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Periplocoside E Inhibits Experimental Allergic Encephalomyelitis by Suppressing IL-12-Dependent CCR5 Expression and IFN-γ-Dependent CXCR3 Expression in T Lymphocytes

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Antigen-Presenting Cell, APC; Blood Brain Barrier, BBB; CC chemokine ligand, CCL; CC chemokine receptor, CCR; CXC chemokine ligand, CXCL; CXC chemokine receptor, CXCR; complete Freund's adjuvant, CFA; central nervous system, CNS; experimental allergic encephalomyelitis, EAE; enzyme-linked immunosorbent assay, ELISA; monoclonal antibody, mAb; myelin oligodendrocyte

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Abstract

Periplocoside E (PSE) was found to inhibit primary T cells activation in our previous study. Now we examined the effect and mechanisms of PSE on the central nervous system (CNS) demyelination in experimental allergic encephalomyelitis (EAE). C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG) were treated with PSE following immunization and continued throughout the study. The effect on the progression of EAE and other relevant parameters were assessed. PSE reduced the incidence and severity of EAE. Spinal cords histopathology analysis demonstrated that the therapeutic effect of PSE was associated with reduced mononuclear cell infiltration and CNS inflammation. As RT-PCR analysis showed, PSE decreased the $CD4^+$, $CD8^+$ and $CD11b^+$ cell infiltration. T cells from lymph nodes of MOG-immunized mice expressed enhanced levels of CCR5 and CXCR3 mRNA compared to T cells from normal mice. However, CCR5 and CXCR3 expressions were suppressed in T cells from PSE-treated mice. In vitro study also demonstrated PSE inhibited IFN-γ-dependent CXCR3 expression in T cells through suppressing TCR-ligation induced IFN-γ production while inhibited IL-12-dependent CCR5 expression through suppressing IL-12 reactivity in TCR-triggered T cells. As a result, the initial influx of T cells into CNS was inhibited in PSE-treated mice. The consequent activation of macrophages/microglia cells was inhibited in spinal cord from PSE-treated mice as determination of chemokine expressions (CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10). Consistently, the secondary influx of $CD4^+$, $CD8^+$ and $CD11b^+$ cell was decreased in spinal cords from PSE-treated mice. These

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findings suggest the potential therapeutic effect of PSE on Multiple sclerosis.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) that serves as a model for the human disease multiple sclerosis (MS), since in both diseases circulating T cells and macrophages penetrate the blood brain barrier (BBB) and damage myelin, resulting in impaired nerve conduction and paralysis (MacFarlin and McFarland, 1983; Alvord et al., 1984). Leukocyte trafficking to the site of inflammation at the CNS was characterized as a sequential multi-step event (Karin et al., 1993). At first, a very limited repertoire of T cells, which we named the primary influx, interacts with their target Ag at the site of inflammation, leading to the activation of the BBB to express various adhesion molecules and thus to increase its permeability to circulating leukocytes (Yednock et al., 1992; Karin et al., 1993). Enhanced permeability of this barrier allows a nonselective influx of leukocytes, which we named the secondary influx. This influx correlates with disease onset (Karin et al., 1993; Brocke et al., 1996).

IL-12 stimulates activated myelin-reactive Th1 cells to selectively up-regulate CCR5 (receptor for CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES), in direct correlation with the acquisition of CNS-infiltrating capacities by these cells (Iwasaki et al., 2001; Bagaeva et al., 2003). CXCR3 (receptor for CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC) resembles CCR5 in that it is selectively expressed on activated myelin-reactive Th1 cells. Nonetheless, CXCR3, in contrast to CCR5, was modulated by IFN-γ rather than IL-12 co-stimulation (Nakajima et al., 2002; Bagaeva et al., 2003).

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In primary influx, only auto-reactive T cells which have been up-regulated CCR5 and/or CXCR3 expression in the peripheral recruitment across the vascular wall (Bagaeva et al., 2003). Lymphocyte interaction with vascular endothelium under flow is mediated by the sequential interaction of cell adhesion molecules, and signals provided by chemokine on the endothelial surface, which bind to their specific receptors on the leukocyte surface. Chemokine receptor signaling leads to an increase in integrin avidity on the leukocyte surface, allowing the leukocyte to firmly bind to the endothelium under flow and subsequently to follow chemokine concentration gradients across the vascular wall (Butcher et al., 1999).

These encephalitogenic T cells which migrated to CNS perivascular sites interacted with MHC class II-associated peptides of MOG in the CNS elicited proinflammatory cytokines such as TNF-a and IFN-γ that have the capability to induce macrophages/microglial cells to express chemokines, which are key mediators in the recruitment of the secondary influx of leukocytes (Merrill et al., 1992; Shrikant et al., 1994; Renno et al., 1995; Sun et al., 1996). CCR5 and/or CXCR3, are expressed on T cells infiltrating EAE and MS lesions as well as on T cells in the cerebrospinal fluid and periphery of MS patients during exacerbations (Balashov et al., 1999; Sorensen et al., 1999; Kennedy et al., 2001). The elevated expressions of CCL3, CCL4 and CCL5 (ligands for CCR5) and CXCL9, CLXL10 and CXCL11 (ligands for CXCR3) were essential for the secondary inflammatory infiltrates of $CCR5^+$ cells and $CXCR3^+$ cells (Kuchroo et al., 1992; Hulkower et al., 1993; Glabinski et al., 1995; Godiska et al., 1995; Karpus et al., 1995; Kennedy et al., 1998).

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Periplocoside E (PSE), a pregnane glycoside had been identified from *Periploca sepium*, which was used for treating rheumatoid arthritis in China. Recent studies have shown that PSE is an immunosuppressive compound, which directly inhibit T cells activation *in vitro* and *in vivo* (Zhu et al. 2006). However, no previous study has examined the use of PSE in the treatment of EAE or other Th1 cell-mediated inflammatory diseases of the CNS. In this study, we have examined the effect and mechanisms of PSE on the pathogenesis of CNS inflammation and demyelination in EAE. Our results showed that PSE inhibits CNS demyelination by suppressing IL-12-dependent CCR5 expression and IFN-γ-dependent CXCR3 expression in T cells, attenuating the primary influx of $CCR5^+$ and/or $CXCR3^+$ T cells into the CNS, down-regulating chemokine expressions elicited by the primary influx and consequently the secondary influx of CCR5-and/or CXCR3-bearing $CD4^+$ and $CD8^+$ T cells and $CD11b^+$ cells.

Materials and Methods

Reagents

Periplocoside E (PSE) was provided by laboratory of Natural Products Chemistry, Shanghai Institute of Materia Medica (Shanghai, P.R. China). The peptide MOG 35-55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Sangon Biological Engineering Technology and Service Co. (Shanghai, P.R. China). Amino acid sequences were confirmed by amino acid analysis and mass spectroscopy. The purity of the peptide was greater than 95%. Complete Freund's adjuvant CFA and *Mycobacterium tuberculosis* H37Ra were purchased from Difco (Detroit, MI, USA). Bordetella pertussis toxin (PTX), dimethylsulfoxide, and 3,3',5,5'-tetramethylbenzidine were supplied by Sigma-Aldrich. (St. Louis, MO, USA). RPMI 1640 was bought from GIBCO/Life Technologies Inc. (Gaithersburg, MD, USA), and fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, Utah, USA). [^{3}H] thymidine was provided by Shanghai Institute of Applied Physics, Chinese Academy of Science (Shanghai, P.R. China). The ELISA kits for IFN-γ and IL-12p40, recombinant-mouse IFN-γ and IL-12, anti-IFN-γ (R4-6A2), anti-CD3 (145-2C11), anti-CD28 (37.51), anti-CD4 (GK1.5), anti-CD8 (2.43) monoclonal antibody (mAb) were purchased from PharMingen (San Diego, CA, U.S.A.).

Induction, Treatment, and Clinical Evaluation of EAE

Female C57BL/6 mice, aged 6 to 8 weeks, were purchased from the Shanghai Experimental Animal Center, the Chinese Academy of Sciences. The animals were

housed in specific pathogen-free conditions. Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica. We used active EAE model as previously described (Youssef et al., 2002; Fu et al., 2006). Briefly, female C57BL/6 mice were immunized on day 0 by subcutaneous injection with 100 μl of an emulsion of MOG 35-55 peptide in CFA, distributed over three sites: one along the midline of the back between the shoulders, and two on either side of the midline on the lower back. Each mouse was immunized by 200 μg MOG 35-55 peptide together with 200 μg *Mycobacterium tuberculosis* H37Ra, and additionally received 400 ng of PTX by intraperitoneal (i.p.) injection in 400 μl of PBS on day 0 and 72 h post immunization (p.i.). To determine the effect of PSE on actively induced EAE, PSE was dissolved in PBS containing 1.6 % ethanol and administered i.p. following MOG 35-55 immunization and continued throughout the study (n=15 mice). The dose of PSE (10 mg/kg/day) was chosen on the basis of previous *in vivo* data (Zhu et al., 2006) and our own preliminary experiments. As a control, an equal volume of PBS containing 1.6 % ethanol was injected daily into control mice i.p. (n=15 mice). Clinical assessment of EAE was performed daily and mice were scored for disease according to the following criteria: 0, no overt signs of disease; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund state or dead (Sakurai et al., 2002). Confirmatory evidence of EAE onset was that the mean clinical score of mice was assessed as 1.

Histopathology analysis

To assess the degree of CNS inflammation and demyelination, C57BL/6 mice treated with PSE following induction of active EAE were euthanized on day 17 (at the peak of the disease) by CO_2 asphyxiation and perfused by intracardiac injection of PBS containing 4% paraformaldehyde and 1% glutaraldehyde. Five-micrometer thick transverse sections were taken from cervical, upper thoracic, lower thoracic, and lumbar regions of the spinal cord (four sections per mouse). The sections were stained with Luxol Fast Blue to assess demyelination and with H&E to assess leukocyte infiltration and inflammation. The signs of inflammation and demyelination in the anterior, posterior, and two lateral columns (four quadrants) of the spinal cord sections were scored under microscope. Each quadrant displaying the infiltration of mononuclear cells was assigned a score of one inflammation and the quadrants that showed perivascular lesion and loss of myelin staining were assigned a score of one demyelination. Thus, each animal had a potential maximum score of 16 points of inflammation and/or 16 points of demyelination, and this study represents the analysis of 6 representative mice from three different groups. The pathologic score (inflammation or demyelination) for each group was expressed as the percentage positive over the total number of quadrants examined (Calida et al., 2001).

Preparation of purified T cells and enriched APC

T cells were purified by using immunomagnetic negative selection to delete B cells and I-A^+ APC as described (Zhu et al., 2006). Lymph node cells were allowed to react with anti-I-$A^{d/b}$ mAb and then incubated with magnetic particles bound to goat

anti-mouse Ig (Polysciences, Inc., Eppelheim, Germany). A T cell population depleted of anti-I-$A^{d/b}$-labeled and surface Ig^+ cells was obtained by removing cell-bound magnetic particles with a rare earth magnet (Polysciences, Inc.). Purity of the resulting T cell populations was examined by flow cytometry and found to be consistently >95%.

Splenic antigen presenting cell (APC)-enriched populations were separated using immunomagnetic negative selection to delete the surface Ig^+ cells (B cells) and T cells as described (Yang et al., 2002): spleen cells were allowed to react with a mixture of rat anti-mouse CD4 (GK1.5) and rat anti-mouse CD8 (2.43) mAb and then incubated with a mixture of magnetic particles bound to goat anti-rat (Advanced Magnetics) and goat anti-mouse Ig. An APC-enriched population was obtained by removing cell-bound magnetic particles. Purity of the resulting APC-enriched populations was examined by flow cytometry and found to consistently remain T cells and B cells less than 1%.

Cytokines assay

In the MOG-immunized T cells system, purified T cells (4×10^5/well) were co-cultured with APC-enriched populations (1×10^5/well) in 96-well flat-bottomed tissue culture plate; In the TCR-trigged T cells system, purified primary T cells (2×10^5/well) were stimulated with immobile anti-CD3 mAb (5 μg/ml) plus anti-CD28 mAb (2 μg/ml) in 24-well flat-bottomed tissue culture plate.

APC-enriched cells were obtained from MOG-immunized mice with or without PSE treatment; Purified T cells were obtained from MOG-immunized mice and

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co-cultured in the presence of 10 μg/ml of MOG or not. Supernatants were harvested at 24 h to measure IL-12 levels by ELISA.

Purified T cells were obtained from MOG-immunized mice with or without PSE treatment; APC-enriched cells were obtained from normal mice and co-cultured in the presence of 10 μg/ml of MOG or 1000 pg/ml of IL-12 as indicated. Supernatants were harvested at 48 h to measure IFN-γ levels by ELISA.

Purified primary T cells were stimulated with immobile anti-CD3/CD28 mAbs for 48 h in the 1st culture. TCR-triggering T cells were harvested, washed and then stimulated with 1000 pg/ml IL-12 in the presence or absence of PSE in the 2nd culture for 24 h. IFN-γ level was measured by ELISA.

CXCR3 and CCR5 mRNA expression assay

Purified primary T cells were stimulated with immobile anti-CD3/CD28 mAbs in the presence of anti-IFN-γ (20 μg/ml), PSE (1~4 μM) or PSE plus IFN-γ (5 ng/ml) for 48 h in the 1st culture. TCR-triggering T cells were harvested, washed and then cultured without additional stimulation in the 2nd culture for 24 h. Cells from the 2nd culture were examined for the expression of CXCR3 mRNA (Nakajima et al., 2002).

Purified primary T cells were stimulated with immobile anti-CD3/CD28 mAbs for 48 h in the 1st culture. TCR-triggering T cells were harvested, washed and then stimulated with 1000 pg/ml IL-12 in the presence of PSE (1~4 μM) in the 2nd culture for 24 h. Cells from the 2nd culture were examined for the expression of CCR5 mRNA (Iwasaki et al., 2001).

RT- PCR

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Cells lysed with Trizol (Gibco-BRL, Gaithersburg, MD) according to the manufacture's protocol and total RNA were extracted and reverse-transcribed into cDNA as described (Zhu et al., 2006). cDNA were amplified. Samples were separated by a 1.2% agarose gel and stained with ethidium bromide. Relative quantitation with real-time PCR was performed with SYBR Green PCR Reagents (Qiagen, Valencia, CA) and a Continuous Fluorescence Detection System (MJ Research, USA), according to the manufacturer's instructions. The mRNA levels were normalized to those of β-actin.

Statistical analysis

Student's *t*-test was used to determine significance between groups where appropriate. $p < 0.05$ was considered significant.

Results

PSE inhibited MOG-induced EAE in C57BL/6 mice

To focus our analyses of the effect and mechanisms of PSE treatment on the initiation of the MOG-specific immune response, as opposed to the later stages of the clinical progression of EAE, PSE was administered by i.p. injection to MOG-immunized C57BL/6 mice beginning at day 0 post immunization (p.i.). The incidence of EAE was significantly reduced in PSE-treated mice, as only 5 of 15 of these mice exhibited disease signs, compared with 15 of 15 mice in the vehicle-treated mice ($p < 0.05$) (Fig.1A). The disease onset was at day 13 p.i. (Fig.1B). PSE markedly decreased the mean severity of EAE at day 17 p.i. (maximum mean clinical score: 3.13± 0.29 vehicle-treated mice versus 1.07 ± 0.42 PSE-treated mice, $p < 0.05$) (Fig. 1B). In addition to reducing the clinical score, PSE also prevented the loss of body weight (Fig. 1C). These results suggested that PSE inhibited the severity and duration of clinical paralysis in EAE.

PSE reduced inflammation and demyelination in the spinal cord tissues of MOG-immunized mice

Spinal cord sections from MOG-immunized mice at the peak of EAE (day 17 p.i.) were analyzed for the infiltration of mononuclear cells (inflammation) and the myelin loss (demyelination). PSE treatment significantly reduced both the infiltration of mononuclear cells (Fig.2A, B and E) and the extent of lesion formation in the spinal cords of MOG-immunized mice (Fig.2C, D and E).

It was conceivable that PSE exerted therapeutic effect in EAE by preventing

inflammatory cells from accumulating in the CNS since the inflammation seen in the EAE model studied here was primarily associated with discrete lesions in the spinal cord (Hooper et al., 2000). We used real-time RT-PCR to assess the levels of mRNA specific for CD4, CD8, as well as CD11b in spinal cord extracts as markers of infiltrating $CD4^+$ T-cells, $CD8^+$ T-cells, and macrophages/microglia cells, respectively. Compared with those of non-immune mice, CD4, CD8 and CD11b mRNA levels were markedly increased in the spinal cord tissues of MOG-immunized, vehicle-treated mice (Fig. 2F). PSE treatment reduced the levels of CD4, CD8 and CD11b mRNA in the spinal cord tissues of MOG-immunized mice (Fig.2F).

To obtain a quantitative assessment of the CNS inflammatory cells infiltration, we performed flow cytometric analysis on spinal cord mononuclear cells, which were isolated from representative mice at the peak of EAE (day 17 p.i.). The results revealed that infiltrating inflammatory cells in spinal cord of MOG-immunized mice were composed of T cells ($CD4^+$ and $CD8^+$) and $CD11b^+$ cells (supplementary Fig. 1A). Not only was there an increase in the total number of infiltrating inflammatory cells in the spinal cords, but there was also an increase in the number of infiltrating T cells and $CD11b^+$ (supplementary Fig. 1B).

CCR5 and CXCR3 inductions on MOG-immunized T cells were decreased by PSE treatment

The pathogenesis of EAE was thought to be associated with the initial migration of auto-reactive T cells into CNS tissues (primary influx), which consequently caused a proinflammatory CNS environment and then a massive infiltration of inflammatory

cells (second influx) (Hartung and Rieckmann, 1997). Thus, PSE may exert therapeutic effect in EAE by preventing the initial auto-reactive T cells from accumulating in the CNS. CCR5 and /or CXCR3 are essential for the primary influx of T cells to bind to the chemokine on the endothelial surface. We examined whether CCR5 and CXCR3 expressions were induced on MOG-immunized T cells and if so, whether the inductions of CCR5 and CXCR3 were influenced by PSE treatment. T cells were prepared from inguinal/axillary lymph nodes of MOG-immunized mice treated with vehicle or PSE at day 8 p.i. T cells from MOG-immunized mice expressed enhanced levels of CCR5 and CXCR3 mRNA compared to T cells from normal mice, indicating the presence of $CCR5^+$ T cells and $CXCR3^+$ T cells (Fig.3). Decreased expressions of CCR5 and CXCR3 mRNA were observed for T cells from PSE-treated mice (Fig.3). Thus, these results indicated that PSE treatment decreased the inductions of CCR5 and CXCR3 on T cells in lymphoid organs from MOG-immunized mice.

PSE suppressed IL-12-dependent CCR5 expression and IFN-γ-dependent CXCR3 expression in T cells

Previously we have suggested that PSE inhibited T cells activation both in vivo and in vitro (Zhu et al., 2006). To confirm the effect of PSE on CCR5 and CXCR3 expressions and study the mechanisms involved in, we used both MOG-immunized T cells system and TCR-trigged T cells system.

IFN-γ signal produced as a result of TCR stimulation is an absolute requirement for CXCR3 induction (Nakajima et al., 2002). In vivo PSE treatment directly inhibited

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MOG-induced IFN-γ production in MOG-immunized T cells (Fig.4A). Similarly, PSE (0.5~4 μM) concentration-dependently inhibited MOG-induced IFN-γ production in MOG-immunized T cells in vitro (Fig.4B). Anti-CD3/CD28 mAbs stimulation leads to the production of IFN-γ from T cells. Neutralization of IFN-γ with anti-IFN-γ mAb resulted in potent inhibition of CXCR3 induction (Fig.4C). PSE inhibited anti-CD3-induced IFN-γ production (Zhu et al., 2006). PSE (1~4 μM) concentration-dependently inhibited the CXCR3 mRNA induction (Fig.4C). The additive recombinant IFN-γ reversed the inhibitory effect of PSE (2 μM) on CXCR3 expression (Fig.4C). It suggested that PSE inhibited IFN-γ-dependent CXCR3 expression in T cells through suppressing IFN-γ production (Fig.4C).

CCR5 mRNA is marginally expressed in resting T cells, and the activation with anti-CD3/CD28 mAbs induces only slightly up-regulation of CCR5 mRNA expression. However, exposure of these TCR-triggered T cells to IL-12 resulted in striking level of CCR5 expression. This is the case with both $CD4^+$ and $CD8^+$ T cells (Iwasaki et al., 2001). PSE treatment had no effect on IL-12 production from MOG-immunized APCs (Fig.5A). However, PSE treatment directly inhibited IL-12-induced responses in MOG-immunized T cells, as assessed by IL-12-induced IFN-γ production (Fig.5B). Similarly, PSE concentration-dependently inhibited IL-12-induced IFN-γ production in TCR-trigged T cells (Fig.5C). We used real-time RT-PCR to determinate whether PSE also inhibited IL-12-dependent CCR5 mRNA expression. The result suggested that PSE (1~4 μM) completely inhibited the IL-12-dependent CCR5 mRNA induction (Fig.5D).

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PSE inhibited the primary T cells infiltration in the spinal cord tissues of MOG-immunized mice

As MOG-induced CCR5 and CXCR3 expressions in T cells were inhibited in PSE-treated mice, we next determine whether the primary influx of T cells into CNS was consistently decreased using RT-PCR. The expressions of CD4, CD8, CCR5 and CXCR3 mRNA were not detected before day 8 p.i. (data not shown). Compared with those of non-immunized mice, CD4, CD8, CCR5 and CXCR3 mRNA levels were increased in the spinal cord tissues of MOG-immunized mice at day 8 p.i., when the mice showed no signs of disease (Fig.6). PSE treatment reduced the expressions of CD4, CD8, CCR5 and CXCR3 mRNA in the spinal cord tissues of MOG-immunized mice (Fig.6). However, CD11b mRNA has not been elevated by MOG-immunization (Fig.6). It suggested at this time point there was only primary T cells infiltration without other inflammatory cells, such as macrophages. PSE showed a marked inhibition in the primary CCR5- and CXCR3-bearing $CD4^+$, $CD8^+$ T cells influx.

PSE inhibited the elevated expressions of chemokine in the spinal cord tissues of MOG-immunized mice

These encephalitogenic T cells which migrated to CNS in the first influx have the capability to induce macrophages/microglial cells, astrocytes to express chemokine (Sun et al., 1997). The kinetics of chemokine expression in the spinal cord parenchyma was addressed from four different time points corresponding to preclinical when no mice were exhibiting disease symptoms (day 8 and day 11 p.i.), disease onset when mice showed first symptoms of EAE (day 13 p.i.), and peak EAE

(day 17 p.i.). Chemokine expressions were never detected in the absence of primary infiltrates (data not shown). At day 11 p.i., CCL3, CXCL9 and CXCL10 showed a low level expression in CNS from some of MOG-immunized mice. After onset of clinical disease (day 13 p.i.), the expressions of CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10 all elevated to a higher level and remained elevated throughout the course of acute clinical disease of EAE (from day 13 to day 17 p.i. in our observation) (Fig.7A). PSE treatment inhibited the up-regulation of chemokine expressions induced by the primary T cells influx (from day 13 to day 17 p.i.) (Fig.7B).

PSE inhibited the secondary influx in the spinal cord tissues of MOG-immunized mice

Spinal cords from four different time points corresponding to preclinical (day 8 and day 11 p.i.), EAE onset (day 13 p.i.), and peak EAE (day 17 p.i.) were evaluated to show the kinetics of mononuclear cells infiltration. Compared with the expressions at day 8 and day 11 p.i., CD4, CD8, CCR5 and CXCR3 mRNA levels were increased in the spinal cords of MOG-immunized mice from day 13 to day 17 p.i. (Fig.8A). CD11b mRNA has also been elevated compared with the expression at day 11 p.i. (Fig.8A). It suggested there were not only secondary T cells infiltration but also other inflammatory cells, such as macrophages, which was different with the primary influx only involving T cells (Karin et al., 1993; Brocke et al., 1996). PSE treatment not only reduced the levels of CD4, CD8, CCR5 and CXCR3 mRNA, but also decreased the levels of CD11b mRNA in the spinal cords of MOG-immunized mice (Fig.8B). The results demonstrated PSE inhibited the secondary influx.

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Discussion

PSE was demonstrated as an immunosuppressor aiming at T lymphocytes activation (Zhu et al., 2006). In this study we showed that PSE exhibited protective effect against EAE, which may through multiple mechanisms. Here we found it may through suppressing IL-12-dependent CCR5 expression and IFN-γ-dependent CXCR3 expression in T cells, thereby inhibiting the primary T cells influx, accordingly decreasing elevated chemokine expressions, therefore inhibiting the secondary influx of inflammatory cells, including T cells and macrophages, and consequently attenuating CNS lesions.

The difference between the primary and secondary influx was that primary influx involved only T cells but secondary influx involved both T cells and macrophages (Karin et al., 1993; Brocke et al., 1996). As shown in Fig.8A, at day 8~11 p.i., the expression of CD11b mRNA in MOG-immunized mice was hold on the base line while the expression of CD4 and CD8 mRNA elevated compared with normal mice. It suggested that day 8~11 p.i. belonged to the primary influx stage. From day13 p.i., the expression of CD11b mRNA was elevated as well as the expression of CD4 and CD8 mRNA in MOG-immunized compared with that at day 8~11 p.i. It suggested that the secondary influx stage came, which was identical with the EAE onset.

All the chemokine detected in the spinal cord parenchyma of MOG-immunized mice were expressed at least 3 days after the appearance of their receptors, CCR5 and CXCR3 (Fig.7A). These results strongly suggested that chemokine expressed in the spinal cord parenchyma were not essential for the initial recruitment of $CCR5^+$ and/or

$CXCR3^+$ T cells. When the $CCR5^+$ and/or $CXCR3^+$ T cells in the initial recruitment across the BBB, CCR5 and CXCR3 bind to the chemokine expressed on the surface of vascular endothelium cells. In our study, we could not detect this kind of chemokine because the spinal cord tissue has been perfused by intracardiac injection of PBS. The chemokine expressed on the surface of vascular endothelium cells may not exist after perfusing by a large amount of PBS. However, several studies in different models have shown a correlation between the EAE onset and the expression of the chemokine in the parenchyma, RANTES, MIP-1α, IP-10, MIP-1β and MCP-1 (Godiska et al., 1995; Hulkower et al., 1993; Ransohoff et al., 1993). The peak expression of chemokine paralleled with that of chemokine receptors at day 13 p.i. (Fig.7A and 8A). It implied that chemokine expressed in the parenchyma are key mediators in the recruitment of secondary influx of leukocytes at the inflamed spinal cord tissues.

PSE treatment reduced the accumulation of T cells and macrophages and the lesion formation in the spinal cords of MOG-immunized mice (Fig.2F). The apparent decrease in inflammatory cells invasion was paralleled with reduced levels of mRNA specific for several inflammatory mediators implicated in the disease pathogenesis, including IL-18, TNF-α, IFN-γ, iNOS and COX-2 (data not shown).

It has been demonstrated that IFN-γ and IFN-γ receptor-deficient mice would develop more severe EAE than wild-type mice (Ferber et al., 1996; Chu et al., 2000). Likewise, administration of anti-IFN-γ to wild-type mice exacerbates EAE (Billiau et al., 1988; Lublin et al., 1993). These mice have a massive expansion of

myelin-specific CD4+ cells, suggesting that the complete loss of IFN-γ results in a diminished capacity to regulate the auto-reactive T cells (Chu et al., 2000) and reinforcing the notion that loss of a particular gene from all cell types can have unforeseen side effects. Although these observations indicate that IFN-γ is not essential for the induction of EAE, they do not negate the fact that encephalitogenic T cells generated in vivo in wild-type mice produce significant amounts of IFN-γ or the fact that in vitro suppression of IFN-γ production during the stimulation of myelin-specific T cells reduces the encephalitogenic capacity of these cells (Olsson, 1992). Taken together, these studies would suggest that suppressing IFN-γ production in the encephalitogenic Th1 cells, while preserving IFN-γ expression in other cell types, may provide therapeutic benefit. In our study, PSE inhibited IFN-γ production from T cells both in vivo (Fig.4A and 5B) and in vitro (Fig.4B and 5C). However, whether PSE also demonstrated inhibitory effects on IFN-γ production from NK cells, NKT cells and even macrophages deserve further studies.

Injection of MS patients with recombinant IFN-γ induced exacerbation of the disease (Panitch et al., 1987); Patients who received anti-IFN-γ mAb treatment showed statistically significant improvement in secondary progressive MS (Skurkovich et al., 2001). Furthermore, increase in IFN-γ produce by PBMC precedes clinical attacks (Dettke et al., 1997) and the inflammatory process in the CSF of patients with MS is characterized by increased IFN-γ expression (Woodroofe and Cuzner, 1993). Taken together, the data are consistent with an important pathogenic role for IFN-γ in inflammatory demyelinating diseases MS. Although so many

treatments are effective in EAE, few of them has been effective in human MS. Benefiting from its potent inhibitory effect on IFN-γ production from T cells, PSE may exhibit a therapeutic effect on MS. We will continue to evaluate the pharmacological effects of PSE in the future studies. Our recently results also demonstrated that oral administration of PSE has the therapeutic effects on this EAE model (data not shown).

In conclusion, this study highlights the fact that PSE inhibits EAE by suppressing IL-12-dependent CCR5 expression and IFN-γ-dependent CXCR3 expression in T cells and suggests its therapeutic effect in the treatment of MS.

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Footnotes

* Authors contributed equally to this work.

Legends for Figures

Fig. 1 PSE inhibited the development of clinical signs of EAE in MOG-immunized C57BL/6 mice. Active EAE was induced in female C57BL/6 mice by immunization with MOG35-55 peptide in CFA. The mice (n=15) were treated with vehicle or PSE at 10 mg/kg/day by i.p. injection from day 0 p.i. as detail in *Materials and Methods*. Mice were monitored for signs of EAE, and the results for all mice, both healthy and sick, were presented as percentage of incidence of disease (A), mean disease score ± S.E.M. (B), and body weight (C). *, p <0.05 compared with vehicle-treated control. Three independent experiments were performed with similar results.

Fig. 2 PSE reduced inflammation and demyelination in the spinal cord tissues of MOG-immunized mice. Spinal cord histology: MOG-immunized mice treated with vehicle (A, C) or PSE (B, D) were sacrificed at day 17 p.i. Spinal cords were harvested after extensive perfusion, and 5 μM sections were stained with H&E(A, B) and Luxol fast blue (myelin stain) (C, D). E. Mean scores of inflammation and demyelination ± S.E.M. of 6 mice. (F). Expression of CD4, CD8 and CD11b in spinal cords. RNA from PBS-perfused spinal cords of MOG-immunized mice treated with vehicle or PSE were analyzed by real-time RT-PCR for expressions of CD4, CD8 and CD11b mRNA. Data are mean ± S.E.M. of 6 mice. ***, p <0.001 compared with vehicle-treated control. The data shown are representative of three independent experiments.

Fig. 3 The expressions of CCR5 and CXCR3 in MOG-immunized T cells. At day 8 p.i., T cells were isolated from draining lymph nodes of MOG-immunized mice

treated with vehicle or PSE. CCR5 and CXCR3 mRNA expressions was analyzed by RT-PCR. Three independent experiments were performed with similar results.

Fig. 4 PSE suppressed IFN-γ-dependent CXCR3 expression in T cells. (A) In vivo PSE treatment directly inhibited IFN-γ production from MOG-immunized T cells. Purified T cells were obtained from MOG-immunized mice treated with vehicle or PSE 10 mg/kg; APC-enriched cells were obtained from normal mice and co-cultured in the presence of 10 μg/ml of MOG or not. Supernatants were harvested at 48 h to measure IFN-γ levels by ELISA. (B) In vitro PSE concentration-dependently inhibited IFN-γ production from MOG-immunized T cells. Purified T cells were obtained from MOG-immunized mice; APC-enriched cells were obtained from normal mice and co-cultured in the presence of 10 μg/ml of MOG with indicated concentration of PSE. Supernatants were harvested at 48 h to measure IFN-γ levels by ELISA. (C) CXCR3 mRNA expression assay. 1st culture: Purified primary T cells were stimulated with anti-CD3/CD28 mAbs for 48 h in the presence of anti-IFN-γ (20 μg/ml), PSE (1~4 μM) or PSE plus IFN-γ (5 ng/ml). 2nd culture: TCR-triggering T cells were harvested, washed and then cultured without additional stimulation for 24 h. Cells from the 2nd culture were examined for the expression of CXCR3 mRNA by real-time PCR. Data are mean ± S.E.M. of three independent experiments. *, p <0.05; **, p <0.01; ***, p <0.001 compared with control. Three independent experiments were performed with similar results.

Fig. 5 PSE suppressed IL-12-dependent CCR5 expression in T cells. (A) PSE has no effect on IL-12 production. APC-enriched cells were obtained from MOG-immunized

mice treated with vehicle and PSE 10 mg/kg; Purified T cells were obtained from MOG-immunized mice and co-cultured in the presence of 10 μg/ml of MOG or not. Supernatants were harvested at 24 h to measure IL-12 levels by ELISA. (B) PSE inhibited IL-12 induced IFN-γ production from MOG-immunized T cells. Purified T cells were obtained from MOG-immunized mice treated with vehicle or PSE 10 mg/kg; APC-enriched cells were obtained from normal mice and co-cultured in the presence of 1000 pg/ml of IL-12 or not. Supernatants were harvested at 48 h to measure IFN-γ levels by ELISA. (C) PSE inhibited IL-12 induced IFN-γ production from TCR-triggered T cells. 1st culture: Purified primary T cells were stimulated with anti-CD3/CD28 mAbs for 48 h. 2nd culture: TCR-triggering T cells were harvested, washed and then stimulated with 1000 pg/ml IL-12 for 24 h with PSE (1~4 μM). IFN-γ level in the supernatants from the 2nd culture was measured by ELISA. (D) CCR5 mRNA expression assay. Cells from the 2nd culture were examined for the expression of CCR5 mRNA by real-time PCR. Data are mean ± S.E.M. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with control. Three independent experiments were performed with similar results.

Fig. 6 PSE inhibited the primary T cells infiltration in the spinal cord tissues of MOG-immunized mice. RNA from PBS-perfused spinal cords of MOG-immunized mice treated with vehicle or PSE at day 8 p.i. were analyzed by real-time RT-PCR for expression of CD4, CD8, CD11b, CCR5 and CXCR3 mRNA. Each lane corresponds to an individual mouse with the clinical score on the day of sacrifice indicated above. Three independent experiments were performed with similar results.

Fig. 7 PSE inhibited the elevated expressions of chemokine in the spinal cords of MOG-immunized mice. RNA from PBS-perfused spinal cords of MOG-immunized mice treated with vehicle or PSE at the indicated times, were analyzed by real-time RT-PCR for expressions of CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, CXCL11 mRNA. Each lane corresponds to an individual mouse with the clinical score on the day of sacrifice indicated above. Three independent experiments were performed with similar results.

Fig. 8 PSE inhibited the secondary influx in the spinal cords of MOG-immunized mice. RNA from PBS-perfused spinal cords of MOG-immunized mice treated with vehicle or PSE at the indicated times, were analyzed by real-time RT-PCR for expressions of CD4, CD8, CD11b, CCR5 and CXCR3 mRNA. Each lane corresponds to an individual mouse with the clinical score on the day of sacrifice indicated above. Three independent experiments were performed with similar results.

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Figure 1

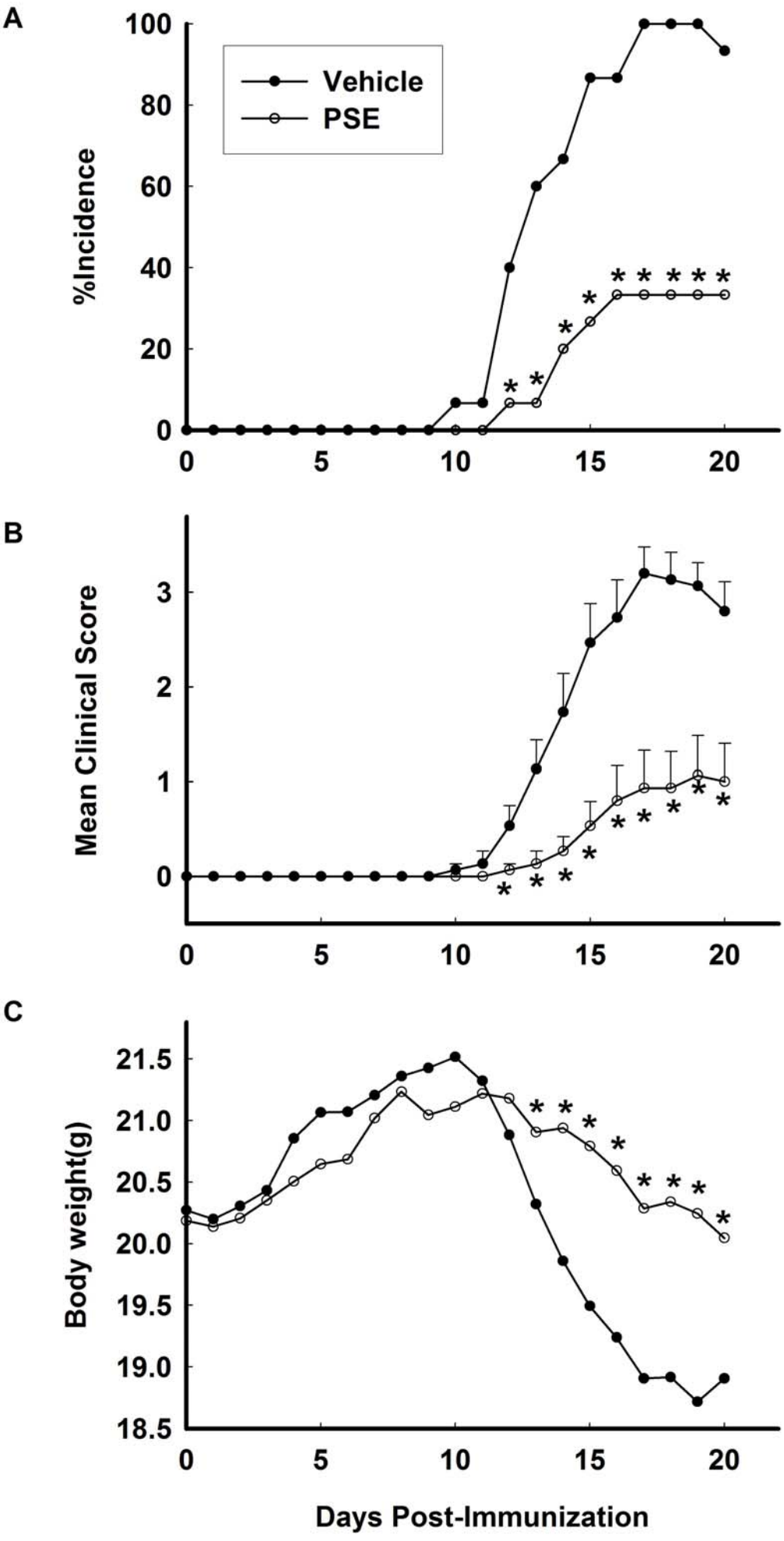

Figure 2

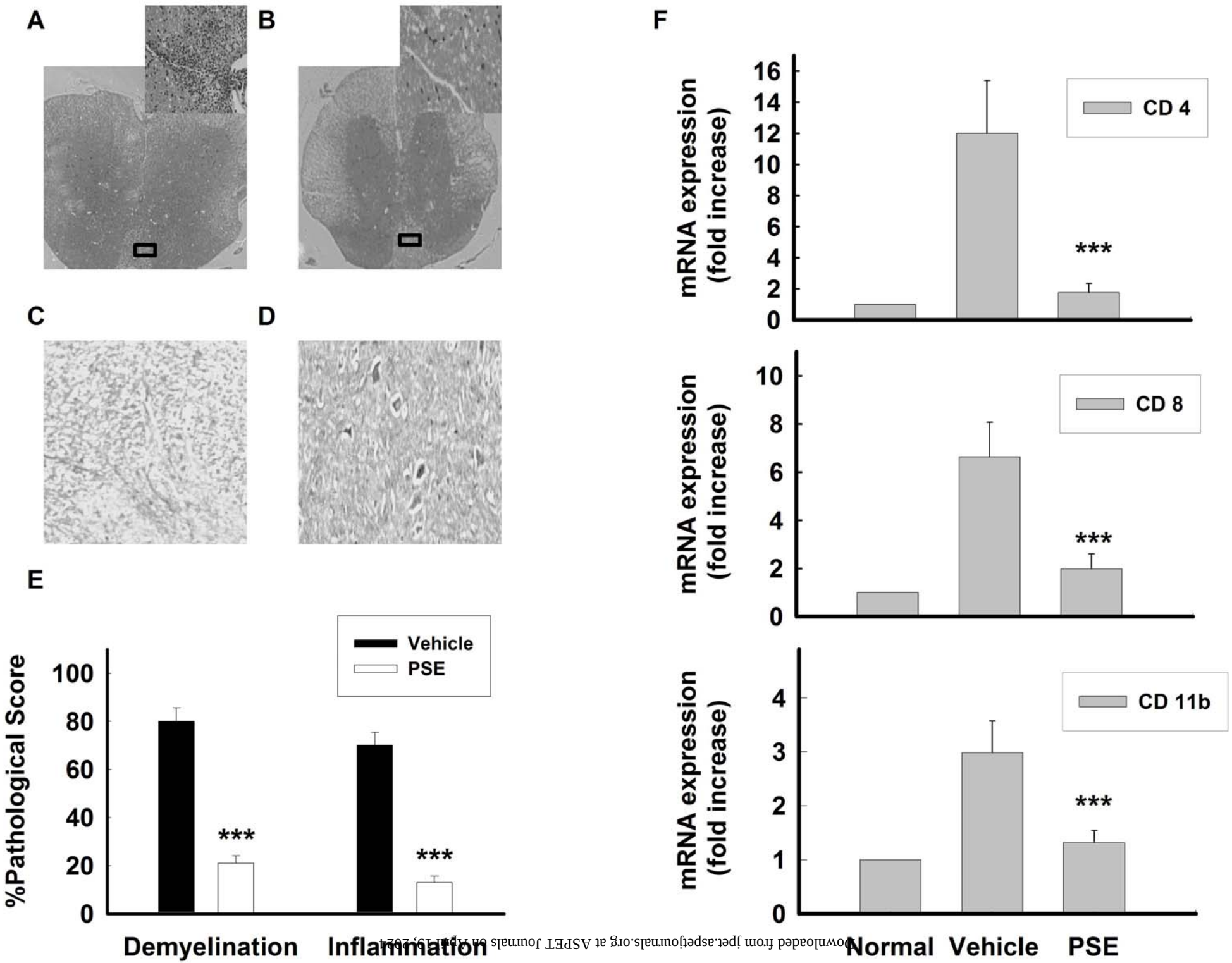

Figure 3

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Figure 4

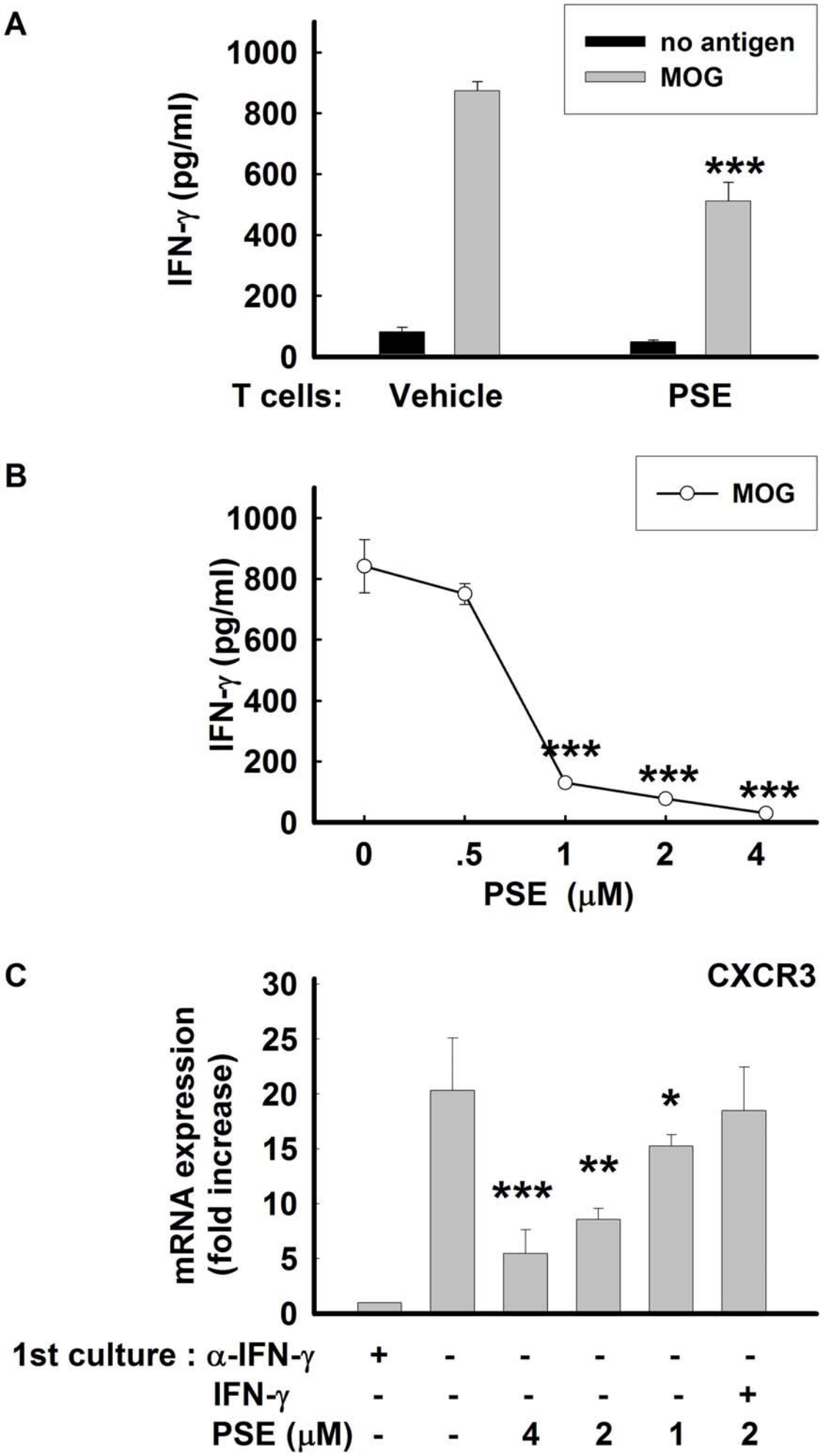

Figure 5

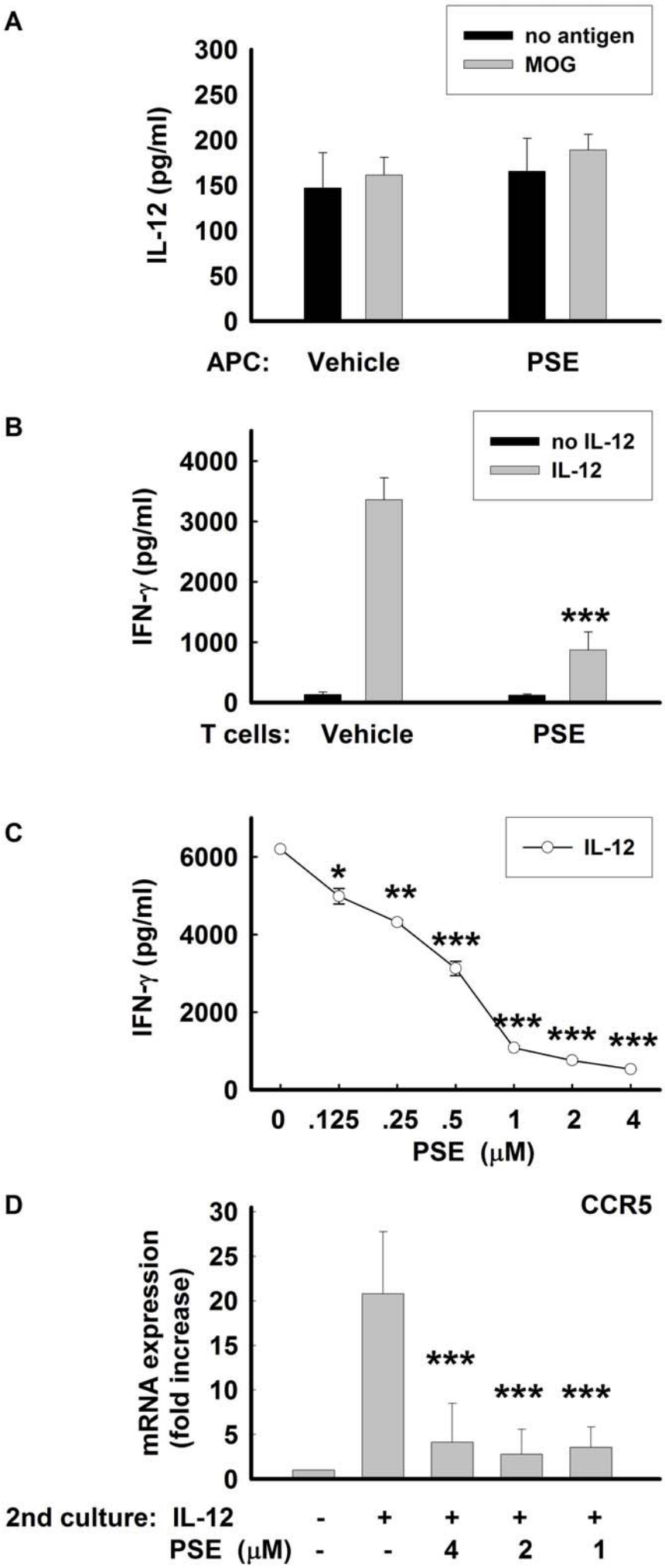

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Figure 6

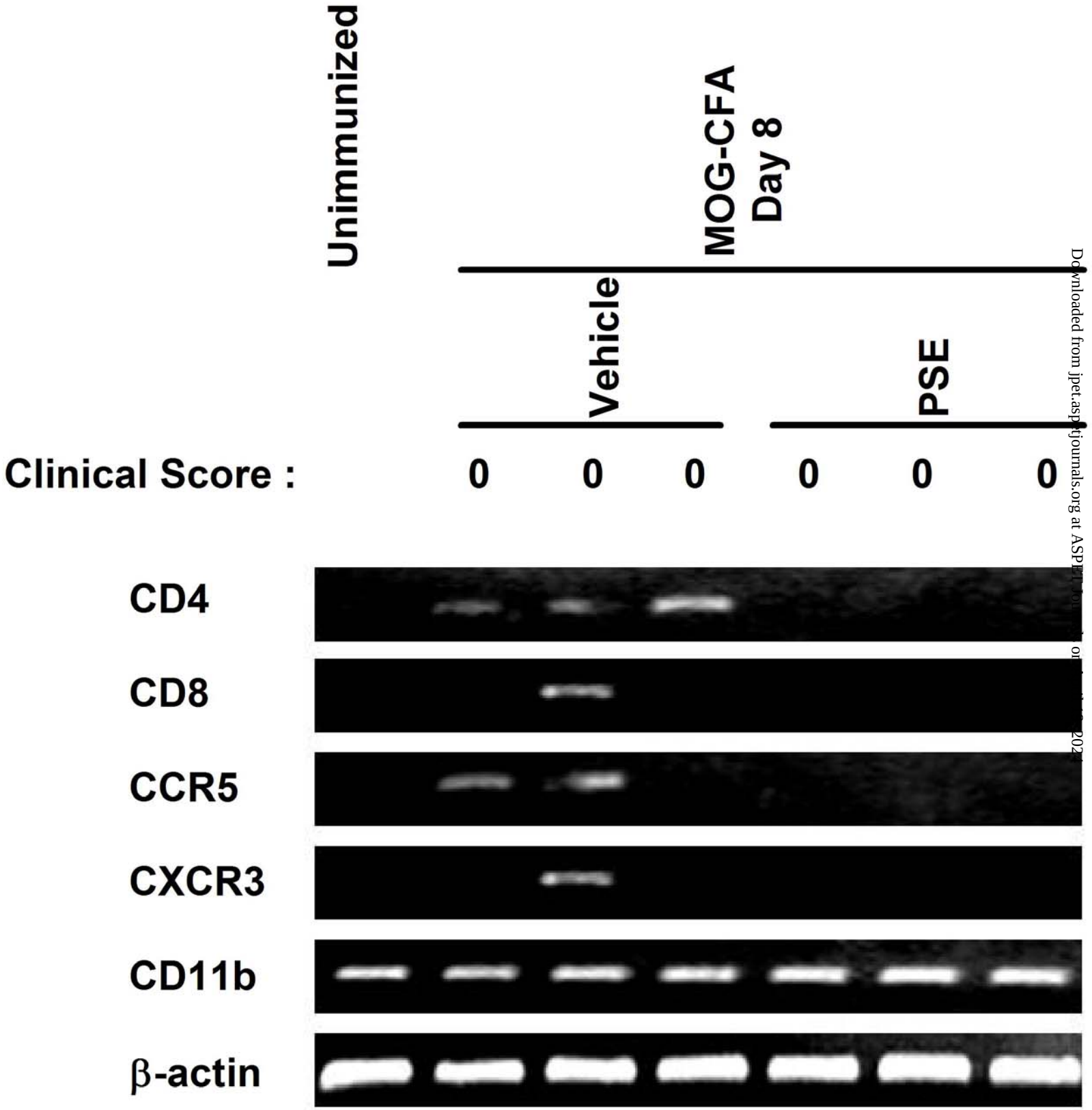

Figure 7

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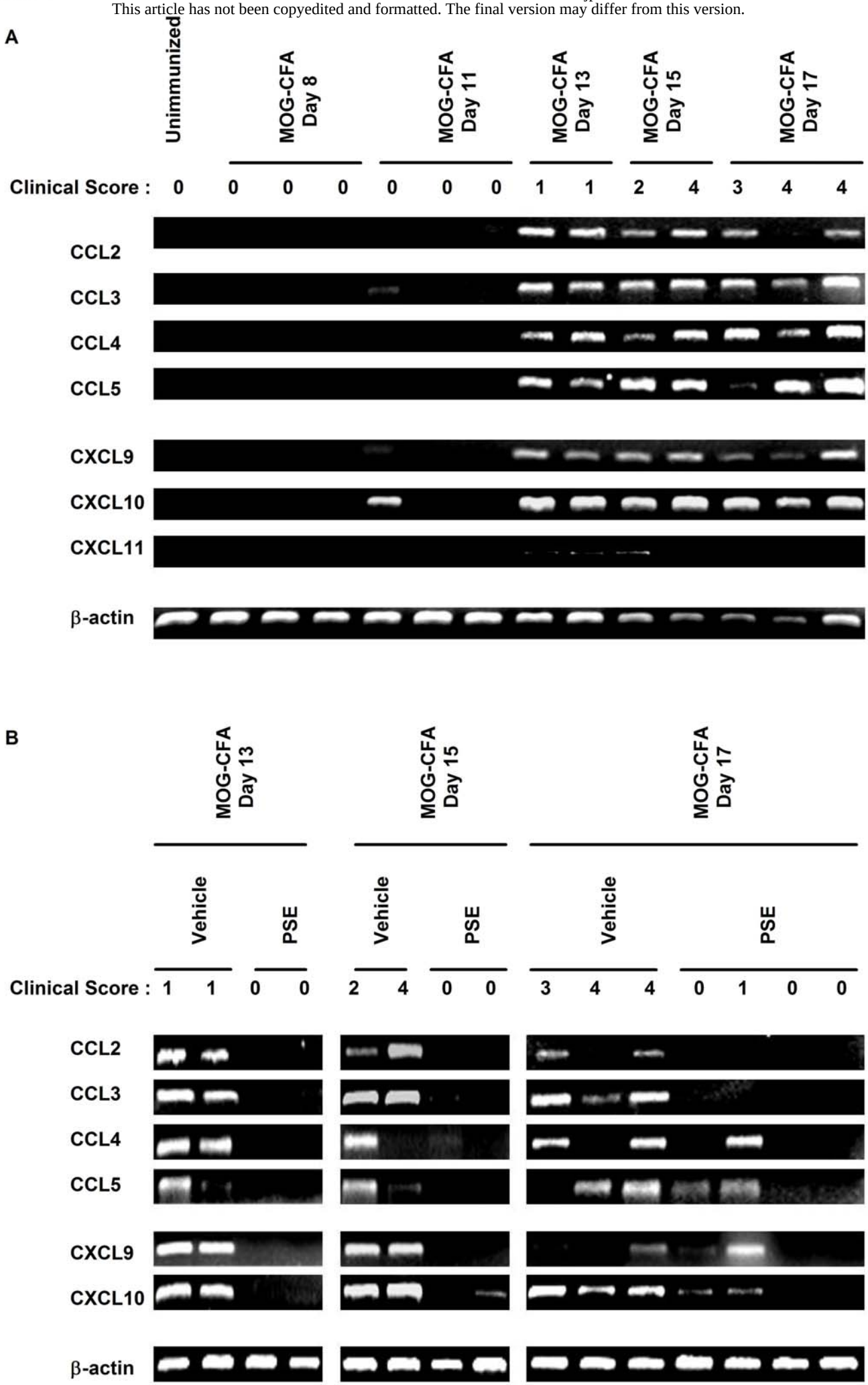

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Figure 8