BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 2046–2056

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

The chemokine receptor antagonist, TAK-779, decreased experimental autoimmune encephalomyelitis by reducing inflammatory cell migration into the central nervous system, without affecting T cell function

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Background and purpose: The C–C chemokine receptor CCR5, and the C–X–C chemokine receptor CXCR3 are involved in the regulation of T cell-mediated immune responses, and in the migration and activation of these cells. To determine whether blockade of these chemokine receptors modulated inflammatory responses in the central nervous sytem (CNS), we investigated the effect of a non-peptide chemokine receptor antagonist, TAK-779, in mice with experimental autoimmune encephalomyelitis (EAE).

Experimental approach: EAE was induced by immunization of C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG) 35–55. TAK-779 was injected s.c. once a day after immunization. Disease incidence and severity (over 3 weeks) were monitored by histopathological evaluation and FACS assay of inflammatory cells infiltrating into the spinal cord, polymerase chain reaction quantification of mRNA expression, assay of T cell proliferation, by [^{3}H]-thymidine incorporation and cytokine production by enzyme-linked immunosorbent assay.

Key results: Treatment with TAK-779 reduced incidence and severity of EAE. It strongly inhibited migration of CXCR3/CCR5 bearing CD4$^+$, CD8$^+$ and CD11b$^+$ leukocytes to the CNS. TAK-779 did not reduce proliferation of anti-MOG T cells, the production of IFN-γ by T cells or CXCR3 expression on T cells. In addition, TAK-779 did not affect production of IL-12 by antigen-presenting cells, CCR5 induction on T cells and the potential of MOG-specific T cells to transfer EAE.

Conclusions and implications: TAK-779 restricted the development of MOG-induced EAE. This effect involved reduced migration of inflammatory cells into the CNS without affecting responses of anti-MOG T cells or the ability of MOG-specific T cells to transfer EAE.

British Journal of Pharmacology (2009) **158,** 2046–2056; doi:10.1111/j.1476-5381.2009.00528.x; published online 26 November 2009

Keywords: experimental autoimmune encephalomyelitis; CCR5; CXCR3; chemokine; T cell migration

Abbreviations: APC, antigen-presenting cell; CCR, C–C chemokine receptor; CXCR, C–X–C chemokine receptor; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NK, natural killer PCR, polymerase chain reaction; Th, T helper

Introduction

In multiple sclerosis (MS) and the corresponding animal model, experimental autoimmune encephalomyelitis (EAE), the development of disease requires the trafficking of effector cells, including macrophages and antigen-specific T cells, into the central nervous system (CNS). These cells initiate lesion

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Received 23 June 2009; revised 12 August 2009; accepted 22 August 2009

formation, and contribute to the inflammation and demyelination characteristic of the disease (Kawakami *et al.*, 2004; Sospedra and Martin, 2005). Numerous cytokines, chemokines and adhesion molecules play varied and complex roles in leukocyte migration during EAE, but a complete account of their function during the massive accumulation of leukocytes in the CNS during EAE has not yet been given.

Over the last decades, a family of chemotactic cytokines, the chemokines, has been found to be involved in the trafficking of leukocytes in both the normal and the pathological states (Pease and Williams, 2006). The expression of these chemokines and their receptors is increased during the acute phase of MS and also in EAE, its animal model (Godiska *et al.*, 1995; Muller *et al.*, 2004). In EAE, there is a tight correlation between expression of these chemokines and their receptors, the distribution of leukocytes infiltrating the CNS and the development of this disease (Hulkower *et al.*, 1993; Glabinski *et al.*, 1995; Godiska *et al.*, 1995; Miyagishi *et al.*, 1997). There is ample evidence for a role of the chemokine receptors, CXCR3, CCR5 and CCR2 (nomenclature follows Alexander *et al.*, 2008), in the pathogenesis of EAE and MS. The frequency of detectable $CCR5^+$ and $CXCR3^+$ T cells is elevated in EAE and MS (Balashov *et al.*, 1999; Fischer *et al.*, 2000). The expression of CCR2 was also increased in CNS tissues obtained from animals with EAE and MS patients (Balashov *et al.*, 1999; Fife *et al.*, 2000; Fischer *et al.*, 2000). These findings provide further support for the view that $CXCR3^+$, $CCR5^+$ and $CCR2^+$ leukocytes may play an important role in EAE and MS.

A non-peptide, synthetic CCR5 antagonist, TAK-779, was initially developed for the treatment of HIV infection (Baba *et al.*, 1999). TAK-779 appears to selectively inhibit CCR5 and CXCR3 (Gao *et al.*, 2003). Interestingly, Tokuyama *et al.* (2005) more recently reported that TAK-779 blocked ligand binding to murine CCR2. In this study, we have demonstrated that treatment with TAK-779 inhibited the development of EAE induced by myelin oligodendrocyte glycoprotein (MOG). Our results showed that this inhibition was mediated by a markedly diminished migration of inflammatory cells into the CNS without affecting the responses of anti-MOG T cells.

Methods

Induction and assessment of EAE

All animal care and experimental procedures 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. Female C57BL/6 mice, aged 6–8 weeks, were purchased from the Shanghai Experimental Animal Center, the Chinese Academy of Sciences, Shanghai, China. The animals were housed in specific pathogen-free conditions. We used the active EAE model as previously described (Ni *et al.*, 2007). Briefly, female C57BL/6 mice were immunized at day 0 by s.c. injection with 100 μL of an emulsion of MOG 35–55 peptide in complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* H37Ra, to three sites: one along the midline of the back between the shoulders, and two on each side of the midline on the lower back. The final dose of MOG 35–55 and *M. tuberculosis* H37Ra were 200 and 400 μg per mouse respectively. Additionally, each mouse received 400 ng of *Bordetella pertussis* toxin in 200 μL of phosphate-buffered saline (PBS) by intraperitoneal injection at day 0 and at 72 h post-immunization. Clinical assessment of EAE was performed daily, 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).

Drug treatment

The mice were immunized with MOG and were treated s.c. with TAK-779 or vehicle. The mice ($N = 10$) were injected s.c. with 150 μg TAK-779 (dissolved in 5% mannitol solution) in a volume of 100 μL, once daily after MOG immunization. TAK-779 injection was started from day 0 after immunization and continued once daily for 22 days. The dose of 150 μg was determined based on the observations in prior experiments that the dose of 50 μg per mouse could not produce inhibition, and a dose of more than 100 μg per mouse was required to produce significant inhibition. The dose of 150 μg per mouse has also been used in other mouse experimental models (Yang *et al.*, 2002), and approximately the same dose was used in allograft rejection and asthma models (Akashi *et al.*, 2005; Suzaki *et al.*, 2008). As a control, an equal volume of PBS containing 5% mannitol was injected daily in the control mice ($N = 10$).

Adoptive transfer

C57/BL6 mice were immunized with MOG 35–55 as previously described, and treated with TAK-779 or with vehicle ($N = 10$). Ten days later, draining lymph nodes from both groups were harvested, and lymphocytes were isolated and cultured with 50 $\mu g \cdot mL^{-1}$ of MOG 35–55 and 20 $ng \cdot mL^{-1}$ of murine recombinant interleukin (IL)-12 (BD). After 4 days of culture, cells were washed and resuspended in PBS for transfer. Recipient C57/BL6 mice ($N = 4$) received 1×10^7 cells i.v., as well as 200 ng of *Pertussis* toxin in 200 μL of PBS twice (i.p. injection) immediately after cell transfer and 48 h later. The mice were observed daily for clinical signs of EAE, up to 24 days after the cell transfer, and scored every day according to the criteria described earlier.

Preparation of purified T cells and antigen-presenting cell (APC)-enriched cells

T cells were purified by using immunomagnetic negative selection to delete B cells and $I\text{-}A^+$ APC as described by Yang *et al.* (2002). Lymph node cells were allowed to react with anti-$I\text{-}A^{d/b}$ mAb and then incubated with magnetic particles bound to goat anti-mouse Ig (Polysciences, Inc., Eppelheim, Germany). A T cell population depleted of anti-$I\text{-}A^{d/b}$-labelled 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 APC-enriched populations were separated using

immunomagnetic negative selection to delete the surface Ig^+ cells (B cells) and T cells (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, Lexington, MA, USA) 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 contamination of T cells and B cells was less than 1%.

Proliferation of lymph node cells

Lymph node cells (2×10^5 per well) prepared from immunized mice with or without TAK-779 treatment were cultured in 96-well flat-bottomed microculture plates in the presence of MOG at the indicated concentration or in medium alone. After 60 h, 0.5 μCi per well [^{3}H] thymidine was added to each well and then incubated for 12 h. The cells were then harvested onto glass fibre filters, and radioactivity was measured in a Microbeta Trilux liquid scintillation counter (PerkinElmer Life and Analytical Science, Boston, MA, USA).

Detection of IFN-γ

Purified T cells (4×10^5 per well) were obtained from MOG-immunized mice with or without TAK-779 treatment; APC-enriched cells (1×10^5 per well) were obtained from normal mice. The T cells and APC-enriched cells were co-cultured in the absence or presence of 10 $\mu g \cdot mL^{-1}$ of MOG. Supernatants were harvested at 48 h to measure IFN-γ levels by enzyme-linked immunosorbent assay (ELISA). All samples were measured in duplicate.

Detection of IL-12

APC-enriched cells (1×10^5/well) were obtained from MOG-immunized mice with or without TAK-779 treatment; purified T cells (4×10^5/well) were obtained from MOG-immunized mice. The APC-enriched cells and the T cells were co-cultured in 96-well flat-bottomed tissue culture plates in the absence or presence of 10 $\mu g \cdot mL^{-1}$ of MOG. The supernatants were harvested at 24 h to measure IL-12 p70 levels by ELISA. All samples were measured in duplicate.

Detection of IL-17

Splenocytes and lymph node cells were isolated from MOG-immunized mice with or without TAK-779 treatment. Cells were cultured in the absence or presence of 10 $\mu g \cdot mL^{-1}$ of MOG. The supernatants were harvested at 72 h to measure IL-17 levels by ELISA. All samples were measured in duplicate.

Reverse transcription–polymerase chain reaction (PCR) and real-time PCR

Cells were lysed with Trizol (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol, and total RNA was extracted and reverse-transcribed into cDNA as described previously (Ni *et al*., 2007). The cDNA was amplified. Samples were separated by a 1.2% agarose gel and stained with ethidium bromide. Relative quantitation of real-time PCR was performed with SYBR Green PCR Reagents (Qiagen, Valencia, CA, USA) and a continuous fluorescence detection system (MJ Research, Waltham, MA, USA), according to the manufacturer's instructions. The mRNA levels were normalized to those of β-actin. Primers used in this study were: CD4 sense, 5′-GCTCACCGTCATCTGCTCTGA-3′; CD4 antisense, 5′-AG GGCCTCCCACTGTGATCT-3′; CD8 sense, 5′-CATCCTGC TTCTGCTGGCATT-3′; CD8 antisense, 5′-TGGGCGCTGAT CATTTGTGAAA-3′; CD11b sense, 5′-AGCCCAAGATCACATG-3′; CD11b antisense, 5′-TGCAGAAGCATAACCC-3′; CCR2 sense, 5′-CCAAACAAGATGATCACCAT-3′; CCR2 antisense, 5′-GGTCTAAGTGCTTGTCAAT-3′; CXCR3 sense, 5′-GAACGT CAAGTGCTAGATGCCTCG-3′; CXCR3 antisense, 5′-GTACAC GCAGAGCAGTGCG-3′; CCR5 sense, 5′-GGATTTTCAAGG GTCAGTTC-3′; CCR5 antisense, 5′-AACCTTCTTTCTGAGA TCTGG-3′; β-actin sense, 5′-TCC-TGA-GCG-CAA-GTA-CTC-TGT-3′; β-actin antisense, 5′-CTG-ATC-CAC-ATC-TGC-TGG-AAG-3′; CCL2 sense, 5′-CCCAATGAGTAGGCTGGAGA-3′; CCL2 antisense, 5′-AAGGCATCACAGTCCGAGTC-3′; CCL3 sense, 5′-AAG-TCT-TCT-CAG-CGC-CAT-ATG-3′; CCL3 antisense, 5′-GTG-GAA-TCT-TCC-GGC-TGT-AG-3′; CCL4 sense, 5′-TTC-TGT-GCT-CCA-GGG-TTC-TC-3′; CCL4 antisense, 5′-CGG-GAG-GTG-TAA-GAG-AAA-CAG-3′; CCL5 sense, 5′-AT C-TTG-CAG-TCG-TGT-TTG-TCA-3′; CCL5 antisense, 5′-TTC-TTG-AAC-CCA-CTT-CTT-CTC-TG-3′; CXCL9 sense, 5′-CGTG GTAAAACACTTGCGGATATT-3′; CXCL9 antisense, 5′- CAA TCATGCTTCCACTAACCGACT-3′; CXCL10 sense, 5′- TGA TTTGCTGCCTTATCTTTCTGA-3′; CXCL10 antisense, 5′-CAG CCTCTGTGTGGTCCATCCTTG-3′; IFN-γ sense, 5′-CTTGG CTTTGCAGCTCTT-3′; IFN-γ antisense, 5′-GGACCTGTG GGTTGTTGA-3′; IL-17 sense, 5′-CACCGCAATGAAGACC-3′; antisense, 5′-CGAAGCAGTTTGGGAC-3′.

Histopathology

To assess the degrees of inflammation and demyelination in the CNS, C57BL/6 mice treated with or without TAK-779, following the induction of active EAE were anaesthetized on day 17 (at the peak of the disease) with pentobarbital sodium, and perfused with PBS containing 4% paraformaldehyde and 1% glutaraldehyde by intracardiac injection. Five-micrometre-thick transverse sections (5 μm) were taken from embedded blocks of spinal cord from cervical, upper thoracic, lower thoracic and lumbar regions (four sections per mouse). The sections were stained with H&E to assess the amount of mononuclear cell infiltration and inflammation. The severity of inflammation in the anterior, posterior and two lateral columns (four quadrants) of the spinal cord was scored under microscopy. Each quadrant displaying infiltration of mononuclear cells was assigned a score of 1–4 on inflammation. Thus, each animal had a potential maximum score of 16 for inflammation. The data in this study were from five mice, from each of the normal, vehicle and TAK-779-treated groups. Inflammation was scored by a single observer, unaware of the treatment status of the animal, and was expressed as a percentage of positive quadrants, relative to the total number of quadrants examined (Bright *et al*., 1999).

Isolation of mononuclear cells from CNS and flow cytometry assay

Isolation of mononuclear cells from the CNS was performed as described previously (Huang *et al.*, 2001). Briefly, the mice were anaesthetized with pentobarbital sodium and perfused with 40 mL of cold PBS. The spinal cords were extruded by flushing the vertebral canal with PBS and rinsed in PBS. Tissues were forced through 70 μm nylon cell strainers (BD Falcon, San Jose, CA, USA), after which the spinal cord cell suspensions were incubated with collagenase (1 mg·mL^{-1}) at 37°C for 30 min, and passed again through 70 μm nylon cell strainers to yield single-cell suspensions. CNS mononuclear cells were isolated by centrifugation (400× *g*) at room temperature for 20 min over a 30/70% discontinuous Percoll gradient. The cells were collected from the interphase, washed in PBS, counted and immediately analysed by flow cytometry as described as follows. Single-cell suspensions from CNS, obtained as described earlier, were washed and resuspended in FACS buffer (1% FCS and 0.1% sodium azide in PBS). After blocking with anti-FcR Abs at 4°C for 20 min, the cells were stained for surface markers with directly conjugated Abs in FACS buffer at 4°C for 30 min. The cells were washed twice and resuspended in the 400 μL of PBS for flow cytometry analysis. The absolute number of a leukocyte subtype was determined by the multiplication of the percentage of that cell type by the total number of infiltrated leukocytes in that sample.

Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance followed by Dunnett's post-test or Student's *t*-test was used to determine the difference between two groups where appropriate. A P value < 0.05 was considered significant.

Materials

The CCR5 and CXCR3 antagonist, TAK-779 (*N*,*N*-dimethyl-*N*-(4-[[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]carbonyl]amino]benzyl)-tetrahydro-2H-pyran-4-aminium chloride; M_r = 531.13) was synthesized with 98% purity, based on the structure shown (Baba *et al.*, 1999). The peptide MOG 35–55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Sangon Biological Engineering Technology and Service Co. (Shanghai, P.R. China). CFA and *M. tuberculosis* H37Ra were purchased from Difco (Detroit, MI, USA). *Bordetella pertussis* toxin, dimethylsulphoxide and 3,3′,5,5′-tetramethylbenzidine were purchased from Sigma-Aldrich (St Louis, MO, USA). The RPMI 1640 medium was bought from Invitrogen (Carlsbad, CA, USA), and the fetal calf serum was obtained from Hyclone Laboratories (Logan, UT, USA). [^{3}H]-Thymidine (0.5 mCi·mL^{-1}) was provided by Shanghai Institute of Applied Physics, Chinese Academy of Science (Shanghai, P.R. China). The ELISA kits for interferon-γ (IFN-γ), IL-12p70 and IL-17, anti-IFN-γ (R4-6A2), anti-CD3 (145-2C11), anti-CD28 (37.51), anti-CD4 (GK1.5), anti-CD8 (2.43), rat anti-mouse CD4-PE, CD8-FITC, CD11b-FITC, CD11b-biotin, CCR5-PE, rabbit anti-mouse CXCR3, avidin-Red 670 and goat anti-rabbit-FITC antibody (Abs) were all purchased from PharMingen (San Diego, CA, USA). Percoll was purchased from Amersham Biosciences (Little Chalfont, UK).

Results

Administration of TAK-779 reduced the incidence and severity of MOG-induced EAE in C57BL/6 mice

The incidence of EAE was significantly reduced in mice treated with TAK-779. All mice (100%) in the vehicle-treated group developed severe EAE, about 2 weeks after immunization (mean day of onset, 15.4 ± 0.73; Figure 1A). In contrast, only 60% of mice treated with TAK-779 showed mild signs of disease with a delay of disease (mean onset, day 17.2 ± 0.8;

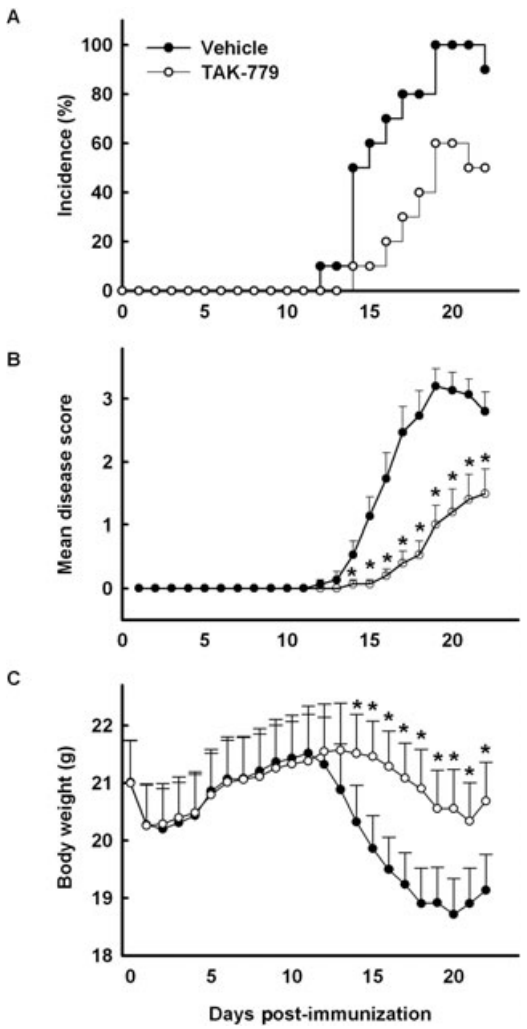

Figure 1 TAK-779 inhibited the development of experimental autoimmune encephalomyelitis (EAE) in myelin oligodendrocyte glycoprotein (MOG)-immunized C57BL/6 mice. Active EAE was induced in female C57BL/6 mice by immunization with MOG 35–55 peptide in complete Freund's adjuvant. The mice were treated with TAK-779 (150 μg per mouse) from day 0 post-immunization. The mice were monitored for signs of EAE, and the results for all mice, both healthy and sick, are presented as percentage incidence of disease (A), mean disease score (B) and body weight (C). Results are expressed as mean ± SEM, n = 10. *, P < 0.05 compared with vehicle-treated group by unpaired Student's test. Three independent experiments were performed with similar results.

$P < 0.05$; Figure 1A). Correspondingly, TAK-779 markedly decreased ($P < 0.05$) the mean severity of EAE in treated mice, with a maximum mean clinical score of 1.47 ± 0.42, compared to 3.13 ± 0.29 in the vehicle group (Figure 1B). In addition to reducing the clinical score, TAK-779 also prevented the loss of body weight in EAE mice (Figure 1C).

Treatment with TAK-779 did not reduce MOG-specific immune responses

Because EAE is a T cell-mediated disease, we next examined whether administration of TAK-779 influenced the generation of anti-MOG T cell responses. Inguinal and axillary lymph nodes were harvested from normal mice, MOG-immunized mice with or without TAK-779 treatment. Draining lymph node T cells from MOG 35–55-immunized mice with or without TAK-779 treatment showed a robust, dose-dependent proliferation in response to MOG 35–55 (Figure 2A). Interestingly, an increased proliferative response towards MOG 35–55 restimulation was observed in T cells from TAK-779 treated mice.

As CXCR3 has also been implicated in T cell production of IFN-γ (Campbell *et al*., 2004), we examined levels of IFN-γ in the supernatants from MOG 35–55-stimulated T cells. Purified T cell populations were prepared from normal or immunized mice with or without TAK-779 treatment respectively. These populations were stimulated with MOG in the presence or absence of an APC-enriched population prepared from normal mice. The results shown in Figure 2B demonstrated that in the presence of normal APC, T cell populations from MOG-immunized mice, but not from normal mice, produced high levels of IFN-γ upon stimulation with MOG *in vitro*. The IFN-γ production was not significantly increased in T cell populations from MOG-immunized mice with TAK-779 treatment.

Previous studies (Bagaeva *et al*., 2003) have shown the role of IL-12 in the development of EAE. We examined whether TAK-779 influenced the capacity of lymphocytes to produce IL-12 following MOG immunization. IL-12 was produced by APC through interactions with TCR-triggered T cells (Shu *et al*., 1995). APCs prepared from immunized mice with or without TAK-779 treatment were co-cultured with T cells from MOG-immunized mice, with or without MOG. The results show (Figure 2C) that there was no difference in IL-12 production.

T cells produce IL-17, a cytokine that plays an important role in the development of EAE (Park *et al*., 2005). Splenocytes

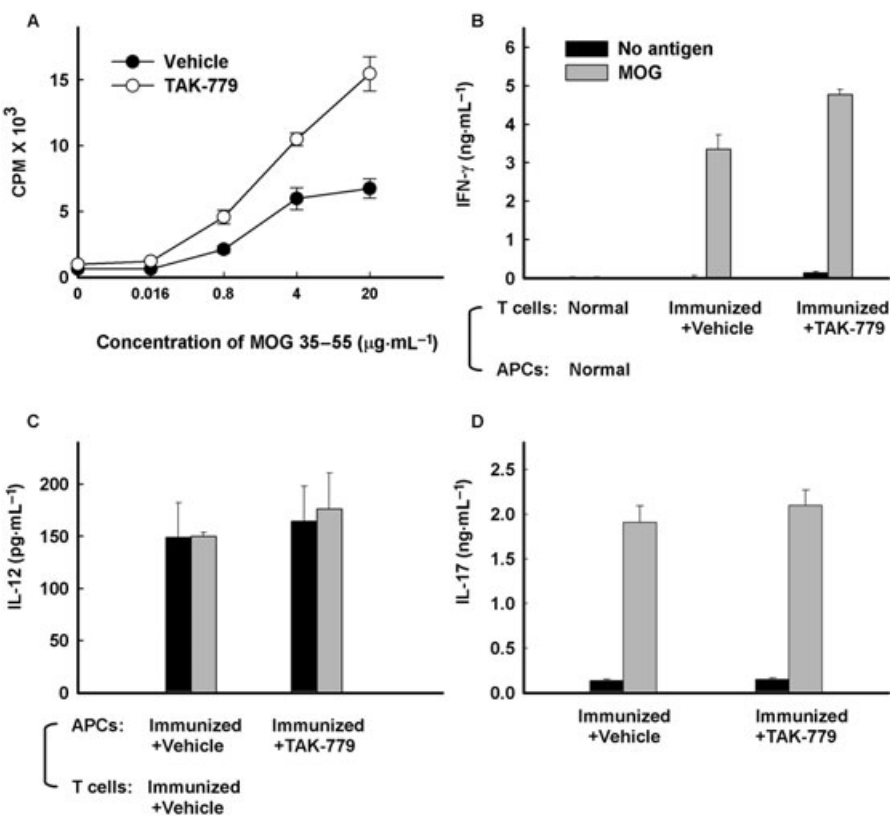

Figure 2 TAK-779 treatment did not affect myelin oligodendrocyte glycoprotein (MOG)-specific immune responses. (A) Draining lymph node cells were isolated from immunized mice treated with or without TAK-779 at day 10 post-immunization. The cells (2×10^5 cells per well) were cultured with indicated concentrations of MOG 35–55 for 72 h to assess proliferation by [^{3}H] thymidine incorporation. (B) Purified T cells (4×10^5 per well) from MOG-immunized mice with or without TAK-779 treatment were co-cultured with antigen-presenting cell (APC)-enriched cells (1×10^5 per well) from normal mice in the presence or absence of MOG (10 μg·mL^{-1}) for 48 h. IFN-γ in cell supernatants was measured by enzyme-linked immunosorbent assay (ELISA). (C) APC-enriched cells (1×10^5 per well) from MOG-immunized mice with or without TAK-779 treatment were co-cultured with purified T cells (4×10^5 per well) from MOG-immunized mice in the presence or absence of MOG (10 μg·mL^{-1}) for 24 h. IL-12p70 in cell supernatants was measured by ELISA. (D) Splenocytes (4×10^5 per well) from MOG-immunized mice with or without TAK-779 treatment were cultured for 72 h in the presence or absence of MOG (10 μg·mL^{-1}). IL-17 in cell supernatants was measured by ELISA. Results are expressed as mean ± SEM, $n = 10$. These experiments were performed three times with similar results.

were obtained from MOG-immunized mice with or without TAK-779 treatment, and then cells were tested for MOG-induced IL-17 production. There was no significant difference in IL-17 production by splenocytes (Figure 2D), between the TAK-779-treated and the vehicle-treated groups. Similar results were also observed in lymph node cells from MOG-immunized mice with or without TAK-779 treatment (data not shown).

Treatment with TAK-779 did not affect the expression of CCR5 and CXCR3 on T cells, but decreased chemokine and cytokine expression in spinal cord

It has been demonstrated that IFN-γ production as a result of T cell receptor (TCR) stimulation is an absolute requirement for induction of CXCR3 (Nakajima *et al*., 2002), and CCR5 is induced on TCR-triggered T cells depending on exposure to IL-12 (Iwasaki *et al*., 2001). Because *in vivo* TAK-779 treatment inhibited neither MOG-induced IFN-γ production in MOG-immunized T cells *in vitro* (Figure 2B), nor IL-12 production of APCs from MOG-immunized mice (Figure 2C), we examined the effects of TAK-779 treatment on the expression of mRNA for CXCR3 and CCR5 in T cells from EAE mice. As shown in Figure 3A, the expression of CXCR3 and CCR5 mRNA in purified T cells from EAE mice was unaffected by TAK-779 treatment.

Elevated expression of CCL2, CCL3, CCL4 and CCL5, and CXCL9 and CLXL10 was essential for the inflammatory infiltration of $CCR5^+$, $CXCR3^+$ and $CCR2^+$ cells (Godiska *et al*., 1995; Kennedy *et al*., 1998; Balashov *et al*., 1999; Huang *et al*., 2000). IFN-γ and IL-17 are two cytokines critical to the development of EAE. These chemokines/cytokines were produced by activated monocytes and T cells. After onset of clinical disease of EAE (13 days after MOG immunization), mRNA for all chemokines (CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10), IFN-γ and IL-17 were highly expressed in the spinal cord. The expression of CCL2, CCL3, CCL4, CXCL9, CXCL10, IFN-γ and IL-17 was significantly decreased by TAK-779 treatment, compared with untreated EAE mice (Figure 3B), but the expression of CCL5 was not changed.

We also found that the expression of mRNA for CCR2 was increased in untreated EAE mice, and this increase was reduced after treatment with TAK-779 (data not shown).

Treatment with TAK-779 inhibited the migration of CCR5 and CXCR3 bearing leukocytes including T cells to CNS

We examined whether the administration of TAK-779 influenced leukocyte migration to the CNS in MOG-immunized mice. Spinal cord sections from MOG-immunized mice following onset of EAE were analysed for the infiltration of mononuclear cells. As shown in Figure 4, the cellular infiltration was significantly less in the spinal cords of MOG-immunized mice after TAK-779 treatment, regardless of whether or not they developed clinical signs of EAE.

Next, we characterized the infiltrated leukocytes in CNS of MOG-immunized mice; infiltrated cells were harvested from spinal cords of mice and subjected to flow cytometric analysis.

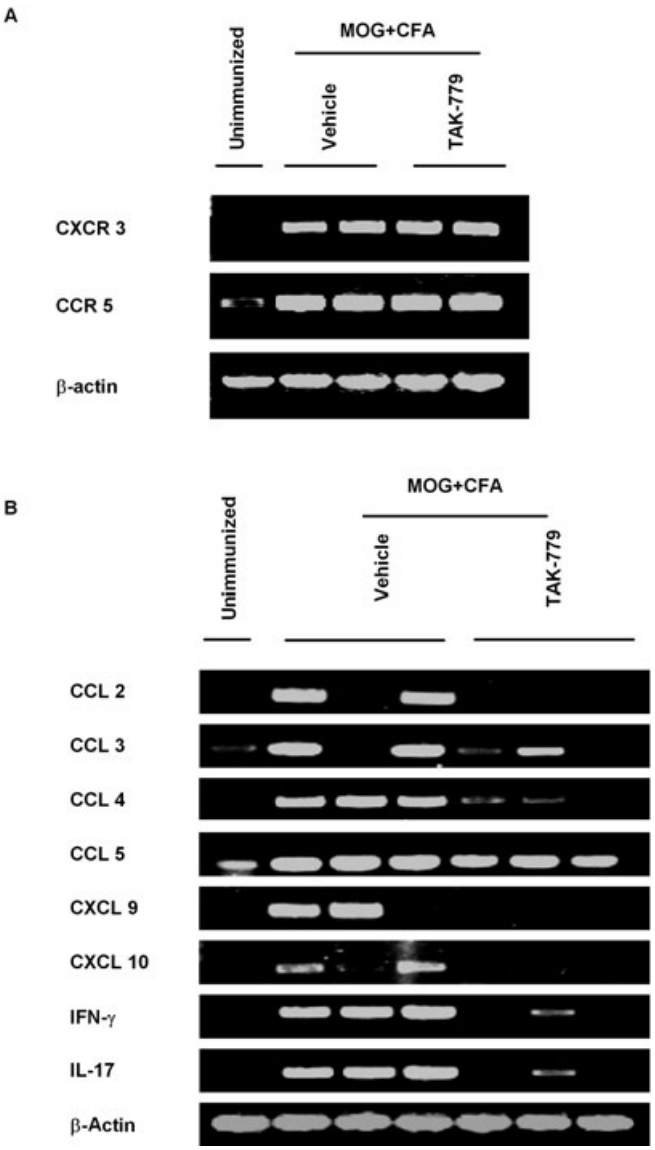

Figure 3 Expression of chemokine receptors in T cells, and of chemokine and cytokines in spinal cord. (A) Expression of CCR5 and CXCR3 in myelin oligodendrocyte glycoprotein (MOG)-immunized T cells. Purified T cells were isolated from draining lymph nodes of MOG-immunized mice with or without TAK-779 treatment at day 8 post-immunization. Expression of mRNA for CCR5 and CXCR3 was analysed by RT-PCR. (B) mRNAs were obtained from phosphate-buffered saline-perfused spinal cord of MOG-immunized mice with or without TAK-779 treatment at day 13 post-immunization. Each lane corresponds to an individual mouse. The expression of mRNA for CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, IFN-γ and IL-17 was analysed by RT-PCR. Three independent experiments were performed with similar results.

As shown in Figure 5A, large numbers of $CD4^+$, $CD8^+$ and $CD11b^+$ leukocytes accumulated in the spinal cord samples from MOG-immunized mice. In the normal (without EAE) mice, a very small number of cells could be detected in spinal cord samples, and they mainly expressed CD11b (Figure 5A,B). Administration of TAK-779 markedly decreased the number of infiltrating cells and especially decreased the proportion and number of $CD4^+$ and $CD8^+$ T cells

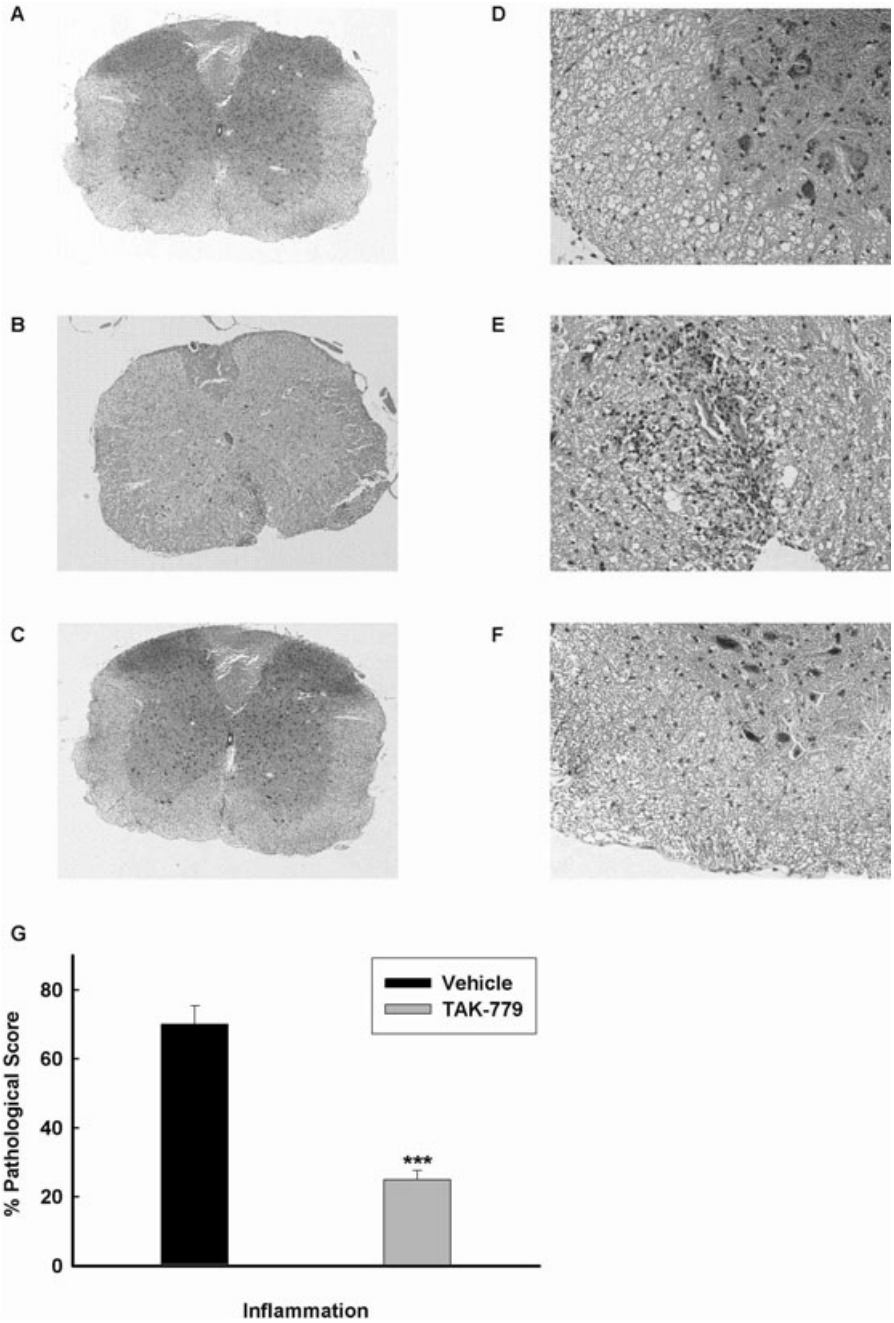

Figure 4 TAK-779 treatment inhibited cellular infiltration into the spinal cord of mice. Spinal cords were perfused by phosphate-buffered saline from myelin oligodendrocyte glycoprotein (MOG)-immunized mice treated with vehicle or TAK-779, and were subjected to histopathological examination. Normal mice (A, D), MOG-immunized mice treated with vehicle (B, E) or with TAK-779 (C, F) were killed at day 17 post-immunization. Spinal cords were harvested after extensive perfusion, and 5 μM sections were stained with H&E; magnification, A–C ×100; D–E ×400. (G) Mean scores of inflammation ± SEM, $n = 5$. ***, $P < 0.001$ compared with vehicle-treated mice (unpaired Student's test). Three independent experiments were performed with similar results.

(Figure 5A,B). To determine whether the infiltrating cells expressed CXCR3 or CCR5, the infiltrating cells were harvested from spinal cords of MOG-immunized mice and subjected to flow cytometric analysis. The flow cytometric analysis results also showed a large proportion of infiltrated cells were $CXCR3^+$ or $CCR5^+$ (Figure 5C). However, we could not detect CXCR3 or CCR5 on infiltrating cells harvested from spinal cords of MOG-immunized mice treated with TAK-779 (Figure 5C).

Then, we assayed the leukocyte lineage-specific expression of mRNA for CD4, CD8, CD11b, CCR5 and CXCR3 in the spinal cord tissues by quantitative real-time PCR. Compared with normal mice, CD4, CD8, CD11b, CCR5 and CXCR3 mRNA levels were markedly increased in the spinal cord tissues of MOG-immunized, vehicle-treated mice. TAK-779 treatment reduced the levels of CD4, CD8, CD11b, CCR5 and CXCR3 mRNA expression in the spinal cord tissues of MOG-immunized mice (Figure 5D).

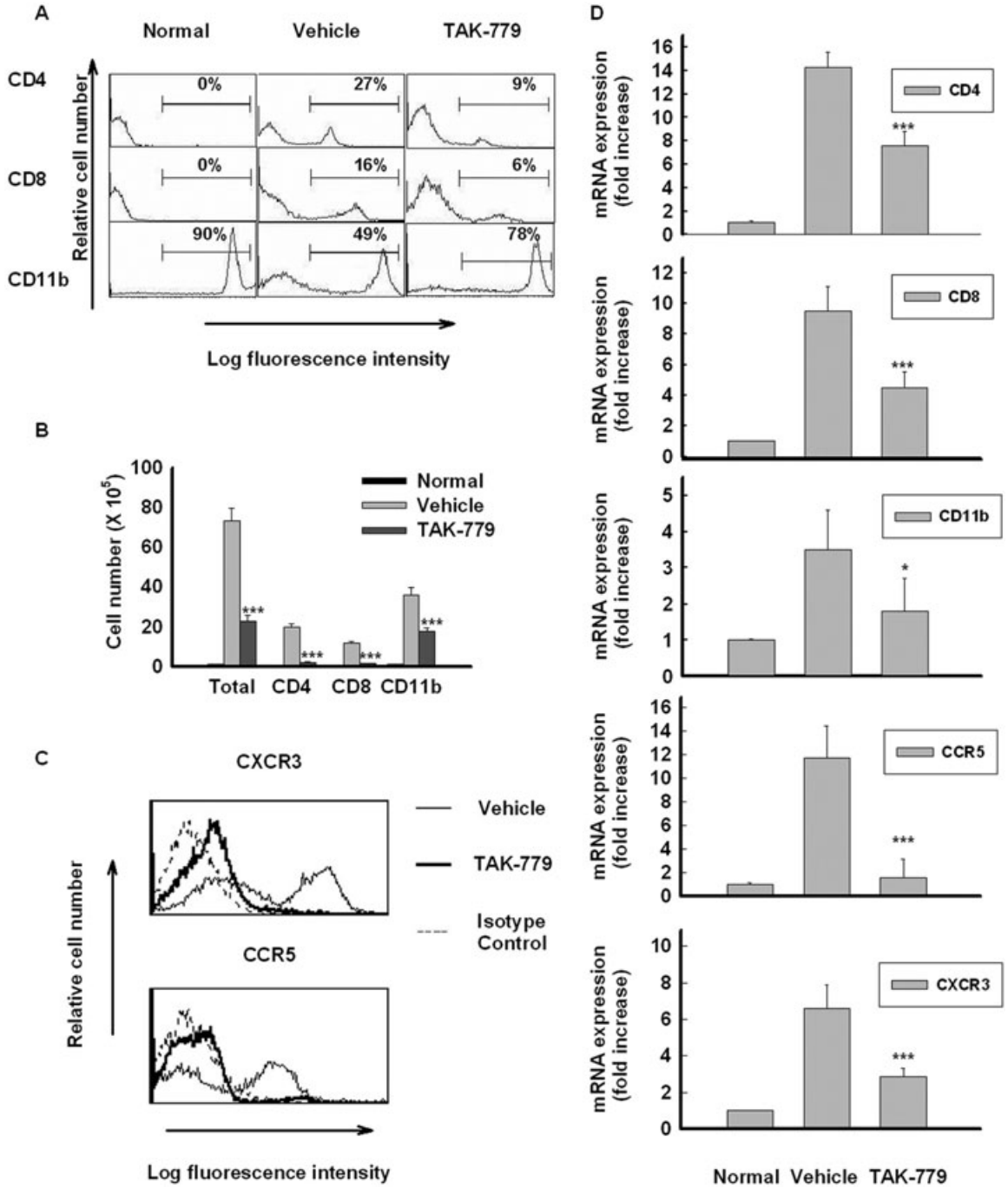

Figure 5 TAK-779 treatment decreased the infiltration of CXCR3 and CCR5 bearing leukocytes into the spinal cord. Experimental autoimmune encephalomyelitis (EAE) was induced by myelin oligodendrocyte glycoprotein (MOG) immunization. When vehicle-treated mice showed peak clinical EAE (day 19), mononuclear cells in spinal cord samples were harvested, enumerated and stained. (A) FACS analysis of infiltrating cells. (B) Number of T cells and $CD11b^+$ cells in spinal cord was decreased by TAK-779 administration. Data are expressed as total cells per mouse. The data show the average number of cells ± SEM from three independent experiments, with six mice per experiment. The average total cell number of the normal mice was $1.1 \pm 0.01 \times 10^6$, and the number of $CD11b^+$ cells was $0.99 \pm 0.009 \times 10^6$. (C) Infiltrating cells from MOG-immunized mice treated with vehicle or TAK-779 were harvested for CXCR3 and CCR5 detection by flow cytometry. (D) Expression of CD4, CD8, CD11b, CXCR3 and CCR5 in spinal cords. RNAs from PBS-perfused spinal cords of MOG-immunized mice treated with vehicle or TAK-779 were examined by real-time RT-PCR for expressions of CD4, CD8, CD11b, CCR5 and CXCR3 mRNA. Results are expressed as mean ± SEM $n = 6$. The data shown are representative of three independent experiments. ***, $P < 0.001$ compared with vehicle-treated mice.

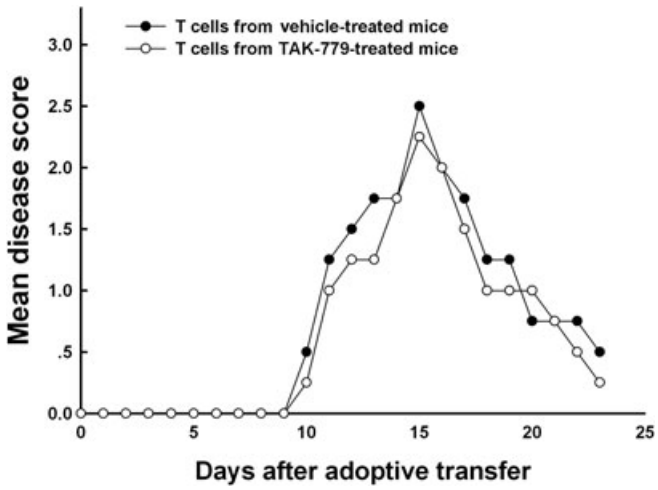

Figure 6 TAK-779 treatment did not affect the potential of T cells to transfer experimental autoimmune encephalomyelitis (EAE). Draining lymph nodes from vehicle- and TAK-779-treated mice were prepared by immunization with myelin oligodendrocyte glycoprotein (MOG) 35–55 and cells (2×10^6 per well), and were cultured with MOG 35–55 (50 $\mu g \cdot mL^{-1}$) and with murine rIL-12 (20 $ng \cdot mL^{-1}$) for 96 h. Each normal C57/BL6 mice was injected i.v. with 1×10^7 T cells. Data are presented as the EAE (disease) score in each group. Each group consisted of four recipient mice.

Treatment with TAK-779 did not affect the potential of MOG-specific T cells to transfer EAE

To determine whether TAK-779 affected the potential of MOG-specific T cells, MOG-specific T cells generated from mice treated with or without TAK-779 were compared for their ability to transfer EAE. Draining lymph nodes from vehicle- and TAK-779-treated mice were prepared by immunization with MOG 35–55, and lymphocytes were transferred following *in vitro* activation with MOG 35–55 and rIL-12 for 96 h. Each normal C57/BL6 mice was injected i.v. with 1×10^7 T cells to induce the transfer of EAE ($n = 4$). The results of this part of the experiment shown in Figure 6 demonstrated that both the recipients of T cells from vehicle-treated donors and the recipients of T cells from TAK-779-treated donors developed EAE. The onset, severity and duration of clinical disease in recipient mice were almost the same in these two groups.

Discussion

We employed the well-established murine model of MOG-induced EAE to assess the role of chemokine receptors in the pathogenesis of this autoimmune disease. We demonstrated that a large proportion of cells, infiltrating into the CNS, expressed CXCR3 or CCR5. TAK-779, a non-peptide chemokine receptor antagonist, prevented MOG-induced EAE by inhibiting the migration of inflammatory cells into the CNS (spinal cord). TAK-779 reduced both the incidence and severity of EAE. However, T cell functions or the potential of MOG-specific T cells to transfer EAE were not reduced by TAK-779 treatment. Meanwhile, the production of IFN-γ by T cells, the production of IL-12 by APCs and the expression of CCR5 or CXCR3 on T cells were all not affected by TAK-779. Migration of CXCR3/CCR5 bearing $CD4^+$, $CD8^+$ and $CD11b^+$ leukocytes into the spinal cord was inhibited by TAK-779 treatment.

These results, demonstrated by histological studies, FACS and real-time PCR analysis, indicated that TAK-779 ameliorated MOG-induced EAE by markedly reducing the migration of inflammatory cells, without affecting their function.

Many studies have shown that chemokines and their receptors play an important role in leukocyte recruitment to inflammatory sites (Cascieri and Springer, 2000; Pease and Williams, 2006). Consistent with this view, several chemokines such as RANTES, MIP-α, MCP-1, IP-10, Mig and I-TAC were identified in CNS, and simultaneously, T cells expressing CXCR3 and CCR5 were detected at the same site in EAE mice (Godiska *et al.*, 1995; Balashov *et al.*, 1999; Huang *et al.*, 2000). However, the requirement for these chemokines and chemokine receptors in the process of T cell recruitment to CNS has remained unclear. Using the CXCR3 and CCR5 antagonist, TAK-779, the present study aimed to determine the functional roles of the chemokine system in T cell recruitment.

Several reports suggested that interaction of CXCR3 and its ligands and/or CCR5 and its ligands had an important role in EAE/MS (Balashov *et al.*, 1999; Fischer *et al.*, 2000; Bagaeva *et al.*, 2003). However, gene inactivation of CXCR3 or CCR5 showed that CXCR3 and CCR5 served as negative regulators of disease onset, severity and resolution. Furthermore, CXCR3–/– or CCR5–/– mice exhibited no effect on T cell recruitment to the spinal cord during EAE (Tran *et al.*, 2000; Liu *et al.*, 2006; Muller *et al.*, 2007). In our present study, blocking both CXCR3 and CCR5 functions showed a beneficial effect on EAE, which seemed to contradict the earlier reports. This apparent discrepancy may be explained by pointing out that earlier work disrupted either of these two genes, one at a time, whereas in our study, both CXCR3 and CCR5 were blocked by TAK-779. Alternatively, it is possible that a small molecule and a genetic deletion can perturb a protein's activity in different ways, leading to different conclusions about the protein's biological function (Knight and Shokat, 2007). Furthermore, our results were consistent with other reports that blocking of chemokines or their receptors inhibited EAE (Karpus *et al.*, 1995; Arimilli *et al.*, 2000; Fife *et al.*, 2001).

EAE is a T cell-mediated autoimmune disease, but diverse types of cells are involved in the process of EAE induction, including $CD11b^+$, $CD11c^+$, $NK1.1^+$, $Gr\text{-}1^+$ and $F4/80^+$ monocytes. In addition, CXCR3 and CCR5 are expressed by T cells, as well as macrophage/monocytes. We also found a large proportion of $CD11b^+$ monocyte/macrophages expressing CXCR3 and CCR5, in the infiltrating leukocytes of the EAE mice (data not shown). $CD11b^+$ monocyte/macrophages play an important role in regulating the invasion of autoactive T cells and the demyelinating pathology in EAE and MS (Tran *et al.*, 1998). For this reason, TAK-779 could also affect the recruitment of $CXCR3^+$ and $CCR5^+$ monocyte/macrophages. Our results showed that there was an extensive mononuclear cell infiltration in spinal cord after MOG immunization and that these infiltrating cells consisted of a large number of $CD4^+$, $CD8^+$ T cells and $CD11b^+$ cells. Therefore, the recruitment and activation of T cells and monocytes/macrophages

appear to be critical steps in the pathogenesis of EAE. These events appear to constitute a self-amplification process whereby active T cells and monocytes/macrophages secrete pro-inflammatory cytokines and chemokines that recruit additional inflammatory cells, including more T cells and monocytes/macrophages into the spinal cord which, in turn, cause severe tissue damage. TAK-779 treatment interrupts this inflammatory sequence by inhibiting the infiltration of CCR5 and CXCR3-bearing leukocytes into the CNS.

Another chemokine receptor, CCR2, plays a crucial role in the pathogenesis of EAE, as mice lacking CCR2 are resistant to EAE and fail to develop mononuclear cell infiltrates. Adoptive transfer of MOG 35–55-specific T cells, lacking expression of CCR2, was able to induce EAE, whereas $CCR2^{-/-}$ recipients of wild-type T cells failed to develop disease (Fife *et al.*, 2000). TAK-779 has been shown to inhibit macrophage chemotaxis mediated by CCL2–CCR2 (Tokuyama *et al.*, 2005), and in our experiments, TAK-779 reduced the expression of CCL2 (Figure 3B) and CCR2 (data not shown) in spinal cord. As the recruitment of monocytes/macrophages to sites of inflammation is dependent on CCR2 (Boring *et al.*, 1997), the reduced number of $CD11b^{+}$ monocytes/macrophages after TAK-779 treatment might partly be due to inhibition of CCR2.

T cells from TAK-779-treated MOG-immunized mice showed a more potent recall response to MOG35-55 *in vitro* (Figure 2A). This might be caused by the different composition of T cells in lymph nodes in MOG-immunized mice, with or without TAK-779. Large numbers of T cells accumulated in the spinal cord of MOG-immunized mice, but fewer were found in spinal cords of MOG-immunized mice after TAK-779 treatment. These infiltrated T cells were highly sensitive to MOG 35–55. Treatment with TAK-779 might prevent MOG-activated T cells entering target sites such as the CNS, resulting in a greater accumulation of MOG-activated T cells in lymph nodes. Thus, these lymph node-derived T cells from TAK-779-treated mice could show an increased proliferative response towards MOG 35–55 *in vitro*. In addition to proliferation, T cells from TAK-779-treated mice had an unchanged potential to transfer EAE (Figure 6). These results indicated TAK-779 did not affect the generation and activation of MOG-specific T cells.

CXCR3 and CCR5 are both Th1-type chemokine receptors, and the Th1 type of inflammatory T cells is generally known to express CXCR3 together with CCR5. Their induction depends on the stimulation by Th1 cytokines, IFN-γ and IL-12, respectively (Bagaeva *et al.*, 2003; Campbell *et al.*, 2004). In this study, the production of IFN-γ and IL-12 was not affected by TAK-779 treatment. Moreover, the levels of CXCR3 and CCR5 mRNA in T cells from MOG-immunized mice were unchanged after TAK-779 treatment. Using adoptive transfer, T cells from TAK-779-treated EAE mice had the same potential as those from untreated EAE mice, to induce the transfer of EAE. Thus, it is clear that TAK-779 treatment did not influence T cell activation, ultimately leading to CXCR3 and CCR5 induction in MOG-immunized mice.

Our present results provide further support for the view that chemokine receptors such as CXCR3, CCR5 and CCR2 have a pivotal role in the migration of activated leukocytes into the CNS in EAE. Administration of chemokine receptor antagonists, such as TAK-779, could ameliorate MOG-induced EAE by reducing the inflammatory cells infiltrating into the CNS. Our data demonstrate an important role for specific chemokine receptors and *in vivo* cellular recruitment during the pathogenesis of EAE. They also open the possibility of using chemokine receptor antagonists for therapy of tissue-specific autoimmune disease.

Acknowledgements

This work was supported by the grants of Shanghai Science and Technology Committee (no. 08XD14053, 08DZ1980200, 064319014).

Conflict of interest

None.

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