Differential Regulation of $CD4^+$ T Helper Cell Subset Responses by 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 in Experimental Autoimmune Encephalomyelitis

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Abstract: Peroxisome proliferator-activated receptor (PPAR) is a family of nuclear receptor transcription factors that regulates immune cell growth, differentiation and homeostasis. We and others have shown earlier that *in vivo* treatment with PPARα, β/δ or γ agonists ameliorates experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS). In this study we show that C57BL/6 mice induced to develop EAE display augmented neural antigen-specific T cell response that was inhibited by PPARγ agonist 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ_2). EAE mice showed elevated expression of IFNγ and IL-17 in the CNS and lymphoid organs compared to naïve mice that decreased significantly following treatment with 15d-PGJ_2. EAE mice also expressed elevated levels of IL-12 and IL-23 that decreased after treatment with 15d-PGJ_2. 15d-PGJ2 also attenuated the expression of IFNγ, IL-17, IL-12 and IL-23 in neural antigen-specific spleen cells *ex vivo* and *in vitro*. Moreover, EAE mice expressed low levels of IL-4, IL-10 and PPARγ compared to naïve that increased significantly following treatment with 15d-PGJ2. However, 15d-PGJ2 failed to change the expansion of $CD4^+CD25^+Foxp3^+$ Treg cells in EAE. These findings suggest that 15d-PGJ2 differentially regulates $CD4^+$ T helper cell subset responses in EAE.

Keywords: Cytokines, EAE/MS, Inflammation, PPARγ, T helper cells, Treg.

INTRODUCTION

Multiple sclerosis (MS) is a neurological disorder that affects more than a million people worldwide [1, 2]. The disease usually begins in young adults and affects women more frequently than men [3]. About 30% of MS patients develop clinical paralysis and become wheel chair-bound for rest of their lives [4]. The manifestation of focal sclerotic lesions with myelin and axonal loss in the central nervous system (CNS) is pathological hall-marks of MS [5]. Although the etiology of MS is not known, it is generally viewed as an autoimmune disease, mediated by myelin-reactive T cells in the CNS [6]. Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the CNS that can be induced in susceptible animals by immunization with neural-antigens such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) or by adoptive transfer of neural antigen-sensitized T cells [7-9]. The clinical and pathological features of EAE show close similarity to human MS, and therefore has been commonly used as a model system to study the mechanism of MS pathogenesis and to test the efficacy of potential therapeutic agents for the treatment of MS.

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptor transcription factors that regulate cell growth, differentiation and homeostasis [10]. PPAR α, β/δ, and γ are three isoforms identified in many different animals with distinct tissue distribution and function [11]. 15-deoxy $\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) is a natural ligand for PPARγ [12] and the thiazolidinedione (TZD) compounds such as rosiglitazone and ciglitazone are synthetic agonists for PPARγ [13]. Upon activation with specific ligands, PPARs undergo conformational changes leading to heterodimerization with retinoid X receptor (RXRα) and binding to promoter regions of target genes [12]. PPARs regulate lipid metabolism and glucose homeostasis and PPARγ agonists are prescription drugs for type 2 diabetes [14]. Earlier studies have shown that *in vitro* treatment with PPARγ agonists, 15d-PGJ_2 and rosiglitazone, repress inflammatory response-genes in activated macrophages [15]. *In vivo* treatments with PPARγ agonists attenuated inflammatory diseases such as experimental colitis, adjuvant-induced arthritis, atherosclerosis, experimental myocarditis and sepsis in mice and rats [16-19]. PPAR agonists inhibit immune and inflammatory responses in culture [20, 21] and *in vivo* treatment with PPARγ agonists ameliorates EAE [7, 22-24]. We have shown earlier that PPARγ agonists, 15d-PGJ_2 and Ciglitazone, ameliorate EAE by blocking IL-12 signaling through the Jak-Stat pathway leading to Th1 differentiation [7]. Further analyses showed that PPARγ deficient heterozygous mice develop an exacerbated EAE in association with an augmented neural antigen-specific Th1 response, suggesting a physiological role for PPARγ in the regulation of inflammation and demyelination in EAE [25]. $PPAR\gamma^{+/-}$ mice and those treated with PPARγ antagonists

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develop an exacerbated EAE in association with augmented neural antigen-specific Th1 response, suggesting a physiological role for PPARγ in the regulation of EAE [9, 25].

In this study, we examined the regulation of different $CD4^+$ T helper subsets by PPARγ agonist in EAE. We found that 15d-PGJ2 inhibits Th1 and Th17 responses involving Th2/Treg cells in EAE, suggesting its significance to the treatment of MS and other autoimmune diseases.

MATERIALS AND METHODS

Animals

Six to eight week old female C57BL/6 mice were obtained from Harlan (Indianapolis, IN) or Jackson Laboratory (Bar Harbor, Maine) and maintained in the animal care facility at Methodist Research Institute. All animal protocols used in the experiments were approved by the Institutional Animal Care and Use Committee at Methodist Research Institute.

Reagents

The PPARγ agonist, 15-deoxy $\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2), was purchased from CalBiochem (La Jolla, CA) or Cayman Chemical Company (Ann Arbor, MI). The 21 amino acid peptide [MEVGWYRSPFSRVVHLYRNGK] corresponding to mouse MOGp35-55 (96.81% pure) was obtained from Genemed Synthesis Inc. (San Francisco, CA). The goat anti-mouse IL-23p19 and goat anti-mouse IL-27p28 were purchased from R&D Systems (Minneapolis, MN). The qRT-PCR primers for IL-17, IFNγ, T-bet, IL-12p35, IL-12p40, IL-23p19, IL-27p28, IL-10, IL-4, PPARγ and 18S were purchased from Applied Biosystems (Foster City, CA). Recombinant mouse IFNγ, IL-17, IL-12, IL-23, IL-4, and IL-10 were purchased from R&D Systems (Minneapolis, MN). Antibodies to IFNγ, IL-17, IL-23p19, IL-4 and IL-10 were purchased from eBioscience (San Diego, CA), while anti-IL-12p40 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotin conjugated antibodies specific to IL-17 was purchased from BD Pharmingen (Franklin Lakes, NJ), IFNγ from Endogen (Woburn, MA), IL-12/23p40 from Biolegend (San Diego, CA), IL-12p70 from Mabtech (Mariemont, OH) and IL-4 and anti-IL-10 from eBioscience (San Diego, CA). Antibodies specific to CD4-PE, IL-17A-FITC, IFNγ-PE, CD25-APCCy7 and Foxp3-FITC were purchased from eBioscience (San Diego, CA).

Induction, Treatment and Evaluation of EAE

To induce EAE, six to eight week old female C57BL/6 mice were immunized (s.c.) with 100 μg MOGp35-55 in 150 μl emulsion of Complete Freund's Adjuvant (Difco, Detroit, MI) at the lower dorsum on days 0 and 7. The mice also received 100 ng pertussis toxins in 100 μl PBS (i.p.) on days 0 and 2. The mice were treated (i.p) with 100 μg 15d-PGJ2 in 25 μl DMSO on every other day from the day of first immunization. The control EAE mice received only 25 μl DMSO. Clinical signs were scored everyday from day 0 to 30 in a blinded manner as follows; 0, normal; 0.5, stiff tail; 1, limp tail; 1.5, limp tail with inability to right; 2, paralysis of one limb; 2.5, paralysis of one limb and weakness of one other limb; 3, complete paralysis of both hind limbs; 4, moribund; 5, death.

T Cell Proliferation Assay

The effect of 15d-PGJ2 on T cell proliferation was measured by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) and ^3H thymidine uptake assays. To determine the *ex vivo* response, EAE mice were treated with 15d-PGJ2 or DMSO on every other day from the day of immunization. The spleen cells were isolated on day 14 and cultured in RPMI medium ($2x10^5$/200 μl/well) with 0, 2.5, 5 and 10 μg/ml antigen in 96 well tissue culture plates. ^3H thymidine (0.5 μCi/well) was added at 72 hrs. WST-1 reagent (Roche Diagnostics, Indianapolis, IN) was added (10 μl/well) after 92 hrs and the OD at 450 nm measured after 96 hrs using a titer-plate reader (Alpha Diagnostics, San Antonio, TX). The cells were then harvested using a Tomtec harvester 96 (Hamden, CT) and ^3H thymidine uptake measured using a Wallac Microbeta Trilux counter (Perkin Elmer, Waltham, MA). To determine the *in vitro* effect of 15d-PGJ2, spleen cells isolated from DMSO treated EAE mice were cultured with 10 μg/ml MOGp35-55 antigen in the presence of 0, 1, 2.5, 5, 10 and 25 μM 15d-PGJ2. Cell viability and proliferation were measured using WST-1 and ^3H thymidine uptake assays.

Quantitative RT-PCR

The effect of 15d-PGJ2 on the expression of inflammatory cytokines in EAE was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). C57BL/6 mice induced to develop EAE were treated with 15d-PGJ2 or DMSO every other day. On day 14, brain, spinal cord, spleen and lymph node samples were isolated. Total RNA was extracted using TRIzol reagent according to manufacturer's instruction (Invitrogen) and reverse transcribed into cDNA using TaqMan reverse transcription kit. The cDNA was then amplified using TaqMan Universal Master Mix with probe and primers in the ABI 7900 Fast Sequence Detection System. The levels of gene expression normalized to 18S were calculated using the relative quantification (delta delta Ct) study software (Applied Biosystems, Foster City, CA) and the results are presented as arbitrary fold change compared to control.

Cytokine ELISA

The effect of 15d-PGJ2 on cytokine secretion was determined by ELISA [25, 37]. To determine the *ex vivo* cytokine response, spleen cells were isolated from EAE mice treated with DMSO or 15d-PGJ2 and cultured in RPMI medium ($1x10^6$/ml) in the presence of 0, 2.5, 5 and 10 μg/ml MOGp35-55 antigen in 12 well plates. To determine the *in vitro* response, spleen cells from DMSO treated EAE mice were cultured in RPMI medium ($1x10^6$/ml) with 10 μg/ml antigen in the presence of 0, 1, 2.5, 5, 10 and 25 μM 15d-PGJ2 in 12 well tissue culture plates. The culture supernatants were collected after 48 hrs and the levels of IFNγ, IL-17, IL-12p70, IL-23, IL-4 and IL-10 measured by ELISA. ELISA plates were coated with capture antibodies in 50 μl/well of 0.06 M Carbonate buffer (pH 9.6) at 4°C overnight. The excess Abs were washed off and the residual binding sites blocked by the addition of 1% BSA in PBS for 1 h. The test samples (culture supernatants) and standards were added to the respective wells and incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween-20 and biotin-conjugated detection antibodies were

added. After incubation at room temperature for 1 hr, the plates were washed and avidin-alkaline phosphatase was added, followed by 1 mg/ml of p-nitrophenyl phosphate. After 30 min incubation at room temperature, the absorbance was read at 405 nm and the cytokine levels in the culture supernatants were calculated from the standard curve.

Immunostaining and Flow Cytometry

The effect of 15d-PGJ2 on intracellular cytokines was determined by flow cytometry. To determine the *ex vivo* response, spleen cells were isolated from EAE mice treated with DMSO or 15d-PGJ2 on day 14 following immunization with MOGp35-55 and cultured in RPMI medium ($1x10^6$/ml) with 10 μg/ml antigen in 12-well plates for 40 hrs. For intracellular cytokine analysis, GolgiStop was added for the last 4 hrs to prevent protein secretion. The cells were washed, fixed and permeabilized in BD Cytofix/Cytoperm solution (BD Bioscience Pharmingen, San Diego, CA) at room temperature for 15 minutes. Samples were washed and incubated in BD Perm/Wash buffer with anti-IFNγ-PE and anti-IL-17-FITC antibodies (1:50) at 4°C for 30 min. For Treg analysis, spleen cells were surface stained with anti-CD4-PE and anti-CD25-APCCy7 antibodies (1:50) at 4°C for 30 min. The cells were then washed and incubated in BD Perm/Wash buffer with anti-Foxp3-FITC antibody (1:50) at 4°C for 30 min. The cells were washed, resuspended in PBS with 0.1% BSA and the data acquired using a three color LSR II flow cytometry instrument (BD Biosciences, Franklin Lakes, NJ). The data were analyzed using FlowJo 8.2.6 software (Ashland, OR).

Statistical Analysis

The values are mean ± SD and the data were analyzed by one-way ANOVA using the GraphPad Prism 5.0 software and the p values expressed as *($p<0.05$), **($p<0.01$) and ***($p<0.001$) comparing EAE to naive and #($p<0.05$), ##($p<0.01$) and ###($p<0.001$) comparing 15d-PGJ2 to DMSO in EAE in the figures.

RESULTS

15d-PGJ2 Inhibits EAE and Neural Antigen-Induced T Cell Proliferation

To understand the mechanism in the attenuation of EAE by 15d-PGJ2, we first examined its effect on the clinical outcome. As shown in Fig. (**1A**), C57BL/6 mice induced to develop EAE showed a mean clinical score of 1.9 on day 14 that decreased to 0.25 (87% inhibition) following treatment with 15d-PGJ2. To study the mechanism in the attenuation of EAE by 15d-PGJ2, we examined the neural antigen-induced T cell proliferation in culture. We found that spleen cells isolated from EAE mice treated with DMSO or 15d-PGJ2 displayed a dose-dependent increase in cell viability and proliferation in response to antigen in culture (Fig. **1B**). The spleen cells from DMSO treated mice cultured in the absence of antigen showed a WST-1 absorbance of 0.5 that increased to 2.1 following addition of 10 μg/ml antigen in culture. In contrast, the spleen cells from 15d-PGJ2 treated mice cultured in the absence of antigen showed a WST-1 absorbance of 0.2 that increased to 0.7 in the presence of antigen. Similarly, spleen cells from DMSO treated mice cultured in the absence of antigen showed a background ^{3}H Thymidine uptake of 1023 cpm that increased to 4589 cpm following stimulation with 10 μg/ml antigen in culture (Fig. **1B**). In contrast, the spleen cells from 15d-PGJ2 treated mice cultured in the absence of antigen showed a background ^{3}H Thymidine uptake of 313 cpm that increased to 1379 cpm following addition of 10 μg/ml antigen in culture. When compared to DMSO control, the spleen cells from 15d-PGJ2 treated mice showed a significant decrease in viability and proliferation in response to antigen *ex vivo*, reaching 65 and 70 percent inhibition. Spleen cells from 15d-PGJ2 treated mice showed lower basal survival and proliferation compared to DMSO treated mice in the absence of antigen in culture. However, the viability and proliferation index of spleen cells from DMSO treated mice were 4.2 and 4.5 that decreased to 3.5 and 4.4 in 15d-PGJ2 treated mice, suggesting a partial inhibition of neural antigen-specific T cell proliferation *ex vivo* (Fig. **1B**).

We then examined the *in vitro* effect of 15d-PGJ2 on neural antigen-specific T cell proliferation in culture. As shown in Fig. (**1C**), spleen cells from EAE mice treated with DMSO showed significant increase in cell viability and proliferation in response to MOGp35-55 antigen that decreased dose-dependently following the addition of 15d-PGJ2 in culture. Spleen cells cultured in the absence of antigen showed a WST-1 absorbance of 0.15 that increased to 0.8 after stimulation with 10 μg/ml antigen and decreased to 0.1 (88% inhibition) following the addition of 25 μM 15d-PGJ2 in culture. Similarly, spleen cells from DMSO treated EAE mice cultured in the absence of antigen showed a background ^{3}H Thymidine uptake of 180 cpm that increased to 1200 cpm after stimulation with 10 μg/ml antigen and decreased to 0 (100% inhibition) following the addition of 25 μM 15d-PGJ2 in culture (Fig. **1C**). These results suggest that 15d-PGJ2 inhibits neural antigen-specific T cell proliferation and viability in culture.

15d-PGJ2 Inhibits IFNγ and IL-17 mRNA Expression in EAE

To further understand the mechanism by which 15d-PGJ2 attenuates EAE, we examined its effect on Th1 and Th17 responses. As shown in Fig. (**2A**), C57BL/6 mice induced to develop EAE showed a significant increase in the expression of IFNγ, IL-17 and T-bet in the CNS and lymphoid organs compared to naïve mice. The expression of IFNγ mRNA in the brain, spinal cord and lymph nodes increased from 1 in the naïve to 5, 6, and 5 fold in DMSO treated EAE mice, and from undetectable to elevated expression in the spleen. Interestingly, *in vivo* treatment with 15d-PGJ2 resulted in a significant decrease in the expression of IFNγ mRNA, reaching 64, 58, 57 and 70 percent inhibition, respectively. Similarly, the expression of IL-17 mRNA in the brain, spinal cord and lymph nodes increased from 1 in naïve to 5, 24 and 20 fold in DMSO treated EAE mice, and from undetectable to elevated expression in the spleen. *In vivo* treatment with 15d-PGJ2 also resulted in a significant decrease in the expression of IL-17 mRNA, reaching 94, 96, 80 and 97 percent inhibition, respectively. We also found a similar increase in the expression of T-bet mRNA in the brain, spinal cord and lymph nodes from 1 in naïve to 9, 9, and 6 fold in DMSO treated EAE mice, and from undetectable in naïve to elevated expression in EAE spleen. *In vivo* treatment with 15d-PGJ2 resulted in a significant inhibition of T-bet mRNA expression, reaching 98, 73, 44 and 100

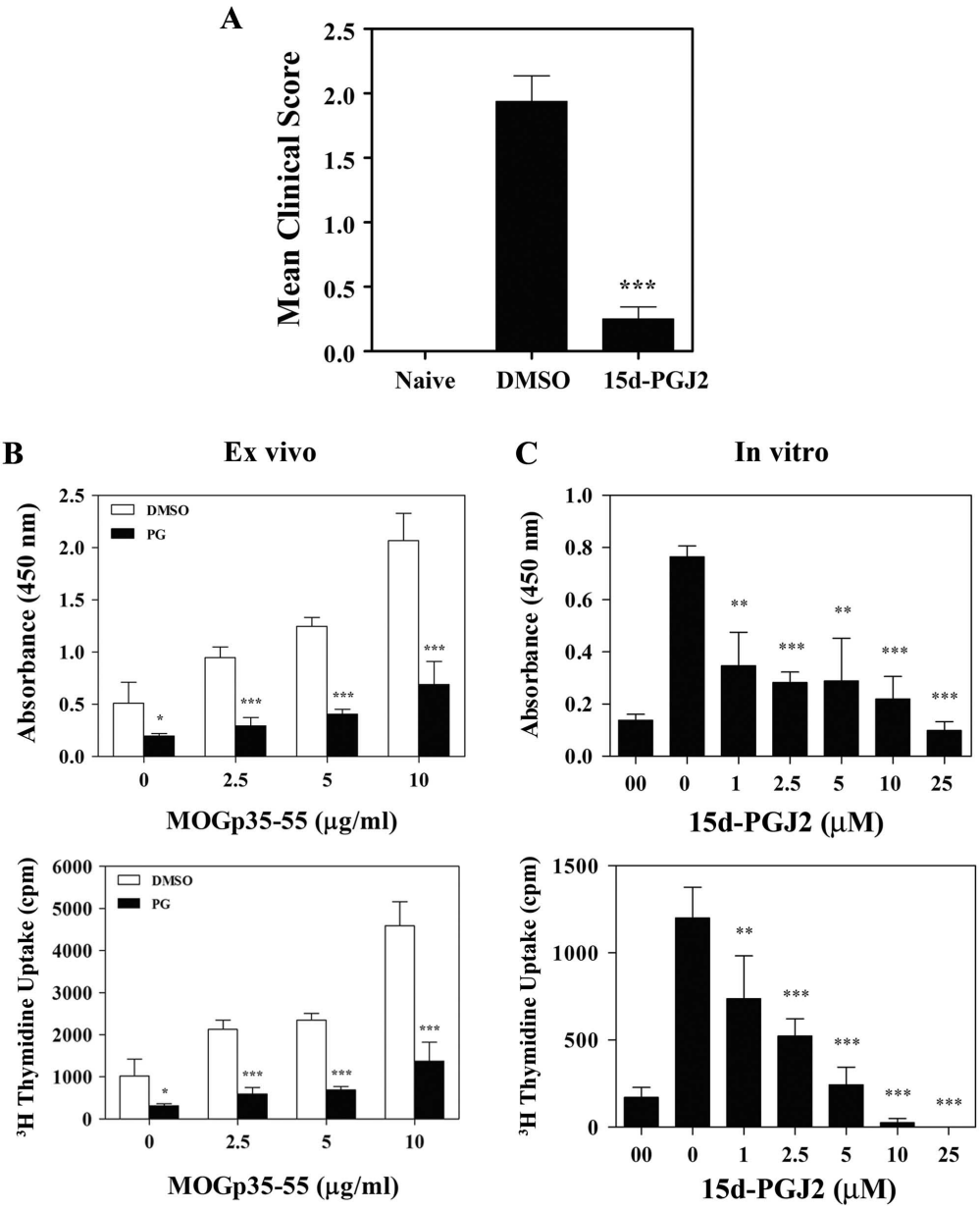

Fig. (1). Inhibition of neural antigen-induced T cell proliferation by 15d-PGJ2 in EAE. C57BL/6 mice induced to develop EAE were treated (i.p.) with DMSO and 100 μg 15d-PGJ2 every other day. (**A**) The clinical signs of EAE in DMSO and 15d-PGJ2 treated mice were scored on day 14. (**B**) The spleen cells were isolated on day 14 and cultured with 0, 2.5, 5 and 10 μg/ml MOGp35-55 antigen *ex vivo*. (**C**) Spleen cells from DMSO treated EAE mice were also cultured with 0 or 10 μg/ml of MOGp35-55 in the presence of 0, 1, 2.5, 5 and 10 μg/ml 15d-PGJ2 *in vitro*. The viable cell count was determined by WST-1 assay and proliferation by ^{3}H thymidine uptake assay (**B**, **C**). The figure is representative of three independent experiments.

percent inhibition, respectively. These results demonstrate that 15d-PGJ2 inhibits the expression of IFNγ, IL-17 and T-bet in the CNS and lymphoid organs, suggesting the regulation of Th1/Th17 responses in EAE.

15d-PGJ2 Inhibits IFNγ and IL-17 Secretion from Neural Antigen-Specific T Cells

We then examined the effect of 15d-PGJ2 on IFNγ and IL-17 production from neural antigen-specific T cells in culture. As shown in Fig. (**2B**), MOGp35-55-immune spleen cells from DMSO and 15d-PGJ2 treated mice showed a dose-dependent increase in the secretion of IFNγ and IL-17 in response to antigen in culture. The spleen cells from DMSO treated EAE mice cultured in the absence of antigen secreted 9 ng/ml IFNγ that increased to 30 ng/ml following stimulation with 10 μg/ml antigen. On the other hand, spleen cells from 15d-PGJ2 treated EAE mice cultured in the absence of antigen secreted 8 ng/ml IFNγ that increased to 17 ng/ml. Similarly, spleen cells from DMSO treated mice cultured in the absence of antigen secreted 1 ng/ml IL-17 that increased to 57 ng/ml. Whereas, spleen cells from 15d-PGJ2 treated mice cultured in the absence of antigen secreted 0.1 ng/ml IL-17 that increased to 2.5 ng/ml in response to antigen (Fig. **2B**). However, when compared to the DMSO treated control group, spleen cells from 15d-PGJ2 treated mice showed significant reduction in the secretion of IFNγ

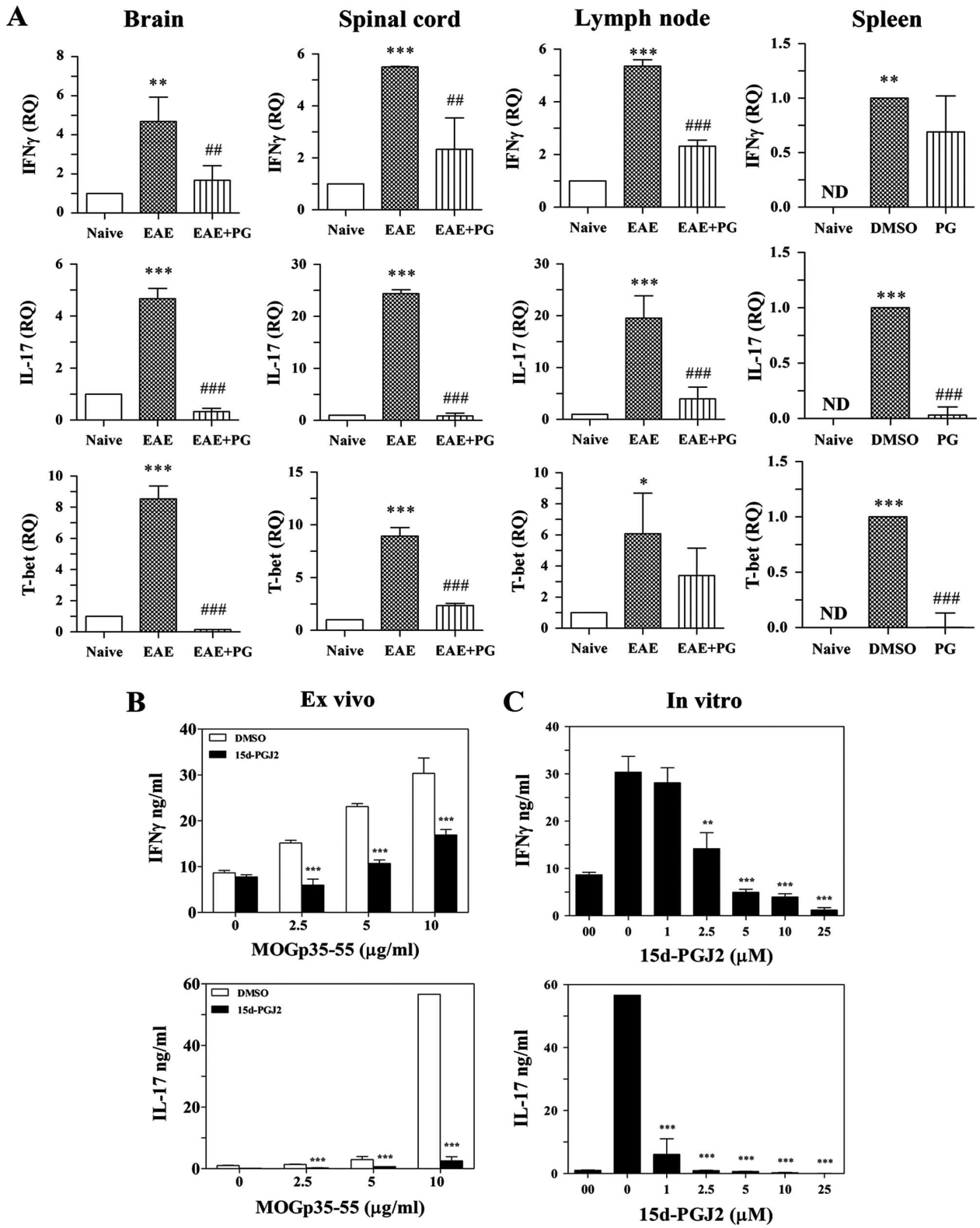

Fig. (2). Inhibition of Th1 and Th17 responses by 15d-PGJ2 in EAE. (**A**) C57BL/6 mice induced to develop EAE were treated with DMSO or 15d-PGJ2. The mice were euthanized on day 14 and total RNA extracted from the brain, spinal cord, lymph node and spleen. The expression levels of IFNγ, IL-17 and T-bet were analyzed by qRT-PCR. The fold change was calculated and shown as mean ± SEM for EAE compared to naïve (*p<0.05, **p<0.01, ***p<0.001) and 15d-PGJ2 compared to DMSO (#p<0.05, ##p<0.01, ###p<0.001). (**B**) Spleen cells from DMSO and 15d-PGJ2 treated EAE mice were cultured with 0, 2.5, 5 and 10 μg/ml MOGp35-55 (*ex vivo*). (**C**) Spleen cells from DMSO treated EAE mice were cultured with 0 and 10 μg/ml antigen in the presence of 0, 2.5, 5, 10 and 25 μM of 15d-PGJ2 (*in vitro*). After 48 hrs, culture supernatants were collected and the levels of IFNγ and IL-17 were determined by ELISA. Tissue samples from 2 mice were used in each experiment (n=2) and the figure is representative of three independent experiments.

and IL-17, reaching 44 and 96 percent inhibition, respectively.

Further analyses showed that *in vitro* culture of spleen cells, from DMSO treated EAE mice, with antigen resulted in a significant increase in the secretion of IFNγ and IL-17 that decreased dose-dependently following addition of 15d-PGJ2 in culture. As shown in Fig. (**2C**), spleen cells cultured in the absence of antigen secreted 9 ng/ml IFNγ that increased to 30 ng/ml following stimulation with 10 μg/ml antigen, which decreased to 1 ng/ml (96% inhibition) following addition of 25 μM 15d-PGJ2. Similarly, spleen cells cultured in the absence of antigen secreted 2 ng/ml IL-17 that increased to 57 ng/ml after stimulation with antigen, which decreased to 0 (100% inhibition) following addition of 15d-PGJ2 (Fig. **2C**). These results further suggest that 15d-PGJ2 inhibits neural antigen-specific Th1/Th17 responses *in vivo* and *in vitro*.

15d-PGJ2 Regulates the Expansion of Neural Antigen-Specific Th1 and Th17 Cells

To determine the mechanism in the regulation of Th1 and Th17 cells by 15d-PGJ2, we then examined $IFN\gamma^+$ and $IL\text{-}17^+$ T cells in culture. As shown in Fig. (**3A**), spleen cells from DMSO treated EAE mice showed significant number of $IFN\gamma^+$ T cells that decreased following treatment with 15d-PGJ2. Spleen cells from mice treated with DMSO cultured in the absence of antigen showed 7% $CD4^+IFN\gamma^+$ cells that increased to 15% following stimulation with 10 μg/ml antigen. On the other hand, spleen cells from mice treated with 15d-PGJ2 cultured in the absence of antigen showed 4% $IFN\gamma^+$ cells (44% inhibition) that increased to 14% (10% inhibition compared to DMSO). Similarly, spleen cells from DMSO treated mice cultured in the absence of antigen showed 6.5% $IL\text{-}17^+$ cells that increased to 15% following stimulation with 10 μg/ml antigen. Whereas, the spleen cells from 15d-PGJ2 treated mice cultured in the absence of antigen showed 6% $IL\text{-}17^+$ cells that increased to 13% (16% inhibition compared to DMSO) (Fig. **3B**). Further analyses showed that *in vitro* culture of spleen cells from DMSO treated EAE mice in the presence of 15d-PGJ2 also resulted in a significant reduction in the percentage of $IFN\gamma^+$ and $IL\text{-}17^+$ T cells (Fig. **3C**). Spleen cells cultured with 10 μg/ml antigen showed 15% $IFN\gamma^+$ T cells (Fig. **3A**) that decreased to 1% (97% inhibition) following addition of 10 μM 15d-PGJ2. Similarly, spleen cells from DMSO treated EAE mice cultured with 10 μg/ml antigen showed 15% $IL\text{-}17^+$ T cells (Fig. **3B**) that decreased to 6% (61% inhibition) following addition of 15d-PGJ2. These results show that 15d-PGJ2 inhibits the expansion of neural antigen-specific Th1/Th17 cells *in vitro*, but not *in vivo*.

15d-PGJ2 Inhibits the Expression of IL-12 Family Cytokines in EAE

To investigate the mechanism by which 15d-PGJ2 regulates Th1/Th17 responses in EAE, we examined its effect on IL-12 family cytokines in the CNS and lymphoid organs. As shown in Fig. (**4A**), EAE mice showed elevated expression of IL-12p35, IL-12p40, IL-23p19 and IL-28p28 that decreased significantly following treatment with 15d-PGJ2. DMSO treated EAE mice displayed 7, 11 and 10 fold increases in the expression of IL-12p35 mRNA in the brain, spinal cord and lymph node with elevated expression in spleen compared to naïve mice that decreased by 98, 79, 48 and 100 percent following *in vivo* treatment with 15d-PGJ2, respectively. Similarly, the expression of IL-12p40 mRNA increased 2, 8 and 6 fold in the brain, spinal cord and lymph node with elevated expression in spleen of EAE mice compare to naïve that decreased by 76, 80, 63 and 100 percent in 15d-PGJ2 treated mice, respectively. Moreover, when compared to naïve, EAE mice showed 6, 8 and 14 fold increase in the expression of IL-23p19 mRNA in the brain, spinal cord and lymph node with elevated expression in spleen that decreased by 98, 34, 92 and 96 percent following *in vivo* treatment with 15d-PGJ2, respectively. We have also found significant increase in the expression of IL-27p28 mRNA reaching 5, 6 and 35 fold in the brain, spinal cord and lymph node with elevated expression in spleen that reduced by 96, 11, 67 and 100 percent in 15d-PGJ2 treated mice, respectively.

We then examined the secretion of IL-12 and IL-23 in spleen cells from EAE mice in culture. As shown in Fig. (**4B**), spleen cells from DMSO or 15d-PGJ2 treated EAE mice showed a dose-dependent increase in the secretion of IL-12 and IL-23 in response to antigen. The spleen cells from DMSO treated EAE mice cultured in the absence of antigen secreted 6 ng/ml IL-12 that increased to 18 ng/ml following stimulation with 10 μg/ml antigen. Similarly, spleen cells from 15d-PGJ2 treated EAE mice cultured in the absence of antigen secreted 6 ng/ml IL-12 that increased to 14 ng/ml. Moreover, spleen cells from DMSO treated EAE mice cultured in the absence of antigen secreted undetectable IL-23 that increased to 49 ng/ml after stimulation with 10 μg/ml antigen. Whereas, spleen cells from 15d-PGJ2 treated EAE mice cultured in the absence of antigen secreted 3 ng/ml IL-23 that increased to 22 ng/ml (Fig. **4B**). However, when compared to DMSO control, EAE mice treated with 15d-PGJ2 showed partial reduction (33%) in the secretion of IL-12 with a significant (55%) inhibition of IL-23 in culture.

We also examined the *in vitro* effect of 15d-PGJ2 on the secretion of IL-12 and IL-23 in spleen cells from EAE mice. As shown in Fig. (**4C**), spleen cells from DMSO treated EAE mice secreted elevated levels of IL-12 and IL-23 in response to MOGp35-55 antigen in culture. *In vitro* treatment with 15d-PGJ2 resulted in a significant decrease in the secretion of IL-12 and IL-23 in culture. The spleen cells cultured in the absence of antigen secreted 7 ng/ml IL-12 that increased to 15 ng/ml in the presence of 10 μg/ml antigen and decreased to undetectable level after treatment with 5 μM 15d-PGJ2. Similarly, the spleen cells cultured in the absence of antigen secreted undetectable IL-23 that increased to 33 ng/ml in the presence of 10 μg/ml antigen and decreased to 0.3 ng/ml at 1 μM 15d-PGJ2. These results suggest that 15d-PGJ2 inhibits IL-12 family cytokines in EAE *in vivo* and *in vitro*.

15d-PGJ2 Modulates Th2 and Treg Responses in EAE

To further determine the mechanism in the attenuation of EAE by 15d-PGJ2, we examined its influence on Th2 and Treg cells. As shown in Fig. (**5**), EAE mice showed significant decrease in the transcription of anti-inflammatory cytokines in the brain and spinal cord compared to naïve mice. In DMSO treated EAE mice the expression of IL-4 mRNA changed 1, 0.1 and 4 fold in brain, spinal cord and lymph

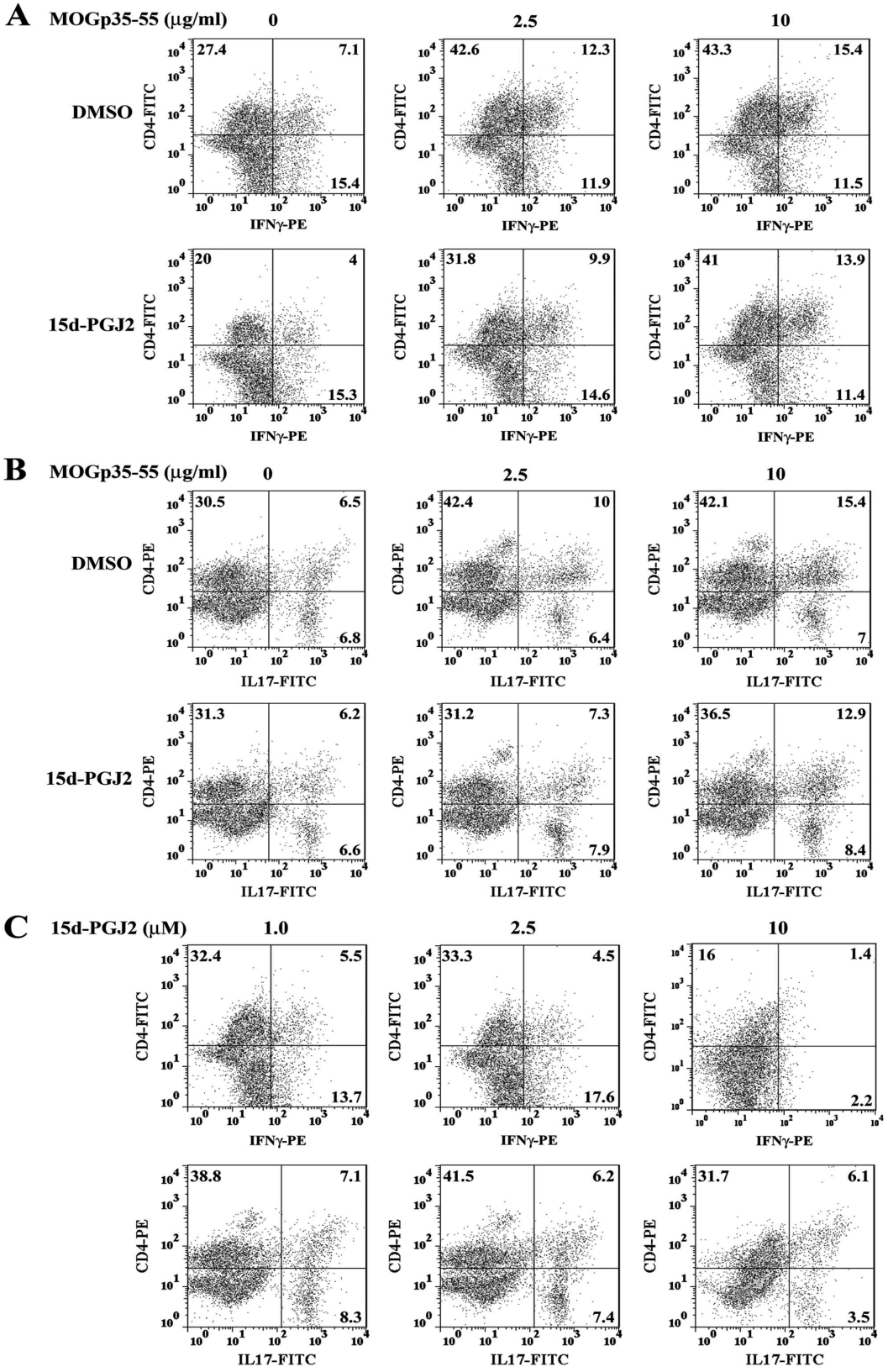

Fig. (3). Inhibition of Th1 and Th17 cell expansion by 15d-PGJ2 in EAE. C57BL/6 mice induced to develop EAE were treated with DMSO or 100 μg 15d-PGJ2. Spleen cells were isolated on day 14 and cultured in the presence of 0, 2 and 10 μg/ml of MOGp35-55. The cells were stained for intracellular IFNγ and IL-17 and the percent $CD4^+IFN\gamma^+$ (**A**) and $CD4^+IL\text{-}17^+$ (**B**) cells were analyzed by flow cytometry. (**C**) Spleen cell from DMSO treated EAE mice were cultured with antigen in the presence of 15d-PGJ2 and $CD4^+IFN\gamma^+$ or $CD4^+IL\text{-}17^+$ cells analyzed by flow cytometry. Spleen cells from 2 mice were used in each experiment (n=2) and the figure is representative of three independent experiments.

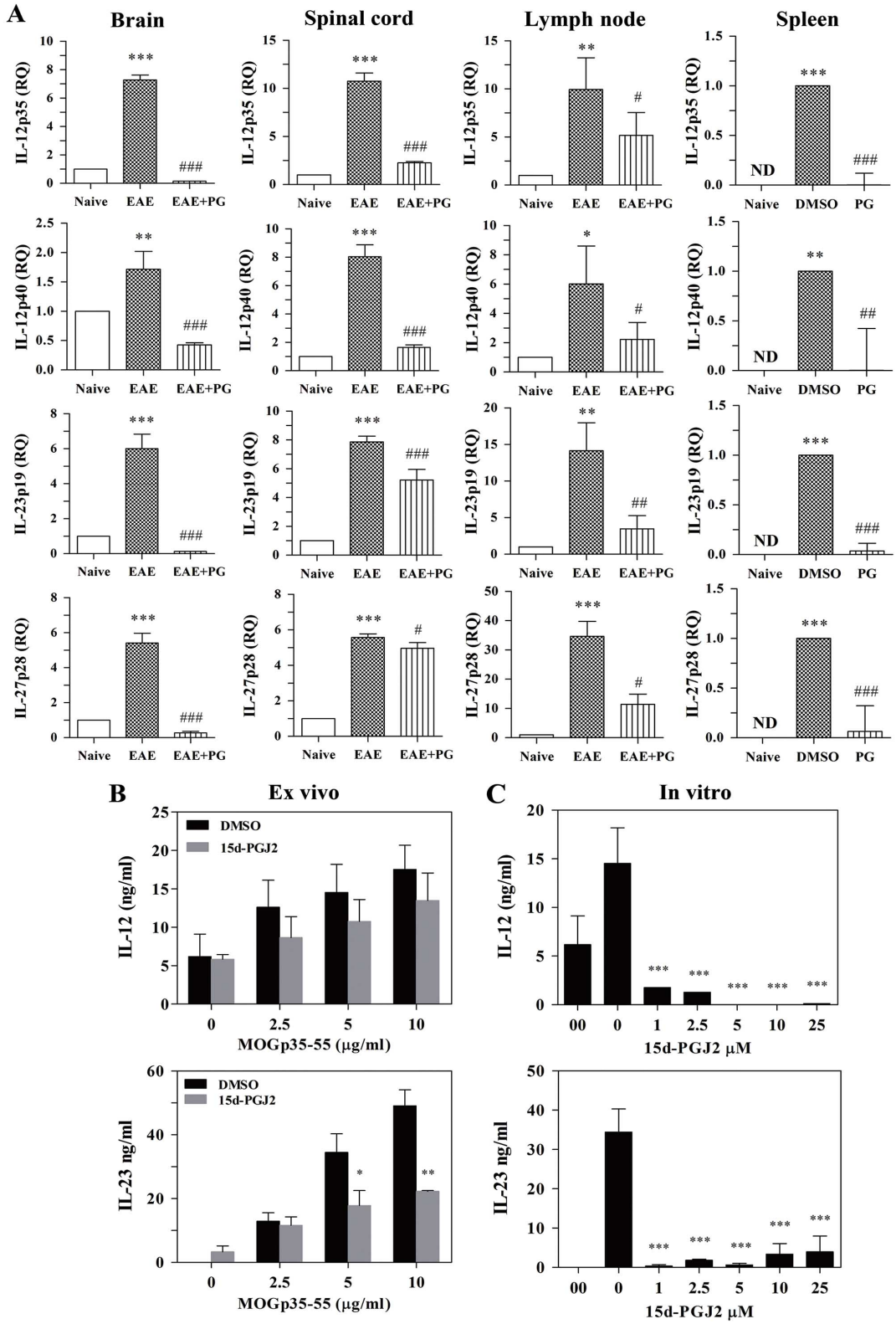

Fig. (4). Inhibition of IL-12 family cytokines by 15d-PGJ2 in EAE. (**A**) C57BL/6 mice induced to develop EAE were treated with DMSO or 15d-PGJ2. On day 14, total RNA was extracted from the brain, spinal cord, lymph node and spleen and the expression of IL-12p35, IL-12p40, IL-23p19 and IL-27 analyzed by qRT-PCR. The fold change in EAE was calculated and shown as mean ± SEM (n=6) for EAE compared to naïve (*p<0.05, **p<0.01, ***p<0.001) and 15d-PGJ2 compared to EAE (# p<0.05, ## p<0.01, ### p<0.001). (**B**) Spleen cells from DMSO and 15d-PGJ2 treated EAE mice were cultured with 0, 2.5, 5 and 10 µg/ml MOGp35-55 (*ex vivo*). (**C**) Spleen cells from DMSO treated EAE mice were cultured with 0 and 10 µg/ml antigen in the presence of 0, 2.5, 5, 10 and 25 µM of 15d-PGJ2 (*in vitro*). After 48 hrs, culture supernatants were collected and the levels of IL-12p70 and IL-23 were determined by ELISA. Spleen cells from 2 mice were used in each experiment (n=2) and the figure is representative of three independent experiments (*p<0.05, **p<0.01, and ***p<0.001).

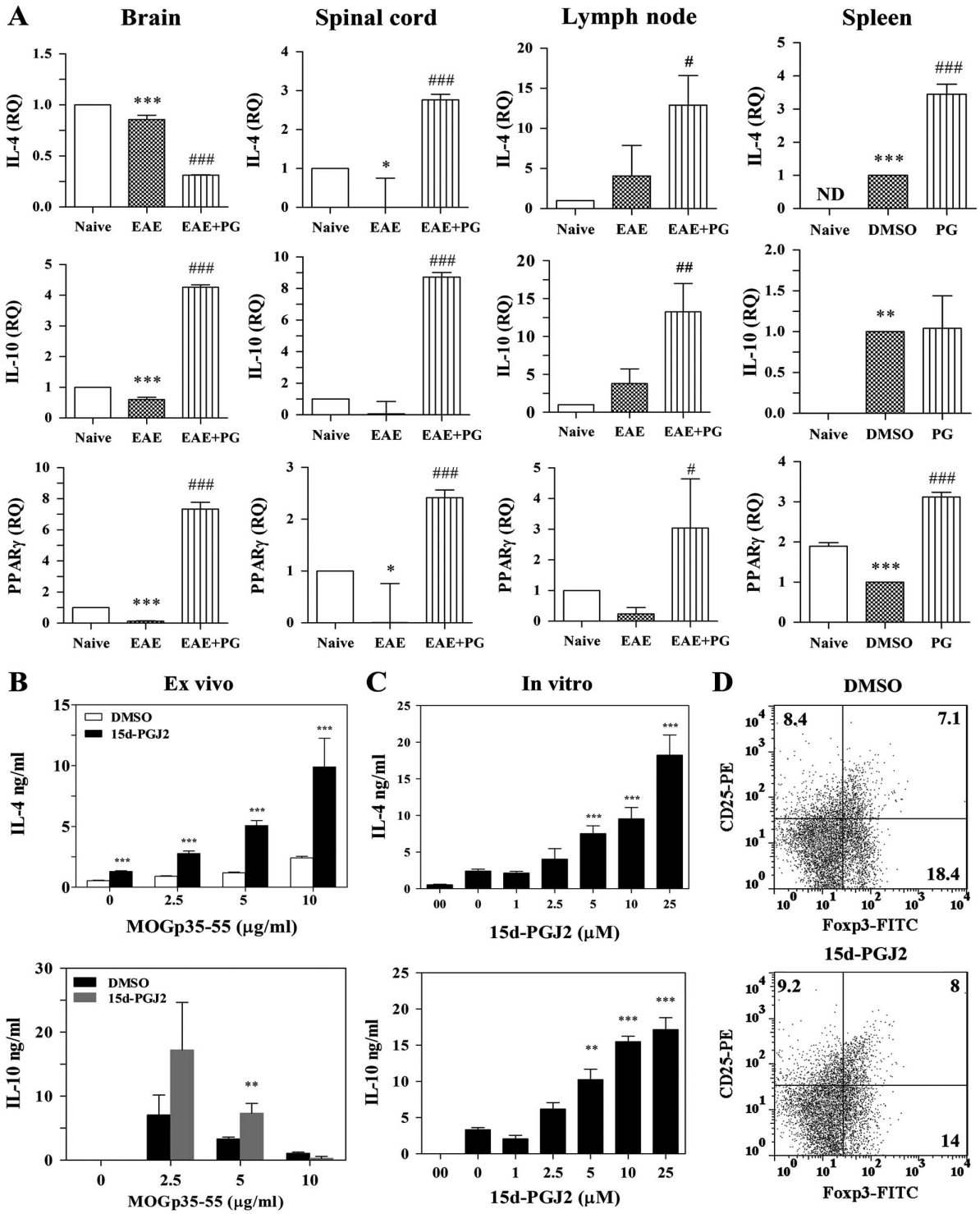

Fig. (5). Regulation of Th2 and Treg responses by 15d-PGJ2 in EAE. (**A**) C57BL/6 mice induced to develop EAE were treated with DMSO or 15d-PGJ2. On day 14 total RNA was extracted from brain, spinal cord, lymph node and spleen. The expression levels of IL-4, IL-10 and PPARγ were analyzed by qRT-PCR. The fold change was calculated and shown as mean ± SEM for EAE compared to naïve (*p<0.05, **p<0.01, ***p<0.001) and 15d-PGJ2 compared to DMSO (#p<0.05, ##p<0.01, ###p<0.001). (**B**) Spleen cells from DMSO and 15d-PGJ2 treated EAE mice were cultured with 0, 2.5, 5 and 10 μg/ml MOGp35-55 (*ex vivo*). (**C**) Spleen cells from DMSO treated EAE mice were cultured with 0 and 10 μg/ml antigen in the presence of 0, 2.5, 5, 10 and 25 μM of 15d-PGJ2 (*in vitro*). After 48 hrs, culture supernatants were collected and the levels of IL-4 and IL-10 determined by ELISA. (**D**) Spleen cells from EAE mice treated with DMSO and 15d-PGJ2 were isolated on day 14 and stained for CD4, CD25 and Foxp3. The percent $CD4^+CD25^+Foxp3^+$ cells were analyzed by flow cytometry. Spleen cells from 2 mice were used in each experiment (n=2) and the figure is representative of three independent experiments (*p<0.05, **p<0.01, and ***p<0.001).

nodes with significant increase in spleen that decreased 0.3 fold in the brain but increased 3, 13 and 4 fold in the spinal cord, lymph nodes and spleen following *in vivo* treatment with 15d-PGJ2, respectively. Similarly, the expression of IL-10 mRNA in EAE mice changed 1, 0.1 and 4 fold in the brain, spinal cord and lymph nodes that increased by 4, 9 and 13 fold following *in vivo* treatment with 15d-PGJ2, respectively. Moreover, DMSO treated EAE mice showed a reduction in the expression of PPARγ mRNA in the CNS and lymphoid organs compared to naïve mice. In DMSO treated EAE mice the expression of PPARγ mRNA changed 0.1, 0.01, 0.2 and 1 fold in brain, spinal cord, lymph nodes and spleen compared to naïve that increased 7, 2, 3 and 3 fold after *in vivo* treatment with 15d-PGJ2, respectively.

To further confirm the results, we examined the secretion of IL-4 and IL-10 in spleen cells from EAE mice in culture. As shown in Fig. (**5B**), spleen cells from DMSO and 15d-PGJ2 treated EAE mice showed significant increase in the secretion of IL-4 and IL-10 in response to antigen in culture. The spleen cells from DMSO treated EAE mice cultured in the absence of antigen secreted 0.5 ng/ml IL-4 that increased to 2.4 ng/ml following stimulation with 10 μg/ml MOGp35-55 antigen. Similarly, spleen cells from 15d-PGJ2 treated EAE mice cultured in the absence of antigen secreted 1.3 ng/ml IL-4 that increased to 9.9 ng/ml after stimulation with 10 μg/ml antigen. Moreover, spleen cells from DMSO treated EAE mice cultured in the absence of antigen secreted undetectable IL-10 that increased to 7 ng/ml after stimulation with 2.5 μg/ml antigen but decreased to 3.3 at 5 μg/ml antigen. Similarly, spleen cells from 15d-PGJ2 treated EAE mice cultured in the absence of antigen secreted undetectable IL-10 that increased to 17 ng/ml after stimulation with 2.5 μg/ml antigen, but decreased to 7 ng/ml at 5 μg/ml antigen (Fig. **5B**). However, when compared to DMSO control the spleen cells from EAE mice treated with 15d-PGJ2 showed significant increase in IL-4 and IL-10 production in response to antigen *ex vivo*.

We have also examined the *in vitro* effect of 15d-PGJ2 on the secretion of IL-4 and IL-10 in spleen cells from EAE mice in culture. As shown in Fig. (**5C**), *in vitro* stimulation of spleen cells from DMSO treated EAE mice with antigen in the presence of 15d-PGJ2 resulted in a dose-dependent increase in IL-4 and IL-10 secretion. The spleen cells cultured in the absence of antigen secreted 0.7 ng/ml IL-4 that increased to 2.4 ng/ml following stimulation with 10 μg/ml antigen and further increased to 18.2 ng/ml by the addition of 25 μM 15d-PGJ2. Moreover, spleen cells cultured in the absence of antigen secreted undetectable IL-10 that increased to 3.3 ng/ml after stimulation with antigen and further increased to 17.2 ng/ml in the presence of 25 μM 15d-PGJ2. However, the spleen cells from DMSO treated EAE mice showed 7.1% $CD4^+CD25^+Foxp3^+$ Treg cells that increased to 8% in mice treated with 15d-PGJ2 (Fig. **5D**). These results suggest that 15d-PGJ2 modulates anti-inflammatory responses in EAE *ex vivo* and *in vitro*.

DISCUSSION

In this manuscript we have shown that 15d-PGJ2 regulates Th1/Th17 responses in EAE. While C57BL/6 mice induced to develop EAE showed elevated Th1 and Th17 responses, *in vivo* treatment with 15d-PGJ_2 attenuated the expression of both IFNγ and IL-17 in the CNS and lymphoid organs. EAE mice also showed elevated expression of IL-12 family cytokines that decreased after treatment with 15d-PGJ_2. *Ex vivo* or *in vitro* treatment with 15d-PGJ2 inhibited neural antigen-induced secretion of IFNγ, IL-17, IL-12 and IL-23, while increasing IL-4, IL-10 and PPARγ in the lymphoid organs. Our findings highlight that 15d-PGJ2 differentially regulate autoimmune T helper cell subsets in EAE.

Earlier studies have demonstrated the potential use of natural and synthetic PPARα, δ and γ selective agonists for the treatment of MS [7, 22, 26, 27]. But the mechanisms by which PPAR agonists modulate CNS demyelination are not fully defined. The pathogenesis of EAE/MS is a complex process involving the activation of neural antigen-specific immune cells in the primary lymphoid organs and their migration, reactivation and secretion of myelinotoxic inflammatory cytokines in the CNS [5, 28-30]. IFNγ and IL-17 are pro-inflammatory cytokines produced by Th1 and Th17 cells, respectively that play distinct roles in the pathogenesis of EAE and MS [31-34]. We have shown earlier that PPARγ agonists inhibit CNS inflammation by modulating neural antigen-specific Th1 cells in EAE [7]. In this study we found that the PPARγ agonist 15d-PGJ2 inhibits the expression of both IFNγ and IL-17 in the CNS and lymphoid organ of C57BL/6 mice with EAE and neural antigen-specific spleen cells in culture. This is consistent with earlier reports on the inhibition of IFNγ and IL-17 by PPARγ agonists in EAE [7, 22-24]. The PPARγ agonist 15d-PGJ2 also inhibited the expression of T-bet in immune cells *ex vivo* and *in vitro*. Thus our findings suggest the regulation of both Th1 and Th17 responses by 15d-PGJ2 EAE. In addition, we have shown earlier that PPARγ deficient heterozygous mice develop an exacerbated EAE with augmented Th1 response in EAE [25]. A recent study showed that PPARγ activation in $CD4^+$ T cells selectively suppress Th17, but not Th1, Th2 or Treg differentiation (Klotz *et al*., 2009). The T cell-specific PPARγ knockout and endogenous ligand activation studies have further confirmed a physiological role for PPARγ in the regulation of Th17 differentiation and development of autoimmunity [35]. Moreover, PPARγ agonists, rosiglitazone and pioglitazone reduced the pathophysiology of asthma by inhibiting IL-17 production, involving NF-κB pathway in animal models [36]. This is consistent with our recent report on the inhibition of Th1 and Th17 responses by PPARδ agonists in EAE [37]. Thus our findings highlight that 15d-PGJ2 ameliorates EAE by modulating Th1/Th17 responses and suggest the use of PPARγ agonists in the treatment of MS and other autoimmune diseases.

The IL-12 family cytokines play critical roles in the differentiation of Th1/Th17 cells and pathogenesis of EAE/MS [27, 31, 38]. We and others have shown earlier that targeted disruption or pharmacological inhibition of IL-12 or IL-23 was sufficient to prevent Th1/Th17 differentiation and pathogenesis of EAE [32, 33, 39]. We have also demonstrated earlier that PPARγ agonists inhibit Th1 differentiation in EAE by blocking IL-12 production and IL-12 signaling in EAE [7]. In this study we found that *in vivo* treatment with 15d-PGJ2 inhibits the expression of IL-12 and IL-23 in the CNS and lymphoid organs of EAE mice. This is consistent with earlier reports on the inhibition of IL-12 production by PPARγ agonists in macrophage, microglia and dendritic cells [40], suggesting this be a mechanism by which 15d-

PGJ2 regulates Th1/Th17 responses in EAE. However, the *in vitro* effects of 15d-PGJ2 were much stronger than *in vivo* and this could be due to the dose response and complex *in vivo* mechanisms compared to *in vitro* culture system.

The Th2/Treg cells and anti-inflammatory cytokines such as IL-4 and IL-10 are critical regulators of inflammation and demyelination in EAE/MS [41]. To further understand the mechanisms by which 15d-PGJ2 inhibits EAE, we examined the expression of IL-4 and IL-10. We found that *in vivo* treatment with 15d-PGJ2 increased the expression of IL-4, IL-10 and PPARγ in the CNS of mice with EAE, suggesting the involvement of anti-inflammatory cytokines in the attenuation of EAE by 15d-PGJ2 [42]. Moreover, a significant reduction in the expression of PPARγ in EAE compared to naïve mice and its reversal to elevated levels following treatment with 15d-PGJ2 suggest its role in the regulation of EAE and perhaps MS. Earlier studies have shown that rosiglitazone increased the level of IL-10 and reduced cisplatin-induced inflammation and cytotoxicity in the kidney [43]. Although a direct causal link between IL-10 and Th1/Th17 responses is not shown in this study, the presence of PPARγ response element (PPRE) in IL-10 gene promoter and its activation associated increase in the production of IL-10 by PPARγ agonists in dendritic cells or $CD4^+$ T cells suggest that IL-10 might be a potential regulator of PPARγ responses in EAE [44]. The $CD4^+CD25^+Foxp3^+$ regulatory T cells are important modulators of Th1/Th17 responses and pathogenesis of EAE [45, 46]. Earlier studies have shown that PPARγ agonists attenuate immune responses by promoting Treg cells through PPARγ in culture [47]. However, a minimal increase in Treg cells observed in mice treated with 15d-PGJ2 suggests the involvement of other mechanisms in the attenuation of EAE. While the activation of NF-κB, Jak-Stat, Mapk and JNK signaling pathways leads to Th1/Th17 differentiation [26], selective inhibition of these inflammatory signaling networks by PPARγagonists attenuate EAE [7, 8]. Our future investigations will further unravel the molecular signatures of inflammatory signaling networks that are targeted by 15d-PGJ2 and other PPARγ agonists in MS and other autoimmune inflammatory diseases.

ABBREVIATIONS

MS = multiple sclerosis

EAE = experimental autoimmune encephalomyelitis

MOG = myelin oligodendrocyte glycoprotein

qRT-PCR = quantitative reverse transcription polymerase chain reaction

PPAR = peroxisome proliferator-activated receptor

Th = T helper

Treg = regulatory T cell

PBS = phosphate buffered saline

FBS = fetal bovine serum

ELISA = enzyme linked immunosorbant assay

IL = interleukin

IFN = interferon

Foxp3 = forkhead box protein 3

15d-PGJ2 = 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2

CD = clusters of differentiation

DMSO = dimethyl sulfoxide

CNS = central nervous system.

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DISCLOSURES

None.

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