A Thesis

For the Degree of Master of Veterinary Medicine

Protective effects of inflammatory demyelinating disease of the central nervous system by an acidic polysaccharide of *Panex ginseng*

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Protective effects of inflammatory demyelinating disease of the central nervous system by an acidic polysaccharide of *Panex ginseng*

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LIST OF ABBREVIATIONS

α -GalCer	α−galactosylceramide
Ab	antibody
CD	cluster of differentiation
CFA	complete freund's adjuvant
CPM	cycles per minute
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
Foxp3	Forkhead box P3
GITR	glucocorticoid-induced TNF-receptor-related protein
GM-CSF	granulocyte monocyte colony-stimulating factor
H&E	hematoxylin and eosin
IFN	interferon
IL	interleukin
i. p.	intraperitoneally
i. v.	intravenous
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NK cells	natural killer cells
PBS	phosphate buffered saline
PE	phycoerythrin
s. c.	subcutaneous
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gels
TCR	T cell receptor
Th cells	T helper cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Tregs	$CD4^+CD25^+$ regulatory T cells
VIP	vasoactive intestinal peptide

An acidic polysaccharide of *Panax ginseng* (APG), so called ginsan, is a purified polysaccharide. APG has multiple immunomodulatory effects of stimulating natural killer (NK) and T cells and producing a variety of cytokines that proved to diminish the proinflammatory response, and protect from septic lethality. To determine APG's role in the autoimmune demyelinating disease, we tested whether APG can regulate inflammatory and encephalitogenic response in experimental autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis (MS). Here, we demonstrate the therapeutic efficacy of the APG which induces the suppression of an encephalitogenic response during EAE. APG significantly ameliorates the progression of EAE by inhibiting the proliferation of autoreactive T cells and the production of such inflammatory cytokines as IFN-γ and IL-17. More importantly, APG promotes the generation of immunosuppressive Tregs through the activation of transcription factor, Foxp3. Furthermore, the depletion of $CD25^+$ cells from APG-treated EAE mice abrogates the beneficial effects of EAE. The capacity of APG to induce clinically beneficial effects furthers our understanding of the basis for its therapeutic immunosuppression of EAE and, possibly, multiple sclerosis (MS). Thus, our results suggest that APG may serve as an effective therapy for MS and other autoimmune diseases.

Key words: Panax ginseng; APG; EAE; $CD4^+CD25^+$ regulatory T cells; IL-17

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory and demyelinating autoimmmune model of diseases in the central nervous system (CNS). EAE bears pathologic and clinical similarities to human multiple sclerosis (MS) and is used as a model to test potential therapeutic agents (1). The objective of such therapies is to control autoreactive T cell responses and to maintain peripheral self-tolerance via immunosuppressive activities (2, 3, 4). Recently, the alteration or reduction of $CD4^+CD25^+$ regulatory T cell (Treg) functions has been reported in patients with MS and other autoimmune diseases (5, 6). Tregs contribute to the maintenance of peripheral tolerance and active suppression of autoimmunity in a T cell receptor (TCR)-dependent manner, as established when their depletion increased the severity of and mortality from EAE (7). However, the endogenous factors and mechanisms controlling the peripheral expansion of Tregs are mostly unknown.

The agent commonly called ginsan is a purified acidic polysaccharide extracted from the roots of *Panax ginseng* (APG) (8). APG stimulates normal lymphoid cells to proliferate and to produce cytokines such as IL-1, IL-2, IFN-γ and granulocyte monocyte colony-stimulating factor (GM-CSF) (8, 9). In addition, APG significantly reduces the production of inflammatory cytokines such as TNF-α, IL-1β, IL-6 IFN-γ, IL-12, and IL-18 at the early phase of sepsis in mice (10). The inflammatory response diminished by APG treatment for sepsis as a

consequence of down-regulated signals that were transmitted through Toll-like receptors (TLRs) (10). TLRs trigger intracellular signaling pathways resulting in the induction of inflammatory cytokines that promote Th1 responses (11). Moreover, TLR agonists can be used to break immunological tolerance and induce Th1-mediated autoimmune diseases such as arthritis (12), EAE (13), and diabetes (14). Therefore, we hypothesized that APG may have clinically beneficial effects on EAE through the reduction of inflammatory cytokine production. Our data demonstrate that APG significantly reduces the severity of EAE by inhibiting the proliferation of autoreactive T cells as well as production of the inflammatory cytokines, IFN-γ and IL-17. We further demonstrate that APG treatment significantly boosts the expression of Foxp3 in the CNS of APG-treated mice during EAE. Moreover, depletion of $CD25^+$ cells abrogates the beneficial effects of APG treatment in mice with EAE. These observations provide a powerful rationale for more thoroughly assessing the efficacy of APG as a novel therapeutic approach to the treatment of multiple sclerosis (MS).

2. Materials and Methods

Animals

C57BL/6 female mice were purchased from Orientbio, Inc. (Sungnam, Korea) and were 6-8 weeks-old and weighed 18-25g when used for the experiments. These mice were housed in conventional animal facilities with a NIH-07-approved diet and water *ad libitum* at a constant temperature (23 ± 1ºC) according to the guidelines for the Care and Use of Laboratory Animals of the Institutional Ethical Committee of Cheju National University.

Antigens and antibodies

Synthetic murine MOG_{35-55} peptide (M-E-V-G-W-Y-R-S-P-F-S-R -V-V-H-L-Y-R-N-G-K) was purchased from Bio Synthesis Inc. (Lewisville, TX, USA). PC61 clone (anti-CD25 monoclonal antibody, mAb) was obtained form BD Pharmingen (San Diego, CA, USA). For depletion of $CD25^+$ cells, 50 μg anti-CD25 mAb was injected intraperitoneally (i.p.) into each mouse 2 days before immunization. Every 5 days thereafter, anti-CD25 mAb was injected i.p. until the termination of experiments (15). Depletion was confirmed by flow cytometry with PE-CD25 mAb (PC61, Pharmingen, San Diego, CA, USA)

Induction and clinical evaluation of MOG_{35-55}-induced EAE

Mice were injected subcutaneously (s.c.) with 200 ㎍ of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide in CFA (Difco, Detroit, MI) containing 500 ㎍ of heat- inactivated *Mycobacterium tuberculosis* on day 0, supplemented by intravenous (i.v.) injections of 200 ng of pertussis toxin on day 2 (List Biologic, Campbell, CA, USA). The mice were observed daily for clinical signs of disease and scored on an arbitrary scale of 0 to 5 with gradations of 0.5 for intermediate scores (16): 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness or abnormal gait; 3, complete hind limb paralysis; 4, complete hind limb paralysis with forelimb weakness or paralysis; 5, moribund or deceased.

APG treatment

For treatment, 200 μg of APG was injected i.p. into each mouse at -1 day and the day of inoculation with MOG_{35-55}. Every 7 days thereafter, 200 μg of APG was injected i.p. until the termination of experiments. Control mice were injected with PBS (17).

Preparation of tissue and histological staining

Murine spinal cords were harvested at the end of each experiment, fixed in 10% formalin, and embedded in paraffin. Five-micrometer paraffin sections were stained with hematoxylin and

eosin (H&E) for visualization of inflammatory infiltrates. Histological findings were graded into four categories (1, leptomeningeal infiltration; 2, mild perivascular cuffing; 3, extensive perivascular cuffing; 4, extensive perivascular cuffing and severe parenchymal cell infiltration).

T cell culture and proliferation

Spleen mononuclear cells were suspended in culture medium containing Dulbecco's modification of Eagle medium (Gibco, Paisley, UK) supplemented with 1% (v/v) minimum essential medium (Gibco), 2 mM glutamine (Flow Laboratory, Irvine, CA, USA), 50 IU/ml penicillin, 50 mg/ml streptomycin and 10% (v/v) FCS (both from Gibco). Spleen mononuclear cell suspensions containing 4×10^5 cells in 200 μl culture medium were placed in 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). 10 μl of MOG_{35-55} peptide (10 μg/ml) or Con A (5 μg/ml, Sigma, St. Louis, MO, USA) was then added into triplicate wells. After 3 days of incubation, the cells were pulsed for 18 hours with 10 μl aliquots containing 1 μCi of ^{3}H-methylthymidine (specific activity 42 Ci/mmol; Amersham, Arlington Heights, IL, USA). Cells were harvested onto glass fiber filters, and thymidine incorporation was then measured. The results were expressed as CPM (18).

Intracellular cytokine staining and FACS analysis

Single cell suspensions were prepared and stained with fluorescently labeled mAbs. Abs were directly labeled with one of the following fluorescent tags; FITC, PE; CD25 (PC61.5), CD4 (H129.19), CD8 (53-6.7) (BD Pharmingen), IFN-γ (XMG1.2), IL-4 (11B11) CTLA-4 (UC10-4F10-11) (BD Biosciences, San Jose, CA, USA) and Foxp3 (FJK-16s) (eBioscience, San Diego, CA, USA). Intracellular flow cytometry for cytokines, Foxp3, and CTLA-4 was performed using Cytofix/Cytoperm (BD Biosciences), according to the manufacturer's instructions. Appropriate isotype controls were always included. All samples were analyzed on a FACSAria^{TM} using Diva software (18).

Western blot analysis

Frozen spinal cord tissue was thawed at room temperature, minced, lysed in a buffer consisting of 40 mM Tris-HCl, pH 7.4, and 120 mM NaCl containing the protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mM Na_3VO_4) and then homogenized. Equal amounts of protein (80 μg/well) were loaded in each lane and electrophoresed under denaturing conditions in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). After electrophoresis, the proteins were electrotransferred onto nitrocellulose transfer membranes (Schleicher and Schuell, Keene, NH, USA). After blocking with 5% nonfat milk, the blots were incubated

with Foxp3 (1:500, eBioscience) for 60 min followed by incubation with HRP-conjugated anti-mouse, rabbit IgG (1:250, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 45 min. Visualization was achieved using Amersham ECL reagents (Amersham Life Science, Buckinghamshire, UK). The results were quantified with a densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

Cytokine ELISA

Murine IFN-γ was measured using ELISA kits (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions. Murine IL-17 was measured using ELISA kits (eBioscience) according to the manufacturer's instruction.

Statistical analysis

The results are presented as means ± S.D. or S.E. Statistical significance was calculated using Student's *t*-test ($P < 0.005$, $P < 0.01$ or $P < 0.05$).

3. Results

APG induced resistance to EAE

To study the effect of APG on the development of EAE, we immunized PBS- or APG-treated EAE mice with MOG peptide and monitored them for signs of EAE for 33 days post-immunization. As Figure 1 depicts, APG effectively reduced the severity of EAE. In PBS-treated mice, EAE developed by day 9 after immunization and reached a maximal clinical score of 2.5 ± 0.08 two weeks later. By contrast, the APG-treated group had a delayed onset of symptoms (day 12) and far less severe disease, reaching a maximal clinical score of only 0.5 ± 0.16 (Fig. 1, Table Ⅰ).

Consistent with these clinical finding, histological examination of the CNS tissues revealed dramatic differences in the two groups. H&E staining revealed that PBS-treated EAE mice had a wide distribution of inflammatory cells infiltrating areas adjacent to the subarachnoid space (Fig. 2A), within the parenchyma (Fig. 2C) in perivascular lesions (Fig. 2E), and in the spinal cord. By contrast, APG-treated EAE mice manifested markedly fewer inflammatory cells (Figs. 2, B, D and F).

APG decreased T cell responses and production of IFN-γ

We first tested antigen-specific T cell proliferation in APG-treated EAE mice. As Figure 3 illustrates, proliferation of antigen-specific T cells in response to the MOG_{35-55} peptide was significantly decreased in APG-treated compared to PBS-treated EAE mice ($P < 0.005$), indicating that APG-induced T cells can regulate MOG_{35-55} -specific T cells and inhibit their autoreactivity. Further, APG-treated EAE mice decreased the number of IFN-γ (Fig. 4A, 1.4 ± 0.30 % vs. 2.2 ± 0.14 % in PBS-treated EAE mice, $p < 0.05$) and the production of IFN-γ and IL-17 (Fig. 4B, $p < 0.01$). However, comparison of APG-treated and PBS-treated EAE mice showed no difference in the frequency of $CD8^+$ T cells and IL-4-producing cells (Fig. 4A). Therefore, APG suppression specifically affected the proliferation of MOG_{35-55} -specific T cells and production of IFN-γ and IL-17.

APG altered the number of Tregs and increased Foxp3 expression in the CNS

To test the possibility that APG-induced T cells can regulate encephalitogenic T cells via induction of Tregs, we quantified the number and frequency of Tregs in APG- or PBS-treated EAE mice. In the APG-treated EAE mice, $CD4^+CD25^+$ cell populations increased only marginally but did not reach a level of statistical significance (4.0 ± 0.47% *vs.* 3.5 ± 0.38% in PBS-treated EAE mice) (Fig. 5).

The transcription factor Foxp3 seems to determine differentiation of the regulatory phenotype and distinguish Tregs from activated T cells (19, 20). Therefore, we next asked whether APG modified the phenotype of Tregs to acquire features distinguishable from those of encephalitogenic T cells. The expression of Foxp3 in relation to $CD4^+$ T cells was also determined. As shown in Figure 6A, the analysis of APG- and PBS-treated EAE mice by flow cytometry showed no difference in the number of Foxp3 in the CNS. However, compared with PBS-treated EAE mice (0.8 ± 0.03%), APG-treated EAE mice exhibited a marked increase in the expression of Foxp3 in the CNS when evaluated by Western blot analysis (Fig. 6B, 1.2 ± 0.03 %, $P < 0.01$). Additionally, in the APG-treated EAE mice, CTLA-4-expressing $CD4^+$ T cell populations increased only marginally, although not at a level of statistical significance (1.7 ± 0.24% *vs.* 1.3 ± 0.21 % in PBS-treated EAE mice) (data not shown). Therefore, APG therapy favorably altered the number of Tregs and induced Foxp3 expression in the CNS during EAE, indicating that APG may have capacity to modify Tregs through the activation of Foxp3.

Depletion of Tregs abrogates the beneficial effects of APG on EAE

To assess the suppressive role of Tregs in EAE, C57BL/6 mice were injected with either 50 μl PBS or anti-CD25 (PC61) i. p. on day -1, 5, and 10 post-immunization with MOG_{35-55} peptide to induce EAE (15, 17). As in Figure 7, the severity and incidence of EAE in anti-CD25 mAb,

APG-treated EAE mice were significantly higher than that of APG-treated EAE mice. That is, the maximal clinical score of anti-CD25 mAb, APG-treated EAE mice significantly exceeded that of APG-treated EAE mice (1.5 ± 0.23 v*s*. 0.5 ± 0.16 %). (Fig. 7, Table Ⅱ). Moreover, after depletion of $CD25^+$ cells, APG-treated EAE mice underwent a marked increase of inflammatory cell infiltration widely distributed adjacent to subarachnoid spaces (Fig. 8B), in parenchyma (Fig. 8D) and perivascular lesions (Fig. 8F) of the spinal cord compared to EAE mice treated with APG alone (Fig. 2, B, D and F). Also, elimination of $CD25^+$ cells resulted in a marked increase in the amount of inflammatory cells infiltrating the CNS of APG-treated EAE mice (Figs. 8A, C and E) compared to that in PBS-treated EAE mice (Fig. 2, A, C and E). After depletion of $CD25^+$ cells, APG-treated EAE mice did not suppress the proliferation of MOG_{35-55}-specific T cells (Fig. 9A) and did not downregulate IFN-γ and IL-17 production (Fig. 9B). Clearly, the depletion of $CD25^+$ cells abrogated the therapeutic effects of EAE and the anti-inflammatory effects in the CNS of APG-treated EAE mice. This outcome suggests that APG-induced Tregs suppress the activation and proliferation of autoreactive T cells in the periphery.

4. Discussion

Recently, Tregs have been reported to play a critical role in controlling autoimmune diseases, including MS (5, 6). Kohm *et al.* (15) have reported that Tregs from conventional C57BL/6 mice suppress active and passive EAE associated with a decrease of autoreactive Th1 cells and that Tregs inhibit both the proliferation and IFN-γ production by a MOG_{35-55}-specific Th1 cell line *in vitro*. This notion is supported by our results demonstrating that APG significantly reduced the disease severity of EAE and inhibits MOG_{35-55}-specific T cell proliferation. Furthermore, depletion of $CD25^+$ cells abrogated the beneficial effects to APG on EAE. The mechanisms underlying these clinical benefits of APG would be the increase in the number of Tregs through the activation of transcription factor Foxp3.

Tregs express high levels of CTLA-4, glucocorticoid-induced TNF receptor-related protein (GITR), CD62L, CD69 (19, 20), and Foxp3 and ,most importantly, Foxp3 seems to determine differentiation of the regulatory phenotype and distinguish Tregs from activated T cells (21, 22). Recent studies have addressed how Tregs suppress effector T cells in various pathological conditions, including inflammation, autoimmunity, and organ transplantation (7, 15). Evidence suggesting the capacity of Tregs to suppress effector T cells altered by multiple elements in EAE is accumulating rapidly (2, 4, 23). These elements include vasoactive intestinal peptide (VIP) (2), α−galactosylceramide (α-GalCer) (23) and rapamycin (4). VIP-induced Tregs

are very strong suppressors of responder autoreactive T cell proliferation. In addition, VIP prevents EAE progression, as noted in $CD25^+$-depleted mice, restores the number of $CD4^+CD25^+$ Treg cells, and induces the generation of $CD4^+CD25^+Foxp3^+$ cells from activated $CD4^+CD25^-$ cells *in vitro* (2). α-GalCer administration in mice with experimental autoimmune myasthenia gravis, increased the size of the Tregs compartment, and augmented the expression of Foxp3 (23). In agreement with these results, APG treatment increased the expression of Foxp3 in the CNS of EAE mice, and suppressed the proliferation of autoreactive T cells.

The previously shown alleviation of inflammatory cytokine expression in sepsis under the presence of APG, therefore, seemed to be mediated primarily by the down-regulation of TLR expression (10). TLR agonists might be useful not only to boost immunity against microbes and tumors but also to progress autoimmune diseases. Indeed, Hansen *et al.* recently reported that multiple TLR agonists could serve as adjuvants to induce EAE in C57BL/6 mice (24). In our study, APG treatment significantly ameliorated the progression of EAE and down-regulated production of the inflammatory cytokines, IFN-γ and IL-17. Recent compelling evidence demonstrated that IL-17-producing T cells comprise proinflammatory T helper cells, termed Th17 cells, which are major contributors to autoimmune disease (25). Intriguingly, pathogenic Th17 and immunosuppressive Tregs from naïve $CD4^+$ T cells are reciprocally induced, contingent upon the presence of either IL-6 or IL-2, respectively, in the presence of TGF-

β (26, 27, 28). Our results support the presumption that down-regulation of IFN-γ and IL-17 is associated with the active suppression of autoimmunity by APG, mediated by $CD4^+CD25^+$ immunoregulatory T cells during EAE. Thus, we would speculate that the reduction of inflammatory cytokine production during EAE is one reasonable explanation for beneficial effects of APG on EAE.

Accordingly, our results provide evidence that APG are potent regulators of inflammatory cytokines, can dampen their inflammatory effect during EAE, and produce several changes in autoreactive T cells. Furthermore, APG may enhance Tregs functions during EAE. Indeed, Tregs may contribute to the therapeutic effects of APG during EAE and perhaps would extend to MS. The mechanism underlying the molecular events linking APG with Tregs is not fully elucidated in this study. However, the ability of APG to enhance functional Tregs could be used as an attractive therapeutic tool in the future. Conclusions from the strong evidence provided here and elsewhere attest to the capacity of APG to promote immunosuppressive effects showing clinically beneficial effects. Therefore, APG may serve as a new therapeutic agent for MS as well as other autoimmune diseases of humans and warrants continued evaluation for translation into therapeutic applications.

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Table Ⅰ. *Clinical and histological evaluation of EAE induced by MOG_{35-55} in C57BL/6 mice*

	Clinical status			Histological Finding
Treatment	Incidence of EAE (%)	Days of onset	Mean of maximal score	Mean of histological score
Vehicle	46.6 (7/15)	18.4 ± 1.12	2.5 ± 0.05	3.1 ± 0.07
APG	30.8 (4/13)	19.0 ± 0.79	0.5 ± 0.16*	1.5 ± 0.42*

PBS (Vehicle)- or APG-treated EAE mice were inoculated with MOG_{35-55} peptide to incite EAE as described in *Materials and Methods*. Results are the mean values ± SE of three independent experiments. The difference in mean of clinical scores and mean of histological score between PBS- and APG-treated EAE mice are statistically significant (* $P < 0.05$).

Table Ⅱ. *Clinical and histological evaluation of APG-treated EAE mice after depletion of $CD25^+$ cells*

	Clinical status			Histological Finding
Treatment	Incidence of EAE (%)	Days of onset	Mean of maximal score	Mean of histological score
Anti-CD25	62.5 (5/8)	17.3 ± 1.32	2.75 ± 0.14	3.8 ± 0.06
Anti-CD25, APG	50.0 (4/8)	16.3 ± 0.62	1.5 ± 0.23	2.13 ± 0.24

For depletion of $CD25^+$ cells, 50 μg anti-CD25 mAb was injected i.p. into each mouse at day 2 before immunization. Every 5 days thereafter, anti-CD25 mAb was injected i.p. until termination of the experiments. Results are the mean values ± SE of three independent experiments.

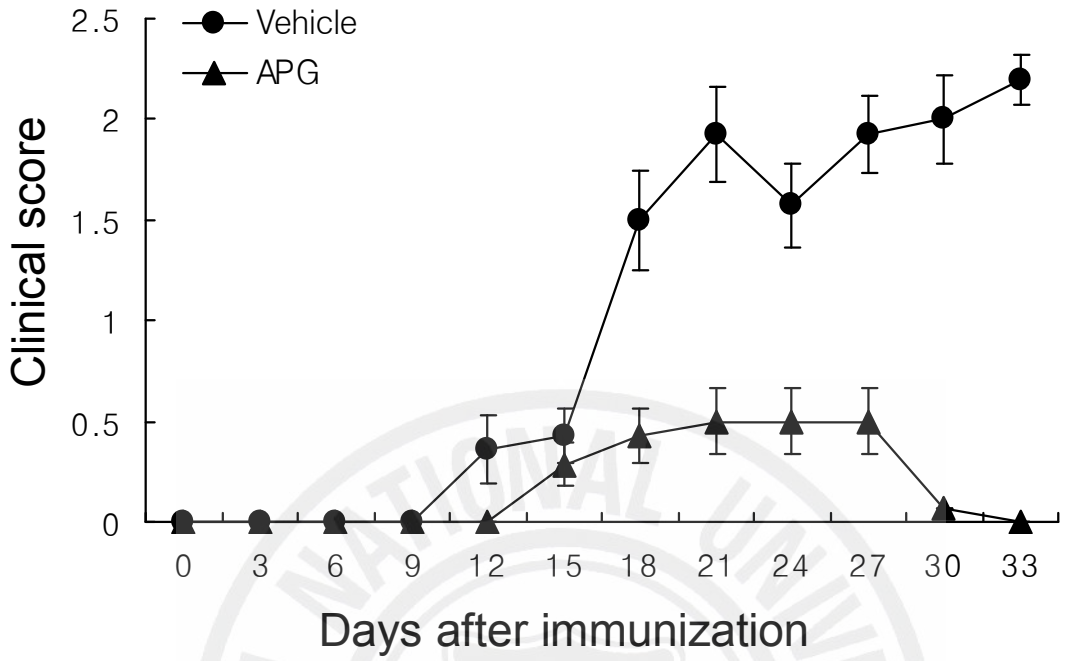

FIGURE 1. APG induced resistance to EAE. PBS (Vehicle, closed circles)- or APG (closed triangules)- treated EAE mice were treated with APG before, during, and after immunization with MOG_{35-55} peptide in CFA as described in *Materials and Methods*. The development of EAE was then monitored. Data shown are the mean clinical scores of disease for 3 or 4 mice per group and are representative of three independent experiments.

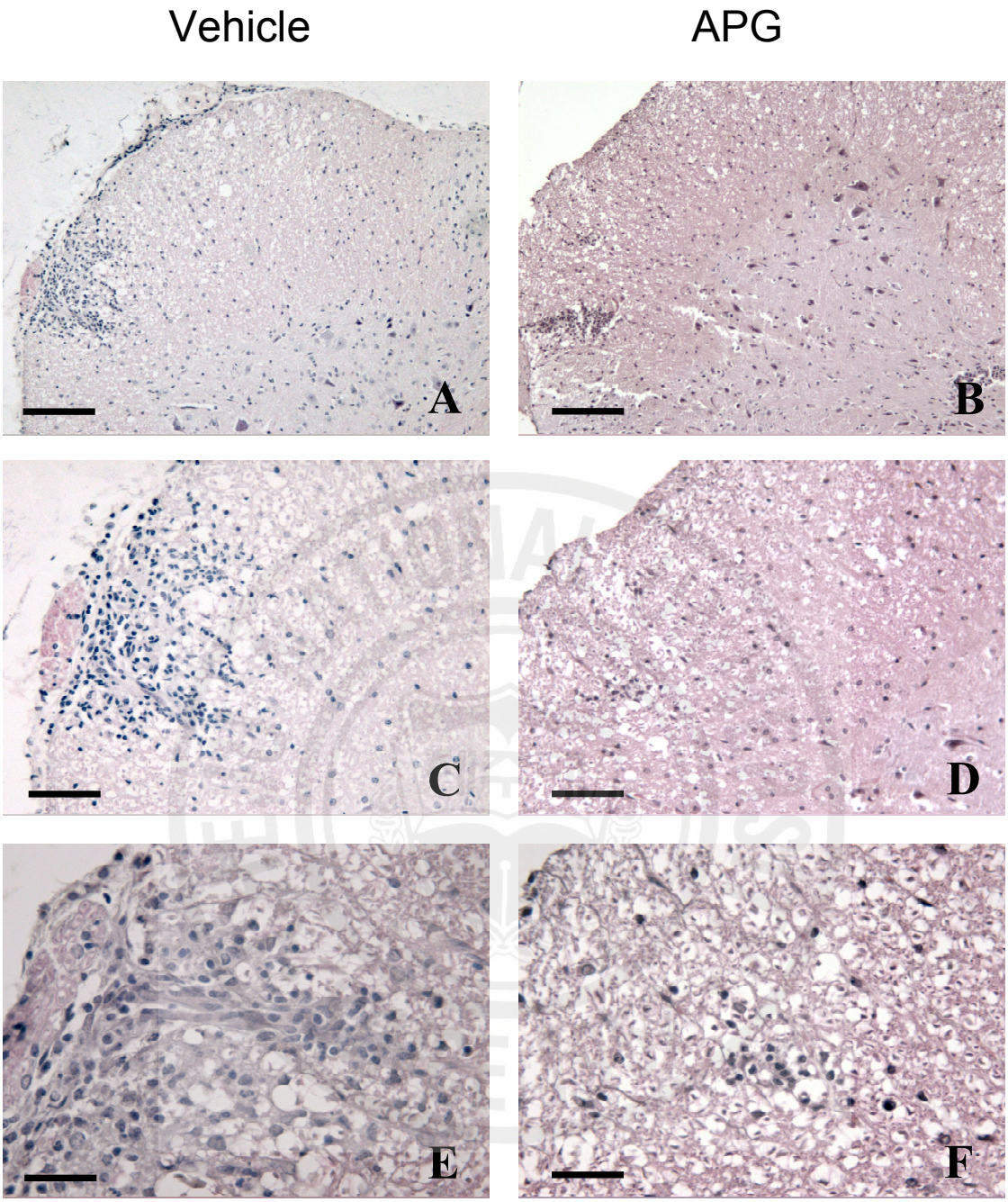

FIGURE 2. APG decreased the infiltration of inflammatory cells into the CNS. PBS (Vehicle)- and APG-treated EAE mice were compared for the presence inflammatory cells in the subarachnoid space (Fig. 2A and B), parenchyma (Fig. 2C and D) and perivascular lesion (Fig. 2E and F) adjacent to the spinal cord as visible by H&E staining. Bars = 120 μm (A, B), 60 μm (C, D), 30 μm (E, F).

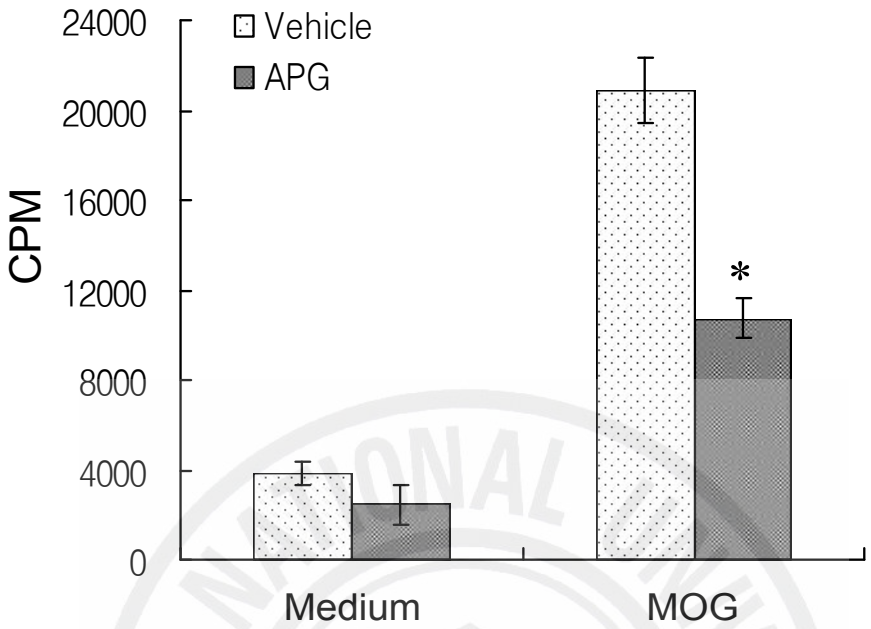

FIGURE 3. APG decreased MOG_{35-55} –specific T cell responses. Mice were killed at day 33 after immunization. Proliferative responses to the antigens (MOG_{35-55} peptide or Con A) shown were assessed in triplicate wells for each experiment. Data are means ± SD of CPM and are representative of three experiments. Statistical evaluation was performed to compare the APG-treated groups and corresponding PBS-treaed groups, respectively (* $P < 0.005$).

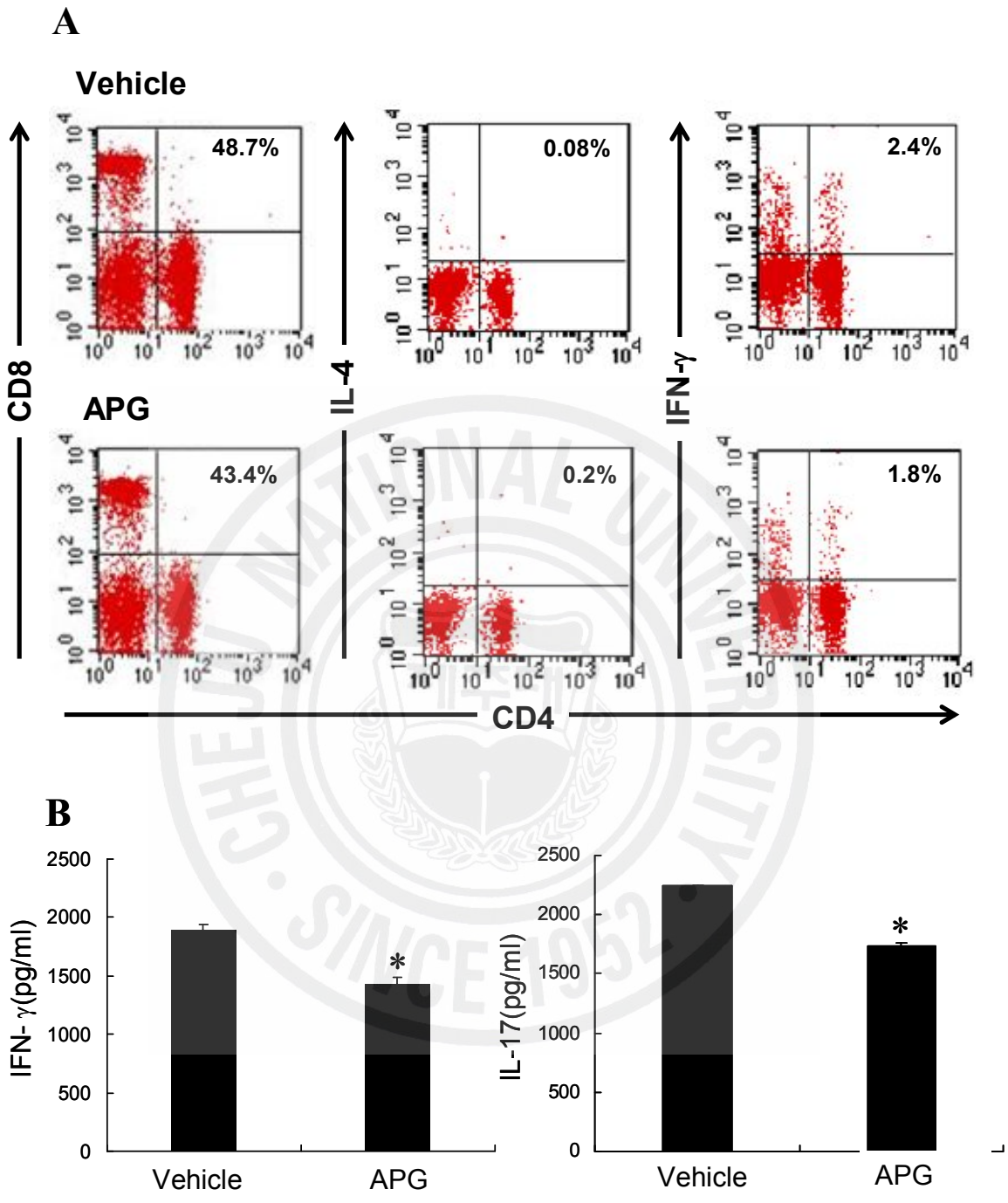

FIGURE 4. APG decreased the number of Th1 cytokine-producing $CD4^+$ T cells and the production of IFN-γ and IL-4. (A) Splenocytes from mice treated with APG or PBS were drawn on day 33 after immunization and prepared as single-cell suspensions. The expression of CD4 vs. production of intracellular IFN-γ and IL-4 by splenocytes was compared with appropriate

isotype controls within this gate. (B) Cytokine profile of purified splenic cells from PBS (Vehicle)- or APG-treated EAE mice. IFN-γ and IL-17 were measured by ELISA. Statistical evaluation was performed to compare the APG-treated groups and corresponding PBS-treated groups (* $P < 0.01$).

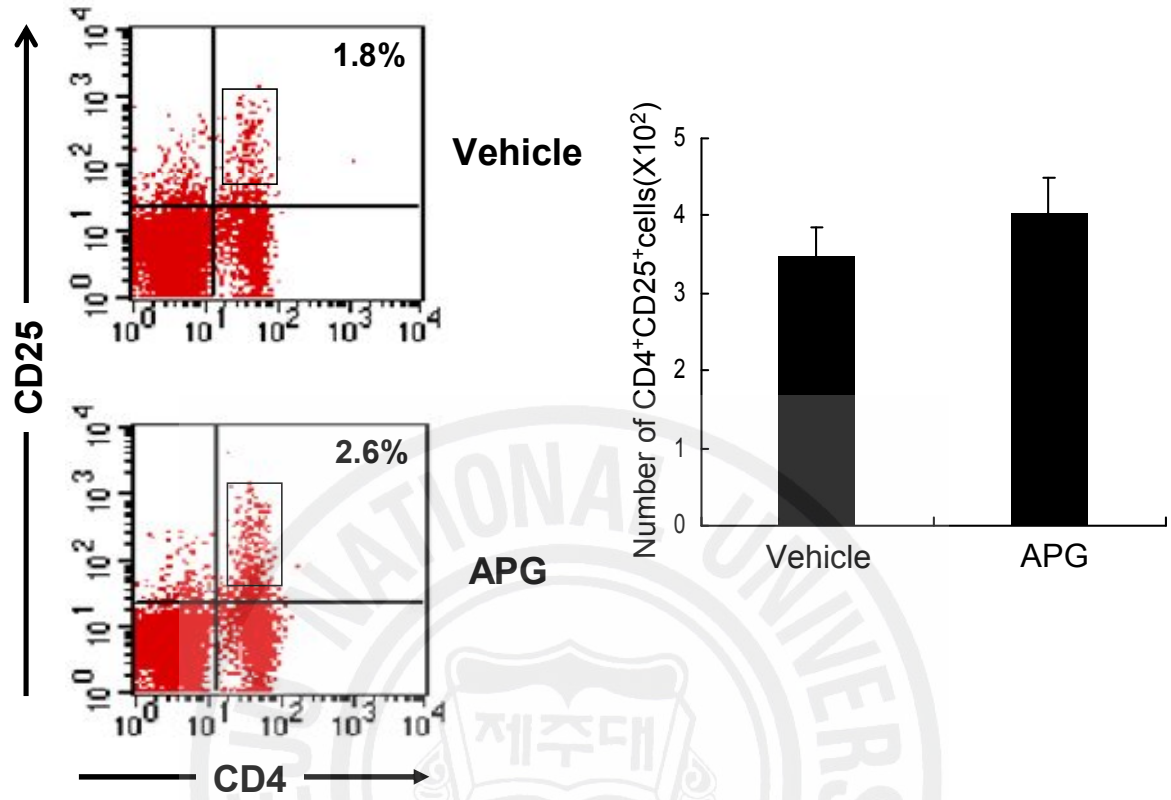

FIGURE 5. APG altered the number of regulatory T cells. Groups of mice were treated with APG and injected with MOG_{35-55} peptide in CFA as described in *Materials and Methods*, then sacrificed on day 33 post-immunization. Spleen cells were prepared, stained with FITC-conjugated anti-CD25 and PE-conjugated anti-CD4, and then analyzed by flow cytometry.

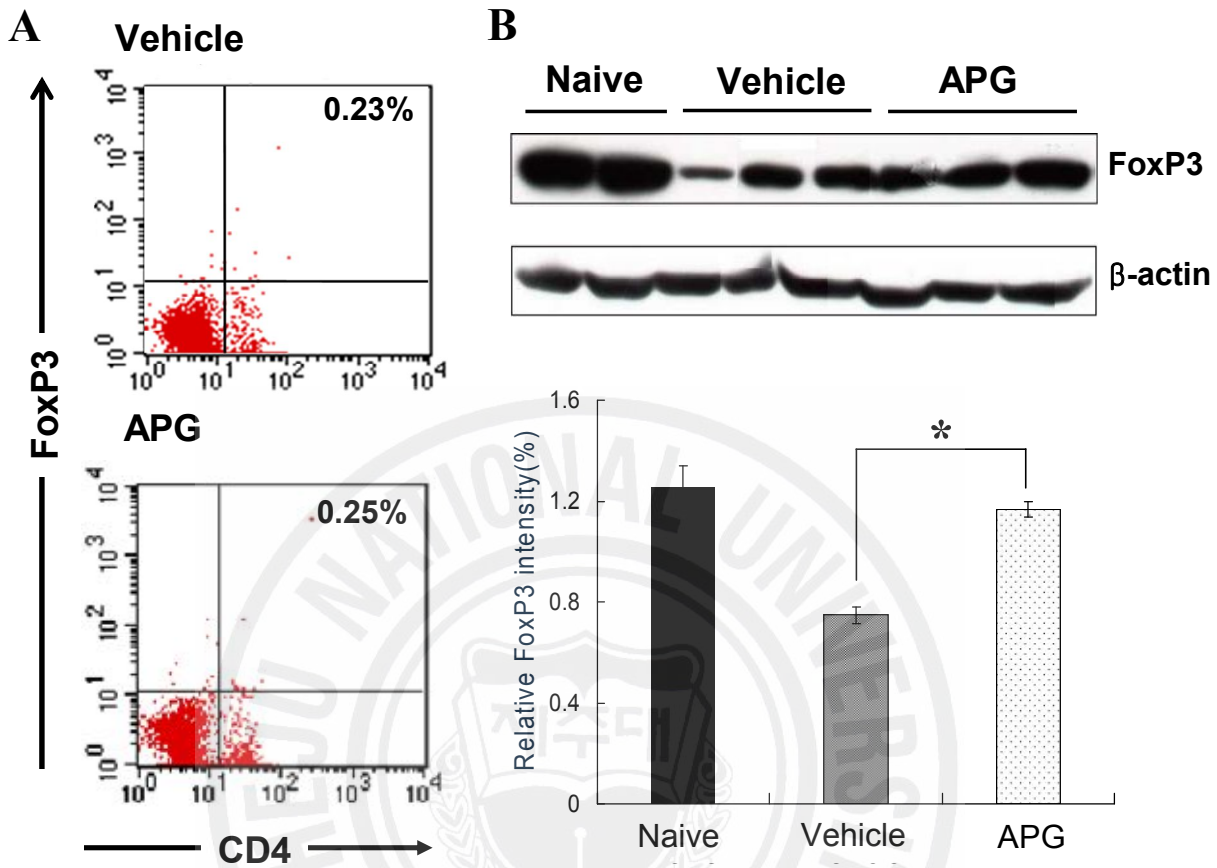

FIGURE 6. APG increased the Foxp3 expression in the CNS. (A) The cells were analyzed by flow cytometry for expression of Foxp3 in the CNS isolated on day 33 after MOG immunization of mice treated with APG or PBS. (B) Western blot analysis of Foxp3 expression modulated by APG in the spinal cord was compared to that from PBS (Vehicle)-treated EAE mice. β-actin expression was used to demonstrate that equal amounts of protein extracts were loaded. Quantitative graphical representation of band densities for Foxp3 protein are shown. Statistical evaluation was performed to compare the APG-treated groups and corresponding PBS-treated groups (* $P < 0.01$).

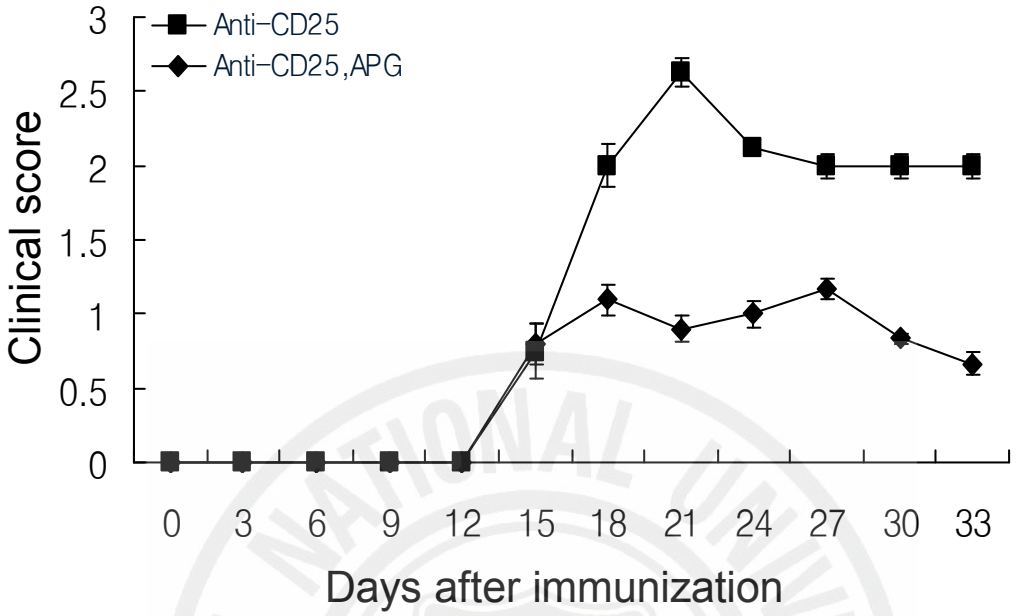

FIGURE 7. Depletion of $CD25^+$ cells abrogated the beneficial effects of APG on EAE. Anti-CD25 (closed squares)- and anti-CD25, APG (closed diamonds)- treated EAE mice were treated with APG and immunized with MOG_{35-55} peptide in CFA as described in *Materials and Methods,* after which the development of EAE was monitored. For depletion of $CD25^+$ cells, C57BL/6 mice were injected i.p. with 50 μg anti-CD25 mAb (PC61) on day 1 before MOG immunization. Every 5 days thereafter, anti-CD25 mAb was injected i.p. until the termination of experiments. Data shown are the mean clinical scores of disease for 3 or 4 mice per group and are representative of three independent experiments.

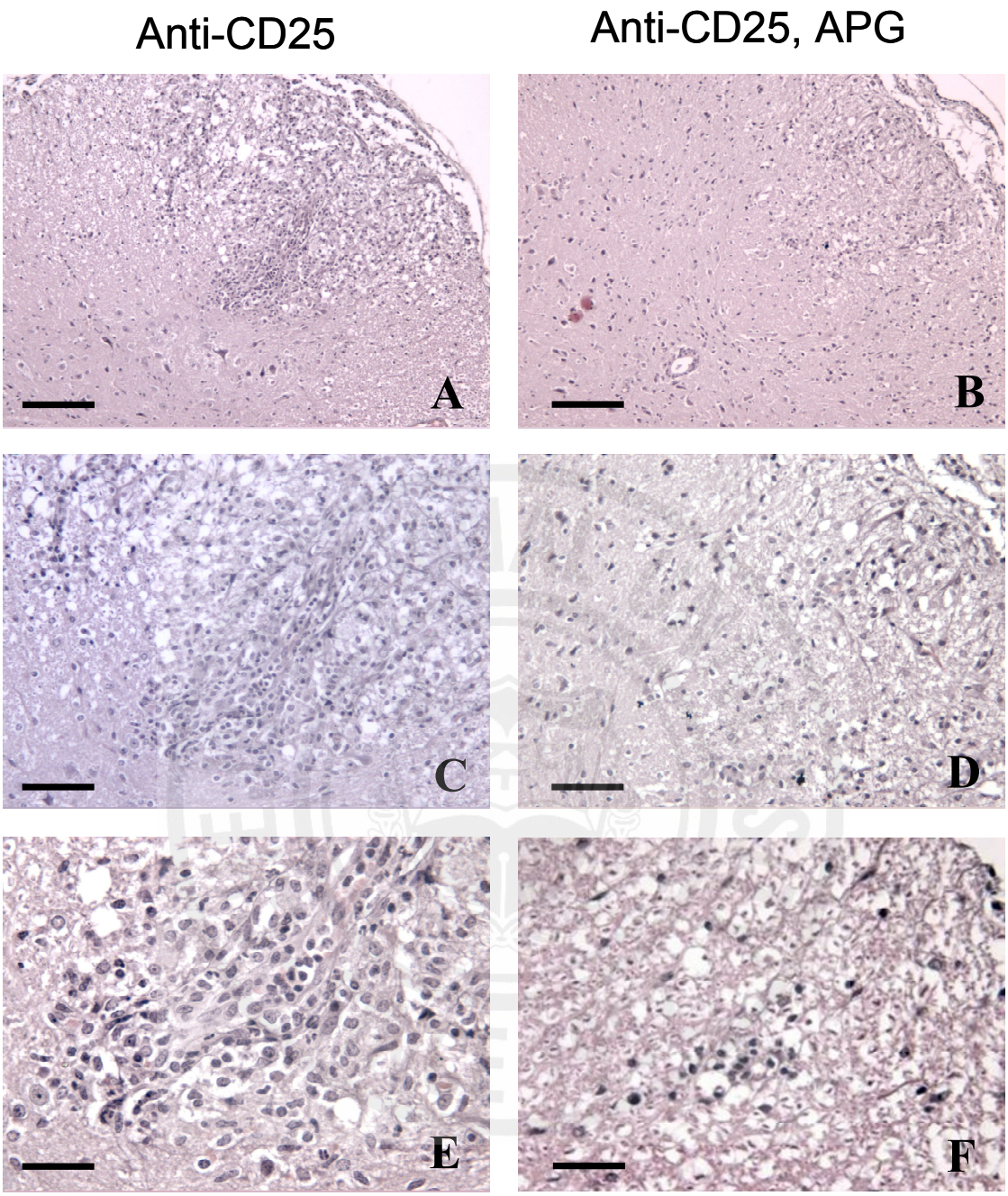

FIGURE 8. An infiltration of inflammatory cells exacerbated in APG-treated EAE mice after depletion of $CD25^{+}$ cells. In samples from anti-CD25- or anti-CD25, APG-treated EAE mice, H&E staining revealed the infiltration of inflammatory cells in the subarachnoid space (Fig. 8A and B), parenchyma (Fig. 8C and D) and perivascular lesion (Fig. 8E and F) adjacent to the spinal cord. Bars = 120 μm (A, B), 60 μm (C, D), 30 μm (E, F).

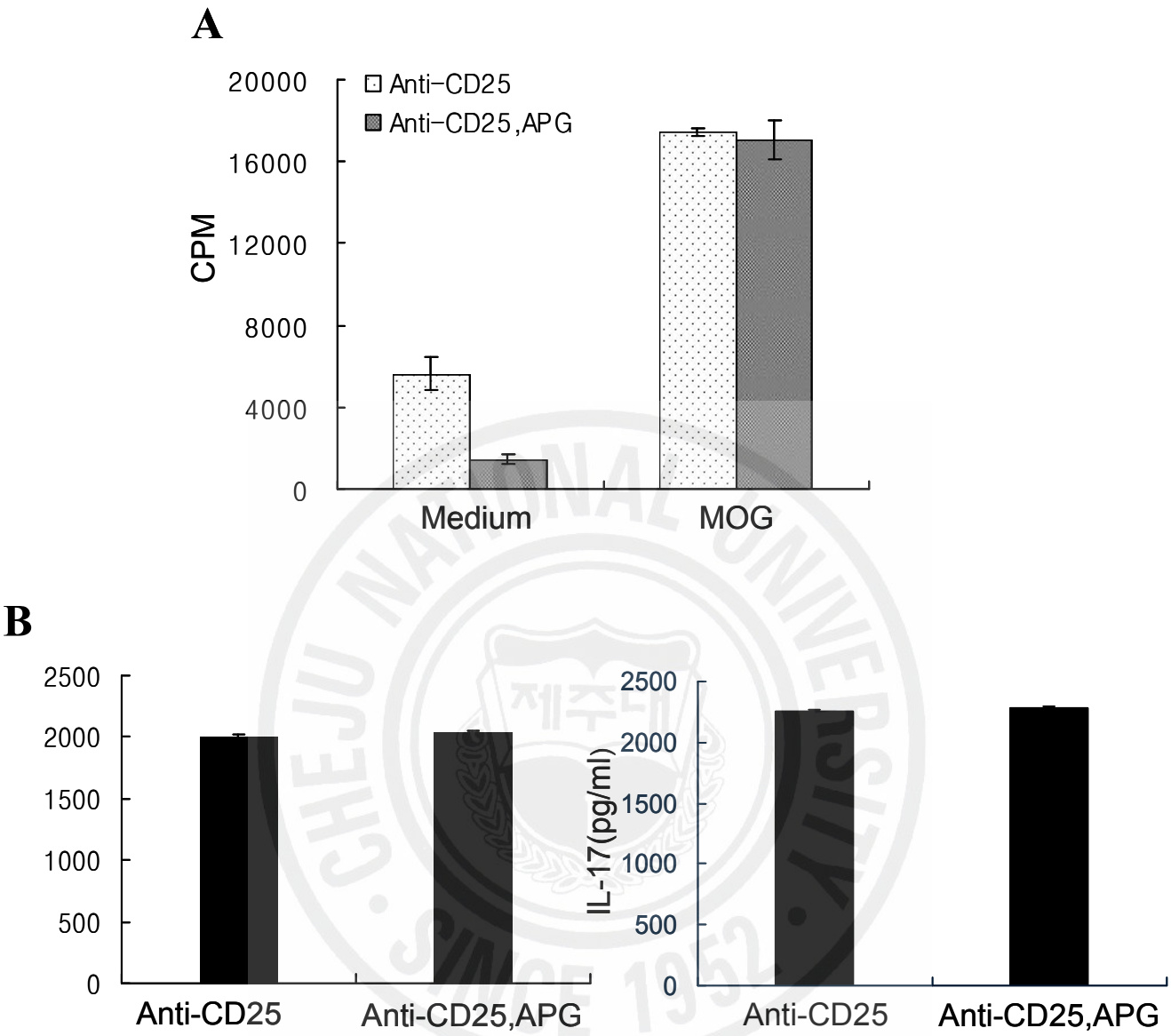

FIGURE 9. Depletion of $CD25^{+}$ cells up-regulated T cell proliferation and Th1 cytokine production in APG-treated EAE mice. (A) Proliferative responses to the antigens (MOG_{35-55} peptide or Con A) shown were assessed in triplicate wells for each experiment. Data are means ± SD of CPM and are representative of three experiments. (B) Cytokine profile of purified anti-CD25- or anti-CD25 on splenic cells from APG-treated EAE mice. IFN-γ and IL-17 were measured by ELISA.

초 록

실험적 자가 면역성 뇌척수염 모델에서 면역 조절자로서의 진산(acidic polysaccharide of *Panax ginseng*)의 역할과 효능

지도교수 : 지영흔

황인선

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실험적 자가 면역성 뇌척수염 (experimental autoimmune encephalomyelitis, EAE)은 중추 신경계에서 T 세포 매개성 탈수초성 질병이며, 이는 사람의 다발성 경화증의 동물 모델로 잘 알려져 있다. 진산 (acidic polysaccharide of *Panax ginseng,* APG) 은 조혈과정 활성화, 살균, 항암, 항산화 효과 등의 다양한 작용을 하며, 면역 작동 세포들의 면역조절자로 잘 알려져 있다. 따라서 본 연구에서는 EAE에 대하여 진산의 면역 조절자로서 치료효과를 가지는지 알아보고자 하였다. 그 결과 EAE 유발 후 진산 투여군에서 임상 증상의 완화, 중추신경 내 염증세포 침윤의 감소와 자가반응성 T 세포의 증식 억제 및 Th1 type 사이토카인인 IFN-γ와 자가면역성 질병 발병에 관여하는 결정적 사이토카인인 IL-17 분비 억제 효과가 있었다. 따라서 이들 결과로부터 진산은 EAE에 대하여 저항성 유도함을 알 수 있었다. 또한 최근 자가면역성 질병의 조절자로 알려진 조절 T 세포가 진산 투여군에서 수적으로 증가함을 관찰할 수 있었는데, 이렇게 증가된 조절 T 세포의 기능학적 변화를 관찰한 결과, 진산 투여군에서 Foxp3 단백질의 발현양의 증가를 볼 수 있었다. 또한 항-CD25 항체 투여한 진산 투여군에서 진산의 치료 효과가 미약해졌음을 확인할 수 있었다. 이상의 결과로 자가 면역성 뇌척수염에서 진산은 조절 T 세포를 매개로 뛰어난 치료학적 효과를 나타냄을 알 수 있었고, 자가반응성 T 세포의 활성 억제, Th1 type cytokine인 IFN-γ의 분비 억제와 함께 자가면역성 질병 발병에 관여하는 결정적 cytokine인 IL-17의 분비를 억제시킴으로 인해 유도될 것으로 사료된다.

주요어: 실험적 자가면역성 뇌척수염 (EAE); 진산 (APG); 조절 T 세포; IL-17

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