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ACCELERATED COMMUNICATION

Sphingosine 1-Phosphate Receptor 1 ($S1P_1$) Upregulation and Amelioration of Experimental Autoimmune Encephalomyelitis by an $S1P_1$ Antagonist[S]

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ABSTRACT

Sphingosine 1-phosphate receptor 1 ($S1P_1$) is a G protein–coupled receptor that is critical for proper lymphocyte development and recirculation. Agonists to $S1P_1$ are currently in use clinically for the treatment of multiple sclerosis, and these drugs may act on both $S1P_1$ expressed on lymphocytes and $S1P_1$ expressed within the central nervous system. Agonists to $S1P_1$ and deficiency in $S1P_1$ both cause lymphocyte sequestration in the lymph nodes. In the present study, we show that $S1P_1$ antagonism induces lymphocyte sequestration in the lymph nodes similar to that observed with $S1P_1$ agonists while upregulating $S1P_1$ on lymphocytes and endothelial cells. Additionally, we show that $S1P_1$ antagonism reverses experimental autoimmune encephalomyelitis in mice without acting on $S1P_1$ expressed within the central nervous system, demonstrating that lymphocyte sequestration via $S1P_1$ antagonism is sufficient to alleviate autoimmune pathology.

Introduction

Sphingosine 1-phosphate receptor 1 ($S1P_1$) plays an important role in many physiologic systems, including vascular development, lymphocyte development, and lymphocyte recirculation (Liu et al., 2000; Allende et al., 2003, 2004; Matloubian et al., 2004; Cyster and Schwab, 2012). $S1P_1$ is required on developing lymphocytes to mature beyond a semimature $CD69^{hi}$, $CD62L^{lo}$ state, rendering the blood and lymph of mice lacking $S1P_1$ on developing lymphocytes largely devoid of T cells. When $S1P_1^{-/-}$ thymocytes are transferred into recipient mice, they are also retained from blood and lymphatic circulation. $S1P_1$ became a relevant drug target in the treatment of autoimmune disease following the discovery that 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol (FTY720; fingolimod, Gilenya), which was known to inhibit lymphocyte recirculation, is a sphingosine 1-phosphate (S1P) receptor prodrug that is phosphorylated in vivo to yield 2-amino-2 [2-(4-octylphenyl)ethyl]-1,3-propanediol, mono dihydrogen phosphate ester (FTY720-P), a potent agonist of $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ (Mandala et al., 2002). $S1P_1$ selective agonists demonstrated that FTY720 acted via $S1P_1$ to induce lymphocyte sequestration (Sanna et al., 2004). The ability of FTY720-P and other $S1P_1$ agonists to induce sustained internalization and/or degradation of $S1P_1$ (Graler and Goetzl, 2004; Gonzalez-Cabrera et al., 2007, 2008), combined with the deficient egress of $S1P_1$-deficient lymphocytes, has led to the hypothesis that $S1P_1$ agonists act as functional antagonists (Graler and Goetzl, 2004). Several $S1P_1$-selective antagonists have also been generated, which inhibit agonist-dependent effects in vitro; stabilize the $S1P_1$ receptor, allowing for its structural determination; and induce pulmonary edema in vivo. In addition, initial antagonists could reverse agonist-induced lymphocyte sequestration while being unable to induce lymphocyte sequestration themselves (Foss et al., 2005; Wei et al., 2005; Sanna et al., 2006; Hanson et al., 2012). Recent work has shown that

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ABBREVIATIONS: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; Ex26, 1-(5′-((1-(4-chloro-3-methylphenyl) ethyl)amino)-2'-fluoro-3,5-dimethyl-[1,1'-biphenyl]-4-ylcarboxamido)cyclopropanecarboxylic acid; FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol; PBS, phosphate-buffered saline; S1P, sphingosine 1-phosphate; $S1P_{1–5}$, sphingosine 1-phosphate receptors 1–5; $S1P_1$-eGRP, $S1P_1$ enhanced green fluorescent protein; RP-001, 3-(4-(5-(3-cyano-4-isopropoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-ylamino)propanoic acid.

$S1P_1$ antagonists can indeed induce lymphocyte sequestration at high plasma concentrations (Tarrason et al., 2011) and $S1P_1$ antagonists can alleviate animal models of autoimmune arthritis (Fujii et al., 2012), cardiac allograft rejection (Angst et al., 2012), and multiple sclerosis (Quancard et al., 2012).

S1P receptor agonists have come of age with the Food and Drug Administration's approval of FTY720 for the treatment of relapsing-remitting multiple sclerosis. The efficacy of FTY720 is not solely dependent on its ability to cause full lymphocyte sequestration via $S1P_1$, as it is effective at doses that maintain ~50% lymphopenia. This efficacy probably involves both $S1P_1$ and other S1P receptors within the central nervous system (CNS) (Cohen and Chun, 2011; Hla and Brinkmann, 2011). $S1P_1$ agonists that can efficiently penetrate the CNS can induce receptor signaling and degradation of $S1P_1$ expressed on neurons and astrocytes (Gonzalez-Cabrera et al., 2012), and require lymphocyte sequestration for only one-third of a dosing interval to reverse experimental autoimmune encephalomyelitis (EAE) in mice. Additionally, mice lacking $S1P_1$ on astrocytes are refractory to developing EAE, and are suggested to be important targets of FTY720 (Choi et al., 2011). Several other S1P receptors are expressed within the CNS, and the activation and/or degradation of these receptors by FTY720 may also play important roles in reversing the immunopathology of multiple sclerosis (Miron et al., 2008, 2010).

In the present study, we demonstrate that $S1P_1$ antagonism sequesters lymphocytes in the peripheral lymph nodes but not the spleen, similar to that observed with $S1P_1$ agonists. $S1P_1$ antagonism also causes significant upregulation of $S1P_1$ expression on peripheral lymphocytes, mature thymocytes, and lung endothelial cells. Additionally, $S1P_1$ antagonism can alleviate EAE in mice despite the inability of the antagonist to penetrate the CNS. Thus, lymphocyte sequestration induced by $S1P_1$ antagonists is sufficient to ameliorate the autoimmune pathology observed in EAE, and does not require antagonism of $S1P_1$ expressed on neurons or astrocytes within the CNS.

Materials and Methods

Compounds and In Vitro Assays. Example 26 [Ex26, 1-(5′-((1-(4-chloro-3-methylphenyl)ethyl)amino)-2′-fluoro-3,5-dimethyl-[1,1′-biphenyl]-4-ylcarboxamido)cyclopropanecarboxylic acid] was synthesized as a racemic mixture according to its published synthesis in the patent literature (Angst et al., 2009). RP-001 (3-(4-(5-(3-cyano-4-isopropoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-ylamino)propanoic acid) was synthesized as previously described (Cahalan et al., 2011). FTY720 was purchased from Cayman Chemicals (Ann Arbor, MI). Ex26 and RP-001 were solubilized in 50 mM Na_2CO_3, while FTY720 was solubilized in H_2O. In vitro assays for S1P receptor function were performed using the following cell lines: $S1P_1$, $S1P_4$, and $S1P_5$: Tango Human Osteosarcoma U2OS cells (Invitrogen, Carlsbad, CA) expressing the indicated receptor; $S1P_2$: Chinese hamster ovary cells expressing $S1P_2$ coupled to a cAMP response element coupled to beta-lactamase reporter; and $S1P_3$: Chinese hamster ovary cells expressing $S1P_3$ coupled to an Nuclear factor of activated T-cells promoter coupled to beta-lactamase reporter through G protein α 16. $S1P_1$ internalization and polyubiquitinylation were evaluated using human embryonic kidney cells expressing $S1P_1$ enhanced green fluorescent protein (eGFP) as previously described (Gonzalez-Cabrera et al., 2007), pretreating cells for 1 hour with Ex26.

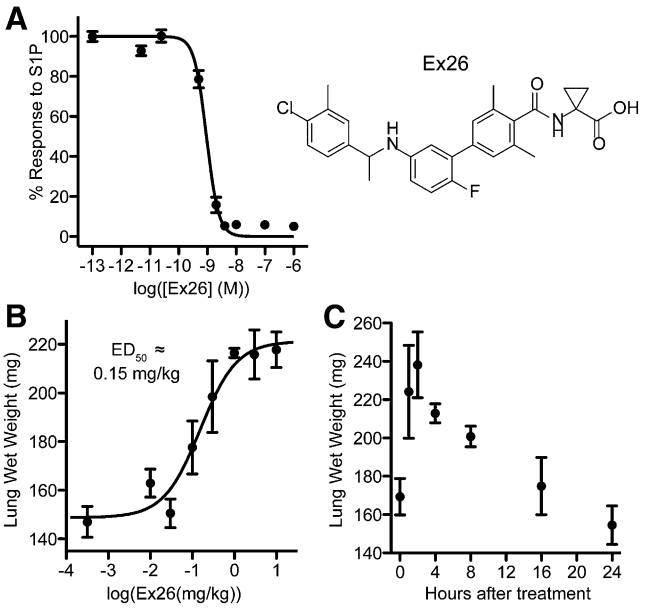

Fig. 1. Ex26 is a potent, selective $S1P_1$ antagonist. (A) Dose response in vitro of Ex26 on $S1P_1$-expressing cells in the presence of 5 nM S1P. The structure of Ex26 is depicted on the right. (B) Ex26 induces dose-dependent pulmonary edema 2 hours following i.p. treatment. (C) Pulmonary edema induced by 3 mg/kg Ex26 i.p. resolves by 16–24 hours following treatment. All data are representative of at least two experiments, with (B) and (C) having four mice per group per experiment. Graphs are plotted as the mean ± S.E.M.

Evaluation of Lymphocyte Sequestration, Pulmonary Edema. Eight-week-old male C57Bl/6J mice were purchased from the The Scripps Research Institute mouse breeding facility (La Jolla, CA) for evaluation of lymphocyte sequestration and pulmonary edema. Mice were injected i.p. with Ex26 or 50 mM Na_2CO_3 vehicle, and blood was removed from the heart following euthanasia. Blood was lysed in 150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; washed with phosphate-buffered saline (PBS) containing 2% fetal bovine serum, 1 mM EDTA, and 0.1% NaN_3; counted using a ViCell-XR counter (Beckman Coulter, Brea, CA); stained with antibodies; and analyzed by flow cytometry. To evaluate pulmonary edema, mice were perfused with 15 ml of PBS through the right ventricle, then the lungs were removed, blotted dry to remove excess PBS, and weighed. All mouse experiments were performed using protocols approved by the Institutional Animal Care and Usage Committee.

Compound Concentrations in Plasma and Tissues. Ex26 plasma concentrations were determined using methanol extraction as previously described (Cahalan et al., 2011), detecting an m/z value of 495.2 for Ex26 using an Agilent 6410 triple quadrupole mass spectrometer coupled to an Agilent 1100LC system (Agilent Technologies, Santa Clara, CA). Ex26 concentrations in the brain were

TABLE 1

Selectivity of Ex26 on S1P receptors

Ex26 displays excellent selectivity for $S1P_1$ over other S1P receptors. It also does not exhibit any detectable agonist activity on any S1P receptor.

Receptor	Antagonist IC_{50}	Agonist EC_{50}
	nM	*nM*
$S1P_1$	0.93	>10,000
$S1P_2$	>10,000	>10,000
$S1P_3$	>10,000	>10,000
$S1P_4$	4900	>10,000
$S1P_5$	3100	>10,000

Ex26, 1-(5′-((1-(4-chloro-3-methylphenyl)ethyl)amino)-2′-fluoro-3,5-dimethyl-[1,1′-biphenyl]-4-ylcarboxamido)cyclopropanecarboxylic acid; S1P, sphingosine 1-phosphate.

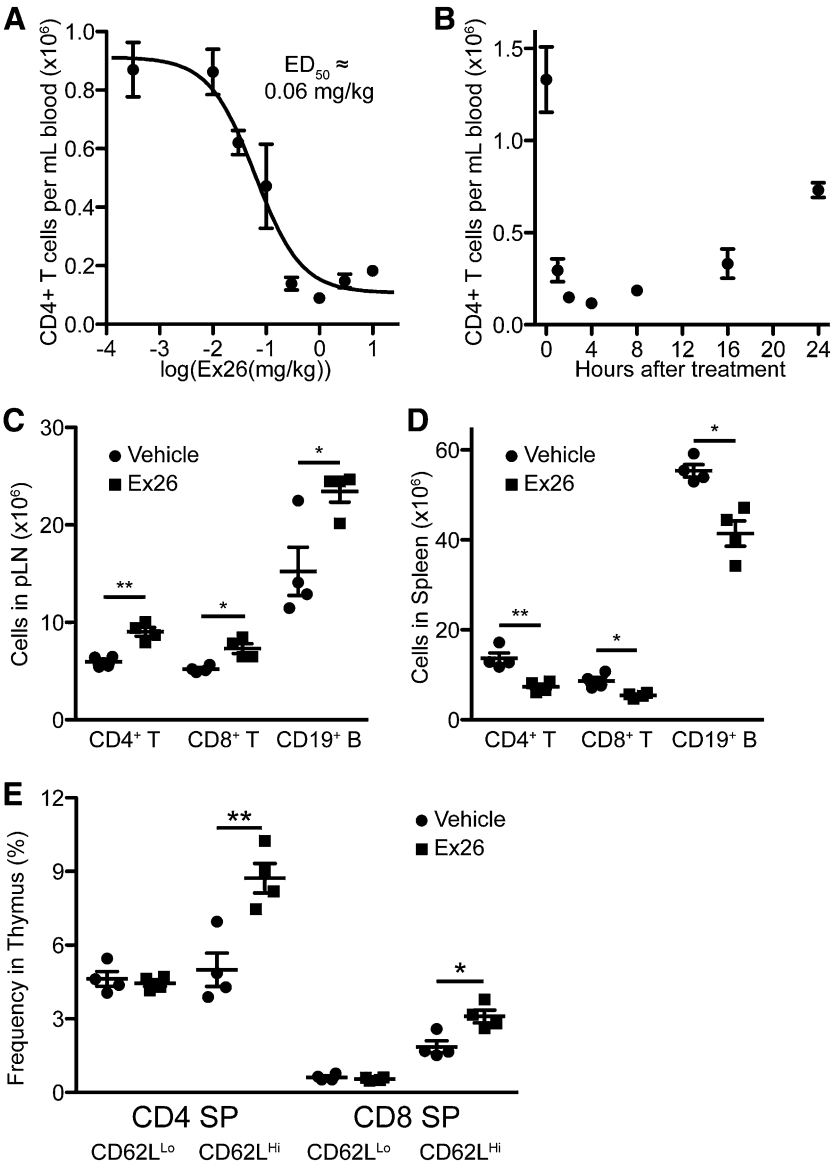

Fig. 2. $S1P_1$ antagonism by Ex26 induces lymphocyte sequestration in the lymph nodes and thymus. (A) Ex26 induces dose-dependent lymphocyte sequestration 2 hours following i.p. treatment. (B) Lymphopenia induced by 3 mg/kg Ex26 i.p. resolves by 24 hours following treatment. (C and D) Continuous administration of Ex26 in 6-week-old mice by micro-osmotic pumps sequesters T and B cells in the peripheral lymph nodes (pLN) (C), leaving the spleen depleted of lymphocytes (D). pLN cell numbers derive from combined inguinal, axillary, and brachial lymph nodes. (E) Ex26 leads to accumulation of mature $CD62L^{Hi}$, but not immature $CD62L^{Lo}$, SP thymocytes. All graphs are representative of three experiments, with 3–4 mice per group per experiment. Graphs are plotted as the mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$ as determined by an unpaired, two-tailed t test.

determined by disruption of brain tissue in water by probe sonication, followed by extraction with acetonitrile and filtration through MultiScreen hydrophilic polytetrafluoroethylene 0.45 μm filters (EMD Millipore, Billerica, MA). Filtrates were analyzed using a API 4000 liquid chromatography-tandem mass spectrometer (AbSciex, Framingham, MA) and quantified using a positive-ion multiple reaction monitoring method (495.1/242.1, m/z).

Continuous Administration of $S1P_1$ Antagonist. Six-week-old $S1P_1$-eGFP mice were anesthetized with isoflurane, and their backs were shaved, cleaned with 70% ethanol to remove any excess hair, then wiped with povidone iodine. An incision was made on the lower back of the mice, and micro-osmotic pumps (Alzet model 1003D; Alzet, Cupertino, CA) containing either 50 mM Na_2CO_3 vehicle or 2 mg/ml Ex26 were implanted, yielding a dose of ~0.1 mg/kg per hour. Mice were given an i.p. dose of 3 mg/kg Ex26 or vehicle immediately following surgery.

Flow Cytometry, $S1P_1$ Expression, and Statistical Analysis. Fluorescently labeled antibodies specific to CD4 and CD8 were obtained from Biolegend (San Diego, CA). Fluorescently labeled antibodies specific to CD19, CD31, CD45.2, CD62L, and CD69 were obtained from Beckton-Dickinson (San Diego, CA). Data were collected using an LSRII flow cytometer (Beckton-Dickinson) and analyzed using FlowJo (Treestar, Ashland, OR). $S1P_1$ expression by flow cytometry was measured using $S1P_1$-eGFP knockin mice (Cahalan et al., 2011). $S1P_1$ expression in the CNS in EAE experiments was evaluated using a C-terminal–specific $S1P_1$ antibody (H-60, Santa Cruz Biotechnology, Santa Cruz, CA; used at 1:500 dilution). All statistical analyses were performed using GraphPad Prism Software (GraphPad, La Jolla, CA).

EAE Induction and Scoring. EAE was induced in female 10-week-old C57Bl/6J mice purchased from Jackson Laboratories (Bar Harbor, ME). EAE was induced using a Hooke Laboratories EAE induction kit (Lawrence, MA; EK-0114 for EAE, CK-0114 for control) according to the manufacturer's instructions. Mice were scored by the following criteria: 0.5 (weak tail), 1 (limp tail), 1.5 (weak tail + weak hind limbs), 2 (limp tail + weak hind limbs), 2.5 (limp tail + unilateral hind limb paralysis), 3 (limp tail + bilateral hind limb paralysis), 4 (limp tail + bilateral hind limb paralysis + partial front limb paralysis), and 5 (moribund or dead). Mice scoring 4 for two consecutive days were euthanized and recorded as 5 for the remaining days of the experiment. Mice were injected i.p. daily with 50 mM Na_2CO_3 vehicle, 30 mg/kg Ex26, or 10 mg/kg FTY720 in a volume of 10 μl per gram weight of mouse beginning the first day on which clinical signs were observed in that mouse.

Results and Discussion

Ex26 is an S1P$_1$ Antagonist that Inhibits Lymphocyte Egress. Most existing S1P$_1$ antagonists are S1P analogs with IC$_{50}$ values in the double-digit nanomolar range that possess relatively short half-lives. Recently, new S1P$_1$ antagonists have been described, including a series of biaryl benzylamines by Novartis (Angst et al. 2009). We synthesized and characterized one of these compounds, Ex26, and confirmed it to be a potent and selective antagonist of S1P$_1$ (Fig. 1A; Table 1), similar to a recently published antagonist (Quancard et al., 2012). Ex26 could inhibit RP-001–induced S1P$_1$ internalization and polyubiquitinylation in vitro (Supplemental Fig. 1). Similar to other previously described S1P$_1$ antagonists, Ex26 induced dose-dependent and time-dependent pulmonary edema in vivo (Fig. 1, B and C), and had a relatively short in vivo half-life of approximately 73.5 minutes (Supplemental Fig. 1C).

Earlier work showed that the S1P-like S1P$_1$ antagonists W146 and VPC44116 reversed agonist-induced lymphocyte sequestration while not causing lymphocyte sequestration (Sanna et al., 2006; Foss et al., 2007). Recent work has found that W146 induces transient lymphocyte sequestration at high doses (Tarrason et al., 2011), which we replicated (unpublished data). Ex26 induced lymphocyte sequestration at low doses, possessing an ED$_{50}$ of ~0.06 mg/kg when examined 2 hours following treatment (Fig. 2A). Lymphocyte sequestration by Ex26 resolved with similar kinetics as did Ex26-evoked pulmonary edema (Fig. 2B). To examine the effects of extended antagonist treatment, we implanted mice with micro-osmotic pumps to continuously deliver Ex26 at a dose of 0.1 mg/kg per hour for 3 days following a loading dose of 3 mg/kg. Extended treatment with Ex26 led to significant retention of T and B cells within the lymph nodes and significant decreases in T and B cells within the spleen, similar to S1P$_1$ agonists (Fig. 2, C and D). Continuous administration of Ex26 also led to thymic retention of mature CD62L^{Hi} single-positive thymocytes, also similar to the effects induced by S1P$_1$ agonists (Fig. 2E). These data demonstrate that disruption of S1P$_1$ signaling by S1P$_1$ antagonism leads to the inhibition of lymphocyte and thymocyte egress.

S1P$_1$ Antagonism Upregulates S1P$_1$ Expression. Since S1P$_1$ agonists downregulate S1P$_1$, we wanted to determine whether S1P$_1$ antagonism could conversely upregulate S1P$_1$. Continuous S1P$_1$ antagonism in mice expressing S1P$_1$-eGFP from the S1P$_1$ locus (Cahalan et al., 2011) for 3 days by

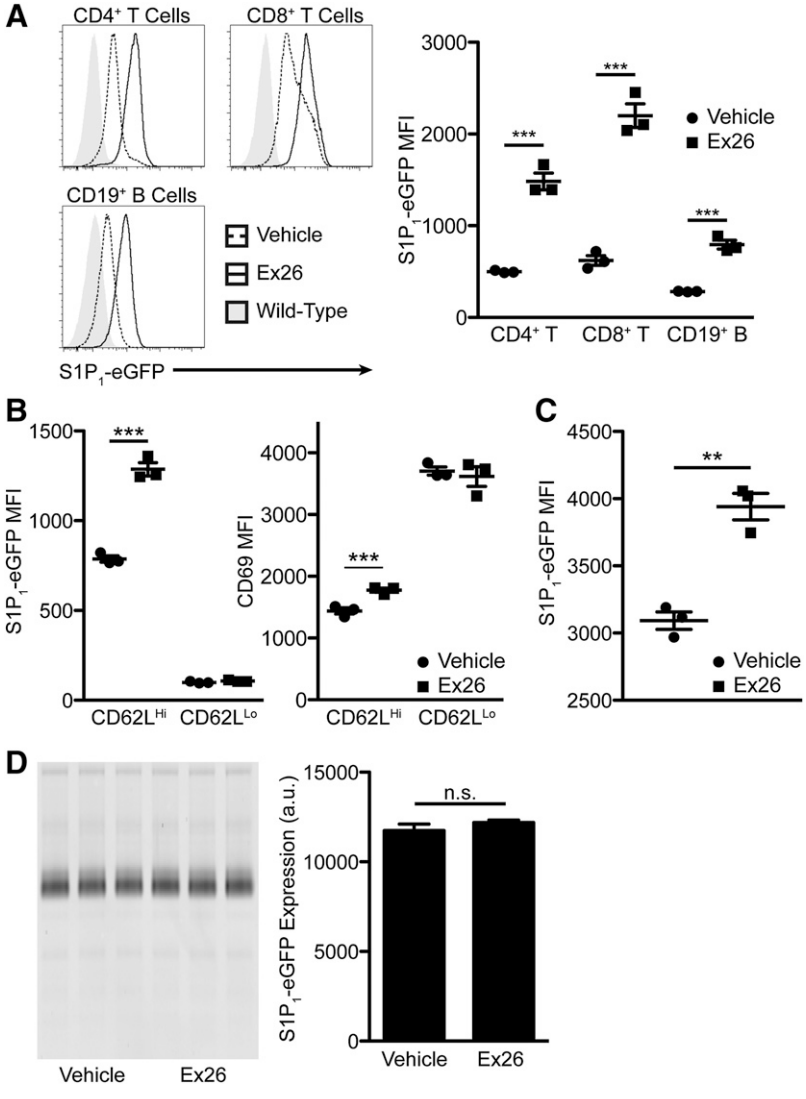

Fig. 3. S1P$_1$ antagonism by Ex26 upregulates S1P$_1$ and CD69, but not in the central nervous system. (A) S1P$_1$-eGFP expression on lymphocytes from lymph nodes from S1P$_1$-eGFP knockin mice continuously administered Ex26 by micro-osmotic pump for 3 days. Gray shaded histograms represent background fluorescence in wild-type mice. The graph on the right represents the mean fluorescence intensity of S1P$_1$-eGFP on the indicated cell type. (B) Mean fluorescence intensity of S1P$_1$-eGFP (left) and CD69 (right) on CD4 SP thymocytes following continuous treatment with Ex26. (C) Mean fluorescence intensity of S1P$_1$-eGFP on lung endothelial cells following continuous treatment with Ex26. (D) Fluorescent scan of SDS-PAGE gel from the brains of mice following 3 days of treatment with Ex26. The graph on the right is obtained by densitometric analysis of the gel on the left. All histograms and graphs are representative of three experiments, with 3–4 mice per group per experiment. Graphs are plotted as the mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ as determined by an unpaired, two-tailed t test. a.u., arbitrary units; MFI, mean fluorescence intensity; n.s., not significant.

micro-osmotic pumps caused significant upregulation of $S1P_1$-eGFP on lymphocytes within the lymph node (Fig. 3A). This suggests that the low concentration of S1P within the lymph node (Schwab et al., 2005) under normal physiologic conditions is sufficient to suppress the expression of $S1P_1$. We observed similar upregulation within the spleen (unpublished data) and a modest upregulation of $S1P_1$-eGFP on fully mature $CD62L^{hi}$ SP thymocytes (Fig. 3B). $S1P_1$ agonists cause a loss of surface expression of CD69 on mature thymocytes (Alfonso et al., 2006). In contrast to the effects seen with agonists, continuous Ex26 treatment led to significant upregulation of CD69 (Fig. 3B), indicating that $S1P_1$ signaling, not only expression of $S1P_1$ (Bankovich et al., 2010), is critical for suppressing the surface expression of CD69; thus, downregulation of CD69 by $S1P_1$ agonists is a measure of agonism, not functional antagonism. Upregulation of $S1P_1$-eGFP was not limited to lymphocytes, as blood endothelial cells within the lung significantly upregulated $S1P_1$-eGFP expression (Fig. 3C). Unlike many $S1P_1$ agonists, including FTY720-P, Ex26 did not cause any changes in the expression of $S1P_1$-eGFP within the brain (Fig. 3D), due to the fact that Ex26 was almost undetectable within the CNS (plasma: 6.8 ± 0.3 μM, brain: 0.01 ± 0.005 μM; 2/3 animals below the level of detection; mean $\pm$ S.E.M.).

$S1P_1$ Antagonism Ameliorates EAE. Because Ex26 did not enter the CNS or cause any change in $S1P_1$ expression within the CNS, it allowed us to determine whether lymphocyte sequestration alone was able to reverse EAE. Whereas 3 mg/kg Ex26 induced relatively short-duration lymphocyte sequestration, we found that a single dose of 30 mg/kg caused lymphocyte sequestration and pulmonary edema that lasted 24 hours in naïve mice (Supplemental Fig. 2, A and B). To examine whether $S1P_1$ antagonism could ameliorate EAE similar to $S1P_1$ agonism, we induced disease using the myelin oligodendrocyte glycoprotein residues 33–55 peptide model, and, upon development of clinical signs of disease, treated mice i.p. once daily with 30 mg/kg Ex26, 10 mg/kg FTY720, or 50 mM Na_2CO_3 vehicle, which we found to be indistinguishable from water, the usual vehicle for FTY720 (unpublished data). We found that treatment of mice with 30 mg/kg Ex26 daily significantly reduced the severity of EAE as assessed by examining clinical signs (Fig. 4A). We observed significant lymphocyte sequestration 3 hours following the last treatment with both 30 mg/kg Ex26 and 10 mg/kg FTY720; however, unlike its effect in naïve mice, 30 mg/kg Ex26 did not cause lymphocyte sequestration that lasted a full 24 hours in mice with EAE, whereas 10 mg/kg FTY720 did (unpublished data), suggesting that treatment with pertussis toxin used in the induction of EAE, or repeated dosing of Ex26, reduced the efficacy of Ex26, potentially by upregulating $S1P_1$ expression on lymphocytes. The reduction in the severity of EAE was seen in the spinal cord, as 30 mg/kg Ex26 inhibited both lymphocyte infiltration and destruction of the white matter in the spinal cord of mice euthanized at the end of the experiment (Fig. 4B). Consistent with the lack of CNS penetration of Ex26, we did not observe any changes in $S1P_1$ expression within the brains of mice euthanized at the end of the experiment that were treated daily with 30 mg/kg Ex26 compared with those treated daily with vehicle, whereas mice treated daily with 10 mg/kg FTY720 exhibited a complete loss in $S1P_1$ within the brain (Fig. 4C). This indicates that antagonism of $S1P_1$ expressed on neurons or astrocytes within the CNS is not required for the amelioration of EAE by $S1P_1$ antagonists, implying that lymphocyte sequestration by $S1P_1$

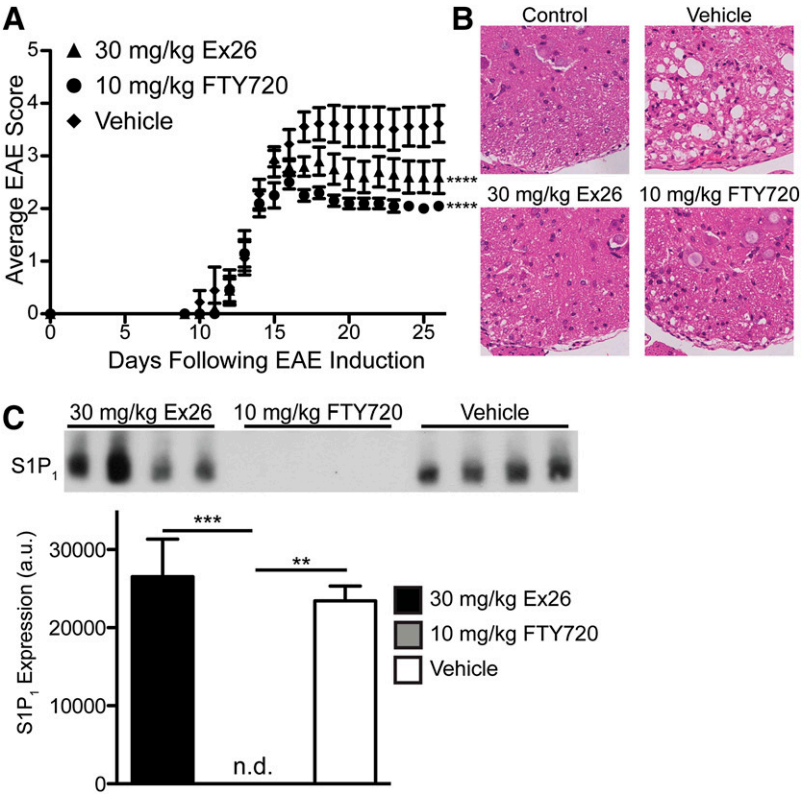

Fig. 4. $S1P_1$ antagonism by Ex26 alleviates EAE. (A) Average EAE scores from myelin oligodendrocyte glycoprotein residues 33-55–induced mice injected daily i.p. with vehicle, 30 mg/kg Ex26, or 10 mg/kg FTY720 following the onset of symptoms. ****$P < 0.0001$ compared with vehicle as calculated by one-way repeated measures analysis of variance with Bonferroni's multiple comparison post test. The graph represents two separate experiments as the mean $\pm$ S.E.M with 9–10 mice per group. (B) Representative spinal cord sections stained with H&E from control mice without EAE (top left) or mice with EAE that had been treated daily as indicated following the onset of clinical signs. (C) Western blot for $S1P_1$ on the brains of mice with EAE treated daily with vehicle (50 mM Na_2CO_3), 30 mg/kg Ex26, or 10 mg/kg FTY720 following the onset of symptoms. The graph represents $S1P_1$ expression as determined by densitometry. a.u., arbitrary units; n.d., not detectable.

antagonists is sufficient to reverse the pathology of EAE, in keeping with the efficacy of lymphocyte migration inhibitory agents, such as natalizumab, that successfully treat multiple sclerosis.

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Authorship Contributions

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

Conducted experiments: Cahalan, Gonzalez-Cabrera, Nguyen, Cisar, Leaf, Brown.

Contributed new reagents or analytic tools: Guerrero, Roberts.

Performed data analysis: Cahalan, Gonzalez-Cabrera, Cisar, Brown, Rosen.

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

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