ELSEVIER

Journal of Neuroimmunology 153 (2004) 108–121

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Sphingosine 1-phosphate receptor agonists attenuate relapsing–remitting experimental autoimmune encephalitis in SJL mice

Michael Webb*, Chui-Se Tham, Fen-Fen Lin, Karen Lariosa-Willingham, Naichen Yu, Jeffrey Hale, Suzanne Mandala, Jerold Chun, Tadimeti S. Rao

Molecular Neuroscience Laboratory, Merck Research Laboratories, 3535 General Atomics Court, San Diego, CA 92121, USA

Received 3 March 2004; received in revised form 23 April 2004; accepted 26 April 2004

Abstract

FTY720 is a prodrug for FTY-phosphate, an agonist at four of the five known receptors for sphingosine-1-phosphate (S1P). We show that administration of either FTY720 or FTY-P to SJL mice with established relapsing–remitting experimental autoimmune encephalitis (EAE) results in a rapid and sustained improvement in their clinical status, and a reversal of changes in expression of mRNAs encoding some myelin proteins and inflammatory mediators. EAE produced by adoptively transferring lymph node cells from immunized mice to naïve hosts is similarly ameliorated by FTY-P. Treatment with FTY-P is accompanied by a dose-responsive peripheral lymphopoenia.

Keywords: Relapsing–remitting EAE; FTY720; Lymphopoenia; Gene expression

1. Introduction

FTY720 is a structural analog of myriocin, a metabolite of the ascomycete fungus *Isaria sinclairia*, with some structural resemblance to sphingosine, an endogenous lysolipid. Sphingosine undergoes phosphorylation via sphingosine kinase leading to the formation of sphingosine-1-phosphate (S1P), the cognate ligand for the family of S1P receptors (S1PR). Activation of S1P receptors results in a plethora of physiological actions such as chemotaxis, cellular differentiation, survival and growth, and regulation of actin-based cytoskeletal reorganization which leads to cell adherence and cell shape changes (Goetzl and An, 1998; Chun, 1999; Fukushima et al., 2001).

FTY720 is a novel immunosuppressive agent which is active in various animal models of graft rejection and autoimmune disease, including graft versus host disease, type 1 diabetes, and rheumatoid arthritis. (Chiba et al., 1996; Suzuki et al., 1996a,b, 1998; Masubuchi et al., 1996; Matsuura et al., 2000). It is currently under development as an immunosuppressive agent for transplantation. It was thought initially that the mechanism of action was through induction of apoptosis in T lymphocytes (Nagahara et al., 2000; Suzuki et al., 1996a,b, 1997), and one effect of in vivo treatment with FTY720 is a profound lymphopoenia in the peripheral blood, with lymphocyte cell counts falling to as low as 5–10% of control levels at therapeutic doses of the compound. However, therapeutic effects are achieved in rats at doses of <1 mg/kg, at which the plasma concentrations are about two orders of magnitude lower than those required to drive T cell apoptosis in vitro (Yanagawa et al., 1998). A further argument against a role of apoptosis in most in vivo situations is provided by the observation that adoptively transferred fluorescently labeled lymphocytes disappear from the peripheral circulation on FTY720 treatment but reappear when drug treatment is discontinued (Pinschewer et al., 2000). It now appears that at least one of the mechanisms by which FTY720 achieves its effects in vivo is by a sequestration of circulating lymphocytes in peripheral lymph nodes (Pinschewer et al., 2000; Brinkmann et al., 2000, 2001a,b; Mandala et al., 2002, Xie et al., 2003).

FTY720 is a substrate for sphingosine kinase-2 (Sanchez et al., 2003) and phosphorylation in vivo (Mandala et al., 2002) has been demonstrated. The resultant ester (FTY-P) has a structure similar to sphingosine-1-phosphate, which is the preferred ligand at a group of G protein coupled

* Corresponding author. Tel.: +1-858-638-7957.
E-mail address: xhua@san.rr.com (M. Webb).

0165-5728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2004.04.015

receptors with five known members (S1P receptors, S1PR). Mandala et al. (2002) showed that FTY720 itself has weak or no activity at any of these receptors but that the phosphate ester is an agonist with low nanomolar potency at four of the five receptors, and these observations were further substantiated by Brinkmann et al. (2002). A nonhydrolysable phosphonate analogue of FTY-P retained sufficient potency at the four S1P receptors to have in vivo efficacy in a lymphopoenia assay. FTY720, sphingosine-1-phosphate, and the phosphonate analogue of FTY-P all caused a rapid and reversible peripheral lymphopoenia in rats and mice, reaching a nadir at 4 h postinjection. The potency of compounds in this assay reflected their intrinsic affinities at the four receptors, indicating that one or more of these receptors is indeed the molecular target of these compounds (Mandala et al., 2002). Recent reports using selective agonists demonstrate that S1P1 is the target for lymphopenia (Forrest et al., 2004; Sanna et al., 2004). Moreover, lymphocytes genetically deleted for S1P1 have thymic emigration and recirculation defects similar to that achieved with the receptor agonists, suggesting that S1P1 is required for egress (Matloubian et al., 2004) and that the agonists induce lymphopenia by downregulating S1P1 on lymphocytes (Graler and Goetzl, 2004).

Two papers have appeared indicating that FTY720 is active in rodent models of experimental autoimmune encephalitis (EAE), an animal model of multiple sclerosis. Brinkmann et al. (2002) treated Wistar rats with FTY720 (0.3 mg/kg/day) from the day of induction of EAE (day 0) in Wistar rats. They showed that rats thus treated did not develop EAE in this monophasic model. In a different acute monophasic rat model, in which myelin basic protein is used as the immunogen in Lewis rats, Fujino et al. (2003) showed once again that dosing rats orally from day 0 almost completely suppressed the development of disease. This was associated with a marked reduction in the number of T lymphocytes infiltrating the spinal cord and a reduction in the levels of the TH1 cytokines IL-2, IL-6, and interferon gamma. While these data are impressive, the efficacy of FTY720 when given at the onset of clinical symptoms or on established EAE is unknown. In the present investigation, we have examined the efficacy of FTY720 and its phosphate ester on an established disease state in the relapsing–remitting EAE model in SJL mouse (SJL rr-EAE), a model which mimics several features of human MS.

EAE can be induced in SJL strain mice by immunizing them with a peptide fragment of the myelin protein, proteolipid protein (PLP), together with pertussis toxin (PTX) treatment (McRae et al., 1992). The resulting EAE shares many features with human multiple sclerosis. It is a chronic disease from which the mice never recover and has a relapsing–remitting pattern similar to that of the major form of human multiple sclerosis (McRae et al., 1992, 1995). In addition to the infiltration of T lymphocytes into the brain and the spinal cord (and unlike the common rat models), these mice also exhibit both demyelination and also axonal damage (Sobel et al., 1990; Marracci et al., 2002). Demyelination has for many decades been regarded as the hallmark of multiple sclerosis, but in recent years, the importance of the axonal damage and eventual neuron loss as the key processes underlying the relentless progression of the disease has been recognised (Trapp et al., 1998, 1999; Wujek et al., 2002). In addition to confirming these features of the SJL-rr model, we also show that in this EAE model, significant changes in the quantitative expression of mRNAs encoding both myelin-related proteins and mediators such as granulocyte-macrophage colony stimulating factor (GM-CSF) and inducible nitric oxide synthase occur.

In this study, we characterise the effects of FTY720 and its phosphorylated derivative on the clinical state, and levels of circulating lymphocytes in the SJL mouse rr-EAE model. Initiating dosing just prior to the onset of clinical signs delays and blunts the first phase of the disease as previously reported in the rat models. More significantly for the clinical setting, if treatment with these compounds is delayed until the peak of the first phase of disease, there is an immediate and rapid improvement in the clinical status of the animals which is maintained for as long as dosing is continued. Treatment with FTY-P is associated with a partial reversal in the gene expression changes seen in untreated animals with the disease. We also show that there is a correlation between the magnitude of clinical improvement and the levels of lymphopoenia achieved at any given dose of FTY-P, but this correlation is incomplete, indicating that nonselective S1P receptor agonism may exert its effects in this model by additional mechanisms. FTY-P is also effective in improving clinical status and preventing mortality in an adoptive transfer version of the disease.

2. Materials and methods

2.1. Experimental autoimmune encephalitis

2.1.1. SJL mouse

Female SJL mice (6–9 weeks old) were obtained from the Jackson laboratory (Bar Harbor, ME). They were housed in a 12-h light / dark cycle with access to food and water ad lib. All animal procedures were conducted in accordance with protocols approved by the local animal care committee.

2.1.2. Active immunization

EAE was induced following the methods of McRae et al. (1992). A 20-mer peptide based on the mouse proteolipid protein (PLP) sequence 139–151 (His–Ser–Leu–Gly–Lys–Trp–Leu–Gly–His–Pro–Asp–Lys–Phe (custom synthesized by American Peptide, Sunnyvale, CA, 98% purity) was used to induce EAE. Peptide was dissolved at 5 mg/ml in distilled water and emulsified with an equal

volume of Freund's adjuvant containing 5 mg/ml of H37Ra *M. tuberculosis* (Difco) following the procedure of Stevens et al. (1999). On the day of initiation of EAE, referred to as day 1 of the experiment, mice received a single injection of 100 ul of emulsion subcutaneously in a skin fold at the back of the neck. On day 1, they were also given 250 ng of pertussis toxin (List Biological Labs, Campbell, USA) from a solution of 50 ug/ml in 0.01 M sodium phosphate buffer pH7.0 with 0.9% NaCl intraperitoneally. This injection was repeated on day 3. Control animals received an injection of water/Freund's emulsion and both pertussis injections.

Mice were weighed daily and assessed using a clinical scale as follows: 0 = healthy mouse, 1 = flaccid tail, 2 = hind limb weakness, 3 = paralysis of one or both hind limbs, 4 = forelimb paralysis, and 5 = death. Animals were euthanased if they reached a score of 4. Mice which appeared to be dehydrated were given 1 ml of saline ip per day, and cages containing mice with clinical scores of above 2 were provided with wet chow on the cage base to ensure that animals could reach their food.

Drugs and experimental compounds were given by intraperitoneal injection or by oral gavage as appropriate. FTY-P was synthesized at the Merck Research Laboratories, Rahway. Mitoxantrone was purchased from Calbiochem as a 10-mg/ml solution and was diluted for use in saline.

In some experiments, animals were bled retroorbitally at a frequency not greater than once per week to provide blood samples for lymphocyte counting. At the termination of experiments, tissues (brain, spinal cord, liver) were taken and snap frozen on dry ice for biochemical analysis. In some cases, tissues to be used for RNA preparation were collected and stored overnight at 4 °C in an RNA preservative solution (RNAlater, Qiagen), and then placed at −70 °C until RNA preparation.

2.1.3. EAE induction in SJL/J mice via adoptive transfer of PLP-reactive lymphocytes

EAE was passively induced by adoptive transfer of PLP-reactive lymphocytes. Thoracic and popliteal lymph nodes were collected 11–12 days after immunization of donor mice with PLP in CFA. Free lymph node cells were obtained by mechanically dissociating the nodes, and red blood cells were hypotonically lysed by a 2-min treatment in 0.144 M (0.95%) ammonium chloride, 17 mM Tris–HCl, pH7.4. These cells were cultured in Click's medium (EHAA) supplemented with 10% foetal calf serum and 50 uM beta mercaptoethanol. Cells were stimulated for 48–72 h in the presence of 25 ug/ml $PLP_{139-151}$ or BSA as a negative control. Proliferation of the stimulated cells was confirmed by [^{3}H]- thymidine incorporation and microscopically by the appearance of blasts. Recipient SJL mice were injected intraperitoneally with PLP-reactive lymphocytes (6–12.5 million cells/mouse in a volume of 0.5 ml phosphate buffered saline per mouse). Clinical evaluation of EAE was conducted using the methods described above.

2.2. Lymphopoenia assays

The lymphopoenia assay was performed using an in vitro microprocessor controlled automatic blood analyzer instrument (H-2000 Hematology Analyzer (Hospitex Diagnostics LP, Webster, TX) using whole blood samples.

Whole blood from EAE animals was collected by retroorbital bleeding, using EDTA as an anticoagulant. The blood was used within 4 h at room temperature for cell counting. Lymphopoenia was defined in our assay as the reduction of lymphocyte cell density (cells/μl) as compared to PLP control vehicle. Data were expressed by plotting cell density as a function of time (days in vivo) and analyzed using software Prism™ 3.0 (San Diego, CA) All values are expressed as mean ± standard error of mean (S.E.M.).

2.3. Molecular biology

2.3.1. Isolation of total RNA and reverse transcription

Total RNA from mouse spinal cord, brain, lung, and heart (LPS-treated) was isolated using an RNeasy® Protect Midi Kit (Qiagen). Total RNA of PLP-treated mouse lymphocytes was isolated using an RNeasy® Protect Mini Kit (Qiagen). RNA was treated with RNase-free DNase I to remove genomic DNA contamination. Total RNA (1.2 μg) from each was reverse transcribed using a RETROscript™ Kit (Ambion). In each reverse transcription reaction, a reaction-omitting reverse transcriptase was included for the assessment of genomic DNA contamination.

2.3.2. Cloning, construction, and DNA sequencing of mini genes

The cDNA encoding myelin components (MAG, PLP, MBP, MOG, and CNPase) and β actin was amplified from mouse brain with polymerase chain reaction (PCR) techniques. The cDNA encoding GM-CSF and iNOS was amplified from mouse lung and LPS-induced heart, respectively. For cloning of mini genes for myelin components, iNOS and β actin, 3 μl of cDNA was amplified with 2.5 U of *PfuTurbo*® DNA polymerase (Stratagene), 200 nM dNTP, and 10 pmol of each primer in a total volume of 50 μl for 30 cycles in GeneAmp® 9700 thermocycler (ABI). Each cycle consisted of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 75 °C. GM-CSF mini gene was amplified from 3 μl of cDNA using 2.5 U of Platinum® *Taq* DNA polymerase High Fidelity (Invitrogen), 200 nM dNTP, 2 mM $MgSO_4$, 1 × High Fidelity PCR buffer, and 10 pmol of each primer in a total volume of 50 μl. PCR was performed using a GeneAmp® 9700 thermocycler (ABI) with incubation at 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 45 s for 30 cycles. The PCR-amplified fragments were subcloned into PCR cloning vectors, pCR®–BluntII-TOPO®, or pCR®II–TOPO® (Invitrogen) using a PCR Cloning Kit (Invitrogen). The integrity of these genes was confirmed by sequencing using an ABI 3100 automated fluorescence

sequencer (ABI). The sequence was analyzed using software Sequencher.

2.3.3. TaqMan® PCR

Quantitative real-time PCR was carried out using an ABI Prism 7900 sequence detector on 1 μl of cDNA samples using 900 nM each primer, 250 nM TaqMan probe, and 25 μl of TaqMan® Universal PCR Master Mix, in a total volume of 50 μl. PCR was carried out with incubation at 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Primers used for these genes were designed from sequences in the Genebank database (Table 1). Additional reactions were performed on each 96-well plate using a known dilution of DNA from mini genes or cDNA as PCR template for constructing a standard curve relating threshold cycle to cDNA concentration. Data were analyzed using software SDS2.0. All data were normalized to β actin and expressed as %control.

2.4. Statistics

All values are expressed as mean ± standard error of mean (S.E.M., $n=4$ individual animals each with 2–3 replicates). Data were analyzed by analysis of variance (ANOVA) followed by post hoc analysis (Neuman–Keul's test; Prizm 3.0, GraphPad, San Diego, CA) and statistical significance inferred at a $p \leq 0.05$.

3. Results

3.1. EAE in SJL mice

Mice injected subcutaneously with a single dose of PLP emulsified in CFA, together with intraperitoneal doses of PTX on the first and third days, began to lose body weight and manifest clinical signs at about day 7. The decline in body weight and increase in clinical scores occurred rapidly, reaching their maxima at about day 14. At this point, mice had typically lost about 30–35% of their body weight, and mean clinical scores were usually about 3. In our hands, the subsequent evolution of the disease showed some interexperiment variations. In some experiments, the first phase of disease would remit to a low clinical score by about day 25, with a slow gain of body weight to about 90% of control values. In those cases where such a clear remission from first phase was observed, mice spontaneously relapsed with subsequent bouts of severe disease, separated by intervening remissions (Fig. 1A). In other experiments, the relapsing–remitting pattern was less obvious, and after a clear first phase with a lower degree of remission, the mean clinical scores showed a chronic secondary type of progression (Fig. 1B).

In both of these patterns, the clinical scores (during remission in the case of clearly rr-EAE) became progressively higher as the disease advanced. After the first phase,

Table 1
Primer and probe sequences used for mouse Taq-Man experiments

Gene	Accession #	Forward primer (F)/TaqMan® probe (TM)/Reverse primer (R)
PLP	M15442	F: AGCGGGTGTGTCATTGTTTG TM: 5′AAACTTGTCGGGATGTCCTAGCCA R: 5′ACAACAGTCAGGGCATAGGTGAT
MBP	M11291; XM_129053; M15062	F: 5′GACCCAAGATGAAAACCCAGTAGT TM: 5′CATTTCTTCAAGAACATTGTGACACCT R: 5′TTGGGATGGAGGTGGTGTTC
MAG	NM_010758	F: 5′CGCACGGTGGAGCTGAGT TM: 5′TCATGTATGCACCTTGGAAGCCC R: 5′CCACCACCGTCCCATTCA
MOG	U64572	F: 5′TGTAGGCCTTGTATTCCTCTTCCT TM: 5′ACGAAGTTTTCCTCTCAGTCTGTGCTG R: 5′GTCCGATGGAGATTCTCTACTTCTG
CNPase	M31810	F: 5′TGTGCTGCACTGTACAACCAAAT TM: 5′CACCACCTCCTGCTGGGCGTATTCT R: 5′AGGCCTTGCCATACGATCTCT
iNOS	U43428	F: 5′AAATCCCTCCTGATCTTGTGTTG TM: 5′ACTCGTACTTGGGATGCTCCATGGTCA R: 5′CAACCCGAGCTCCTGGAA
GM-CSF	X02333	F: 5′AGAAGTCGTCTCTAACGAGTTCTCCTT TM: 5′CGGGTCTGCACACATGTTAGCTTCTT R: 5′GTAGACCCTGCTCGAATATCTTCAG
β actin	X03672	F: 5′CGATGCCCTGAGGCTCTTT TM: 5′CCAGCCTTCCTTCTTGGGTATG R: 5′TTTCATGGATGCCACAGGATT
INFγ	K00083	F: 5′GCATAGATGTGGAAGAAAAGAGTCTC TM: 5′CATCCTTTTGCCAGTTCCTCCAGA R: 5′GCTCTGCAGGATTTTCATGTCA

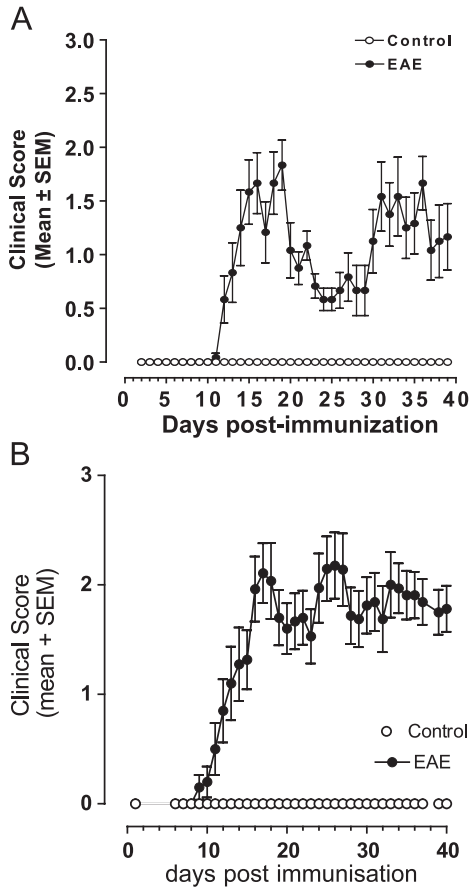

Fig. 1. Representative time course of clinical evolution of EAE in two separate experiments. (A) A typical relapsing–remitting time course is seen for animals induced for EAE by immunization with PLP and treatment with pertussis toxin. (B) A less clear remission is seen after phase 1, and the animals remain chronically sick at a clinical score of about 2.

body weight was little affected during subsequent relapses. Mortality was seen at levels which varied between experiments from between about 10% and 30%, but almost all mortality occurred in the first phase, and animals surviving this phase were unlikely to die subsequently.

3.2. Gene expression changes during EAE

We used the Taq-Man technique to examine quantitative changes in the expression of several myelin-related and inflammation-related genes during the course of EAE. In these studies, gene expression was initially expressed as a percentage of the expression of the β actin-encoding gene, whose expression was essentially constant between different samples. We then derived a final value for the expression in EAE samples by expressing the values as a percentage of the levels observed in disease-free CFA/PTX-treated controls taken at the same time. Fig. 2A represents the clinical evolution of the disease in the EAE experiment from which these time course samples were derived. There was, as was always observed in this model, a clear first phase, beginning at about day 7 and peaking with a mean clinical score of 2 at day 15, and this was followed by a relatively small remission to a mean score of about 1.25 by day 20, at which point the animals remained stable until day 30. At day 30, the clinical scores worsened, but in this experiment, a chronic secondary phase was seen without further evidence of clear relapses and remissions.

We studied the expression of the myelin-related proteins PLP, MBP (Fig. 2B and C), and also CNPase, MAG, and MOG (data not shown). The change in expression of the latter three mRNAs was very similar to that shown for MBP and PLP. The first phase of the disease was marked by a profound reduction in the level of these mRNAs, all of which fell to 26% or less than control levels at day 14. There was a subsequent recovery of expression at day 27, somewhat prior to the minor improvement in clinical score that began at day 30. Thereafter, expression levels again declined at day 44, at which time the clinical status was slowly worsening.

The expressions of the three inflammatory mediators IFNγ, iNOS, and GM-CSF were similarly markedly elevated during the first phase (Fig. 2D–F) and significantly reduced in the first small remission. In all three cases, they remained somewhat higher during the later phases of the disease, with GM-CSF showing the largest elevation (>1000-fold at all time points) over control levels at these later phases.

3.3. Clinical effect of mitoxantrone, FTY720, and FTY-P

We examined the effect in this model of mitoxantrone, a clinically used drug. Because of inherent toxicity, we were able to dose only for 6 days. When the drug was given for 6 days from day 6–12, it delayed the onset of disease and blunted the severity of the first phase, but at later time points, there appeared to be little difference in the condition of these animals from that of untreated controls (Fig. 3).

We initiated once-daily dosing of mice with FTY720 at 3 mpk, beginning at day 7, 14, or 25 after immunization. Animals dosed from day 7 showed a delay in onset of clinical signs of a few days, and the severity of the first phase was blunted compared with control mice (Fig. 4A). The degree of weight loss in this group was also reduced as compared with vehicle-treated mice. After resolution of this mild first phase, the mice remained at a very low and stable clinical score for the duration of the compound dosing. Much more dramatic effects were seen if doing was initiated at the peak of the first phase, when clinical scores were at their highest (Fig. 4B). A rapid improvement in the clinical score was initiated and maintained over several days, until by day 22, the mice had reached a similar low score as the mice treated from day 7. In this experiment, the remission at day 25 in the untreated control animals was relatively modest, and the clinical scores of mice which received FTY from this point declined to a level significantly below

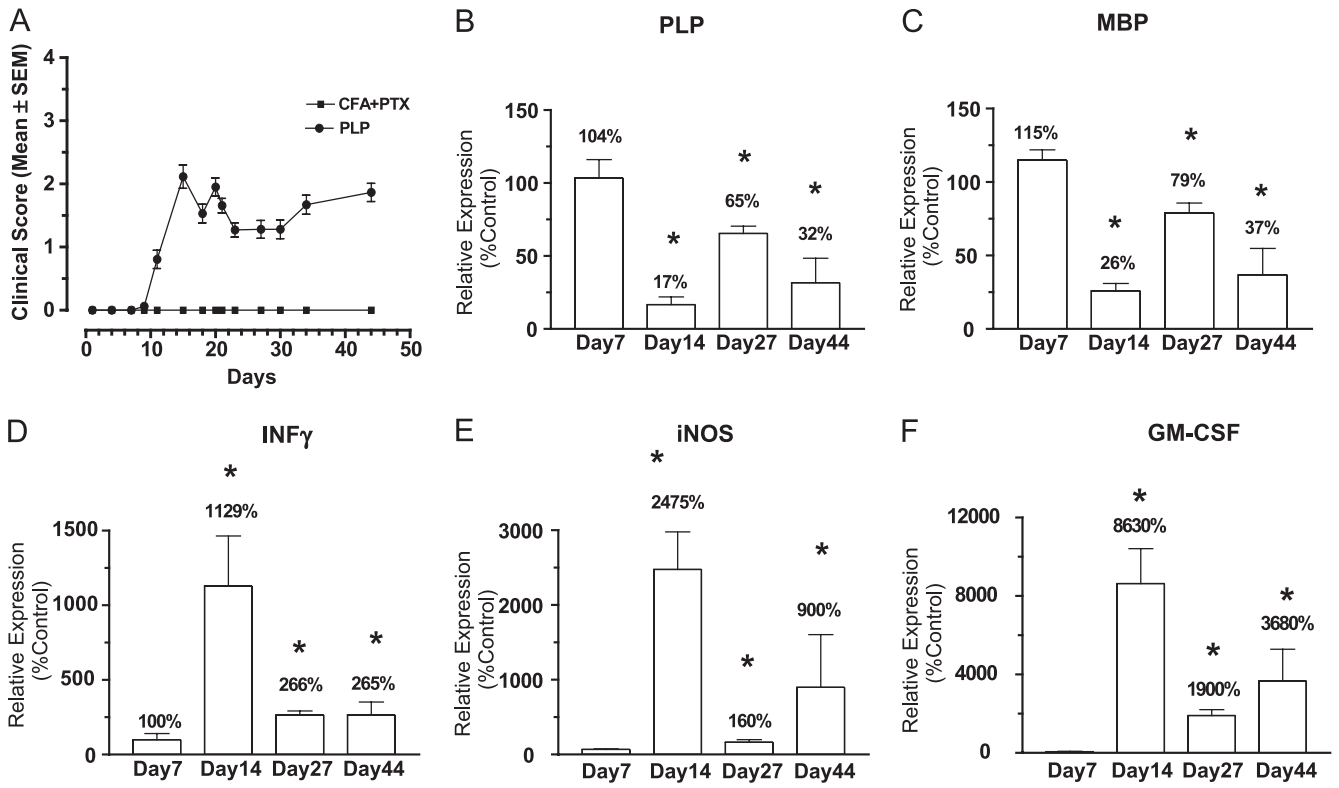

Fig. 2. Gene expression changes during development of EAE. (A) Time course of EAE experiment yielding samples for gene expression studies. (B–F) Taq-Man quantitation of changes in spinal expression of the myelin-related genes for proteolipid protein (PLP; B), myelin basic protein (MBP; C), and for the inflammatory mediators interferon gamma (D), iNOS (E), and GM-CSF (F). Individual samples were initially expressed as a percentage of β actin expression determined in the same Taq-Man reaction and then finally expressed as percentages of the values for the same genes obtained from CFA and PTX treated non-EAE control samples at the same time point. Values are means ± S.E.M., $n = 4$. Statistical significance is calculated by reference to the day 7 time point in each data set.

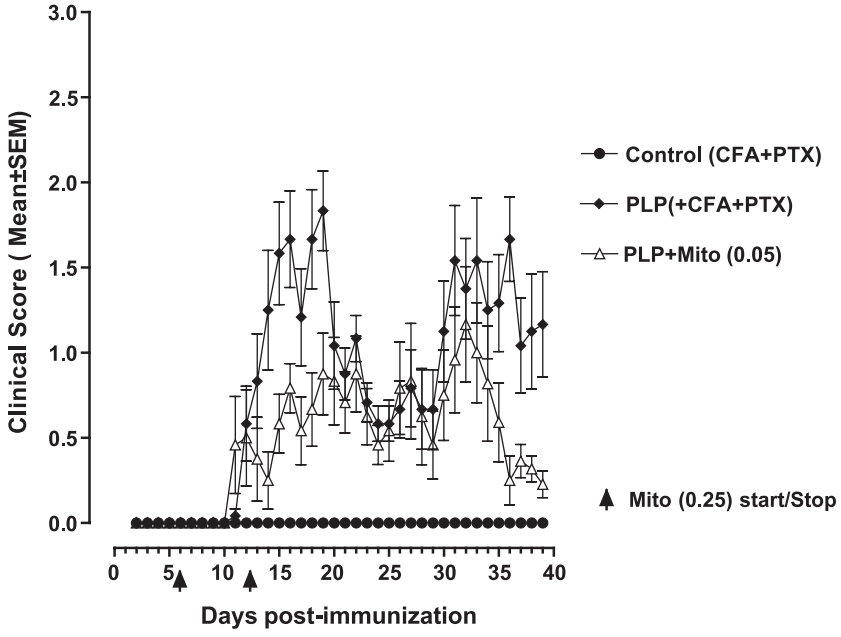

Fig. 3. Effect of mitoxantrone (0.25 mg/kg, ip once daily between days 6 and 12) on clinical evolution of EAE.

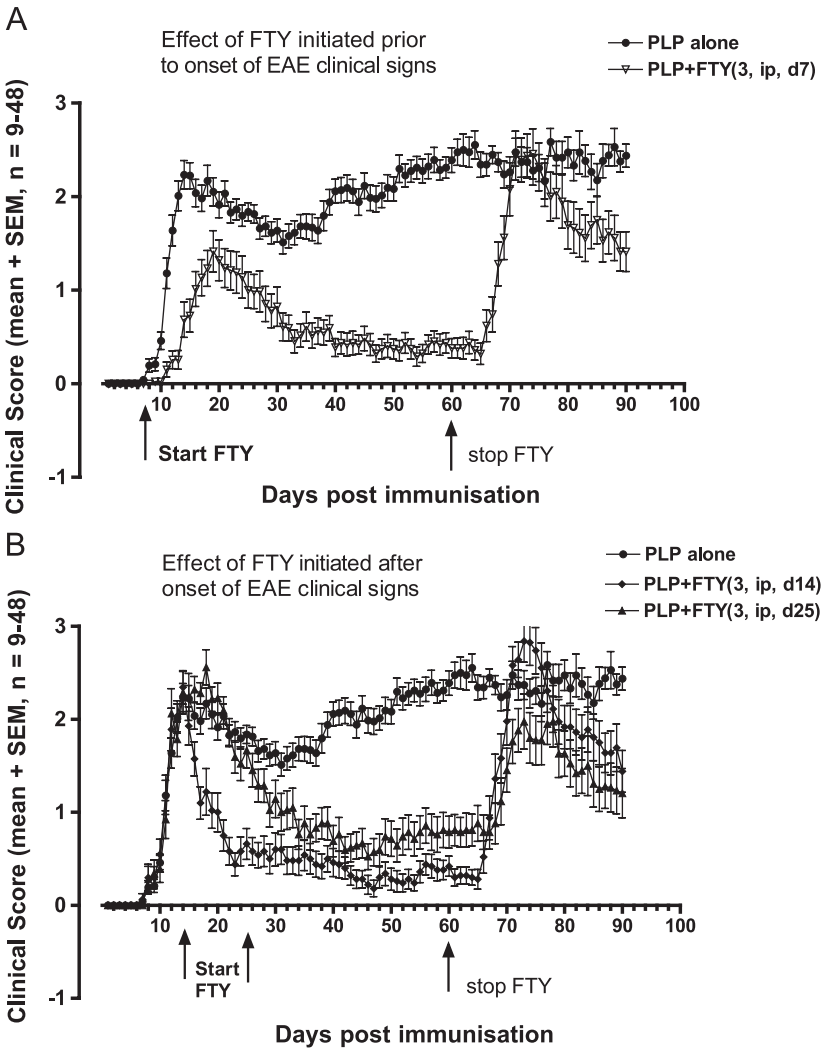

Fig. 4. Effect of once-daily intraperitoneal dosing with FTY720 initiated at different times in the development of the disease. (A) FTY720 (3 mg/kg) was given daily by ip from day 7 until day 60. Dosing was resumed at day 70. (B) FTY720 (3 mg/kg) was given daily by ip injection between days 14 or 25 until day 60. Dosing was resumed at day 70.

the lowest achieved by nondosed vehicle-treated controls. Once again, on continued dosing, the mice were stably maintained at this low level of clinical sign. FTY720 dosing was interrupted for a 10-day period at day 60. After a delay of a few days, clinical signs reappeared in mice in all treatment groups, which relapsed over a period of days to similar disease scores to those seen in untreated controls. On resumption of dosing, these signs once again began to ameliorate. When dosing was initiated at day 25, at which point the animals were expected to have fully remitted from the first phase, the clinical scores were again reduced to a level much below that of untreated controls and then maintained (Fig. 4B), although the eventual maintained clinical scores were not quite as low as when dosing was initiated at day 14 or earlier.

It has been shown (Mandala et al., 2002) that the effects of FTY720 are a result of the generation of the metabolite FTY-P, which is an agonist at four of the five known S1P receptors. We therefore used FTY-P in further experiments to examine the dose response for clinical efficacy and peripheral lymphopoenia, and the relationship between these two phenomena. In initial experiments, FTY-P was given from day 14 to mice induced for EAE and cumulative clinical scores were determined.

The pattern of clinical response was similar to that described for FTY720 itself and was dose responsive. There was a rapid initial improvement in clinical scores of the mice in the highest dosage group, with lower doses giving intermediate effects. On cessation of dosing at day 25, there was an initial delay of about 2 days, after which the mice rapidly relapsed to disease scores similar to or more severe than those seen in untreated controls (Fig. 5A). Clinical scores were added cumulatively during the dosing period, and the data indicate that the level of clinical efficacy obtained was proportional to the dose of FTY-P administered (Fig. 5B).

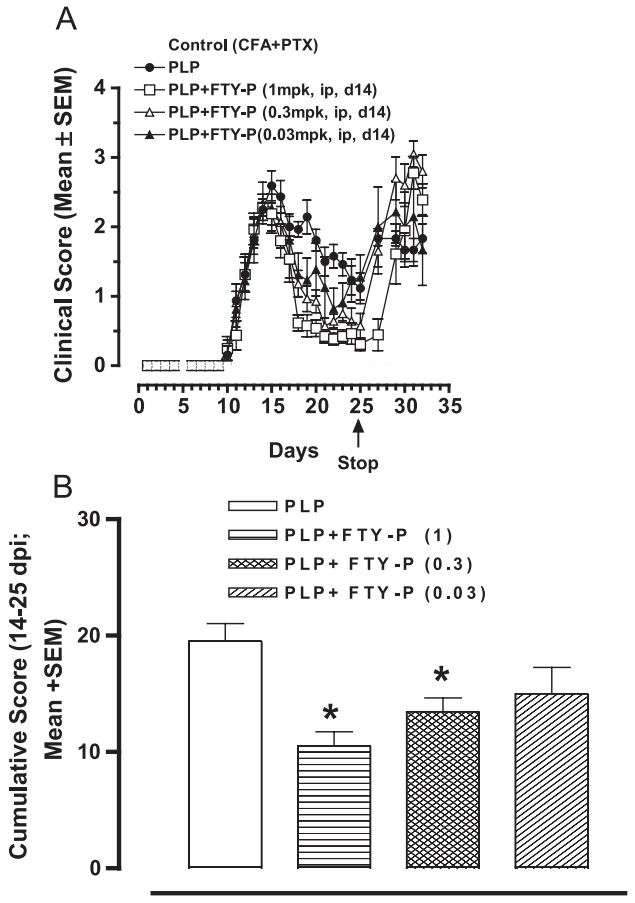

Fig. 5. Effect of FTY-phosphate on clinical state of mice induced for EAE. (A) Time course of EAE. FTY-P was given daily by ip injection between days 14 and 25 at 0.03, 0.3, or 1 mg/kg. (B) Cumulative clinical scores between days 15 and 25 for mice treated daily with 0.03, 0.3, or 1 mg/kg FTY-P between days 14 and 25.

3.4. Lymphopoenia in FTY-treated mice

A reversible sequestration of lymphocytes in peripheral lymph nodes has been described in animals treated with FTY720 (Chiba et al., 1998; Pinschewer et al., 2000; Mandala et al., 2002; Xie et al., 2003). This sequestration has been proposed to be at least one of the mechanisms by which FTY720 achieves its therapeutic efficacy. We therefore measured the changes in numbers of lymphocytes in peripheral blood of the mice receiving the FTY-P throughout the dosing regimen. As has been reported previously, (Sewell and Andrews, 1989), treating mice with pertussis toxin, as was done routinely in the EAE induction protocol, results in a large increase in the levels of circulating peripheral lymphocytes, probably because these cells are unable to home to the peripheral lymphoid organs. Densities of circulating lymphocytes were approximately 7000/mm^3 in naïve animals. Fifteen days after PTX treatment, these levels were substantially elevated and continued to fall until reaching normal levels about 30 days after PTX treatment. Actively immunized animals showed somewhat higher levels of peripheral lymphocytes when compared with sham-immunized (but PTX-treated) controls (Fig. 6A). Because of the profound effect of PTX treatment alone in elevating numbers of circulating lymphocytes, in subsequent experiments, we expressed the peripheral lymphocyte density data as a percentage of the lymphocyte in drug-naïve PLP/PTX-treated controls at each time point. After the correction for the effect of pertussis toxin, we found an FTY-P dose-dependent decrease in the numbers of peripheral blood lymphocytes which was first detectable 24 h after the first dose at the highest concentration (Fig. 6B). Five days after initiation of

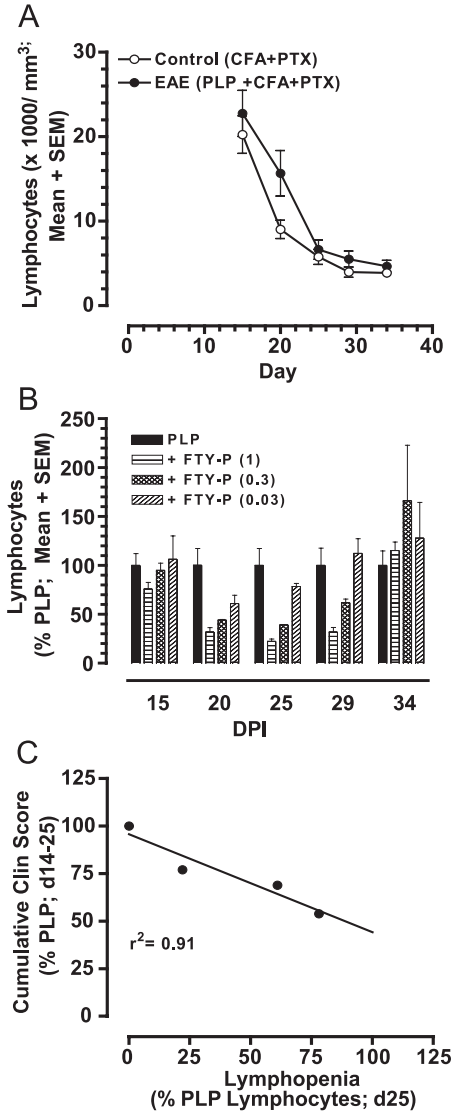

Fig. 6. Lymphopoenia produced by FTY-phosphate treatment. (A) Time course of effect of pertussis toxin treatment with or without PLP immunization on numbers of peripheral lymphocytes in blood samples. (B) Changes in numbers of peripheral blood lymphocytes (expressed as percentage of PTX treated but nondiseased controls at the same time point) at days 15, 20, 25, 29, and 34 in mice treated daily with FTY-P (0.03, 0.3, 1 mg/kg) between days 14 and 25. C. Dose–response correlation between cumulative clinical scores (days 15–25) and lymphopoenia for mice treated with FTY-P.

dosing, a dose-dependent lymphopoenia was seen in all groups, with levels of peripheral lymphocytes continuing to fall in the two highest dose groups until day 24. Lower doses produced a lower magnitude of effect at all time points. At all doses, the nadir was attained by day 25, where the maximum depletion at the highest dose was about 75% of control levels. Cumulative clinical scores for the period 14–25 days correlated with the level of lymphopoenia ($r^2 = 0.91$, Fig. 6C).

When FTY-P dosing was terminated at day 25, the lymphopoenia was reversed. This occurred with a significant delay compared with the reemergence of clinical signs, such that at the highest dose, the numbers of peripheral lymphocytes at day 30 remained as low as they had been at day 20 (Fig. 6B). However, by this day, the clinical scores in this group had already reached a mean of about 2 (compare with <0.5 at day 20, Fig. 5A) and thus there was an incomplete temporal match between the measures of lymphopoenia and clinical score.

3.5. Effect of FTY-P on gene expression in EAE

We examined the effect of FTY-P on the changes in expression of some of the genes which were described previously. In this EAE experiment, animals dosed from day 14 with 1-mg/kg FTY-P had recovered to a very low clinical score by day 25 (Fig. 7A), while untreated animals,

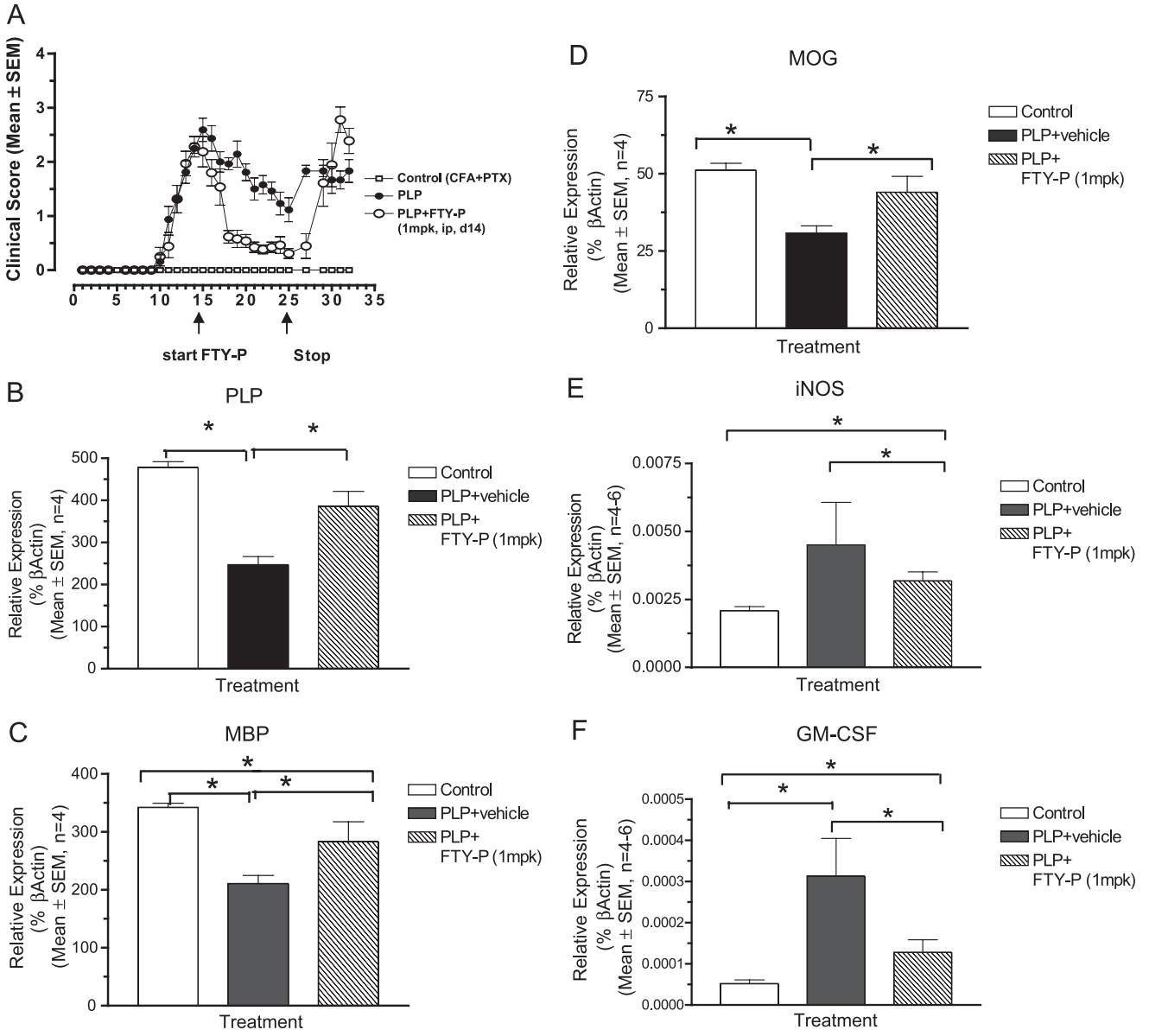

Fig. 7. Effect of FTY-phosphate treatment on gene expression changes at day 25 of EAE. (A) Time course of EAE for study of effect of FTY-P on spinal gene expression changes. (B–F) Effect of FTY-P (daily, 1 mg/kg from day 14) on changes in spinal expression of genes related to myelin (B, PLP; C, MBP; D, MOG) and inflammation-related (E, iNOS; F, GM-CSF) at day 25. *Y* axis, mean and S.E.M., $n = 4$ (MOG, MBP, PLP; $n = 4–6$, iNOS, GMCSF) of expression relative to β actin determined in same samples.

although in remission, still maintained a mean score of >1. In spinal cord samples, the previously observed reduction in expression of myelin-related genes was seen in EAE control mice (Fig. 7B–D), but in each case, treatment with 1-mg/kg FTY-P significantly increased expression levels. As seen previously for iNOS and GM-CSF, expression levels were elevated at day 25, relative to controls, but in this case, FTY-P treatment significantly attenuated the expression of both genes (Fig. 7E,F).

3.6. Effect of FTY-P in adoptive transfer EAE

We examined the effect of FTY-P in EAE induced in recipient SJL mice by adoptive transfer of lymph node cells from actively immunized mice. In this model, neither the donor nor the host mice are exposed to PTX. Initial experiments indicated that adoptive transfer of 2.5×10^7 cells produced an extremely aggressive disease, in which all the recipient mice died by the twelfth day after cell transfer. A severe disease but with less mortality was produced when the cell dose was reduced to 0.625×10^7 cells, in which case approximately 50% of the mice had died by day 12 (Fig. 8B,D). When FTY-P was given daily from day 0, the day of cell transfer, doses of 0.3, 1.0, or 3 mg/kg protected 100% of the mice from mortality by day 15, in contrast to the untreated control group where mortality exceeded 50% at this time (Fig. 8B). A reduction in mean clinical scores was also seen on FTY-P treatment at all three doses tested (Fig. 8A), but the scatter in the data precludes conclusions about dose responsiveness.

We also examined the effect of varying the time of the initiation of daily dosing with respect to the time of cell transfer, defined as day 0. Initiating dosing either between days −1 and 4 resulted in a complete protection from mortality (Fig. 8D), but the effect on clinical scores varied markedly with the day of initiation. Days −1 or +4 resulted

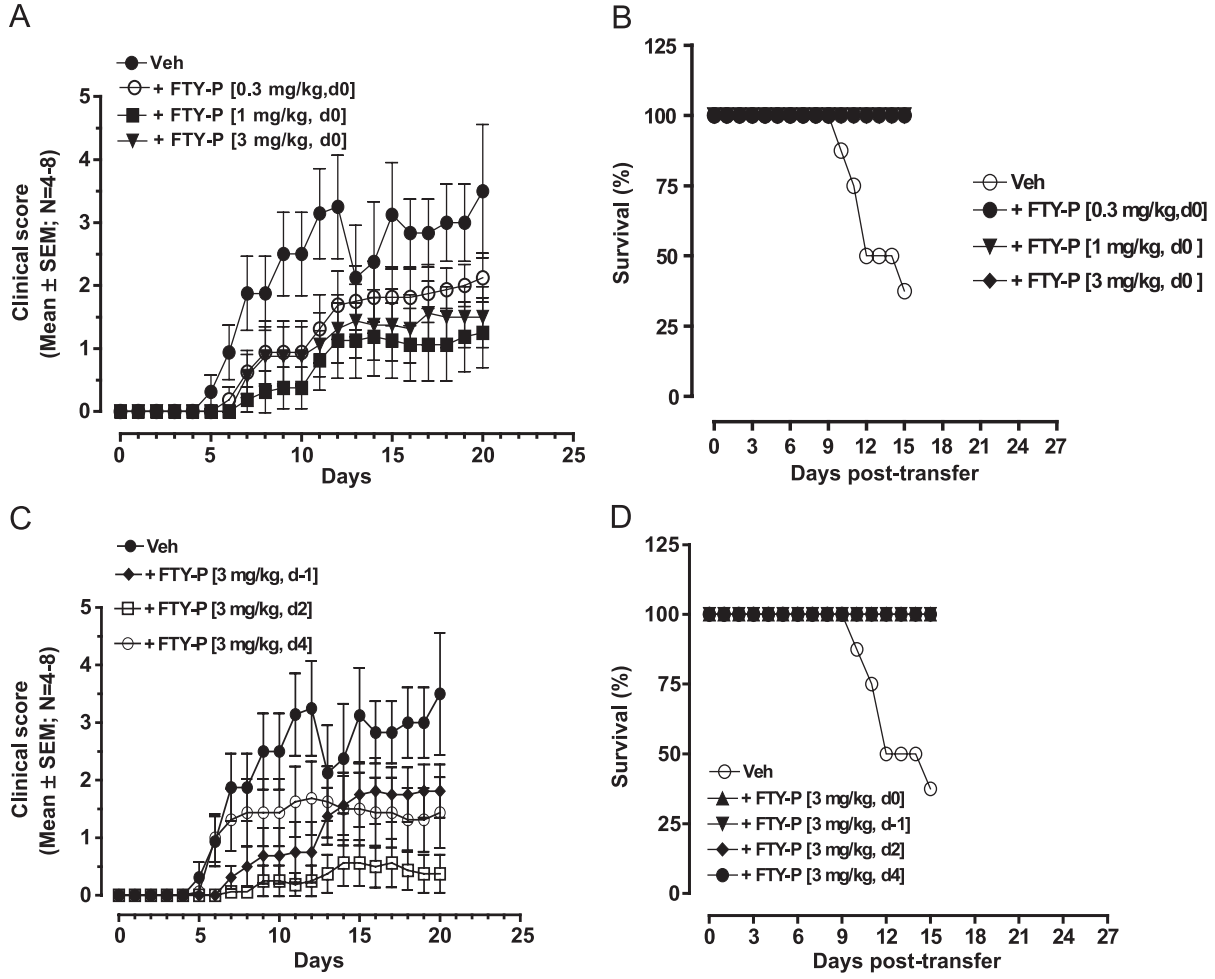

Fig. 8. Effect of FTY-P on an adoptive transfer induced EAE in SJL mice. (A) FTY-P was initiated (once daily, ip injection, 0.3, 1, or 3 mg/kg) starting from the day of cell transfer. Clinical score evolution is shown. (B) Complete protection from mortality is seen up to day 15 by all three doses. (C) Effect on evolution of clinical scores of varying time of initiation of dosing with FTY-P (once daily, ip 3 mg/kg) between day −1 and day +4 (day of cell transfer = day 0). (D) Although there is a profound difference in effect on clinical score, initiating FTY-P treatment at any time between day −1 and day +4 completely protects against mortality.

in a significant level of improvement in clinical scores, but a profound (~5-fold) reduction was seen only when dosing was initiated at day +2 (Fig. 8C).

4. Discussion

FTY720 has been reported previously to be effective in ameliorating several autoimmune diseases, including EAE (Chiba et al., 1996; Suzuki et al., 1996a, 1998; Masubuchi et al., 1996; Matsuura et al., 2000) Two previous studies have examined the effect of immunomodulation with FTY on rat models of EAE. Brinkmann et al. (2002) reported a prophylactic effect of FTY720 on the development of EAE in Wistar rats when administered from the day of immunization (0.3 mg/kg daily). A similar protection was seen by Fujino et al. (2003) in a Lewis rat model, in which administration of FTY (1 mg/kg daily po) from day 0 prevented mortality and almost abolished the clinical signs of EAE. The work we report here extends these observations in several ways.

The SJL mouse EAE model, unlike the monophasic acute models used in the two previous studies, is a chronic disease with a spontaneous pattern of relapses and remissions (McRae et al., 1992, 1995; Sobel et al., 1990; Marracci et al., 2002). This disease pattern is similar to the predominant form of human multiple sclerosis. We observed this pattern in many of our experiments (e.g., Figs. 1 and 5), although in others, the chronic disease was less punctuated by marked episodes of remission and relapse (e.g., Figs. 2 and 4). In addition, the mouse exhibits histopathology in lesioned areas similar to that shown in active plaques of multiple sclerosis patients. The major hallmarks of this histopathology are (1) infiltration of peripheral leucocytes, including autoaggressor T cells, (2) reactive gliosis, (3) demyelination, and (4) substantial axonal loss and neurodegeneration (Brown et al., 1982). This latter feature is now accorded a major role in producing the relentless progression of the human disease and is a prominent feature of the mouse model.

The chronic nature of the SJL mouse EAE allows experiments with an extended time course in which the effect of compound on an already established disease can be examined. We have shown that administration of FTY or its phosphate ester to mice prior to the appearance of clinical signs results in a delay and blunting of the first phase of the disease. Thereafter, mice may be maintained at low and stable clinical scores without relapse for as long as adequate dosing is continued. Here we report for the first time that initiation of dosing at the peak of the first phase of the disease results in a rapid decline of clinical signs. This is evident from the first day after dosing and is sustained until the mice reach a low and stable score similar to that of preonset-dosed mice. If dosing is delayed until day 25, at which point some degree of spontaneous remission is evident, the clinical scores fall to levels significantly below untreated controls, and mice so treated do not subsequently relapse while dosing is continues. These data demonstrate that FTY720-sensitive mechanisms have an unusually long time window for therapeutic intervention. In addition, these data also contrast with agents such as mitoxantrone which appear to have a narrow time window in which beneficial effects can be demonstrated.

Once dosing was discontinued, we observed a reappearance of clinical signs in mice, which relapsed over a period of days to clinical scores similar to non-FTY720-treated controls at the same time point. This observation may indicate the survival of a substantial pool of activated T lymphocytes in drug-treated animals. It may also be relevant to note that SJL strain mice are significantly impaired in their ability to mount T helper 2 mediated regulatory responses (Hutchings et al., 1986), which under normal circumstances would be expected to play a role in blunting the severity of the disease. When dosing with FTY720 was restarted in such animals, their clinical scores once again began to decline. Thus, whatever the mechanism by which FTY720 achieves its effect, this is still operative in animals that have previously been exposed to the compound.

As has been reported previously, we saw a dose-dependent and reversible lymphopoenia on treatment with FTY720 or FTY-P. This reached a maximum of about 70–80% depletion at the highest doses used. This level of lymphopoenia was established over several days after the initiation of dosing, and we were able to show that cumulative clinical scores over the dosing period correlated with the plateau levels of lymphopoenia achieved for each specific dose. This would be expected if, as has been claimed, the lymphopoenia is a manifestation of the mechanism of action of FTY720, a reversible sequestration of autoaggressor T lymphocytes in peripheral lymph nodes (Xie et al., 2003). Because EAE is known to be a T cell-dependent disease, such sequestration, by preventing the entry of T cells with specificity for myelin components into the CNS, would account for the therapeutic efficacy. If the sequestered lymphocytes remain viable in the peripheral lymph nodes, their release from these sites on drug withdrawal would account for the relatively rapid relapses seen on withdrawal of compound. It would be an interesting extension of this work to examine the in vitro responses to specific antigen challenge of cells recovered from spleen and peripheral lymph nodes at various time points during and after cessation of dosing, and this remains a direction for future investigation.

In dose response experiments, we found that a threshold of about 70% depletion of peripheral lymphocytes was required to see any efficacy, and thereafter, the dose–response relationship between clinical benefit and lymphopoenia was very steep. In spite of these observations, we did observe disconnection between lymphopoenia and clinical scores. This was particularly seen at the initiation and termination of dosing. At initiation of dosing in sick animals, we saw a rapid onset of clinical improvement

which was evident prior to the establishment of substantial levels of lymphopoenia. On withdrawal of compound, there was a delay of 1–2 days before the clinical signs began to increase. Nevertheless, animals were seen to have relapsed to clinical scores of 2 or above while levels of lymphopoenia remained the same as they were when the animals had scores little above baseline (0.5). The correlation between lymphopoenia and clinical efficacy is thus imperfect, and although the lymphopoenia is a biomarker correlated with clinical efficacy and may be a contributory mechanism to this efficacy, additional mechanisms may also be involved in producing the overall therapeutic benefit seen in models of transplant and autoimmune disease.

In an adoptive transfer version of the disease, PTX was not employed at any stage. Thus, the potential difficulties of studying agonists at a class of receptors, some of which couple to Gi, while abolishing signaling through this G protein can be avoided. In our hands, this was a much more severe disease than that produced by active immunization, and at high cell doses (2.5×10^7), resulted in complete mortality by day 15. Reducing the cell dose to either 1.25 or 0.625×10^7 cells reduced the mortality at day 15 to about 70%. It was remarkable that even under these conditions of high mortality, FTY-P was effective even at the lowest dose tested (0.3 mg/kg) in completely preventing death up to day 15. In addition, there was a dose-dependent reduction in clinical scores of surviving mice. Dosing could be initiated between days −1 and +4 relative to cell transfer to achieve the protection against mortality, but we observed that initiating at day +2 was very much more effective than at any other time. We do not at present understand the basis of this observation because it would reasonably be expected that while there might be a later limit for initiating treatment with resultant efficacy, it is difficult to comprehend why delaying the first dose until day 2 should produce a much greater effect than initiating from day −1. The observation may be relevant to the kinetics of lymphocyte homing to target tissue or to additional effects of FTY on aggressor cells which depend in some way on the temporal relation to in vivo activation.

During the characterization of the model, we examined changes in gene expression for several myelin proteins, cytokines, and inflammatory mediators. The expression of five myelin protein mRNAs behaved similarly (PLP, MBP, as shown in Fig. 2; MOG, CNPase, and MAG, data not shown). There was a major decrease in expression to about 25% of control levels in the first phase, after which some degree of recovery was seen by day 27. Subsequently, levels of expression fluctuated somewhat but never attained the levels of controls. In the particular EAE experiment from which these samples were taken, there was not a clear pattern of relapse–remission after the first phase, and so it is difficult to be certain that these fluctuations are correlated with the subsequent clinical status of the mice, although a close inspection of Fig. 3A suggests that the increase in expression between days 44 and 62 may be related to a minor improvement in clinical status between these two times.

It seems likely that these changes in gene expression are a direct measure of loss of myelin-forming cells during the disease. The implication of the increased (although still attenuated relative to controls) gene expression after the first phase is that some level of recovery of oligodendrocyte number may occur. If this is the case, it still remains to be demonstrated that any oligodendrocyte repopulation is accompanied by significant remyelination. Treatment with FTY-P substantially reversed the decrements in expression of these myelin-related genes at day 25, and it is tempting to suggest a correlation between this observation and the improvement in the clinical status of the FTY-P treated mice.

The expressions of the genes for iNOS, IFNγ, and GM-CSF were upregulated during the course of EAE, with the greatest increase being seen at the peak of the first phase. Thereafter, these remained upregulated but at lower levels. iNOS has previously been described to be upregulated in both EAE and in human MS patients, and iNOS inhibitors have been claimed to ameliorate the course of EAE.

GM-CSF is of particular interest in the context of EAE. Local delivery of rGM-CSF by retrovirally transduced T cells leads to a severe EAE in normal mice (Marusic et al., 2002). GM-CSF knockout mice are resistant to the induction of EAE, but susceptibility can be restored by treating them with rGM-CSF (McQualter et al., 2001). Furthermore, antibody against GM-CSF reverses established EAE in a mouse model (McQualter et al., 2001). It has been shown that GM-CSF treatment of microglia in vitro promotes their differentiation into a cell with a dendritic-like phenotype (Re et al., 2002), a cell type which is about two orders of magnitude more potent in presenting antigen to T cells. If such cells are differentiating in the CNS under the influence of GM-CSF, they will strongly potentiate the activation of invading T cells by presenting fragments of newly released myelin proteins from regions in which some level of demyelination is under way. This hypothesis is strengthened by the observations of Liu et al. (2002) that 17b oestradiol treatment, which suppresses EAE induction, suppresses the ability of mature dendritic cells to present antigen to primed T cells in vitro.

We noted a substantial decrease in GM-CSF gene expression in animals induced for EAE and subsequently treated with FTY-P. The effect on GM-CSF gene expression may be a consequence of the improvement in clinical status, reflecting a decreased infiltration of GM-CSF-producing T cells as a result of lymphocyte sequestration away from the CNS. Alternatively, it is possible that FTY-P has some direct effect either on the production of GM-CSF or on cellular responses to the cytokine, possibilities that remain to be investigated.

The phosphate ester of FTY720 is an agonist with nanomolar potency at four of the five known receptors for the lysophospholipid sphingosine-1-phosphate (Mandala et

al., 2002; Brinkmann et al., 2002). Activity at S1P1 is thought to mediate lymphocyte sequestration (Forrest et al., 2004; Sanna et al., 2004, Matloubian et al., 2004), however, activity at other S1P receptors may contribute to clinical efficacy. Because FTY-P gains access to CNS tissue which expresses multiple forms of S1PR, including S1PR5 which is exclusively expressed on oligodendrocytes, we cannot yet exclude that some actions of the compound may be mediated centrally in EAE models.

References

Brinkmann, V., Pinschewer, D., Chiba, K., Feng, L., 2000. FTY720: a novel transplantation drug that modulates lymphocyte traffic rather than activation. Trends Pharmacol. Sci. 21, 49–52.

Brinkmann, V., Chen, S., Feng, L., Pinschewer, D., Nikolova, Z., Hof, R., 2001a. FTY720 alters lymphocyte homing and protects allografts without inducing general immunosuppression. Transplant. Proc. 33, 530–531.

Brinkmann, V., Pinschewer, D.D., Feng, L., Chen, S., 2001b. FTY720: altered lymphocyte traffic results in allograft protection. Transplantation 72, 764–769.

Brinkmann, V., Davis, M.D., Heise, C.E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C.A., Zollinger, M., Lynch, K.R., 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J. Biol. Chem. 277, 21453–21457.

Brown, A., McFarlin, D.E., Raine, C.S., 1982. Chronologic neuropathology of relapsing experimental allergic encephalomyelitis in the mouse. Lab. Invest. 46, 171–185.

Chiba, K., Hoshino, Y., Suzuki, C., Masubuchi, Y., Yanagawa, Y., Ohtsuki, M., Sasaki, S., Fujita, T., 1996. FTY720, a novel immunosuppressant possessing unique mechanisms: I. Prolongation of skin allograft survival and synergistic effect in combination with cyclosporine in rats. Transplant. Proc. 28, 1056–1059.

Chiba, K., Yanagawa, Y., Masubuchi, Y., Kataoka, H., Kawaguchi, T., Ohtsuki, M., Hoshino, Y., 1998. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats: I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J. Immunol. 160, 5037–5044.

Chun, J., 1999. Lysophospholipid receptors: implications for neural signaling. Crit. Rev. Neurobiol. 13, 151–168.

Forrest, M., Sun, S.Y., Hajdu, R., Bergstrom, J., Card, D., Doherty, G., Hale, J., Keohane, C., Meyers, C., Milligan, J., Mills, S., Nomura, N., Rosen, H., Rosenbach, M., Shei, G.J., Singer, I.I., Tian, M., West, S., White, V., Xie, J., Proia, R.L., Mandala, S., 2004. Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. J. Pharmacol. Exp. Ther. 309, 758–768.

Fujino, M., Funeshima, N., Kitazawa, Y., Kimura, H., Amemiya, H., Suzuki, S., Li, X.K., 2003. Amelioration of experimental autoimmune encephalomyelitis in Lewis rats by FTY720 treatment. J. Pharmacol. Exp. Ther. 305, 70–77.

Fukushima, N., Ishii, I., Contos, J.J., Weiner, J.A., Chun, J., 2001. Lysophospholipid receptors. Annu. Rev. Pharmacol. Toxicol. 41, 507–534.

Goetzl, E.J., An, S., 1998. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine-1-phosphate. FASEB J. 12, 1589–1598.

Graler, M.H., Goetzl, E.J., 2004. The immunosuppressant FTY720 downregulates sphingosine 1-phosphate G-protein-coupled receptors. FASEB J. 18, 551–553.

Hutchings, P.R., Varey, A.M., Cooke, A., 1986. Immunological defects in SJL mice. Immunology 59 (3), 445–450 (Nov).

Liu, H.Y., Buenafe, A.C., Matejuk, A., Ito, A., Zamora, A., Dwyer, J., Vandenbark, A.A., Offner, H., 2002. Estrogen inhibition of EAE involves effects on dendritic cell function. J. Neurosci. Res. 70, 238–248.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C.L., Rupprecht, K., Parsons, W., Rosen, H., 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 296, 346–349.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkman, V., Allende, M.L., Proia, R.L., Cyster, J.G., 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427, 355–360.

Marracci, G.H., Jones, R.E., McKeon, G.P., Bourdette, D.N., 2002. Alpha lipoic acid inhibits T cell migration into the spinal cord and suppresses and treats experimental autoimmune encephalomyelitis. J. Neuroimmunol. 131, 104–114.

Marusic, S., Miyashiro, J.S., Douhan III, J., Konz, R.F., Xuan, D., Pelker, J.W., Ling, V., Leonard, J.P., Jacobs, K.A., 2002. Local delivery of granulocyte macrophage colony-stimulating factor by retrovirally transduced antigen-specific T cells leads to severe, chronic experimental autoimmune encephalomyelitis in mice. Neurosci. Lett. 332, 185–189.

Masubuchi, Y., Kawaguchi, T., Ohtsuki, M., Suzuki, C., Amano, Y., Hoshino, Y., Chiba, K., 1996. FTY720, a novel immunosuppressant, possessing unique mechanisms: IV. Prevention of graft versus host reactions in rats. Transplant. Proc. 28, 1064–1065.

Matsuura, M., Imayoshi, T., Chiba, K., Okumoto, T., 2000. Effect of FTY720, a novel immunosuppressant, on adjuvant-induced arthritis in rats. Inflamm. Res. 49, 404–410.

McQualter, J.L., Darwiche, R., Ewing, C., Onuki, M., Kay, T.W., Hamilton, J.A., Reid, H.H., Bernard, C.C., 2001. Granulocyte macrophage colony-stimulating factor: a new putative therapeutic target in multiple sclerosis. J. Exp. Med. 194, 873–882.

McRae, B.L., Kennedy, M.K., Tan, L.J., Dal Canto, M.C., Picha, K.S., Miller, S.D., 1992. Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. J. Neuroimmunol. 38, 229–240.

McRae, B.L., Vanderlugt, C.L., Dal Canto, M.C., Miller, S.D., 1995. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. J. Exp. Med. 182, 75–85.

Nagahara, Y., Enosawa, S., Ikekita, M., Suzuki, S., Shinomiya, T., 2000. Evidence that FTY720 induces T cell apoptosis in vivo. Immunopharmacology 48, 75–85.

Pinschewer, D.D., Ochsenbein, A.F., Odermatt, B., Brinkmann, V., Hengartner, H., Zinkernagel, R.M., 2000. FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory. J. Immunol. 164, 5761–5770.

Re, F., Belyanskaya, S.L., Riese, R.J., Cipriani, B., Fischer, F.R., Granucci, F., Ricciardi-Castagnoli, P., Brosnan, C., Stern, L.J., Strominger, J.L., Santambrogio, L., 2002. Granulocyte-macrophage colony-stimulating factor induces an expression program in neonatal microglia that primes them for antigen presentation. J. Immunol. 169, 2264–2273.

Sanchez, T., Estrada-Hernandez, T., Paik, J.H., Wu, M.T., Venkataraman, K., Brinkmann, V., Claffey, K., Hla, T., 2003. Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. J. Biol. Chem. 278, 47281–47290.

Sanna, M.G., Liao, J., Jo, E., Alfonso, C., Ahn, M.Y., Peterson, M.S., Webb, B., Lefebvre, S., Chun, J., Gray, N., Rosen, H., 2004. Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. J. Biol. Chem. 279, 13839–13848.

Sewell, W.A., Andrews, P., 1989. Inhibition of lymphocyte circulation in mice by pertussis toxin. Immunol. Cell Biol. 67, 291–296.

Sobel, R.A., Tuohy, V.K., Lu, Z.J., Laursen, R.A., Lees, M.B., 1990. Acute

experimental allergic encephalomyelitis in SJL/J mice induced by a synthetic peptide of myelin proteolipid protein. J. Neuropathol. Exp. Neurol. 49, 468–479 (Sep).

Stevens, D.B., Chen, K., Seitz, R.S., Sercarz, E.E., Bronstein, J.M., 1999. Oligodendrocyte-specific protein peptides induce experimental autoimmune encephalomyelitis in SJL/J mice. J. Immunol. 162, 7501–7509.

Suzuki, S., Enosawa, S., Kakefuda, T., Shinomiya, T., Amari, M., Naoe, S., Hoshino, Y., Chiba, K., 1996a. A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. Transplantation 61, 200–205.

Suzuki, S., Li, X.K., Enosawa, S., Shinomiya, T., 1996b. A new immunosuppressant, FTY720, induces bcl-2-associated apoptotic cell death in human lymphocytes. Immunology 89, 518–523.

Suzuki, S., Li, X.K., Shinomiya, T., Enosawa, S., Amemiya, H., Amari, M., Naoe, S., 1997. The in vivo induction of lymphocyte apoptosis in MRL-lpr/lpr mice treated with FTY720. Clin. Exp. Immunol. 107, 103–111.

Suzuki, K., Yan, H., Li, X.K., Amemiya, H., Suzuki, S., Hiromitsu, K., 1998. Prevention of experimentally induced autoimmune type I diabetes in rats by the new immunosuppressive reagent FTY720. Transplant. Proc. 30, 1044–1045.

Trapp, B.D., Peterson, J., Ransohoff, R.M., Rudick, R., Mork, S., Bo, L., 1998. Axonal transection in the lesions of multiple sclerosis. N. Engl. J. Med. 338, 278–285.

Trapp, B.D., Ransohoff, R., Rudick, R., 1999. Axonal pathology in multiple sclerosis: relationship to neurologic disability. Curr. Opin. Neurol. 12, 295–302.

Wujek, J.R., Bjartmar, C., Richer, E., Ransohoff, R.M., Yu, M., Tuohy, V.K., Trapp, B.D., 2002. Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. J. Neuropathol. Exp. Neurol. 61, 23–32.

Yanagawa, Y., Sugahara, K., Kataoka, H., Kawaguchi, T., Masubuchi, Y., Chiba, K., 1998. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats: II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. J. Immunol. 160, 5493–5499.

Xie, J.H., Nomura, N., Koprak, S.L., Quackenbush, E.J., Forrest, M.J., Rosen, H., 2003. Sphingosine-1-phosphate receptor agonism impairs the efficiency of the local immune response by altering trafficking of naive and antigen-activated CD4+ T cells. J. Immunol. 170, 3662–3670.