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Pertussis toxin modulates microglia and T cell profile to protect experimental autoimmune encephalomyelitis

Jun-xiang Yin [a], Zhiwei Tang [a], Yan Gan [a], Lejun Li [a], Fudong Shi [a], Stephen Coons [b], Jiong Shi [a,*]

[a] Department of Neurology, Barrow Neurological Institute, 350 W Thomas Road, Phoenix, AZ 85013, USA
[b] Department of Neuropathology, Barrow Neurological Institute, 350 W Thomas Road, Phoenix, AZ 85013, USA

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ABSTRACT

Pertussis toxin (PTx) has various effects in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). This study was designed to explore the protective effects of PTx of different doses and subunits. EAE model was induced with myelin oligodendrocyte glycoprotein (MOG35–55, 200 ug) plus complete Freund's adjuvant in 6–7 week-old female C57BL/6 mice. PTx reduced clinical deficits of EAE by 91.3%. This reduction in clinical deficits was achieved by attenuating demyelination by 75.5%. Furthermore, PTx reduced the lymphocyte infiltration, deactivated microglia activation and changed T cell profile by increasing T helper (type 1 and 2) and T regulatory cells.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). Multiple lines of evidence support the notion that T cells play a critical role in both mediating and regulating MS pathogenesis. Therapeutic strategies for MS have been focused on understanding factors that control T cell function (O'Brien et al., 2010; Severson and Hafler, 2010). It is believed that autoimmune T cells mediate the early steps of new MS lesions although the specific targets of autoreactive T cells remains uncertain. Studies on both animal models and MS patients indicate involvement of not only T helper cells and also regulatory T cell populations in modulating demyelination and axonal loss by regulating proinflammatory cytokines (De Santi et al., 2011; Disanto et al., 2012; Gandhi et al., 2010; McFarland and Martin, 2007). While autoreactive T cells are involved in disease induction, cells of myeloid lineage, antibodies and complement seem to dictate the effector stages of tissue damage (Disanto et al., 2012).

Pertussis toxin (PTx) is commonly used to induce experimental autoimmune encephalitis (EAE), an animal model of MS. Its adjuvant effect in EAE is attributed to its ability to increase permeability of the blood–brain barrier that allows proinflammatory cells to enter the CNS (Bruckener et al., 2003; Wolf and Andreoni, 1982). We serendipitously found that a single large dose of PTx administered intracerebroventricularly (icv) attenuated demyelination, axonal loss and neurological deficits in EAE (Yin et al., 2010). This paradoxical protective effect of PTx was later confirmed by another group using a chronic treatment regimen (Weber et al., 2010). We also found PTx could protect neurological function by enhancing neuronal vascular endothelial growth factor (Tang et al., 2013). However, the neuroprotective mechanism of a single large dose of PTx is not completely understood. In this study, we investigated whether systemic administration of PTx would have a similar protective effect in EAE. We further studied the underlying mechanism involving microglia activation, lymphocyte infiltration and T cell profile.

2. Materials and methods

2.1. EAE induction

C57BL/6 mice (6–7 weeks old) were purchased from Taconic Laboratory (Hudson, NY). EAE was induced by myelin oligodendrocyte glycoprotein (MOG_{35-55};

Abbreviations: PTx, pertussis toxin; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; Treg cells, Regulatory T cells; Th1 cell, T helper cell type 1; Th2, T helper cell type 2; APC, antigen presenting cell.

* Corresponding author. Department of Neurology, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, 500 W Thomas Road, Suite 300, Phoenix, AZ 85013, USA. Tel.: +1 4807203005.
E-mail address: jiong.shi@dignityhealth.org (J. Shi).

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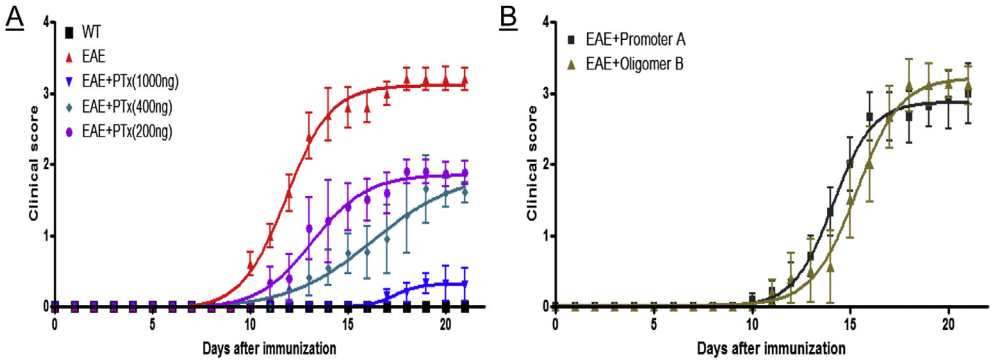

Fig. 1. PTx treatment attenuated neurological deficits in EAE. Clinical scores of all mice were evaluated daily ($n = 9$–12/group). Mean ± SEM was analyzed by using the method of Boltzmann sigmoid of nonlinear regression. (A) Shown was a dose-dependent response to PTx treatment after it was injected ip at day 7. (B) Shown was lack of response when two PTx subunits were used separately. $^{**}p < 0.01$ compared with EAE group. WT: wild type; EAE: Experimental autoimmune encephalomyelitis; EAE + PTx: EAE with pertussis toxin treatment.

MEVGWYRSPFSRVVHLYRNGK, Bio-synthesis Inc. Lewisville, TX) with complete Freund's adjuvant containing 0.5 mg of heat killed Mycobacterium tuberculosis (CFA, Difco Laboratories, Detroit, MI). On the day of immunization (day 0) and 48 h later (day 2), 200 ng pertussis toxin (List Biological laboratories Inc. Campbell, CA) was injected iv (Tu et al., 2009; Yin et al., 2010) http://www.ncbi.nlm.nih.gov/pubmed/10975822. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Barrow Neurological Institute (Protocol number 309) and performed according to the Revised Guide for the Care and Use of Laboratory Animals. The animals were kept in groups on a 12:12 h light/dark cycle with food and water ad libitum.

2.2. Treatment groups and evaluation of clinical deficits

Mice were randomly divided into 7 groups ($n = 9$–12/per group): Control (wild type without EAE or treatment), EAE, EAE with PTx treatment at three doses (200 ng, 400 ng, and 1000 ng, *i.p.*), EAE with different PTx subunits (Promoter A 500 ng *i.p.* and Oligomer B 800 ng *i.p.*). On day 7 after EAE induction, mice in the treatment groups were given PTx or its subunits *i.p.* Clinical deficits were assessed by an examiner blinded to the treatment status. We uses a five-point standardized rating scale to evaluate motor deficits: 0 no deficit; 1 tail paralysis; 2 unilateral hind limb weakness; 3 incomplete bilateral hind limb paralysis and/or partial forelimb weakness; 4 complete hind limb paralysis and partial forelimb weakness; 5 moribund state or death (Tonra et al., 2001; Yin et al., 2010). The scores were collected daily and were analyzed with Boltzmann Sigmoidal Equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + \exp((T50 - X)/\text{Slope}))$; In the equation, Y means clinical score, X means days after immunization, and there are four parameters: Top (Maximum clinical score), T (1/2) (halftime, the time required to reach half of the maximum clinical score) and Slope (the speed of reaching a certain clinical score).

2.3. Immunohistochemistry

Mice were euthanized at day 21 after EAE induction. They were perfused by 4% paraformaldehyde. The cervical spinal cord was embedded in paraffin and sliced coronally into serial 6-μm thick sections. Ionized calcium Binding Adaptor molecule 1 (Iba-1, 1:1000, Wako Chemicals Inc. LA), a marker for microglia, was stained with their respective primary antibodies, followed by the peroxidase–antiperoxidase procedure with 3, 3′-diaminobenzidine as a cosubstrate. Respective negative controls that omit primary antibodies and positive controls were applied for each case. Digital images were collected by using an Axoplan microscope (Zeiss, Thornwood, NY) under the bright field setting with a 40× objective. Cells were quantified on three randomly selected coronal sections of the cervical spinal cord and brain.

2.4. Western blot

Aliquots of equal amount of proteins from the cervical spinal cord were loaded onto a 12% SDS-polyacrylamide gel. After gel electrophoresis, blots were subsequently probed by primary antibodies. For detection, horseradish peroxidase-conjugated secondary anti-rabbit antibody was used (1:10,000, #7074, Cell signaling technology; Danvers, MA), followed by enhanced chemiluminescence development (ECL kit, #34077, Thermo Scientific Pierce, Rockford, IL). Normalization of results was achieved by running parallel Western blots with β-actin antibody (1:25,000 #ab49900, Abcam Inc; Cambridge, MA). The optical density was quantified using an image densitometer (Model GS-670, BioRad, Hercules, CA). The data are presented as a percentage of target protein relative to β-actin.

2.5. Analysis of spleen lymphocyte proliferation

To test the effect of PTx on lymphocyte proliferation, spleen lymphocytes were isolated from the wild type and cultured with the appropriate antigens for 72 h. Bromo-2′-deoxyuridine (BrdU) labeling reagent (final concentration, 10 μM) was added 24 h before the harvest. After incubation, each suspension was stained with fluorescent antibodies specific for cell surface markers: CD3-APC, CD4-PE, CD11b-PE-Cy7, CD11c-APC, CD19-PE, and CD45-PE. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm Buffer and BD Cytoperm Permeabilization Buffer Plus. After washing, the suspensions were treated with DNase (300 μg/mL in DPBS) and followed by incubation with FITC conjugated anti-BrdU. All antibodies were purchased from BD Pharmingen. Appropriate isotype controls were included. All samples were analyzed on BD FACSAria (BD Biosciences, San Jose, CA). Data were analyzed on BD Diva Software. The number of mononuclear cells per mouse spleen was counted on hemocytometer and the absolute number of a cell subset was calculated based on the percentage of cells stained for the appropriate markers.

2.6. Flow cytometry analysis

To phenotype subsets of $CD4^+/CD25^+/Foxp3^+$, $CD45^+/CD11b^+$, $CD45^+/CD11c^+$, $CD3^+/CD4^+/IL\text{-}4^+$ and $CD3^+/CD4^+/IFN\text{-}\gamma^+$ cells, spleen mononuclear cell was prepared from each group from two sets, one from different group mice on day 21, another set from normal spleen cell treated with different antigens. Single cell suspensions (1×10^6 cells/5 ml BD tube) were incubated with combinations of fluorescent antibodies for 30 min at 4 °C: CD3-APC, CD4-FITC, CD25-PE-Cy7, CD11b-PE-Cy7, CD11c-APC, and CD45-FITC. After fixation and permeabilization with BD Cytofix/Cytoperm Buffer, designated groups were incubated with IFN-γ-PE-Cy7 and IL-4-PE. Foxp3-PE was applied following treatment with BD mouse Foxp3 fixation and permeabilization buffers. Appropriate isotype controls were included. Data were analyzed as described above.

2.7. Statistical analysis

For the mean and SEM of clinical scores, we used the method of Boltzmann sigmoid of nonlinear regression (curve fit). For the data of western blot and flow cytometry, we applied one way analysis of variance (ANOVA) to determine the significance of the difference among the experimental groups. $p < 0.05$ was considered significant using SPSS version 10 for windows.

Table 1
Parameter analysis of neurological function after EAE. All seven groups were evaluated daily for neurological deficits ($n = 9$–12/group). Mean ± SEM was analyzed by using the method of Boltzmann sigmoid of nonlinear regression. Max clinical score showed the most severe deficit in each group. $T(1/2)$ depicted the time (days) it took to reach half of the Max clinical score. Slope represented the speed of progression of neurological deficits. $^*p < 0.05$,$^{**}p < 0.01$ compared with EAE group. WT: wild type; EAE: Experimental autoimmune encephalomyelitis; EAE + PTx: EAE with pertussis toxin treatment.

	WT	EAE	EAE + PTx (1000 ng)	EAE + PTx (400 ng)	EAE + PTx (200 ng)	EAE + promoter A	EAE + oligomer B
Max clinical score (top)	0	3.12 ± 0.41	0.27 ± 0.11**	1.87 ± 0.15*	1.86 ± 0.05*	2.87 ± 0.54	3.15 ± 0.33
$T(1/2)$ (days)	–	11.63 ± 0.36	18.69 ± 0.79**	16.24 ± 0.46*	13.19 ± 0.20	14.11 ± 0.07	15.32 ± 0.45
Slope	–	1.14 ± 0.09	0.63 ± 0.10	2.16 ± 0.31	1.39 ± 0.17	1.01 ± 0.06	1.13 ± 0.10

3. Results

3.1. PTx attenuated neurological deficits of EAE

We assessed the clinical scores over a period of 21 days after EAE induction. In EAE mice (without PTx treatment), neurological deficits started to appear on day 8 (0.31 ± 0.12), peaked on day 16 (3.12 ± 0.42) and reached a plateau afterward. PTx treatment reduced clinical score in a dose dependent manner. The most effective dose is at 1000 ng. It delayed the onset to day 17 (0.15 ± 0.12), and reduced maximal score to 0.27 ± 0.11 ($p < 0.01$). PTx consists of two subunits: promoter A (S1) and oligomer B (S2–S5). The function of promoter A depends on the binding of oligomer B to the target cell surface (Locht et al., 2011). When mice were treated with either promoter A or oligomer B, the onset was slightly delayed to day 10 but the maximal scores were not reduced (2.87 ± 0.54 and 3.15 ± 0.33, respectively) compared with EAE group. We calculated the halftime which represented how fast the disease progressed. It was 11.63 ± 0.36 days in EAE compared with 18.68 ± 0.79 days in PTx treated EAE ($p < 0.01$), suggesting PTx treatment slowed down the progression of demyelination. In lower dose treatment groups (PTx 400 ng and 200 ng), there was a trend of slowing but not significant (Fig. 1 and Table 1).

3.2. PTx attenuated inflammatory reaction and demyelination in the spinal cord of EAE mice

In the cervical spinal cord of EAE mice, abundant inflammatory cells around the foci of demyelination were observed in the white mater. PTx treatment ameliorated inflammation reaction and demyelination in a dose-dependent pattern (images in Fig. S1 and S2), with the highest dose (1000 ng) having the best effects. At the dose of 1000 ng, PTx reduced inflammation reaction by 81.6% and demyelination by 75.5% (bar graphs in Fig. S1 and S2). The lower doses had protective effects but to a lesser degree. The two subunits when used alone didn't demonstrate protect effects.

3.3. PTx inhibited microglia activation and regulated proinflammatory cytokine expression in the spinal cord

In the cervical spinal cord of EAE mice, there were abundant Iba-1 positive cells. They were amoeboid-shaped, suggestive their activated status (Fig. 2A). By contrast, in wild type and PTx treated EAE mice, there were few ramified or resting state microglia. The total number of Iba-1 positive cells was reduced by 69.6% with PTx treatment ($p < 0.01$). Consistently, western blot demonstrated a reduction of Iba-1 immunodensity by 69.7% with PTx treatment ($p < 0.01$) (Fig. 2B). PTx not only reduced the total number of microglia but also inhibited its activation. IL-6 is secreted by macrophages. PTx significantly enhanced the staining of interleukin-6 (IL-6) (Fig. 2C). Tumor necrosis factor beta (TNFβ) is a proinflammatory cytokine to stimulate immune response. Its staining was reduced by PTx treatment ($p < 0.01$) (Fig. 2D).

3.4. PTx modulated T cell and antigen presenting cell (APC) profile

EAE is a T cell dominant animal model. Since we observed abundant infiltrating inflammatory cells in the spinal cord (Fig. S1), we further analyzed their subpopulation by flow cytometry analysis. High dose of PTx (1000 ng) stimulated production of Treg ($CD4^+CD25^+Foxp3^+$), Th1 ($CD3^+CD4^+INF\text{-}\gamma^+$) and Th2 ($CD3^+CD4^+IL\text{-}4^+$) in the context of EAE. The increase in Treg cells was 247.9%, Th1 239.5% and Th2 235% ($p < 0.01$) (Fig. 3A, B and C). We further assessed antigen presenting cells, namely macrophage ($CD45^+CD11b^+$) and dendritic cells ($CD45^+CD11c^+$). PTx increased

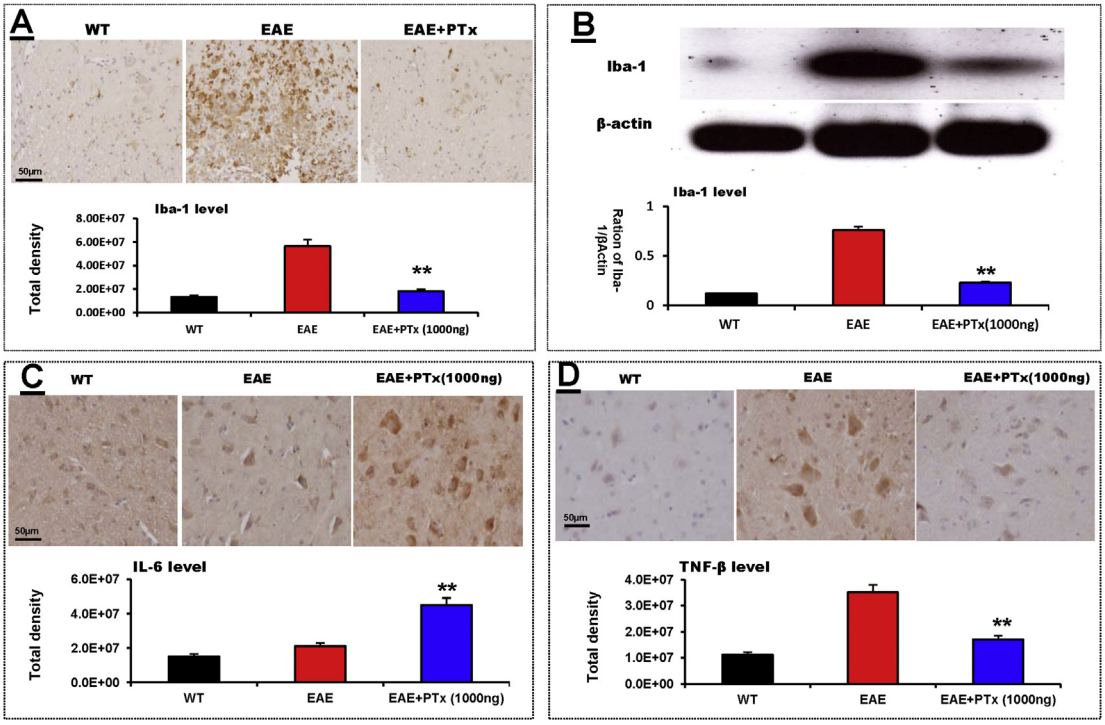

Fig. 2. PTx treatment reduced microglia and inhibited its activation in the spinal cord. (A) The total number of Iba-1, a marker for microglia, positive staining cells in EAE mice was much more than that of WT and EAE + PTx mice in the spinal cord (**$p < 0.01$). We chose the 1000 ng PTx for analysis since it showed the most protective effects. (B) Shown was the representative western blot of Iba-1. (C) IL-6 is secreted by macrophages. IL-6 positive staining cells were increased in EAE + PTx (**$p < 0.01$). (D) TNFβ, a proinflammatory cytokine, was increased after EAE, but suppressed by PTx treatment (**$p < 0.01$). WT: wild type; EAE: Experimental autoimmune encephalomyelitis; EAE + PTx: EAE with pertussis toxin treatment. Data were presented as Mean ± SEM.

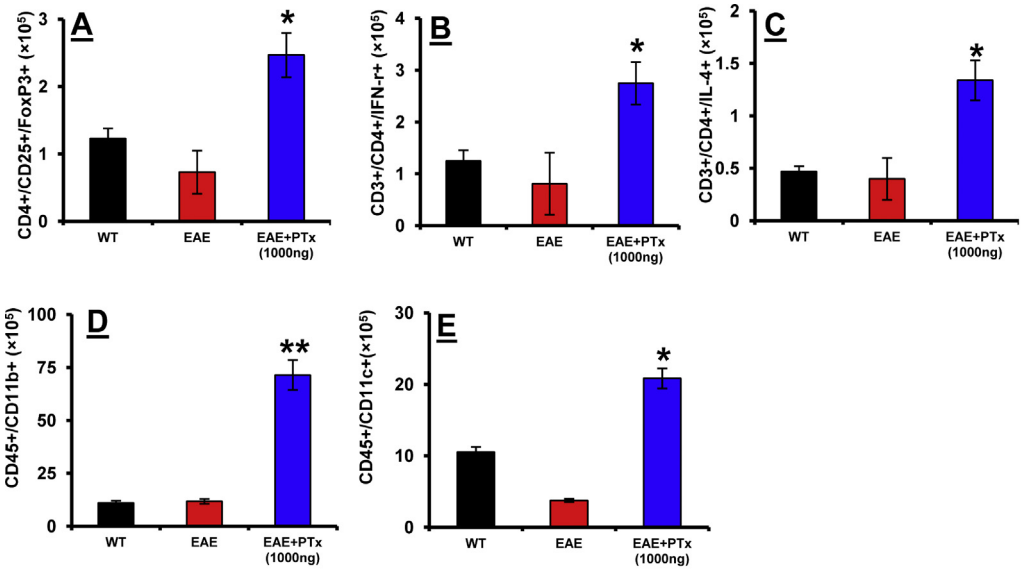

Fig. 3. PTx treatment modulated T cells and antigen presenting cell (APC) profile. Shown were analyses of flow cytometry data of subpopulation of T lymphocytes based on their specific markers ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. EAE). PTx 1000 ng was chosen for analysis since it showed the most protective effects. WT: wild type; EAE: Experimental autoimmune encephalomyelitis; EAE + PTx: EAE with pertussis toxin treatment.

macrophage by 6.1 fold, and dendritic cells by 5.5 fold ($p < 0.01$) (Fig. 3D and E).

3.5. PTx regulated APC and lymphocyte proliferation in vitro

Macrophage and dendritic cells are APCs and have been identified as key regulators in demyelinating diseases of the central nervous system. Infiltrating macrophages are thought to contribute to axonal loss in MS and in EAE (Hendriks et al., 2005; Murray and Wynn, 2011). We used splenocyte culture to assess the direct effect of PTx on APC and lymphocyte proliferation *in vitro*. Compared with MOG treatment group, PTx treatment increased APC and Th cells in a dose-dependent manner. At the high dose (1000 ng), PTx increased T cell by 25.5%, macrophage by 282.7% and dendritic cell by 358.3% (compared with control, $p < 0.01$). At lower doses, PTx increased these cells to a lesser degree. There was no significant change in B cells (Table 2).

4. Discussion

PTx has various effects on the immune system. In EAE, the animal model for MS, it has been considered an immune adjuvant responsible for a more frequent and severe course of disease in animals not previously susceptible. PTx breaches the integrity of BBB and induces the infiltration of effector T cells and macrophages to create a proinflammatory milieu (Chen et al., 2006; Fissolo et al., 2012; Herrero-Herranz et al., 2008; Locht et al., 2011). Paradoxically, a large dose of PTx was found to improve neurological function in EAE by preventing axonal loss and demyelination of the spinal cord (Weber et al., 2010; Yin et al., 2010). In this study, we studied the effects of PTx on EAE by different dosages and subunits. We have shown dose-dependent protective effects of PTx against MOG induced EAE. At the high dose and with the integrity of all subunits PTx ameliorates clinical deficits by reducing inflammatory infiltration and demyelination in the spinal cord.

Macrophages and dendritic cells are derived from monocytes which migrate from the circulation and extravagate through the endothelium. They are the primary antigen presenting cells that drives T cell-dependent immune responses (Murray and Wynn, 2011; Randolph et al., 2008). The first-responder macrophages usually exhibit an inflammatory phenotype and secrete pro-inflammatory cytokines (such as IL6), while the resident tissue macrophages maintain homeostasis by clearing apoptotic cells and debris and by promoting epithelial repair (Murray and Wynn, 2011). IL-4 produced by Th2 cells is sufficient to cause local macrophage proliferation (Murai et al., 2009; Smith et al., 2011). We have found that PTx increases peripheral APC production *in vivo* and *in vitro*. However, APC in CNS is reduced and inactivated by PTx. This suggests PTx inhibits APC migration to the CNS. This is consistent with our previous report that PTx inhibits microglia migration (Yin et al., 2010).

Treg cells play critical roles in the induction of peripheral tolerance to self and foreign antigens. Naturally occurring $CD4^{+}CD25^{+}$ Treg cells express a characteristic transcription factor forkhead box protein P3 (Foxp3). The Foxp3 is not only a unique marker but also required for the development and function of Treg

Table 2
PTx regulated APC and lymphocyte proliferation *in vitro*. Spleen cells were isolated from wild type mice and cultured with the appropriate antigens for 72 h. Bromo-2′-deoxyuridine (BrdU) labeling reagent was added 24 h before harvest. Flow cytometry analyzed the percentage and number of spleen mononuclear cell proliferation. Data were presented as Mean ± SEM. $^{*}p < 0.05$,$^{**}p < 0.01$ vs. EAE.

Lymphocyte subunits	Control	Control + MOG	Control + PTx (1000 ng)	Control + PTx (500 ng)	Control + PTx (100 ng)
$CD4^{+}$ (10*6)	38.8 ± 4.5	41.2 ± 3.2	48.7 ± 3.6*	43.3 ± 3.6	42.4 ± 3.2
B cell ($CD3^{-}CD19^{+}$) (10*6)	19.5 ± 2.3	23.4 ± 2.0	29.7 ± 1.9*	24.1 ± 2.6	19.55 ± 1.8
$CD45^{+}/CD11b^{+}$ (10*6)	5.2 ± 1.1	10.2 ± 1.4	19.9 ± 1.1**	16.8 ± 1.4*	11.7 ± 0.9
$CD45^{+}/CD11c^{+}$ (10*6)	1.2 ± 0.2	1.8 ± 0.2	5.5 ± 0.6**	1.6 ± 0.2	1.3 ± 0.2

cells (Jang et al., 2011). Treg cells are dominant in suppression of autoimmune pathology. The rescue of Treg cells can prevent and reverse autoimmune disease. Impairment in Treg cells leads to a failure in immune tolerance and triggers autoimmunity (Long and Buckner, 2011). In this study, we have shown not only Th ($CD4^+$) cells but also Treg ($CD4^+CD25^+FoxP3^+$) cells are increased by PTx treatment.

In summary, we have demonstrated a dose-dependent effect of PTx in regulating APC, Th and Treg cells, which in turn ameliorate demyelination in an animal model of autoimmune disease. This provides a platform in further studies on therapeutic intervention of MS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.01.027.

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