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Title page

$S1P_1$ receptor modulation with cyclical recovery from lymphopenia ameliorates mouse model of multiple sclerosis

Pedro J. Gonzalez-Cabrera, Stuart M. Cahalan, Nhan Nguyen, Gor Sarkisyan, Nora B. Leaf, Michael D. Cameron, Tomoyuki Kago, and Hugh Rosen.

Department of Chemical Physiology (P.J.G-C., S.M.C., N.N., G.S., N.B.L., T.K., H.R.) and Department of Immunology (S.M.C.), The Scripps Research Institute, La Jolla, CA; Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, FL (M.D.C).

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Running Title Page

Running Title: EAE efficacy of $S1P_1$ agonism without sustained lymphopenia

Corresponding Author:

Hugh Rosen

The Scripps Research Institute

La Jolla, CA, 92037

Phone: 858-784-2396

Fax: 858-784-2988.

E-mail: hrosen@scripps.edu

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Non-standard abbreviations:

MS, multiple sclerosis; S1P, sphingosine-1 phosphate; MOG, myelin oligodendrocyte glycoprotein; EAE, experimental allergic encephalomyelitis; i.p., intraperitoneally; eGFP, enhanced green fluorescent protein; PTX, pertussis toxin; PFA, paraformaldehyde; H&E,

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hematoxilin and eosin; LFB, Luxol fast blue; PML, progressive multifocal leukoencephalopathy; CYM-5442, 2-(4-(5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-yl amino) ethanol.

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Abstract

Multiple sclerosis therapies modulate T cell autoimmune pathology in the CNS but may exacerbate latent infections. Fingolimod, a non-selective S1P receptor agonist that induces sustained lymphopenia and accumulates in CNS represents a new modality of MS therapy. We hypothesized that sustained lymphopenia is not required for efficacy and that a selective, CNS-penetrant, but peripherally short-acting $S1P_1$ agonist would show full efficacy in a mouse model of MS. Using daily treatment with 10 mg/kg CYM-5442, at onset of clinical signs in MOG_{35-55}-induced EAE, we assessed clinical scores, CNS cellular infiltration, demyelination and gliosis for 12 days for CYM-5442, vehicle and fingolimod. CYM-5442 levels in CNS and plasma were determined at experiment termination and blood lymphopenia was measured at 3- and 24 h following last injection. Plasma levels of cytokines were assayed at protocol end. Changes in $S1P_1$-eGFP expression on neurons and astrocytes during active EAE and upon CYM-5442 treatment were directly quantified by flow cytometry and Westerns using native-locus-eGFP tagged $S1P_1$ mice. $S1P_1$ agonism alone reduced pathology equivalent to fingolimod (maximally lymphopenic throughout) despite full reversal of lymphopenia within each dosing interval. CYM-5442 levels in CNS were sustained, but not in plasma. Neuronal and astrocytic $S1P_1$ expression in EAE was suppressed by CYM-5442 treatment relative to vehicle mice, whereas key cytokine levels such as IL-17A were also significantly reduced in drug treated mice. $S1P_1$-selective agonists inducing reversible lymphopenia while persisting in CNS may be effective MS treatments while limiting adverse consequences of long-term lymphopenia.

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Introduction

MS is a chronic inflammatory disease of the CNS characterized by demyelination, axonal severing, axonal loss, and astrogliosis, causing severe neurological dysfunction. Although direct etiology is unknown, establishment of a chronic autoimmune response in the CNS requires that T cells autoreactive to myelin components escape negative selection and peripheral suppression to enter the CNS initiating a cascade of inflammation, demyelination, phagocytosis of myelin debris, and astrogliosis. These processes culminate in the sclerotic plaques that are pathognomonic of MS (Compston and Coles, 2008).

Several treatments for MS inhibit lymphocyte entry into the CNS. Tysabri, a monoclonal antibody against VLA-4, prevents integrin-mediated invasion of T cells into CNS (Yednock et al., 1992), but is associated with potentially fatal recrudescence of JC virus infection known as PML (Kleinschmidt-DeMasters and Tyler, 2005). Most recently, fingolimod (FTY720, GILENYA®), whose active phosphate fingolimod-P acts on S1P receptors $S1P_{1, 3, 4, 5}$ (Mandala et al., 2002), prevents the egress of lymphocytes from secondary lymphoid organs, was approved as an oral treatment (Brinkmann et al., 2010). Fingolimod induction of lymphocyte sequestration is entirely dependent on its actions on $S1P_1$ (Graler, 2010; Mehling et al., 2008). However, S1P receptors are expressed on a wide variety of cells other than lymphocytes, including neurons (Kimura et al., 2007), oligodendrocytes (Miron et al., 2008), macrophages (Singer et al., 2005), microglia (Durafourt et al., 2011), and astrocytes (Malchinkhuu et al., 2003). S1P receptors in primary CNS cells directly affect morphology, survival, proliferation and differentiation (Brinkmann et al., 2010). Astrocytes, which migrate to S1P through S1P receptors (Mullershausen et al., 2007), may play a critical role in the pathology of MS, as mice lacking $S1P_1$ on astrocytes do not develop as severe symptoms in $MOG_{35\text{-}55}$-induced EAE (Choi et al.,

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2011). Due to the lack of selectivity of fingolimod-P, insights into the specific receptors involved in its efficacy in treating MS have been limited.

Here, we explore the role of $S1P_1$ in the amelioration of EAE symptoms by using the selective $S1P_1$ agonist CYM-5442 (Gonzalez-Cabrera et al., 2008) in combination with $S1P_1$-eGFP knock-in mice (Cahalan et al., 2011), allowing direct analysis of receptor expression, signaling, and sub-cellular localization. We demonstrate that daily CYM-5442 administration significantly reduces MOG_{35-55}-induced EAE in mice despite cyclical return of blood lymphocytes to normal levels within each dosing interval. Additionally, we show that $S1P_1$-eGFP expression during daily CYM-5442 treatment leads to degradation of $S1P_1$-eGFP on neurons and astrocytes, but not peripheral lymphocytes. Thus, cyclical restoration of normal lymphocyte recirculation during each dosing interval, with a selective $S1P_1$ agonist that persists within the CNS, provides a foundation of adequate efficacy that may preserve the ability of the host to maintain sufficient lymphocyte surveillance.

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Materials and Methods

Mice. Six to 8-week old female C57BL/6J (B6) mice and congenic Edg1$^{eGFP/eGFP}$ ($S1P_1$-eGFP) mice were used (Cahalan et al., 2011). All procedures were approved by the Animal Care and Use Committee at The Scripps Research Institute (La Jolla, CA).

EAE Induction and Clinical Scoring. Mice, lightly anesthetized with isofluorane (Isothesia, Butler, IL), received a 200µl intradermal lumbar inoculation of immunogen (2 mg/ml $MOG_{35\text{-}55}$ peptide (Cedarlane, Burlington, NC) dissolved in water, emulsified 1:1 in incomplete Freund's Adjuvant (DIFCO, Detroit, MI) supplemented with 4 mg/ml heat-inactivated Mycobacterium tuberculosis (DIFCO, strain H37 RA). Immediately following immunization, and on day 2 post immunization, mice received 0.1ml i.p. injections of PTX (2 mg/ml) in PBS. Animals were weighed daily and scored for neurological signs as described (Miller et al. 2010): 0, no symptoms; 1, complete loss of tail tone or hind limb weakness; 2, loss of tail tone plus hind limb weakness; 3, partial hind-limb paralysis; 4, full hind limb paralysis; 5, moribund. Animals exhibiting scores of 5 were euthanized and were included in the clinical scoring.

$S1P_1$ Agonist Administration. CYM-5442 was synthesized as shown (Gonzalez-Cabrera et al., 2008) and dissolved in sterile water. At the onset of clinical symptoms (days 10-13 post immunization), mice were randomized into two groups receiving daily i.p. injections of $S1P_1$ agonists, dosed at 10 mg/kg (CYM-5442), 3 mg/kg (fingolimod, Cayman Chemical, Ann Arbor, MI) or equal volumes of vehicle for an additional 8-12 days, and euthanized for further studies.

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Histology and Immunofluorescence. Histological examination of brain and spinal cord from vehicle and drug treated groups was performed both at study end, and on days 18 to 19 following EAE induction, when significant therapeutic windows in clinical scoring between drug-treated and vehicle-treated mice were typically observed. Following euthanasia, animals were perfused with PBS and ice-cold 4% PFA, and spinal cords and brains carefully removed and incubated for 1 h on ice in 4% PFA, followed by 72 h in 30% sucrose at 4C. Cellular infiltration and anatomy was assessed on paraffin embedded CNS tissue sections, cut at 10 μm and stained by H&E. Luxol fast blue and FluoroMyelin Red (Molecular Probes) staining was performed in spinal cords to assess for demyelination. Phase-contrast images of H&E and LFB sections were acquired using an Olympus BX51 microscope.

Colocalization studies in spinal cords of $S1P_1$-eGFP mice were done in frozen sections from tissues processed as above. Tissues were embedded in Tissue-Tek (Torrance, CA), frozen in a dry ice/2-methyl-butane bath, and sectioned at 10 μm using a cryostat. Slides were blocked at room temperature for 1h in PBS containing 1% fetal bovine serum (FBS), 1% normal-goat serum, 0.01% fish gelatin, and 0.1% Tween-20. Tissues were then incubated overnight at 4C with antibodies against GFP (Pierce, 1:10,000), the vascular endothelial marker CD31/PECAM (BD Pharmingen, 1:50), the neuronal marker MAP2 (Abcam, 1:10,000), the astrocyte marker GFAP (Abcam, 1:1000), or the oligodendrocyte marker, MBP (Millipore, 1:100). Slides were washed 3 times with PBS containing 0.1% Tween-20 (PBST), then incubated for 1h at room temperature with secondary antibodies conjugated to 546 nm or 633 nm Alexa Fluor dyes (Molecular Probes). Slides were washed with PBST, incubated with 0.5μg/mL DAPI in PBST, rinsed in PBS, and mounted using Vectashield (Vector labs, Burlingame, CA). Staining of spinal

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cord sections with the $S1P_1$ carboxyl-terminus recognizing antibody (Santa Cruz Biotechnology, clone H-60, 1:50) or an isotype control was done in paraffin embedded tissue.

Measurement of Blood Lymphocyte Counts. Cardiac blood was taken from mice in each treatment group and left to rotate for 2 h in EDTA tubes. Red blood cell lysis was done using 2 washes in 1M TAC buffer for 15 min at 37C. Samples were resuspended in 900 µl FACS buffer, counted with a Coulter Counter, and stained with APC-CD4, PerCP-Cy5.5-CD8 and PE-CD19 antibodies (BioLegend, San Diego, CA) to collect T and B cell numbers. Analysis was performed with FlowJo (Treestar).

Cellular Isolation and Flow Cytometry. Brains from wild-type or $S1P_1$-eGFP mice were manually dissociated in Hank's Buffered Salt Solution containing 1% FBS, 500uM EDTA, and 25mM HEPES, and myelin was removed from the samples using a myelin removal kit (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions. Lymph nodes were manually dissociated in the same buffer. Samples were then stained with one or more of the following antibodies: PE F4/80 (BioLegend), APC GLAST-1 (Miltenyi), APC-Cy7 CD11b (BD), PE-Cy7 CD4 (eBioscience), PerCP-Cy5.5 CD8 (BD), Pacific Blue B220 (BD) and data was collected using an LSR II flow cytometer (BD). Calculation of mean (neurons and astrocytes) or modal fluorescence intensity (B220 and CD4 cells) was performed by the method and for the statistical reasons described in detail in the supplemental materials of the original description of the S1P1-eGFP mouse (Cahalan et al., 2011).

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Protein Electrophoresis and Western Blotting. Processing of whole brain and spinal cord tissues from EAE mice for electrophoresis was done as described (Cahalan et al., 2011). After electrophoresis, gels were scanned using a Typhoon (Invitrogen) in-gel scanner using a FITC filter to identify $S1P_1$-eGFP. The Santa Cruz, H-60 anti-$S1P_1$ antibody, was additionally used to confirm $S1P_1$ expression in brains of $S1P_1$-eGFP mice. Detection of $S1P_1$-eGFP ubiquitylation in CNS samples was done as referenced (Gonzalez-Cabrera et al., 2007).

Statistical Analysis. All analyses were performed using either unpaired, two-tailed, Student's t-test or ANOVA. The data bars and error bars indicate mean ± standard error mean. (s.e.m).

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Results

Modulation of EAE by CYM-5442. Since long-lived non-selective S1P receptor agonist pro-drugs such as fingolimod ameliorate EAE, and selective $S1P_1$ agonists inhibit the development of experimental autoimmune neuritis (Zhang et al., 2009), a similar demyelinating disease, we sought to determine whether the short-acting $S1P_1$ agonist CYM-5442 could modulate EAE severity in mice with clinical signs. Daily treatment of mice with 10 mg/kg CYM-5442 from onset of clinical signs following EAE induction with MOG_{35-55} peptide significantly attenuated both clinical signs and weight loss (Figs 1A and B). Maximal clinical score differences between vehicle- and CYM-5442-treated mice were observed at day 16 post-immunization (Vehicle: 2.38 ± 0.16, n=9, CYM-5442: 1.11 ± 0.29, n=9, $p<0.0001$). A significant reduction of infiltrating lymphocytes into the brain parenchyma in CYM-5442-treated but not vehicle-treated mice tracked with improved clinical scores. This was particularly evident in the optic tract (Opt) and within the peri-vasculature feeding the white matter of lumbar and thoracic spinal cord sections (Fig 1C). Spinal cords of mice treated with CYM-5442 showed preservation of myelination within the outmost white matter, as demonstrated by FluoroMyelin Red staining (Fig 1D) and LFB staining (Supplementary Fig 1A). LFB staining in brains of EAE mice showed that CYM-5442 treatment also protected the myelinated tracts lining the Opt (Supplementary Fig 1B), and inhibited astrogliosis (Supplementary Fig 1C). Efficacy was also seen in severe EAE with high mortality (Supplementary Fig 2) as measured by clinical scores (Vehicle: 4.25 ± 0.25, n=4, CYM-5442: 2.28 ± 0.36, n=7, $p = 0.0043$). Thus, CYM-5442 administration after disease onset inhibits cellular infiltration, neuronal damage and astrogliosis during MOG_{35-55} -induced EAE, which is reflected in improved clinical scores, decreased CNS parenchymal cell infiltration, reduced demyelination, and reduced mortality.

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Cyclical Lymphopenia by CYM-5442 is Sufficient for Efficacy in MOG-induced EAE.

CYM-5442 has a high CNS to plasma ratio, and a short half-life in circulation (Gonzalez-Cabrera et al., 2008). Similar to CYM-5442, fingolimod also displays high CNS to plasma ratio (Foster et al., 2007); however, fingolimod has been shown to induce sustained lymphopenia by single dosing (Luo et al., 1999). Rapid clearance of CYM-5442 from circulation led to short duration lymphopenia where blood lymphocyte counts reached nadir at 4 h following treatment and returned to pretreatment levels between 16 to 24 h post-treatment (Fig 2A). Single low dose of 0.2 mg/kg of fingolimod i.p. induced maximal lymphopenia for at least 48 h (Fig 2B). Thus, we compared CYM-5442 and fingolimod to assess whether efficacy-driven by transient lymphopenia would be similar to that achieved by prolonged lymphopenia.

Mice with EAE and treated daily with either 10 mg/kg CYM-5442 or 3 mg/kg fingolimod from onset of symptoms showed equivalent attenuation in mean clinical score relative to vehicle treatment (Fig 2C). Maximal clinical score differences between vehicle- and CYM-5442-treatement and vehicle- and fingolimod treatment were observed at day 21 post-immunization (Vehicle: 2.33±0.10, n=6, CYM-5442: 0.86±0.14, n=7, fingolimod: 0.83±0.42, n=6, $p<0.001$).

Lymphocyte sequestration kinetics by CYM-5442 and fingolimod on the last day of EAE treatment are shown in Fig 2D. Both drugs showed similar B- and T-lymphocyte sequestration at 3 h following the last injection versus vehicle, but only CYM-5442 treatment showed significant recovery of lymphocyte counts to near untreated levels by 24 h. In contrast, fingolimod maintained nadir lymphopenia between the 3- and 24-h groups. These data suggest that sustained lymphocyte sequestration by $S1P_1$ agonism is not required for efficacy in MOG_{35-55}-induced EAE in mice. Further analyses in the plasma of EAE animals at 24 h post last injection, indicated that both CYM-5442 and fingolimod led to significant reduction in the concentration of

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IL-17 and IL-1ß, two pro-inflammatory cytokines involved in the progression of EAE (Fig 2E). Since fingolimod-P uptake into CNS has been associated with cellular targeting and amelioration of EAE (Foster et al., 2007), we determined the kinetics of CYM-5442 during EAE amelioration. CYM-5442 accumulated in CNS relative to plasma (Table 1), with high concentrations found 3 h following the last CYM-5442 treatment (Brain: 5.91 ± 0.17 µmol/mg tissue, Spinal cord: 4.32 ± 0.16 µmol/mg tissue). CYM-5442 persisted in the CNS in significant concentrations 24 h following the last CYM-5442 treatment (Brain: 352 ± 32 nmol/mg tissue, Spinal cord: 360± 72 nmol/mg tissue). In contrast, CYM-5442 in plasma was cleared below the concentration required to maintain lymphopenia by 24 h (3 h: 320 ± 30 nM, 24 h: 10 ± 1 nM). Thus, persistent CYM-5442 presence in the CNS may play an important role in the amelioration of EAE.

$S1P_1$-eGFP Expression in the CNS. $S1P_1$ is thought to be expressed on several cell types involved in the pathology of MS: lymphocytes which invade and attack the CNS, endothelial cells that normally provide a barrier to entry of these lymphocytes into CNS parenchyma (Cahalan et al., 2011), neurons that are targeted for destruction by autoreactive lymphocytes (Nishimura et al., 2010), and astrocytes, which play a major role in inflammation and CNS scarring associated with MS (Sorensen et al., 2003). We analyzed $S1P_1$-eGFP protein expression on these different cell types by immunofluorescence utilizing $S1P_1$-eGFP mice, which retain full physiological and pharmacological functions from the native $S1P_1$ locus (Cahalan et al., 2011). $S1P_1$-eGFP expression within the spinal cord was primarily localized in gray matter (Fig 3A). Within the spinal cord white matter, $S1P_1$-eGFP was expressed on CD31-expressing endothelial cells and MAP2-expressing neurons, while expression on MBP-expressing oligodendrocytes within the white matter was below limits of detection (Fig 3B). $S1P_1$eGFP expression in the

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brain was widespread over cell bodies and neurites, with the highest levels in cerebellum as well as the cortex, hippocampus, and caudate-putamen (Supplementary Fig 3).

CYM-5442-induced $S1P_1$-eGFP degradation during EAE treatment. We induced EAE in $S1P_1$-eGFP mice to examine the effects of EAE induction, and subsequent daily treatment with CYM-5442, on the expression of $S1P_1$-eGFP. CYM-5442 administration during 8 days reduced EAE clinical scores in $S1P_1$-eGFP mice relative to vehicle treatment (Vehicle: 3.75±0.25, n=4, CYM-5442: 2.20±0.2, n=5, p = 0.0017, Supplementary Fig 4A). In naïve conditions (no EAE), a single 10 mg/kg CYM-5442 dose induced similar maximal lymphocyte sequestration in both B6 and $S1P_1$-eGFP mice (Supplementary Fig 4B), whereas lymphocyte subsets were significantly reduced 3 h in $S1P_1$-eGFP mice following the last CYM-5442 injection of active EAE (Supplementary Fig 4C). Since CYM-5442 accumulates in the brain, and numerous $S1P_1$ agonists cause cellular $S1P_1$ internalization and subsequent ubiquitin-dependent $S1P_1$ down-regulation (Graler and Goetzl, 2004; Gonzalez-Cabrera et al., 2007) we examined the ability of CYM-5442 to modulate $S1P_1$-eGFP expression within the brain during active EAE, at 3 h following the last CYM-5442 treatment. Treatment with CYM-5442 caused significant loss of $S1P_1$-eGFP in spinal cords and brains of EAE mice (Fig 4). This loss was clearly evident from immunofluorescence examination of spinal cord frozen sections of agonist treated $S1P_1$-eGFP mice at 488nm, or in paraffin spinal cord sections using an antibody against the C-terminus of $S1P_1$ (Fig 4B and 4C). The concomitant increase in poly-ubiquitinylated receptor in brain from EAE $S1P_1$-eGFP mice (Fig 4C) indicate sustained agonist-induced $S1P_1$ internalization and subsequent $S1P_1$ down-regulation in the CNS.

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$S1P_1$-eGFP expression also appeared to increase in the gray matter following induction of EAE (Fig 4A and 4B), however, we found that injecting PTX alone in the span of 2 days, to resemble the EAE protocol, was able to up-regulate $S1P_1$-eGFP expression in brain and spinal cords of naïve mice (Supplemental Figure 5).

We used flow cytometry for single cell analysis of $S1P_1$-eGFP expression on specific cell types within the brain and lymph nodes two weeks following EAE induction with or without daily CYM-5442 dosing. Following dissociation of myelin, astrocytes, identified as FSC-A^{lo}SSC-A^{hi}CD11b^-GLAST-1^+ cells, and neurons, identified as FSC-A^{lo}SSC-A^{hi}CD11b^-GLAST-1^- cells, up-regulated $S1P_1$-eGFP following induction of EAE, likely due to the direct PTX effect. In addition, both of these cell types showed $S1P_1$-eGFP down-modulation by daily CYM-5442 treatment. In contrast, CYM-5442 treatment did not decrease $S1P_1$-eGFP expression in lymphocytes isolated from the lymph nodes 3 h after the last injection of CYM-5442, nor did EAE induction induce changes in $S1P_1$-eGFP expression in lymphocytes (Fig 4B), in spite of the significant lymphocyte sequestration seen with CYM-5442 at this time point. Supplementary Fig 6 shows internalization of membrane associated $S1P_1$-eGFP into cytoplasmic vesicles by an acute 30 min CYM-5442 treatment in neuronal progenitor cells isolated from day-13 $S1P_1$-eGFP embryos.

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Discussion

Fingolimod has ushered in a new era of oral, disease modifying MS treatments based upon endpoints of clinical score progression, annualized relapse rates and the accrual of gadolinium-enhancing lesions upon magnetic resonance imaging. All current MS treatments have significant adverse effects, some of which may be life threatening. Tysabri, for example, has a significant increased risk of progressive multifocal leukoencephalopathy, the result of inadequate control of pre-existing JC virus infection stemming from deficits in immune surveillance caused by Tysabri inhibition of memory T cells entry into tissues through post-capillary venules (Kleinschmidt-DeMasters and Tyler, 2005). The long-term safety of the non-selective S1P receptor pro-drug fingolimod, which suppresses lymphocyte recirculation for 4-6 weeks following withdrawal (Johnson et al., 2010) as a result of its week-long half-life, has yet to be determined. However, it does not sequester circulating effector T cells efficiently from the circulation (Xie et al., 2003), and thus may allow for effective immuno-surveillance.

Understanding the molecular basis of S1P receptor agonist efficacy in CNS inflammatory disease provides important insights into approaches that could yet improve the safety-efficacy window for patients. Administration of shorter-acting agents, where lymphopenia can return towards normal levels during the dosing interval (Cahalan et al., 2011; Gonzalez-Cabrera et al., 2008), may offer some advantages over the intermittent administration of long-acting agents, which cause sustained lymphocyte sequestration. Here, we demonstrate this using a short-acting, selective, CNS-penetrant $S1P_1$ agonist and $S1P_1$-eGFP knock-in mice in a murine EAE model, permitting tracking of ligand and physiologically and pharmacologically active receptor in both peripheral and CNS tissues. This has allowed an accounting of cell- and tissue-specific receptor distribution in physiology, disease and treatment.

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The detailed quantitative distribution of $S1P_1$ in the CNS of normal, inflamed, and CYM-5442 treated mice is important because agonism of $S1P_1$ alone shows significant efficacy across all measures of EAE. While expression of S1P receptors in the CNS has been explored using mRNA profiling (Chae et al., 2004) and sometimes by antibody-detection of $S1P_1$ protein (Akiyama et al., 2008) (Sinha et al., 2009) , $S1P_1$-eGFP mice allow a high-resolution view of $S1P_1$ expression at a protein level within the CNS. This enables the documentation of significant up-regulation of $S1P_1$-eGFP on neurons and astrocytes in mice with EAE. Because S1P *in vitro* enhances neurite extension (Toman et al., 2004) and can dampen neuronal excitability through G_i-coupled suppression of intracellular cAMP (Zhang et al., 2006), $S1P_1$ up-regulation may be a compensatory mechanism to limit excitatory neuronal damage in the inflammatory processes of EAE.

We have shown here that $S1P_1$ agonism alone is sufficient to ameliorate EAE. CYM-5442 is an important pharmacological tool compound in understanding EAE because its pharmacokinetics produce strong lymphopenia that persists for 6-8 hours, while returning peripheral blood lymphocyte numbers to basal levels within 24 hours of treatment. Given the association of PML with Tysabri, and the reporting of some CNS herpetic infection with fingolimod, it is helpful to understand the boundary conditions for the degree of lymphopenia that are essential for efficacy. It was possible that the full recovery of lymphocyte recirculation within a dosing interval, as observed for CYM-5442 might result in lower efficacy when compared with longer-duration agonists that induce full sequestration for days. This was not the case for CYM-5442 treatment where treatment outcome was indistinguishable from that of fingolimod in both mild and severe models of EAE. Peripheral lymphopenia, while a convenient surrogate marker for efficacy, is clearly not the sole contributor to the effective amelioration of

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both demyelination and leukocyte infiltration in the brain and the spinal cord parenchyma during EAE.

While lymphocyte recirculation was restored in each dosing interval of CYM-5442 treatment, the expression of $S1P_1$-eGFP in the CNS following daily treatment was down-regulated, consistent with the higher, maintained levels of CYM-5442 within the CNS. Although we observed decreased, but not absent, expression of $S1P_1$-eGFP on neurons and astrocytes, evidence for receptor down-regulation and degradation demonstrates a long-term alteration in $S1P_1$ signaling tone in CNS and does not definitively demonstrate functional antagonism by CYM-5442 on these cells *in vivo*. Despite having lower total $S1P_1$ expression, the agonist present may lead to continual $S1P_1$ signaling within the CNS, where any receptor that reaches the cell surface is rapidly activated and internalized, consistent with poly-ubiquitylation of $S1P_1$-eGFP detected in mice treated with CYM-5442. Degradation of $S1P_1$-eGFP in the CNS was in contrast to what was observed in peripheral lymphocytes, which reflects the preferential distribution to CNS tissue of CYM-5442 contrasted with relatively rapid clearance from plasma and secondary lymphoid organs. The lack of $S1P_1$-eGFP degradation on peripheral lymphocytes observed with CYM-5442 is also in contrast to what is found using either fingolimod-phosphate (Oo et al.2011) or the recently published short-duration agonist RP-001 (Cahalan et al., 2011), a closely related compound to CYM-5442. This disparity demonstrates that agonist-induced changes in $S1P_1$ expression are dependent on both the cell type on which $S1P_1$ is expressed, as well as the specific agonist used, and that the properties of pharmacological probes must therefore be examined in detail.

Although we have demonstrated that $S1P_1$ agonism is sufficient to alleviate EAE, the relative quantitative contributions of lymphocyte sequestration, sustained $S1P_1$ signaling, and

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$S1P_1$ down-regulation require additional study. Cell-specific deletion of $S1P_1$ on astrocytes has suggested that astrocytic $S1P_1$ contributes to fingolimod efficacy in models of EAE at the peak of disease severity (Choi et al., 2011). Continued development of additional selective $S1P_1$ agonists with differences in tissue distribution, potency, and capacity to down-regulate receptor will allow for separation of these components. Of particular interest would be identifying agonists that do not cross the blood-brain barrier, and evaluating the ability of such agonists to reverse EAE when given either systemically, excluding the role for CNS-expressed $S1P_1$, or intracranially, which could exclude the role for peripherally expressed $S1P_1$. The combination of chemical and genetic approaches to systematic mapping of S1P receptor signaling in physiology, pathology and therapeutics will likely help develop better therapeutics for the treatment of MS, that achieve clinical efficacy, while best preserving host defense against latent viruses within CNS.

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Authors' contributions:

Participated in research design: Gonzalez-Cabrera, Cahalan, Rosen

Conducted experiments: Gonzalez-Cabrera, Cahalan, Nguyen, Sarkisyan, Leaf, Cameron, Kago

Performed data analysis: Gonzalez-Cabrera, Cahalan, Nguyen, Leaf, Cameron

Wrote or contributed to the writing of the manuscript: Gonzalez-Cabrera, Cahalan, Rosen

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a) This work was supported by US National Institutes of Health Grants [NIH U01, AI074564, R01 AI055509] and a Molecular Libraries Probe Production Centers Network [Grant U54 MH084512].

b) Reprint requests should be addressed to Hugh Rosen, Department of Chemical Physiology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA, USA, 92037.
hrosen@scripps.edu

c) [1]Pedro J. Gonzalez-Cabrera and Stuart M. Cahalan contributed equally to this work.

d) Current address: Kyorin Pharmaceutical Co., Japan (T.K.)

e) Potential conflict of interest: Hugh Rosen is a co-founder of Receptos, Inc.

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Figure Legends

Figure 1. Daily CYM-5442 treatment alleviates EAE A) Plot of mean clinical scores for the indicated treatment in EAE. Mice were induced for EAE using a MOG_{35-55}/ Incomplete Freunds Adjuvant/Mycobacterium tuberculosis emulsification. Mice were injected i.p. daily with 10 mg/kg CYM-5442 in water, or equal volume water (Vehicle). B) CYM-5442 prevents weight loss in mice induced with EAE. Graph indicates mean weights of both animal groups from onset of treatment C) CYM-5442 treatment inhibits inflammatory cell infiltration into the Opt of the brain, and into perivascular cuffs of the spinal cord (scale bar, 40μm) of mice with EAE. Images are of H&E stained sagittal sections of brains and spinal cords. Left image is from a naive mouse (scale bar, 200μm). D) CYM-5442 ameliorates demyelination in the spinal cord of EAE mice. FluoroMyelin Red staining of spinal cord sections taken at end of protocol shows that outermost demyelination is reduced in CYM-5442 treated mice; scale bar, 20μm; blue, DAPI. All curves and images are representative of 3 experiments, at least 6 mice per group per experiment. PC, pyramidal cell; DC, dentate gyrus.

Figure 2. CYM-5442 induces short-duration lymphocyte sequestration. A) Dosing of naïve mice once with 10 mg/kg CYM-5442 induces both T and B lymphocyte sequestration from the blood that recovers by 24 h. B) Fingolimod induces sustained maximal lymphopenia for 48 h in naïve mice administered with a single 0.2 mg/kg i.p. dose. C) Plot of mean clinical scores for the indicated treatment in EAE. Mice were induced for EAE using a MOG_{35-55}/ Incomplete Freund's Adjuvant/ Mycobacterium tuberculosis emulsification. Mice were injected i.p. daily with either 10 mg/kg CYM-5442, 3 mg/kg fingolimod in water, or equal volume water. D) Blood from EAE-induced mice was removed at day 23 either 3 h or 24 h following the last fingolimod or

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CYM-5442 dose, demonstrating that in mice with EAE, blood lymphocyte counts recover only in the CYM-5442 group, even after many daily doses of CYM-5442. E) Reduction of cytokine levels in plasma of CYM-5442 and fingolimod treated mice on day 23 of EAE. Graphs for A are representative of 2 experiments, 4 mice per group per experiment. Graph for B represents 1 experiment with at least 6 animals per group. C is representative of 2 experiments, at least 3 mice per group per experiment. Graph for D represents 1 experiment, at least 3 animals per group.

Figure 3. Expression of $S1P_1$-eGFP within the spinal cord. $S1P_1$-eGFP is highly expressed within the spinal cord. A) Fluorescent microscopy images of spinal cord from wild type and $Edg_1^{eGFP/eGFP}$ mice. $S1P_1$-eGFP is preferentially expressed within the gray matter (GM) in the spinal cord. Scale bar, 100µm. B) $S1P_1$-eGFP is expressed on endothelial cells and neurons but not oligodendrocytes within the spinal cord. Spinal cord sections from $Edg_1^{eGFP/eGFP}$ mice were stained with antibodies against CD31 (endothelial cells; scale bar, 20µm), MBP (oligodendrocytes; scale bar, 20µm) or MAP2 (neurons; scale bar, 100µm). WM, white matter.

Figure 4. Daily CYM-5442 treatment leads to degradation of $S1P_1$-eGFP specifically in the CNS. A) Expression of $S1P_1$-eGFP in the spinal cord is significantly reduced following daily CYM-5442 treatment. Expression of $S1P_1$ within spinal cord was assessed by immunofluorescense (488 nm; scale bar, 20µm) using $S1P_1$-eGFP mice or by immunohistochemical staining of wild-type tissue sections with an antibody against the carboxyl-teminus of $S1P_1$ (C-t-$S1P_1$; scale bar, 100µm). Left image is from a naive mouse. B) $S1P_1$-eGFP is degraded in CNS of EAE-induced mice treated daily with 10 mg/kg CYM-5442. Both astrocytes and neurons upregulate $S1P_1$-eGFP following EAE induction, and degrade $S1P_1$-eGFP following daily CYM-5442 treatment. Gray, wild-type mouse; green, naïve $S1P_1$-eGFP

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mouse; red, vehicle $S1P_1$-eGFP EAE mouse; blue, CYM-5442 $S1P_1$-eGFP EAE mouse. Astrocytes were identified as FSC-A^{lo}SSC-A^{hi}CD11b-GLAST-1^{+}, while neurons were identified as FSC-A^{lo}SSC-A^{hi}CD11b-GLAST-1^{-}. Lymphocytes in the lymph node demonstrate negligible $S1P_1$-eGFP degradation following daily CYM-5442 treatment. Mean (neurons and astrocytes) or modal (Bcells and CD4 T cells) fluorescence intensities are indicated for each group C) Brain $S1P_1$-eGFP degradation by CYM-5442 is associated with increased $S1P_1$-eGFP polyubiquitylation. Receptor expression was measured by in-gel fluorescence imaging of brain lysates and densitometry or by Western blotting using the anti-C-t $S1P_1$ antibody, whereas poly-ubiquitinylation was measured by immunoprecipitation (IP) of brain lysates with GFP followed by immunoblotting (IB) for ubiquitin (P4D1).

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Table 1. CYM-5442 is retained highly in brain and spinal cord. Values are representative of 2 experiments, 3 mice per group per experiment.

Time after last injection	Brain (nmol/mg tissue)	Spinal cord (nmol/mg tissue)d	Plasma (nM)
3 h	5910 ± 170	4320 ± 160	320 ± 30
24 h	352 ± 32	360 ± 72	10 ± 1

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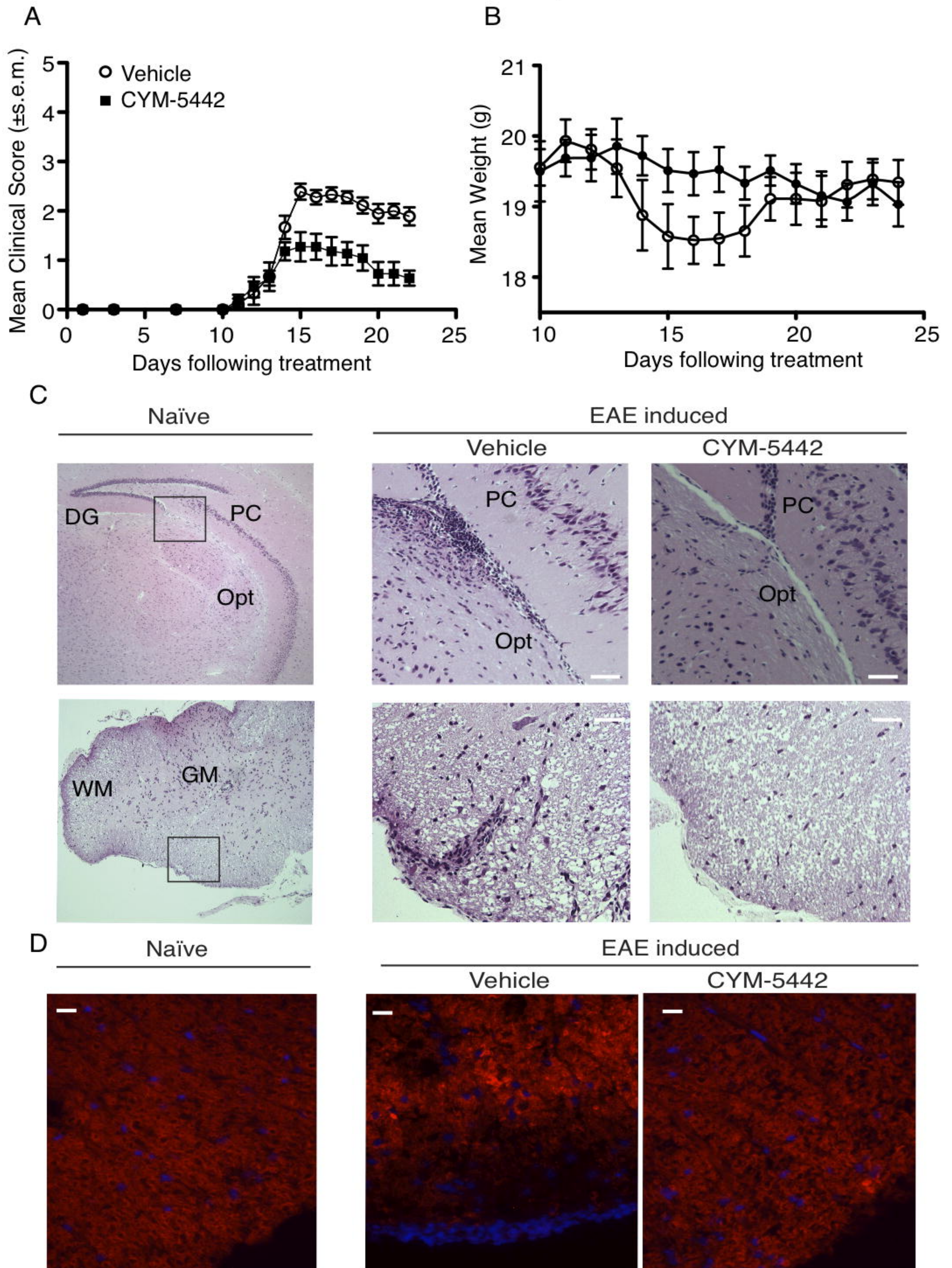

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Figure 2 EAE efficacy of $S1P_1$ agonism without sustained lymphopenia

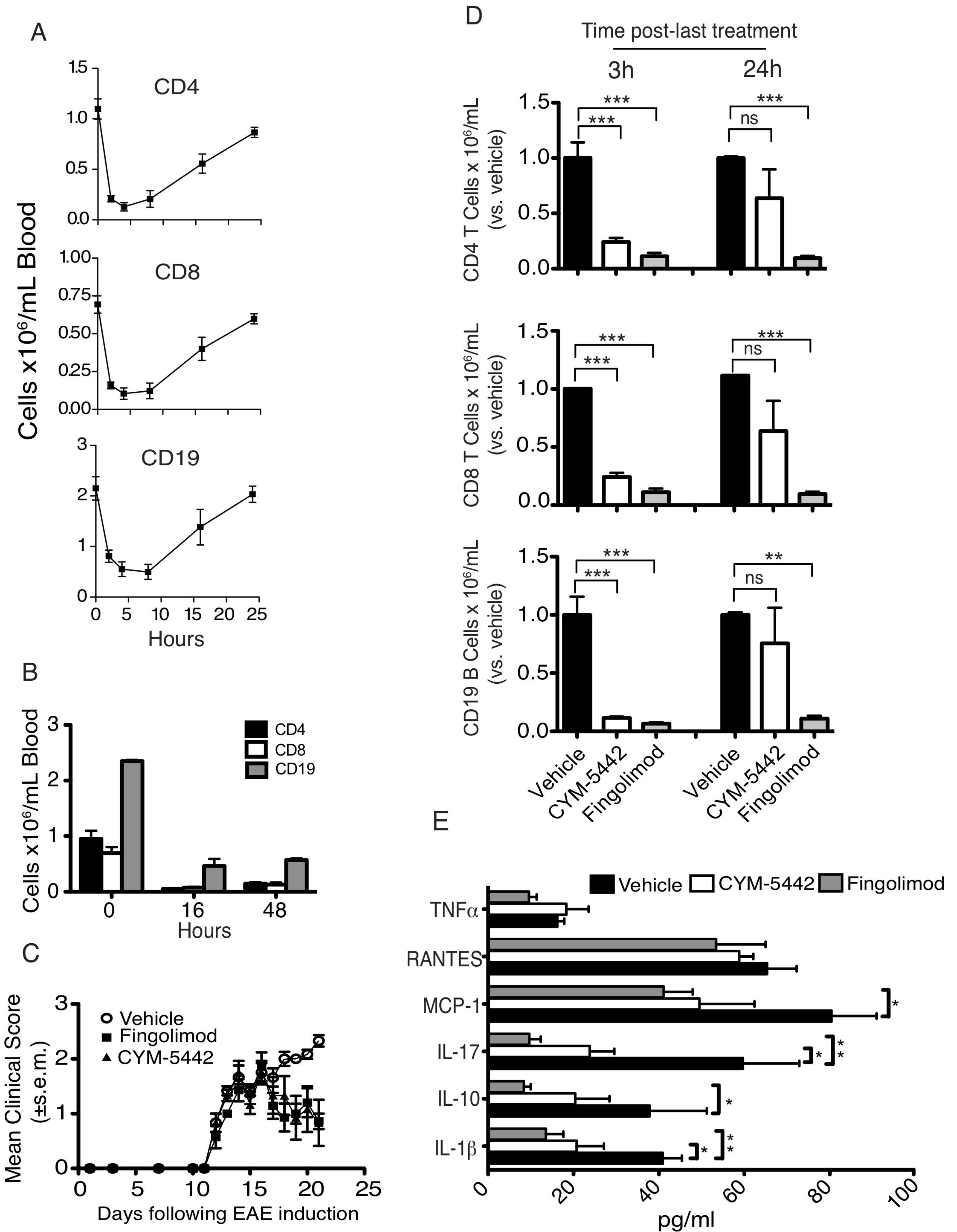

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Figure 3 EAE efficacy of $S1P_1$ agonism without sustained lymphopenia

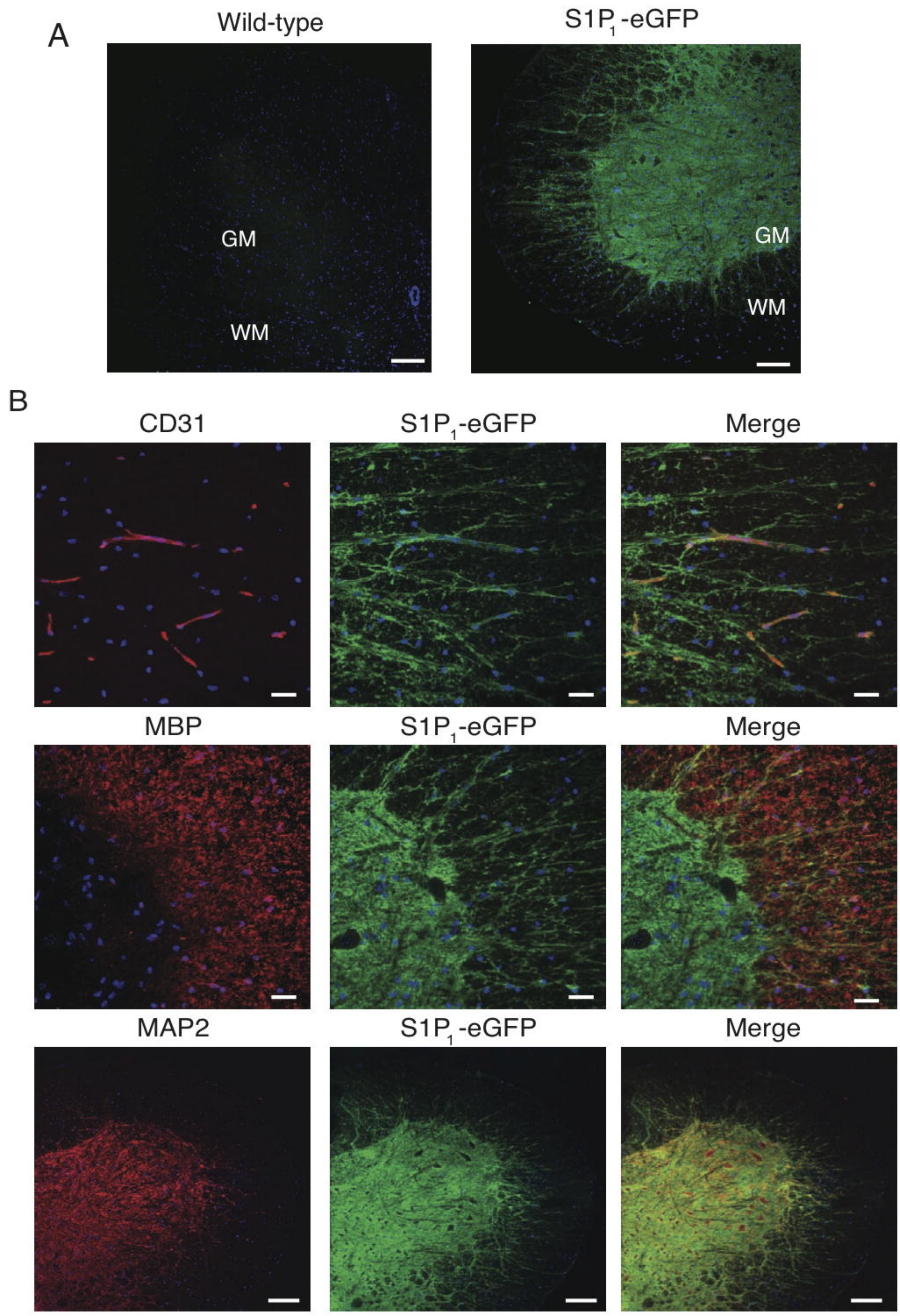

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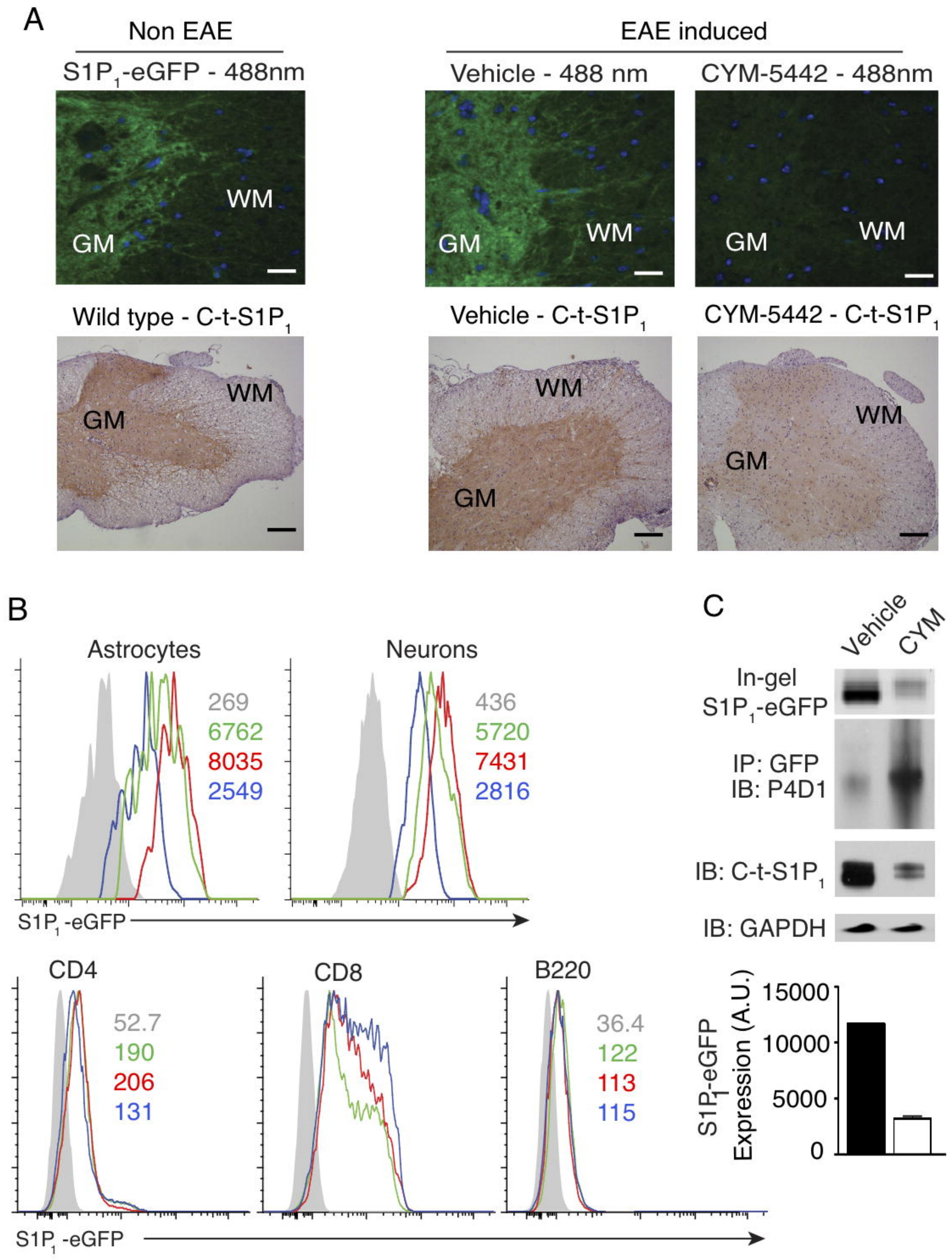
Figure 4 EAE efficacy of $S1P_1$ agonism without sustained lymphopenia



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