciarelli,* Sabine Desbois,* Jan Bauer,[†] Daniel Gonzalez-Dunia,* and Roland S. Liblau*

Pertussis toxin (PTx) is a bacterial toxin used to enhance the severity of experimental autoimmune diseases such as experimental autoimmune encephalomyelitis. It is known to promote permeabilization of the blood-brain barrier, maturation of APC, activation of autoreactive lymphocytes and alteration of lymphocyte migration. In this study, we show that i.v. injection of PTx in mice induces a decrease in the number of splenic CD4⁺CD25⁺ regulatory T cells (Treg cells). Furthermore, PTx not only induces a depletion of the dominant CD4⁺CD25⁺Foxp3⁺ subpopulation of splenic Treg cells, but also reduces to a similar extent the CD4⁺CD25⁻Foxp3⁺ subpopulation. On a per cell basis, the suppressive properties of the remaining Treg cells are not modified by PTx treatment. The reduction in splenic Treg cells is associated with preferential migration of these cells to the liver. Additionally, Treg cells exhibit a high sensitivity to PTx-mediated apoptosis in vitro. Finally, in vivo depletion of Treg cells by injection of an anti-CD25 Ab, and PTx treatment, present synergistic experimental autoimmune encephalomyelitis exacerbating effects. Therefore, we identify a new effect of PTx and provide an additional illustration of the influence of microbial components on the immune system affecting the balance between tolerance, inflammation and autoimmunity. *The Journal of Immunology*, 2006, 177: 1552–1560.

Pertussis toxin (PTx)³ has been used for over three decades as an adjuvant allowing to enhance experimental autoimmune encephalomyelitis (EAE) severity in permissive strains and even to render resistant strains susceptible (1, 2). Its disease-enhancing properties are not restricted to CNS autoimmunity, but also apply to other animal models of autoimmunity such as orchitis (3), uveitis (4, 5), or inflammatory myopathy (6). This 105-kDa protein complex is produced by *Bordetella pertussis*, the bacterium responsible for whooping cough in humans. PTx is immunogenic and involved in the pathogenesis of *B. pertussis* infection (7, 8) and, therefore, has been included in some acellular pertussis vaccines in a chemically or genetically detoxified form (9).

The five proteins that compose PTx (S1, S2, S3, S4, and S5) are encoded by genes organized in a single operon (10). PTx belongs to the A-B class of exotoxins. The B subunit, which is a pentamer (S2, S3, S4, and S5 with a 1:1:2:1 stoichiometry) presents sequence homologies with selectins. It binds glycoproteins (N-linked oligosaccharides and sialylated glycoconjugates) and glycolipids (glucosylceramide, lactosylceramide) expressed on the surface of many eukaryotic cell types (11–13). The A subunit, a single pro-

tein (S1), is then released into the cytoplasm and mediates ADP-ribosylation of the α -subunit of Gi proteins. Thereby, it interferes with the inhibitory activity of Gi proteins on adenylate cyclase, inducing an increase of intracellular cAMP level, and uncouples G-coupled receptors from their signaling pathway (14, 15).

Numerous immunological effects have been attributed to PTx. On the innate immune system, PTx decreases the production of IL-6 and IL-10 by mast cells (16), promotes maturation of APC leading to the up-regulation of MHC class II or costimulatory molecules and production of IL-12 (4, 9, 17, 18). On the adaptive immune system, PTx increases both Th1 and Th2 responses (19–21), and inhibits chemokine-induced lymphocyte migration (22, 23). Classically, the exacerbating effect of PTx on EAE was attributed to increased sensitization to histamine (24, 25), and permeabilization of the blood-brain barrier (24, 26). More recently, PTx was shown to enhance rolling and adhesion of activated T cells on pial vessels. This effect was related to cerebrovascular induction of P-selectin expression, and depended on TLR4 signaling (27, 28). Thus, following administration of PTx, several mechanisms concur to exacerbate autoimmune diseases through enhanced activation of autoaggressive T cells (29) and increased infiltration of the target tissue.

Given the pleiotropic effects of PTx on the immune system, we hypothesized that it might affect CD4⁺CD25⁺ regulatory T (Treg) cells and thereby contribute to its EAE-enhancing properties. Indeed, Treg cells represent a natural population of regulatory cells that plays a major role in the maintenance of peripheral tolerance (30). They develop in the thymus and the transcription factor Foxp3 is essential for Treg lineage specification and function (30, 31). They have been shown to play an important role in the protection against EAE (32–35). Moreover, altered functions of Treg cells from blood of multiple sclerosis patients have recently been identified, inferring that they could also be crucial in regulating immune responses in this disease (36, 37). We thus investigated whether PTx could quantitatively or qualitatively affect Treg cells

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³ Abbreviations used in this paper: PTx, pertussis toxin; EAE, experimental autoimmune encephalomyelitis; Treg, regulatory T; PI, propidium iodide; MFI, mean fluorescence intensity.

and whether this effect could contribute to its adjuvant activity in EAE.

Materials and Methods

Mice

Female C57BL/6 mice of 6–12 wk of age were purchased from Charles River Laboratories and were bred at the IFR30 animal facility under specific pathogen-free conditions. Congenic CD45.1 mice were either purchased from CDTA (Centre National de la Recherche Scientifique) or from our animal house facility (IFR30, Toulouse). All experimental protocols were approved by the local ethic committee on animal experimentation and are in compliance with European Union guidelines.

Abs and PTx

PTx (~99% pure) was from List Biological Laboratories. Anti-CD4-FITC (RM4-5), anti-CD8-FITC (53-6.7), anti-CD45.2-FITC (104), and anti-B220-PE (RA3-6B2) mAbs were obtained from BD Pharmingen. Anti-CD25-PE (PC61), anti-CD4-PE (CT-CD4) mAbs, and Streptavidin Tri-Color conjugate were purchased from Caltag Laboratories. Anti-CD4-allophycocyanin (RM4-5) and anti-Foxp3-PE (FJK-16s) mAbs were obtained from eBioscience. Abs used for cell purification were produced in the laboratory (anti-CD8, anti-B220, anti-MHC class II, and anti-Mac-1).

Cell purification

For the purification of CD4⁺CD25⁺ or CD4⁺CD25[−] T cells, spleen and peripheral lymph nodes were recovered. Negative selection of CD4⁺ cells was performed by incubating the single cell suspension with anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-MHC class II (M5-114), and anti-Mac-1 (M1/70) mAbs, followed by depletion with magnetic beads coated with anti-rat IgG (DynaL Biotech). Cells were then incubated with a biotinylated anti-CD25 mAb (7D4), and positively selected using streptavidin-conjugated microbeads from Miltenyi Biotec. Cell separation was performed on MS columns from Miltenyi Biotec. Purity was ~80% for the CD4⁺CD25[−] population or the CD4⁺CD25⁺ population.

For the *in vitro* assessment of Treg cell function, CD4⁺CD25^{bright} cells were purified from PBS- or PTx-treated mice. To this end, negatively selected CD4⁺ cells were incubated with PE-conjugated anti-CD25 (PC61; BD Pharmingen), washed, and then incubated with anti-PE-conjugated microbeads (Miltenyi Biotec). After enrichment on MS columns, CD4⁺CD25^{bright} isolation was achieved based on CD25-PE and CD4-FITC labeling on a High Speed Cell Sorter Epics ALTRA (Beckman Coulter). Purity was above 98% for CD4⁺CD25⁺ T cells isolated from either PBS- or PTx-treated mice.

Flow cytometry analyses

Splenocytes or lymph node cells (3×10^6 cells/50 μ l) were incubated in FACS buffer (PBS containing 10 g/L BSA and 0.2 g/L Na₂N₃) with fluorochrome-conjugated Abs for 30 min on ice and then washed. For blood cell staining, 50 μ l of peripheral blood was incubated for 30 min on ice with a mix of Abs; RBC were lysed with FACS Lysing solution (BD Biosciences), and samples were washed three times in FACS buffer. For intranuclear staining using anti-Foxp3-PE mAb, we followed the manufacturer's instructions.

For annexin V and propidium iodide (PI) staining, fractionated CD4⁺ T cells were recovered after 4 h of incubation at 37°C in the presence or absence of PTx, washed in FACS buffer, and stained with PE-conjugated anti-CD4 Ab. T cells were then washed and incubated with 50 μ l of Annexin V-FITC (Annexin V-FLUOS Staining kit; Roche) for 15 min at room temperature. Cells were washed, resuspended in FACS buffer, and analyzed on the cytometer within 2 h. PI was added at 170 ng/ml just before sample collection.

Data were collected on an Epics XL4C (Beckman Coulter) or a FACSCalibur (BD Biosciences) instrument, and analysis was performed using the CELLQuest software (BD Biosciences).

In vitro T cell proliferation and suppression assays

25,000 CD4⁺CD25[−] T cells purified from naive mice were stimulated with 0.5 μ g/ml anti-CD3 mAb (145 2c11) and 200,000 irradiated (2,500 rad) spleen cells (APC) in the presence of increasing numbers (from 0 to 25,000) of highly purified CD4⁺CD25^{bright} T cells isolated from PBS-treated or PTx-treated mice. Cultures were performed in round-bottom 96-well plates in complete medium: RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin-streptomycin (Eurobio), 10 mM HEPES (Invitrogen Life

Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), and 50 μ M 2-ME (Sigma-Aldrich Chimie). After 72 h of culture, 1 μ Ci of [³H]thymidine (Amersham Biosciences) was added to the wells for the last 12 h of culture. Cultures were harvested onto glass fiber filters (Packard), and [³H]thymidine uptake was measured with a MATRIX 9600 Direct Beta Counter (Packard).

Cytokine detection by quantitative RT-PCR

The expression of several cytokines was determined by quantitative RT-PCR on cDNA prepared from highly purified CD4⁺CD25^{bright} T cells. RNA was extracted with the RNeasy Mini kit (Qiagen) and genomic DNA was digested with the RNase-free DNA Set kit (Qiagen). First-strand cDNA was synthesized with SuperScript III (Invitrogen Life Technologies). PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the manufacturer's protocol, and SYBR Green Reagent (Eurogentec). Primer sequences were the following: for IFN- γ , 5'-TCAAGTGGCATAGATGTGGAAGAA-3' and 5'-TGGCCTCTGCAGGATTTTCATG-3'; for TGF- β 1: 5'-TGACGTCCTGGAGTTGTACGG-3' and 5'-GGTTCATGTCATGGATGGTGC-3'; for IL-10, 5'-GGTTGCCAAGCCTTATCGGA-3' and 5'-ACCTGCTCCACTGCCTTGCT-3'; for IL-2, 5'-CCTGAGCAGGATGGAGAATTACA-3' and 5'-TCCAGAACATGCCGACAGAG-3'; for β -actin, 5'-CAATAGTGATGACCTGGCCGT-3' and 5'-CACTGCCGATCCTCTTCCCTCC-3' (Prologo). Conditions for PCR were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles consisting of 15 s at 95°C, 1 min at 60°C.

The $\Delta\Delta C_t$ was calculated as following: $\Delta\Delta C_t = (C_t \text{ target gene} - C_t \beta\text{-actin})_x - (C_t \text{ target gene} - C_t \beta\text{-actin})_y$, where x = sample to analyze and y = sample arbitrarily chosen to normalize. Results were expressed as N-fold changes in target gene copies, according to the following equation: amount of target = $2^{-\Delta\Delta C_t}$. Two independent experiments were conducted for each gene and sample, and in each experiment, each sample was run in duplicate.

Analysis of tissue lymphocytes

Mice were perfused intracardially with 20 ml of PBS 3 days after the first injection of either PBS or PTx. Bone marrow was recovered by flushing medium through one femur. Brains were successively passed through a 150- and a 60- μ m cell strainer. Cells were resuspended in 20 ml of RPMI 1640 containing 30% Percoll (Amersham Biosciences) and deposited on 25 ml of RPMI 1640 containing 70% Percoll. After centrifugation for 20 min at 3000 rpm, mononuclear cells were recovered at the interface. Livers were mechanically dissociated, passed through a 70- μ m cell strainer, and lymphocytes were isolated after Percoll separation. Lungs were cut and incubated in RPMI 1640, 0.5 mg/ml Liberase TI (Roche Diagnostic), and 0.5 mg/ml DNase I (Sigma-Aldrich Chimie) at 37°C for 1 h. The suspension was passed through a 70- μ m cell strainer and washed in complete medium. Colons were washed in HBSS, cut, and incubated at room temperature for 15 min in HBSS containing 1 mM dithiothreitol. The tissue was then incubated in HBSS and 0.75 mM EDTA for 90 min at 37°C; the suspension was passed through a 70- μ m cell strainer and intraepithelial lymphocytes were recovered in the filtrate. The remaining tissue was washed in HBSS, and incubated for 6 h at 37°C in complete medium and 0.05 mg/ml collagenase A (Sigma-Aldrich Chimie). The suspension was then filtered to recover lamina propria lymphocytes. Results are presented as pooled analyses of intraepithelial and lamina propria lymphocytes.

In vivo depletion of CD25⁺ cells

C57BL/6 mice were injected i.p. with 0.5 or 1 mg of anti-CD25 mAb (PC61), or control rat IgG (Sigma-Aldrich) diluted in PBS. Where indicated, i.p. injection of PC61 or control rat IgG was performed 7 days before EAE induction.

Induction and scoring of EAE

Female C57BL/6 mice were injected s.c. at the base of the tail with 200 μ l of an emulsion containing 100 μ g of MOG_{35–55} peptide in CFA (Difco Laboratories) supplemented with 500 μ g of *Mycobacterium tuberculosis* H37RA (Difco Laboratories). The MOG_{35–55} peptide was synthesized by Mimotopes (Clayton Victoria) with a purity of 93%. Mice received an i.v. injection of 400 ng of PTx in PBS on the day of immunization and another injection of 200 ng on day 2. Clinical signs of EAE were monitored daily using a score graded as follows: 0, no clinical disease; 0.5, loss of tail tonicity; 1, tail paralysis; 2, hind limbs weakness; 3, hind limbs paralysis; 4, quadriplegia; and 5, moribund or dead animals. Moribund mice were sacrificed.

Histology

Immunized mice were sacrificed by injection of a lethal dose of sedatives, and perfused intracardially with 20 ml of 4% paraformaldehyde in PBS. Brain and spinal cord were embedded in paraffin, and 13- μ m spinal cord sections were stained with H&E. The inflammation index represents the average number of inflammatory infiltrates per spinal cord section, with 10–15 sections studied per mouse.

Statistical analysis

Data are presented as means \pm SEM. Statistical analysis was performed using Student's *t* test. When values displayed a non-Gaussian distribution, the Mann-Whitney *U* test was used. For EAE experiments, the log-rank test was used to assess differences in kinetics and prevalence of disease. A *p* value of <0.05 was considered significant.

Results

PTx treatment selectively reduces the size of the splenic CD4⁺CD25⁺ T cell population

PTx has been shown to induce pleiotropic effects on the immune system. To assess whether PTx could have an influence on Treg cells, we injected C57BL/6 mice i.v. with 400 ng PTx at day 0, and 200 ng at day 2, a regimen used for EAE induction. Control mice received PBS with the same schedule. Mice were sacrificed on day 3 and the cellular composition of lymphoid organs was analyzed by FACS. Total numbers of splenocytes did not display any significant variation upon PTx treatment ($133 \pm 18 \times 10^6$ in PTx-treated mice, $n = 11$, vs $110 \pm 11 \times 10^6$ in PBS-treated mice, $n = 11$; $p = 0.2$). Similarly, no differences were found between groups in the percentage or absolute numbers of CD8⁺ splenocytes (Fig. 1, A and D), whereas there was a modest but significant decrease in CD4⁺ T cell frequency ($p = 0.038$) but not absolute numbers ($p = 0.35$) (Fig. 1, B and E). However, we found a 33% decrease in the percentage of CD4⁺ splenocytes expressing CD25 in PTx-treated mice ($p = 0.0002$) and a 41% decrease in the absolute numbers of CD4⁺CD25⁺ splenocytes ($p = 0.002$) (Fig. 1, C and F). This decrease was associated with a reduced expression level of CD25 on the remaining CD4⁺CD25⁺ T cells (mean fluorescence intensity (MFI) 177 ± 18 vs 214 ± 23 , $p = 0.002$) (Fig.

1G). In contrast, the absolute numbers of CD4⁺CD25[−] splenocytes was unaffected by PTx treatment ($13.4 \pm 2.8 \times 10^6$ in PTx-treated vs $14.0 \pm 1.7 \times 10^6$ cells in PBS-treated mice, $p = 0.79$). Similarly, the percentage of CD4⁺CD25[−] cells among splenocytes was not significantly altered by PTx treatment (10.5 ± 0.8 vs $12.6 \pm 0.8\%$, $p = 0.10$), suggesting that the decrease in frequency of CD4⁺ splenocytes can at least partially be explained by the deletion in CD4⁺CD25⁺ cells.

We also showed that the bioactive PTx holotoxin, but not the heat-inactivated PTx nor the purified B subunit, could induce a reduction of splenic Treg cell numbers (data not shown). These results strongly argue that bioactive PTx is responsible for the described effect, and suggest that this effect depends on the ADP-ribosyltransferase activity of PTx.

In agreement with published data (22), lymph nodes from PTx-treated mice showed a great decrease in total cell numbers ($3.2 \pm 0.7 \times 10^6$, $n = 7$, vs $14.6 \pm 2.2 \times 10^6$, $n = 7$; $p = 0.002$) as well as in the number of CD4⁺CD25⁺ T cells, although a modest increase in the percentage of CD4⁺CD25⁺ T cells ($13.2 \pm 1.1\%$, $n = 9$, vs $11.3 \pm 0.7\%$, $n = 9$; $p = 0.036$) was found. No significant differences were seen in frequency of CD4⁺CD25⁺ T cells among CD4⁺ blood cells nor CD4⁺CD8[−] thymocytes of PTx-treated and PBS-treated mice (data not shown).

We then investigated the duration of the depletion of CD4⁺CD25⁺ T cells in spleen following PTx treatment. Mice were treated as above and the size of the CD4⁺CD25⁺ splenic compartment was analyzed at day 3, and 1, 2, or 4 wk after the first PTx injection. After a significant decrease at day 3, the proportion of splenic CD4⁺CD25⁺ T cells increased from day 7. At 4 wk, there was a full recovery and even a modest increase in CD4⁺CD25⁺ frequency ($113 \pm 11\%$) in PTx-treated relative to control mice (Fig. 2).

PTx treatment depletes regulatory CD4⁺Foxp3⁺ T cell populations

The expression of the transcription factor Foxp3 in T cells is tightly associated with regulatory functions. Indeed, whereas

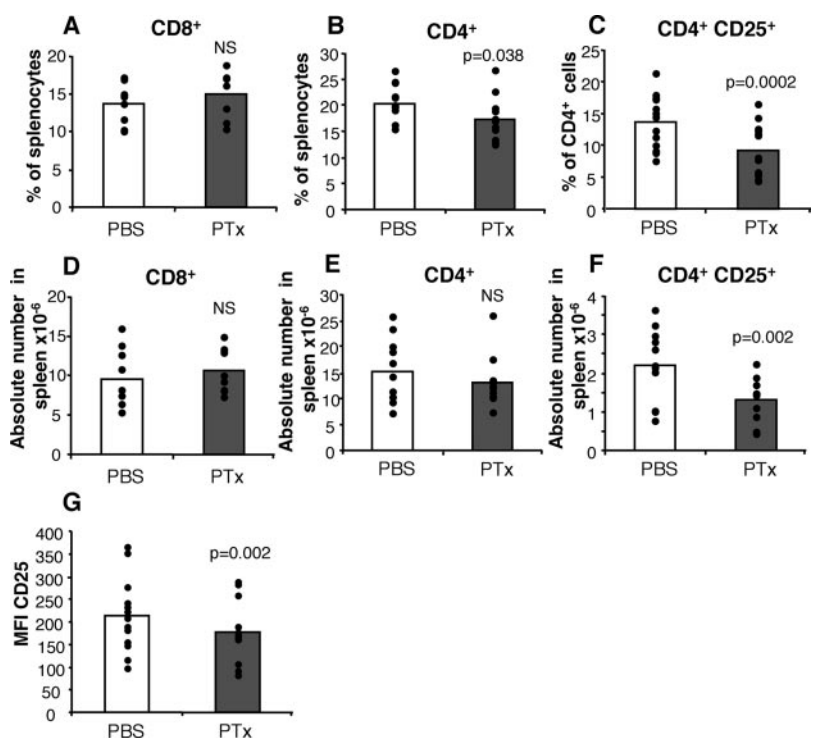


FIGURE 1. Reduction in the relative and absolute numbers of CD4⁺CD25⁺ T cells in the spleen of PTx-treated mice. Mice injected i.v. with 400 ng of PTx or PBS at day 0 and 200 ng of PTx or PBS at day 2 were sacrificed on day 3. Spleen cells were stained with anti-CD4 and anti-CD25, or anti-CD8 mAbs. Percentages (A) and absolute numbers (D) of CD8⁺ cells. Percentages (B) and absolute numbers (E) of CD4⁺ cells. C, Percentages of CD25⁺ among CD4⁺ cells. F, Absolute numbers of CD4⁺CD25⁺ cells. G, MFI of CD25 on splenic CD4⁺CD25⁺ cells from PTx- or PBS-treated mice. Results are from 9 to 13 mice/group.

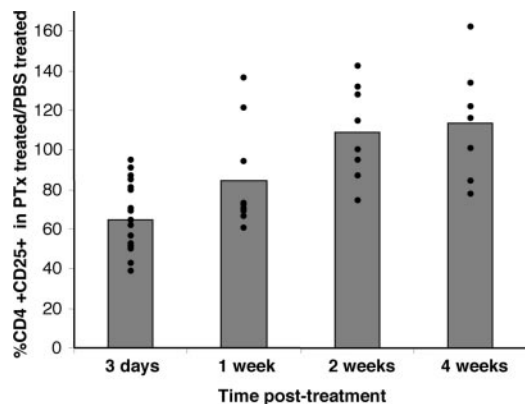


FIGURE 2. PTx-induced $CD4^+CD25^+$ T cell depletion in the spleen is a transient phenomenon. Mice were treated with PTx or PBS as described in Fig. 1 and were sacrificed 3 days ($n = 19$ /group), 1 wk ($n = 9$), 2 wk ($n = 8$), or 4 wk ($n = 7$) later. Spleen cells were stained with anti-CD4 and anti-CD25 mAbs. Data are shown as the ratio of percentages of $CD4^+$ T cells expressing CD25 in PTx-treated mice and PBS-treated mice.

$CD4^+CD25^+Foxp3^-$ T cells are activated effector cells, both $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ T cell subsets display regulatory properties (31). To ensure that PTx was indeed depleting Treg cells, we performed anti-Foxp3 intranuclear staining on splenocytes from PTx- or PBS-treated mice. Interestingly, a clear decrease ($>50\%$) in the percentage of both $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ T cell subsets was observed at day 3 post PTx-treatment (Fig. 3). We observed a 26% decrease in the MFI of Foxp3 staining in Treg cells from PTx-treated mice. A profound decrease was also found in the absolute numbers of both $CD4^+CD25^+Foxp3^+$ ($0.48 \pm 0.10 \times 10^6$

in PTx-treated vs $1.27 \pm 0.17 \times 10^6$ in PBS-treated mice, $p = 0.0025$) and $CD4^+CD25^-Foxp3^+$ ($0.26 \pm 0.03 \times 10^6$ in PTx-treated vs $0.62 \pm 0.06 \times 10^6$ in PBS-treated mice, $p = 0.0002$) Treg populations. Contrasting with these results, the number of $CD4^+CD25^+Foxp3^-$ cells in PTx-treated mice were not decreased, and even slightly increased, as compared with PBS-treated mice, consistent with the previously described effects of PTx on T cells (19, 38).

Collectively, our data show that PTx treatment induces a reduction in both the percentage and the absolute numbers of splenic Treg cells, which applies to $CD4^+Foxp3^+$ Treg cells regardless of their CD25 expression.

PTx does not affect the in vitro functional properties of $CD4^+CD25^+$ Treg cells

We next examined the influence of in vivo PTx treatment on the functional properties of highly purified $CD4^+CD25^{bright}$ T cells. $CD4^+CD25^{bright}$ T cells purified by FACS sorting from PBS- or PTx-treated mice were stimulated with anti-CD3 and APC alone (Fig. 4A) or in coculture with $CD4^+CD25^-$ responder T cells (Fig. 4B). $CD4^+CD25^{bright}$ T cells from both PBS- and PTx-treated mice were hyporesponsive in vitro to TCR triggering. Moreover, the addition of $CD4^+CD25^{bright}$ T cells from either group of mice strongly inhibited $CD4^+CD25^-$ T cell proliferation with similar dose-response suppressive activities. These results indicate that the function of the remaining $CD4^+CD25^{bright}$ Treg cells is not affected by the PTx treatment. Consistent with this statement, their cytokine mRNA content, as determined by real-time quantitative RT-PCR, did not reveal important differences between $CD4^+CD25^+$ T cells from PBS vs PTx-treated mice (Fig. 4C).

Investigation of the mechanisms of PTx-induced reduction of splenic Treg cells

The apparent PTx-induced depletion of splenic Treg cells could be attributed to several mechanisms: 1) loss of expression of their characteristic markers; 2) direct or indirect toxicity of PTx on Treg cells; and/or 3) modification of their migratory properties.

To investigate whether the observed reduction of splenic Treg cells is due to decreased CD25 expression or to a physical loss of Treg cells, we transferred either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells from CD45.2 mice into CD45.1 congenic mice before PTx or PBS treatment. Recipient mice were sacrificed on day 3 and the transferred cells were identified based on CD45.2 expression (Fig. 5A). Of note, CD45.2 expression levels on transferred cells ($CD4^+CD25^+$ and $CD4^+CD25^-$ T cells) was unaffected by PTx treatment (data not shown). The absolute numbers of donor $CD45.2^+CD4^+CD25^+$ T cells was reduced by $38 \pm 11\%$ in the spleen of PTx-treated mice compared with PBS-treated mice, whereas there was no difference between groups regarding the number of $CD4^+CD25^-$ T cells of donor origin (Fig. 5B). As expected, there was a reduction of endogenous $CD45.2^-CD4^+CD25^+$ T cells in both groups of PTx-treated recipient mice (data not shown). These data, using a congenic surface marker unaffected by PTx treatment, confirm the reduction of Treg cells in the spleen of PTx-treated mice and show that this reduction results from a loss of cells and is not merely attributed to a down-regulation of the CD25 molecule.

We next investigated whether this physical loss of Treg cells could be due to apoptosis induction by PTx. $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells purified from untreated mice were incubated in vitro with increasing concentrations of PTx and stained for annexin V and PI. A short incubation of 4 h was selected since, upon longer incubations, Treg cells exhibited poor survival capacities in vitro (39). Treg cells were significantly more sensitive than

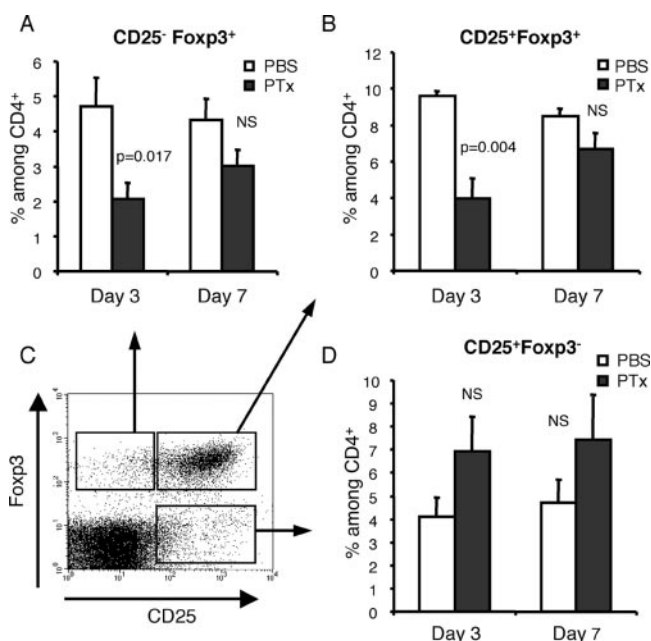


FIGURE 3. Decrease in $Foxp3^+CD4^+$ T cells following PTx treatment. Mice were treated with PTx (■) or PBS (□) as described in Fig. 1, and the percentage of $CD4^+CD25^-Foxp3^+$ (A), $CD4^+CD25^+Foxp3^+$ (B), and $CD4^+CD25^+Foxp3^-$ (D) among splenic $CD4^+$ T cells was determined, 3 and 7 days after treatment, by a combination of surface and intranuclear FACS staining. C, A representative CD25 and Foxp3 staining gated on $CD4^+$ splenocytes illustrating the cell populations analyzed in A, B, and D. Six PTx-treated and 5 PBS-treated mice were analyzed at day 3, and 11 PTx-treated and 11 PBS-treated mice were analyzed at day 7.

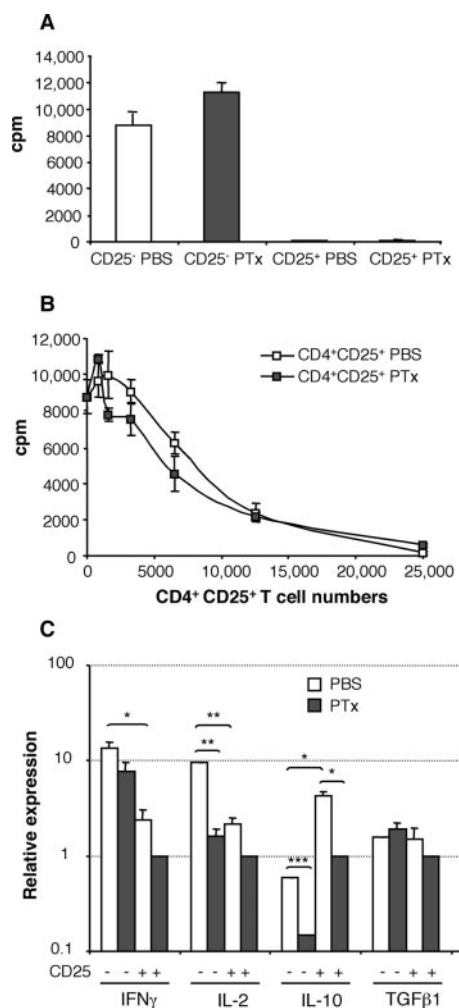


FIGURE 4. PTx treatment does not affect the functional properties of CD4⁺CD25⁺ T cells. **A**, CD4⁺CD25^{bright} or CD4⁺CD25⁻ were purified from PTx- or PBS-treated mice 3 days after treatment, and the proliferative capacity of each cell population (25,000 cells/well) was assessed following stimulation with 0.5 μ g/ml anti-CD3 and 200,000 APC. **B**, A total of 25,000 responder CD4⁺CD25⁻ T cells from untreated mice was stimulated with 0.5 μ g/ml anti-CD3 and 200,000 APC in the presence of increasing numbers of highly purified CD4⁺CD25^{bright} T cells from PTx-treated (gray symbols) or PBS-treated (white symbols) mice. Unstimulated proliferation was minimal, ranging from 13 to 35 cpm. These experiments are representative of four independently performed experiments. **C**, Expression of IFN- γ , TGF- β 1, IL-10, or IL-2 mRNA in CD4⁺CD25⁺ or CD4⁺CD25⁻ freshly purified from PBS (\square)- or PTx (\blacksquare)-treated mice. Results are presented as fold increase relative to CD4⁺CD25⁺ cells from PTx-treated mice. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

CD4⁺CD25⁻ T cells to PTx-induced apoptosis in vitro (Fig. 5C). Therefore, PTx can directly promote apoptosis of Treg cells in vitro.

To evaluate whether differential tissular migration of the Treg cells could explain their reduction from the spleen after PTx treatment, infiltrating lymphocytes were isolated 3 days after treatment from a large number of tissues, and stained for CD4, CD25 and Foxp3. These experiments revealed a significant decrease in CD4⁺CD25⁺Foxp3⁺ cells in both spleen and bone marrow of PTx- vs PBS-treated mice (Table I). In addition, an increase in the absolute numbers of CD4⁺CD25⁺Foxp3⁺ cells was found in the liver ($p < 0.005$), and in the lungs and brain (not reaching statistical significance, possibly as a result of β error) of PTx-treated animals (Table I).

Synergistic effect of PTx and PC61 treatment on EAE

Previous studies have underlined the important role played by Treg cells in the control of EAE (32–35). Because our results indicated that PTx induces a partial depletion in Treg cells, we investigated the contribution of this PTx-induced reduction of Treg cells on EAE induction, relative to the other disease-potentiating effects of PTx.

We first evaluated the effect of a profound in vivo depletion of Treg cells on the course of EAE. A single i.p. injection of 500 μ g of the anti-CD25 mAb PC61 in C57BL/6 mice induced a durable depletion of CD4⁺CD25⁺ T cells (by 83%) in blood of treated mice detectable as early as day 2 after injection and lasting for >17 days (Fig. 6A). Then, C57BL/6 mice were treated with 500 μ g of PC61 or control rat IgG or PBS 7 days before a full protocol of EAE induction. PC61-treated mice displayed a marked increase in the incidence and severity of the disease but not an earlier onset (Fig. 6B) in agreement with previous work (40, 41). Moreover, upon analysis at day 32, the CNS of PC61 ascitis-treated animals showed much higher infiltrates than untreated controls (inflammation index: 3.6 ± 0.3 , $n = 2$ treated mice, vs 0.1 ± 0.0 , $n = 3$ untreated mice, $p = 0.0006$). Taken together, these results indicate a major role for Treg cells in controlling inflammation and tissue damage during CNS autoimmunity.

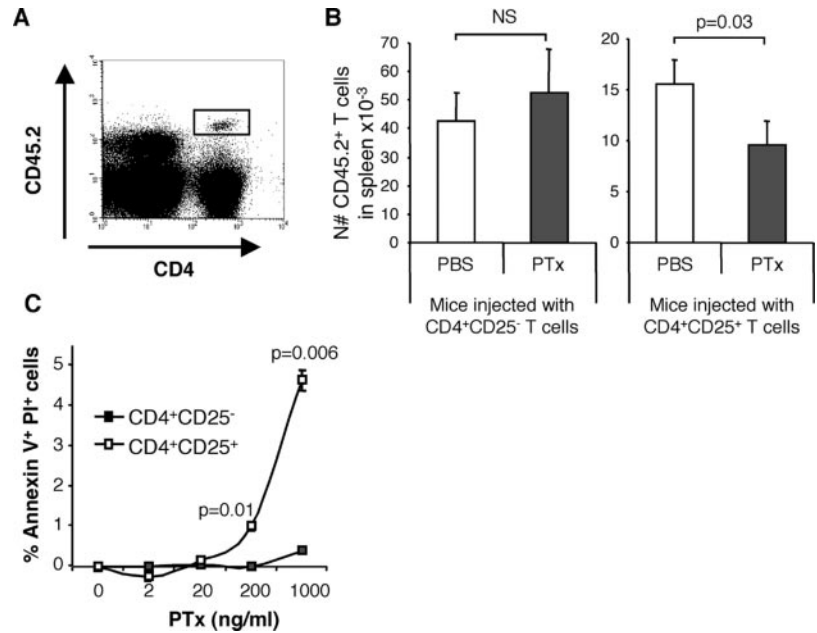
We next evaluated whether the EAE-enhancing effect of PTx was dependent on its action on Treg cells. To this end, mice pre-injected with PC61 or control IgG were immunized with MOG_{35–55} in CFA and were treated or not with PTx. In the absence of PTx, PC61 treatment induced a moderate disease (Fig. 7A), despite profound and durable Treg cell depletion (Fig. 6A). PTx treatment alone, although it resulted in a less pronounced Treg cell reduction, promoted a more severe disease. Importantly, PC61 administration and PTx treatment acted in a synergistic manner on EAE severity (Fig. 7A) indicating that they are acting on nonredundant pathways lowering the threshold for clinical autoimmunity. This synergy was even more striking when we used IFA instead of CFA for MOG_{35–55} immunization (Fig. 7B). Indeed, in this context, PC61 administration alone failed to induce clinical disease. Similarly, PTx only induced a minor and transient disease in 25% of the mice. However, the combined treatment with PTx and PC61 induced a severe disease in 100% of the mice (Fig. 7B).

These results indicate that enhancement of EAE by PTx treatment does not solely rely on its effect on CD4⁺CD25⁺ Treg cells. In addition, they reveal a potent synergistic effect of PTx treatment and in vivo depletion of Treg cells using the PC61 Ab.

Discussion

In this report, we show that administration of PTx to C57BL/6 mice, following a regimen used to induce EAE, caused a specific depletion of both CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁻Foxp3⁺ Treg subpopulations from the spleen. As a consequence, both the percentage and absolute numbers of splenic Treg cells were decreased following PTx injection. Similar results were obtained in BALB/c mice (data not shown). This decrease in Treg cells did not affect evenly all tissues, involving mostly the spleen and to a lesser extent the bone marrow, whereas the liver showed higher Treg cell numbers after PTx treatment. This phenomenon was associated with a preferential sensitivity of Treg cells to the toxicity of PTx in vitro. Experiments in which induction of EAE was performed with or without PTx and/or PC61 treatment indicated that depletion of CD4⁺CD25⁺ T cells is not the major mechanism by which PTx participates to EAE development. However, our results show a clear synergy between PC61, *Mycobacterium* and PTx for the

FIGURE 5. PTx preferentially depletes CD4⁺CD25⁺ T cells. CD45.1 congenic C57BL/6 mice received 2–4 × 10⁶ CD4⁺CD25⁺ or CD4⁺CD25[−] T cells from CD45.2 mice and were treated with PTx or PBS 3 days later. Recipient mice were sacrificed on day 3 after treatment and spleen cells were stained with anti-CD45.2, anti-CD4, and anti-CD25 mAbs. **A**, Identification of transferred cells based on CD45.2 staining. **B**, Numbers of transferred cells present in spleen per million of injected cells. Results are pooled from three independent experiments and groups were compared using one-tailed Student's *t* test. **C**, CD4⁺CD25⁺ T cells are more sensitive than CD4⁺CD25[−] T cells to PTx-induced cell death. 200,000 CD4⁺CD25⁺ (white symbols) or CD4⁺CD25[−] (black symbols) cells were incubated in vitro with different concentrations of PTx for 4 h. Cells were then recovered and stained with annexin V and PI. The percentages of Annexin V⁺PI⁺ cells are presented after subtraction of the background apoptosis present in medium only cultures. This experiment is representative of three independent experiments.



induction and severity of EAE, illustrating how multiple adjuvants can maximize activation of the immune system.

PTx led to a decreased expression of the CD25 molecule on splenic CD4⁺ T cells. This decrease in CD25 MFI (Fig. 1G) may be explained either by a preferential elimination of CD4⁺CD25^{bright} T cells, or by a down-regulation of CD25 expression on Treg cells following PTx treatment. The adoptive transfer experiments indicate physical loss of Treg cells following PTx administration on the basis of a congenic marker whose expression is not modified upon PTx treatment. Second, down-modulation of the CD25 molecule would result in an increase, rather than the observed decrease, of the CD4⁺CD25[−]Foxp3⁺ subpopulation. Moreover, our in vitro studies suggested that the PTx-induced reduction of Treg cells might in part be related to the preferential sensitivity of these cells to direct PTx toxicity. However, the relative contribution of this PTx-induced apoptosis to the Treg cell depletion observed in vivo is at present unclear. PTx-induced apoptosis of Treg cells in vivo might be difficult to assess because of rapid elimination of dying cells.

Our results suggest that depletion of Treg cells is not the major mechanism by which PTx participates in EAE induction or aggra-

vation (Fig. 7). However, a contribution of this effect in EAE enhancement cannot be excluded. Indeed, whereas PC61 only depletes the dominant CD25⁺ Treg population, PTx reduces to the same extent both the CD25⁺ and the CD25[−]Foxp3⁺ regulatory populations (Fig. 3, A and B).

By interfering with Gi protein activity, PTx can affect both survival and migration of Treg cells. The susceptibility of Treg cells to apoptosis is controversial. Human Treg cells were shown to be apoptosis-prone because of lower expression of the anti-apoptotic molecule Bcl-2 than conventional lymphocytes (39). On the other hand, murine Treg cells are resistant to apoptosis induced by Fas (42) as well as by dexamethasone (43). Why should Treg cells be more sensitive to PTx toxicity than conventional T cells? An interesting hypothesis could be that the Treg cell survival specifically depends on a PTx-sensitive receptor. Two major molecules are considered essential to Treg cell survival: TGF-β and IL-2. TGF-β plays an important role in induction, maintenance (44, 45) and activity of Treg cells (46, 47), and a recent study reveals a lack of response to TGF-β in Gai2-deficient mice (48). Thus, a possible explanation for PTx-induced apoptosis of Treg cells could be an inhibition of survival signals provided by TGF-β. Disruption of

Table I. Quantitative analysis of Treg cells in several tissues following PTx treatment^a

		Spleen	Thymus ^b	Bone marrow ^c	Blood	
% CD25 ⁺ Foxp3 ⁺	PBS	10.22 ± 0.25	2.30 ± 0.14	32.75 ± 0.95	2.82 ± 0.38	
	PTx	5.76 ± 0.60***	2.54 ± 0.15	22.70 ± 0.60***	2.96 ± 0.30	
Absolute numbers of CD25 ⁺ Foxp3 ⁺ CD4 ⁺ cells	PBS	1.22 ± 0.12 × 10 ⁶	1.68 ± 0.24 × 10 ⁵	4.55 ± 0.54 × 10 ⁴		
	PTx	0.66 ± 0.10 × 10 ⁶ **	1.04 ± 0.11 × 10 ⁵ *	3.03 ± 0.67 × 10 ⁴		
		Peritoneum	Brain ^d	Liver ^e	Lungs	Colon
% CD25 ⁺ Foxp3 ⁺	PBS	9.85 ± 2.81	7.01 ± 0.41	3.49 ± 0.17	5.44 ± 0.33	8.80 ± 1.43
	PTx	8.98 ± 0.73	8.30 ± 1.06	4.41 ± 0.37	4.69 ± 0.69	5.88 ± 1.04
Absolute numbers of CD25 ⁺ Foxp3 ⁺ CD4 ⁺ cells	PBS		6.09 ± 1.65 × 10 ²	3.32 ± 1.04 × 10 ⁴	8.56 ± 2.01 × 10 ³	1.08 ± 0.68 × 10 ²
	PTx		19.82 ± 5.74 × 10 ²	6.92 ± 1.40 × 10 ⁴ **	17.09 ± 5.07 × 10 ³	0.13 ± 0.04 × 10 ²

^a Numbers of mice analyzed per group: spleen, *n* = 16; thymus, *n* = 8; bone marrow, *n* = 8; blood, *n* = 8; peritoneum, *n* = 3; brain, three experiments with two to three pooled mice per group; liver, *n* = 7; lungs, *n* = 7; colon, *n* = 7. *, *p* < 0.05; **, *p* < 0.005; ***, *p* < 0.0005.

^b Gated on CD4⁺CD8[−] single-positive thymocytes.

^c Gated on CD4⁺TCRβ⁺ cells; absolute numbers given for one femur.

^d Absolute numbers per mouse.

^e Gated on CD4^{high}TCRβ^{high} cells.

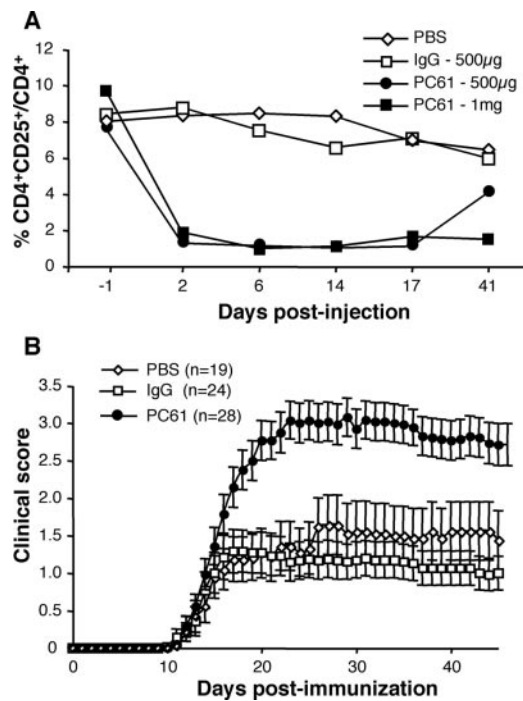


FIGURE 6. Depletion of CD4⁺CD25⁺ T cells exacerbates EAE. *A*, Mice were injected i.p. with PBS (◇), 500 µg of control rat IgG (□), or 500 µg (●) or 1 mg (■) of PC61 mAb at day 1. Blood was collected at different time points, and FACS staining was performed using anti-CD4 and anti-CD25 (7D4) mAbs. The percentage of CD25⁺ among CD4⁺ cells from serially sampled mice was plotted. *B*, Mice were treated with PBS (◇), 500 µg of rat IgG (□), or PC61 (●) and were immunized 7 days later to induce EAE. Pooled results from six experiments are shown. The log-rank test analysis showed that the PBS-treated and the IgG-treated mice display similar EAE curves, whereas the PC61-treated mice have exacerbated disease compared with either PBS or IgG-treated mice ($p < 0.001$).

IL-2R signaling in Treg cells could represent a second mechanism explaining this effect. Indeed, IL-2 has proved to be crucial in the survival of Treg cells in the periphery (49, 50). Although the IL-2R is not directly coupled to a Gi protein, a PTx-sensitive GTP-binding protein has been shown to be activated following the ligation of IL-2 on lymphocytes (51). This signaling pathway has to our knowledge never been explored further, but its inhibition by PTx may have consequences on T cell biology.

Our quantification of Treg cells in a variety of tissues indicates that there is no generalized reduction of Treg cells following PTx injection, but rather that this decrease is selective, affecting mostly the spleen, and is accompanied by migration and/or retention of Treg cells in the liver. Whether the Treg cells accumulating in the liver following PTx injection are destined to die (52) or are only transiently trapped in this tissue is currently uncertain.

Interestingly, several PTx-sensitive chemokine receptors, such as CCR2 (53), CCR4 (54), CCR5 (55), CCR6 (56), and CCR7 (57), seem important for Treg cell function and recruitment in tissues. However, the consequences of PTx-induced impairment of chemokines-induced signaling remain ambivalent. Indeed, the inhibition of chemokine receptors by PTx can lead to a reduced migration of pathogenic T cells to their target tissue and thus to an inhibition of inflammatory diseases (23, 58). Nevertheless, we propose that as PTx inhibits the entry of all lymphocytes in lymph nodes, and induces a decreased frequency of Treg cells in spleen, the probability of encounter between Treg cells and the population of conventional lymphocytes, that contains autoaggressive cells, is limited, so the balance is biased toward activation of effector cells.

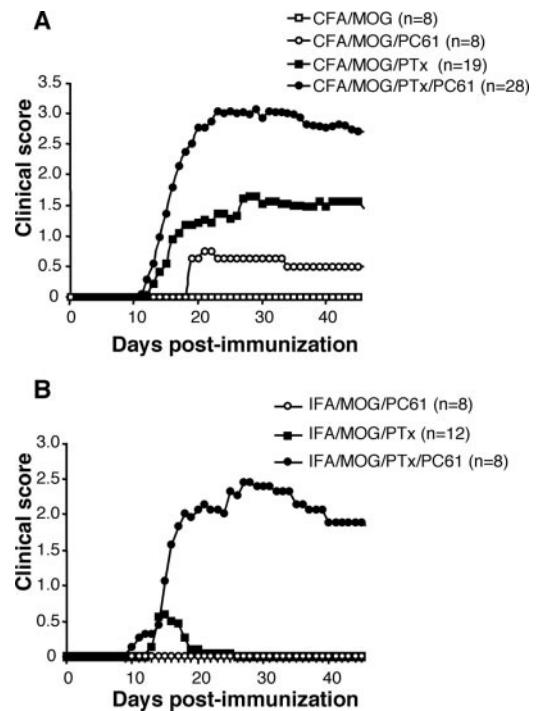


FIGURE 7. Synergy between Ab-mediated depletion of CD4⁺CD25⁺ T cells and PTx administration for EAE induction. *A*, Mice that have been pretreated or not with PC61 7 days earlier were immunized subcutaneously with 100 µg of MOG₃₅₋₅₅ in CFA. These mice further received either PTx or PBS treatment. *B*, A similar protocol was performed except that we substituted IFA for CFA. In this series of experiments, the IFA/MOG₃₅₋₅₅ group (without PTx nor PC61 injection) was omitted because it consistently fails to induce EAE (21, 65, 67).

Interestingly, this effect of PTx may be linked to PTx-induced cell death, because chemokines receptors can be implicated in both apoptotic signaling pathway and migratory patterns (59, 60).

An alternative hypothesis that could explain the effects of PTx on Treg cells involves the inhibition of the PTx-sensitive sphingosine 1-phosphate receptors S1P₁ and S1P₄ signaling. Indeed, the triggering of these receptors on T cells by their ligand leads to increased survival, and increased or decreased chemotaxis depending on the ligand concentration (61). Strikingly, treatment of mice with a pharmacological agonist of this receptor, FTY720, doubles the number of Treg cells in the spleen, and thus appears to differentially affect the homing properties of Treg cells (62).

A synergistic effect on EAE severity was exerted by PTx and PC61 treatment (Fig. 7), a phenomenon that has also recently been described in a passive model of EAE (63). We also noted a synergy between CFA and PTx on EAE triggering. Thus, development of EAE results from the integration of an autoantigen-specific activation signal acting on T cells, and of several non-Ag-specific signals. Among the non-Ag-specific signals, PTx has been shown to promote clonal expansion and differentiation of autoreactive T cells, possibly by providing maturation signals to APC (19–21). Permeabilization of the blood-brain barrier by PTx would then facilitate infiltration of pathogenic cells into the CNS, where they are reactivated, promote recruitment of other types of immune cells and start tissue injury. It is interesting to note that both adjuvants (*Mycobacterium tuberculosis* and PTx) used to potentiate EAE are bacterial compounds. Other microbial molecules have been shown to influence EAE susceptibility or severity (64–67). The mechanisms of these EAE-aggravating effects of infectious

agents are thought to implicate TLR triggering. Indeed, direct activation of TLR9 by CpG oligonucleotides, or TLR4 by LPS, increases the incidence of EAE in a proteolipid protein-TCR transgenic mouse model, however to a lower degree than PTx (66). Besides their known consequences on dendritic cells, the triggering of TLRs could also affect the biology of Treg cells. Actually, Treg cells express TLR4, TLR5, TLR7, and TLR8 (68); ligands of TLR8 have been shown to reverse Treg cell suppressive activity (69), while CpG, ssRNA, or LPS have been shown to modify Treg cell activity following TLR ligation, although the effect varies greatly depending on the experimental models (68–71). Interestingly, a recent report demonstrates that PTx can signal through TLR4, leading to increased permeabilization of the pial vessels to leukocytes (27, 28). In addition, it has been shown that the triggering of TLR4 induces a refractoriness of conventional lymphocytes to the suppressive activity of Treg cells, by a mechanism dependent on IL-6 secretion by APC (70). Thus, although it has been suspected for a long time that signals provided by microbial agents can favor self tolerance disruption leading to autoimmune diseases (72), accumulating evidence indicate that they may target Treg cells directly, particularly through TLR, in animal models and possibly in humans.

To conclude, we have revealed an unknown selective effect of PTx on Treg cells, which could participate in the pathogenesis of autoimmune diseases in animal models. PTx-induced selective elimination of Treg cells, reduces the relative and absolute numbers of Treg cells in the spleen of PTx-treated mice. The discovery of this new effect of PTx, among its multiple ones on the organism, highlights the numerous and complex consequences that environmental agents can display on the control of immunity and the development of autoimmune diseases.

Note added in proof. While this manuscript was undergoing the reviewing process, an independent study similarly reported a decrease in splenic Treg cells following PTx treatment (73).

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Disclosures

The authors have no financial conflict of interest.

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