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A Sphingosine-1-Phosphate Receptor 1-Directed Agonist Reduces Central Nervous System Inflammation in a Plasmacytoid Dendritic Cell-Dependent Manner

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Gradients of the sphingolipid sphingosine-1-phosphate (S1P) are responsible for the egress of lymphocytes from lymph nodes by activating the S1P1 receptor expressed on the surface of lymphocytes. Small molecule drugs that downregulate S1P receptors induce the sequestration of lymphocytes within lymph nodes, thus preventing lymphocytes from accessing sites of inflammation. In particular, FTY720, a pan-S1P receptor agonist, has been efficacious in the treatment of multiple sclerosis as well as its animal model, experimental autoimmune encephalomyelitis (EAE), by virtue of its ability to restrain lymphocytes within the lymph nodes, thus precluding their migration into the CNS. However, multiple leukocyte subsets express S1P receptors of varying types, and although it is beneficial to prevent transmigration of proinflammatory lymphocytes into the CNS, allowing access of regulatory leukocyte subsets to the CNS is desirable. In this study, we show that an S1P1-specific agonist (AUY954) is clinically efficacious in ameliorating pre-established EAE in SJL/J mice. Efficacy of AUY954 correlated with a reduction of lymphocytes in the CNS, but access of plasmacytoid dendritic cells (pDCs) to the CNS was unimpaired, and the presence of pDCs was found to be an important cofactor in mediating the clinical efficacy of AUY954. These results indicate that pDCs are important in quieting autoimmune responses during EAE, and that trafficking inhibitors that are permissive for pDC accumulation in the CNS may be of therapeutic value for the treatment of multiple sclerosis. The Journal of Immunology, 2012, 189: 3700–3706.

Although the precise etiology of multiple sclerosis (MS) remains unclear, the prevailing view is that MS is initiated when autoreactive T lymphocytes are primed and subsequently migrate to the CNS where they recognize myelin and induce inflammation. Within the CNS, protracted inflammation and disease require the presence of infiltrating T cells that are continually stimulated by cells of the innate immune system that present Ag, in particular dendritic cells (1, 2). Inhibition of lymphocyte entry into the CNS is an effective means of curtailing inflammation in both MS and experimental autoimmune encephalomyelitis (EAE), an animal model of MS (3). Such trafficking inhibitors can have effects on both pro- and anti-inflammatory leukocyte subsets; therefore, developing agents that selectively prevent migration of some but not all leukocytes across the blood–brain barrier (BBB) is of clinical importance for effective treatment of MS.

Plasmacytoid dendritic cells (pDCs), the major producers of type I IFN, have been shown to play a role in modulating the severity of EAE. Specifically, pDC depletion in PLP-immunized SJL/J mice, either during the acute phase or during EAE relapse, exacerbates clinical symptoms concomitant with an increase in Th1 and Th17 cells in the CNS (4). Similarly, pDC depletion of myelin oligodendrocyte glycoprotein-immunized C57BL/6 mice during the symptomatic phase of disease results in exacerbated EAE, although pDC depletion prior to disease onset had the opposite effect (5). Consistent with the anti-inflammatory function of pDCs in EAE, pDCs are reduced in the peripheral blood of MS patients as compared with healthy controls (6). Therefore, as putative modulators of inflammation in the CNS, an ideal trafficking inhibitor would prevent lymphocyte entry into the CNS while sparing pDC migration.

Sphingosine-1-phosphate (S1P) plays an essential role in the immune system through five different G protein-coupled receptors (S1P1–5). The trafficking and migration of lymphocytes is controlled by S1P via the S1P1 receptor (7). FTY720, a drug that is used to treat MS (8), is a pan-S1P receptor agonist that rapidly induces lymphocyte retention in the lymph node (LN) by downregulating S1P receptors on the surface of lymphocytes, thus preventing their egress to the blood and into the CNS (9). Our previous work showed that FTY720 also induces the retention of pDCs in the LN, but an S1P1-specific agonist does not have this effect. This is consistent with the finding that mouse pDCs do not express high levels of the S1P1 receptor, but rather express S1P4 (10).

Given the putative anti-inflammatory function of pDCs in EAE (4, 5), and possibly in MS (6), we reasoned that agents that specifically modulate the S1P1 receptor, but preserve S1P4 activity, would prove efficacious in EAE. To test this hypothesis, we have examined
the clinical efficacy of a small molecule agonist that specifically targets S1P1 receptor activity (AUY954) in the context of SJL/J relapsing–remitting EAE. AUY954 has previously been shown to prevent acute allograft rejection (11), to modulate allergic responses (12), and to reduce neuroinflammation in acute and chronic settings (13, 14). We found that AUY954 treatment ameliorated the clinical symptoms of relapsing–remitting EAE in SJL/J mice, concomitant with reduced appearance of cytokine-producing lymphocytes in the CNS. Whereas lymphocyte accumulation was reduced, we found that AUY954 treatment permitted access of pDCs into the CNS, and, indeed, the presence of pDCs within the CNS was found to be required for clinical efficacy of this small molecule S1P1 receptor agonist. These data suggest that S1P1-directed therapies may prove therapeutically valuable for treating MS owing to their selective effect on specific leukocyte subsets.

Materials and Methods

Mice and induction of EAE

Wild-type SJL/J (SJL/JCrHsd) mice were obtained from Harlan Laboratories (Indianapolis, IN) owing to their reliable relapsing–remitting EAE disease trajectory (15). SJL/J mice were housed in specific pathogen-free conditions. In all cases, 6-wk-old age-matched female mice were used for EAE studies. Mice were immunized with an emulsion containing 150 μl IFA (Difco, Detroit, MI), supplemented with 100 μg PLP139–151 (HSLGKWLGHPDKF) and 200 μg Mycobacterium (Difco; ground by mortar and pestle in PBS) in a total volume of 300 μl with 100 μl injected s.c. in three spots on the back. No pertussis toxin was used. Some variability in EAE severity was observed that was related to the Mycobacterium lot. We judged severe EAE to be when the average clinical score at the peak of disease was >10, and moderate EAE when the average clinical score at the peak of disease was <10. EAE mice were weighed and scored daily for clinical disease according to a 16-point scale (16). Briefly, individual scores for loss of tail tone (0–2), disability in each limb (0–3), and impaired righting reflex (0–2) were added for a maximum daily score of 16 (with 16 being assigned for moribund mice that were promptly euthanized). Specifically, scores were as follows: for tail tone, normal, 0; partial loss, 1; complete loss, 2; for limbs, no disability, 0; weakness, 1; paresis, 2; complete paralysis, 3; for righting reflex, normal, 0; delayed, 1; impaired, 2. All experiments were performed according to the University of Toronto approved animal use protocols.

Preparation and administration of AUY954

AUY954 suspended in H2O/1% carboxymethylcellulose was administered intragastrically (10 μg/g body weight) for 3 d starting 1 d after EAE onset. Control mice received the same volume of H2O/1% carboxymethylcellulose.

Preparation and administration of anti-pDC Ag-1

Mice received 250 μg anti-pDC–depleting Ab i.p. (anti-mouse pDC Ag-1 [PDCA1] clone JF05-1C2.4.1; rat IgG2b; Miltenyi Biotec) in 200 μl sterile PBS twice: at the onset of the disease and 2 d after disease onset. Control mice received rat anti-Gal Ab IgG2b.

Assessment of immunopathology in the spinal cord

Immune-mediated inflammation was analyzed at day 14 postimmunization. Briefly, at the time of sacrifice mice were perfused with ice-cold PBS. Spinal cords intended for histology were subsequently fixed in formalin and embedded in paraffin. Paraffin-embedded tissues were subsequently stained with Luxol fast blue and H&E.

Evaluation of lymphocyte subsets and cytokine-producing cells by FACS

Mice were sacrificed 1 d following the final AUY954 (or anti-PDCA1) treatment. Brain and spinal cord were homogenized in collagenase D buffer (10 mM HEPES, 150 mM NaCl, 1 mM MgCl2, 5 mM KCl, and 1.8 mM CaCl2 in HBSS), and tissue was digested following the addition of 1 mg/ml collagenase D and 60 μg/ml DNaseI and incubation for 45 min at 37˚C. Lymphocytes were purified with a 30% Percoll solution and resuspended in 10% media (10% FBS, l-glutamine, sodium pyruvate, penicillin G, streptomycin sulfate, and 2-ME). Cells for intracellular cytokine analysis were restimulated with PMA and ionomycin in the presence of GolgiPlug and incubated at 37˚C for 5 h. Cells were permeabilized and fixed using the BD Cytofix/Cytoperm kit and incubated with 1 μg/ml FC-block in PBS plus 2% PBS. Cells were then stained with CD4-FITC, TNF-α-allophycocyanin, IL-17-PE, or IFN-γ-PE-Cy7 (eBioscience). Pu-

FIGURE 1. Kinetics of lymphocyte and pDC appearance in the CNS during EAE. SJL/J mice were immunized with PLP139–151 in CFA, and brain, spinal cord, and blood were analyzed for lymphocytes as well as pDCs (see Supplemental Fig. 1 for gating strategy). Sampling of these tissues was performed at various phases of EAE (see Supplemental Fig. 2). (A) pDCs and lymphocytes accumulate in the CNS during symptomatic periods of EAE but are reduced during remissions. (B) During the acute phase of EAE, lymphocyte entry into the CNS precedes pDCs by ∼2 d. Four to five mice were used per time point.
rified CNS cells intended for subset analysis were stained with PDCA1-FITC, B220-allophycocyanin or B220-PE, CD19-PerCp-Cy5.5, TCRβ-PerCp-Cy5.5, or CD11c-PE or CD11c-allophycocyanin. Flow cytometry acquisition was performed using the FACSCanto and FACSCalibur, and FlowJo software (version 9.2) was used for analysis. Fluorescence-minus-one controls were used to judge background stain.

Statistical analysis

For EAE, difference in clinical severity of EAE between groups was calculated by an ANOVA between groups of the mean daily EAE score. For all other statistical measurements we used the Mann–Whitney test with the exception of Fig. 6, where a Student t test was applied, as the data distribution were determined to be Gaussian. All tests were performed using Prism software (GraphPad Software).

Results

Lymphocyte entry precedes pDC entry into the CNS during symptomatic periods of EAE

To evaluate the role of pDCs in SJL/J EAE, we first assessed the dynamics of appearance of pDCs versus B and T lymphocytes during various phases of relapsing–remitting EAE (see Supplemental Fig. 1 for FACS analysis gates and Supplemental Fig. 2 for disease profiles using a 16-point clinical scale) (16). As expected, B and T lymphocytes appeared in the CNS slightly before the onset of symptoms (presymptomatic phase), accumulated at the peak of the acute phase, were reduced during remissions, but accumulated again during relapses. In contrast, during the entire disease course the level of lymphocytes in the blood remained roughly stable. Although not present in the CNS during the presymptomatic phase of disease, there was a remarkable accumulation of pDCs in the CNS during the acute phase of disease, and to a lesser degree during relapse. Interestingly, pDCs increased transiently in the blood prior to disease onset (Fig. 1A).

Focusing on the acute phase of disease where we observed the most dramatic increase in pDCs, we next evaluated the dynamics of pDC versus T cell accumulation between day 8 and day 13 of the SJL/J EAE disease course. T cells were observed at low numbers as early as day 8 postimmunization and peaked at approximately days 11–12 postimmunization. In contrast, pDC were not observed in the CNS until day 10–11 postimmunization (Fig. 1B). Therefore, pDCs are actively recruited to the CNS during symptomatic EAE, but appearance of lymphocytes precedes that of pDCs in the CNS during the acute phase of the disease.

S1P1 receptor agonist AUY954 effectively reduces the severity of pre-established EAE

We next asked whether S1P1 agonists could reduce the clinical severity of pre-established SJL/J EAE. To do this, we administered AUY954, a selective agonist of S1P1 receptors, at day 11 postimmunization of SJL/J mice with PLP139–151 in CFA. Based on our kinetics analysis, lymphocyte migration into the CNS would be established and ongoing at this time point, but pDC accumulation would be just beginning. Three daily administrations of AUY954 resulted in a statistically significant reduction in clinical severity of pre-established EAE (Fig. 2)
AUY954 treatment reduces the accumulation of T and B cells, but not pDCs, in the CNS

Administration of the pan-S1P agonist FTY720 has been shown to induce the accumulation of lymphocytes (9) and pDCs (10) in LNs by preventing lymphocytes from responding to an S1P gradient within the medullary cords/lymph. Blockade of lymphocyte egress from LNs results in a reduction in lymphocyte accumulation in peripheral tissues, such as the CNS, in EAE (18). To examine the dynamics of lymphocyte recruitment to the CNS in response to an S1P1 agonist, we counted lymphocyte numbers in the blood and in the CNS during the acute phase of EAE after the mice had been treated on 3 consecutive days with AUY954. We observed a marked reduction in both T and B lymphocyte numbers in the blood following AUY954 treatments as compared with control treated mice, consistent with an important role for S1P1 in regulating lymphocyte egress from LNs (9). Interestingly, we did not observe a reduction in pDCs in the blood, in agreement with our previous observations that pDCs do not express high levels of S1P1 (10) (Fig. 3).

Because we observed that the numbers of pDCs in the blood following AUY954 treatment were unaffected (Fig. 3), we postulated that agonism of the S1P1 receptor would not prevent pDC recruitment to the CNS. In agreement with this hypothesis, AUY954 treatment did not result in a reduction in pDCs in the brain or spinal cord during EAE (Fig. 4). During more moderate EAE (average peak EAE score was <10), pDCs were actually found to accumulate in the spinal cord upon AUY954 treatment (Supplemental Fig. 4). Taken together, the efficacy of AUY954 treatment during the acute phase of EAE positively correlates with the persistence of pDCs in the CNS.

The efficacy of AUY954 during EAE depends on the presence of pDCs

The correlation between sustained migration of pDCs to the CNS and decreased disease severity is consistent with a regulatory role in pDCs during EAE. This was demonstrated by the findings that depletion of pDCs using a neutralizing antibody was able to abrogate the efficacy of AUY954 treatment during the acute phase of EAE (Fig. 5). These results suggest that pDCs play a key role in the pathogenesis of EAE, and that targeting S1P1 in pDCs may be a promising strategy for the treatment of this autoimmune disease.

AUY954 treatment results in reduced B and T lymphocytes, but not pDCs, in the spinal cord during the acute phase of EAE.

SJL/J mice were immunized with PLP139–151 in CFA followed by treatment with AUY954 the day after onset of symptoms for 3 subsequent days. The numbers of B cells, T cells, and pDCs were enumerated in the brain and spinal cord. This experiment examined seven mice per group. The experiment, which provoked EAE with an average clinical score of 10, was performed twice with similar results on a total of 14 mice per group.

FIGURE 4. AUY954 treatment results in reduced B and T lymphocytes, but not pDCs, in the spinal cord during the acute phase of EAE. SJL/J mice were immunized with PLP139–151 in CFA followed by treatment with AUY954 the day after onset of symptoms for 3 subsequent days. The numbers of B cells, T cells, and pDCs were enumerated in the brain and spinal cord. This experiment examined seven mice per group. The experiment, which provoked EAE with an average clinical score of 10, was performed twice with similar results on a total of 14 mice per group.

FIGURE 5. Efficacy of AUY954 treatment during the acute phase of EAE is nullified by pDC depletion. SJL/J mice were immunized with PLP139–151 in CFA. At the onset of symptoms, pDCs were depleted using anti-PDCA1 mAb and 1 d after the onset of symptoms, mice were treated on 3 consecutive days with AUY954. Clinical scores were monitored daily (A) and the cumulative clinical score during the treatment period was tabulated (B). This experiment is the combination of two comparable experiments for a total of 14 mice per group. *p < 0.05, by Mann–Whitney test.
of pDCs for curtailing inflammation during EAE, as has been described by others (4, 5). To examine whether pDCs are required for the mechanism of action of AUY954 during EAE, we pre-administered a mAb (anti-PDCA1) 1 d prior to AUY954 treatment. This agent effectively depleted pDCs in the CNS (Supplemental Fig. 4). Strikingly, when anti-PDCA1 was coadministered with AUY954, no clinical efficacy was observed during the 3-d treatment period (Fig. 5A), and in contrast to AUY954 treatment, the sum of clinical scores during this period for mice that received both AUY954 and anti-PDCA1 was not statistically different from what was observed with vehicle administration (Fig. 5B). In dual-treated mice, B cells were significantly reduced in the brain and spinal cord, and T cells trended toward lower numbers in the CNS (Supplemental Fig. 4). This suggests that the reduction in lymphocyte accumulation in the CNS provoked by AUY954 treatment is not prevented by anti-PDCA1 treatment.

**AUY954 treatment reduces the number of CNS-resident cytokine-producing CD4+ T cells in a pDC-dependent manner**

The manifestation of clinical disease during the acute phase of EAE is at least in part mediated by the production of inflammatory cytokines such as IL-17, IFN-γ, and TNF-α within the CNS. A reduction in levels of cytokines produced by CD4+ T cells is associated with clinical efficacy of FTY720 treatment in EAE (19). Similarly, treatment with AUY954 during the acute phase of EAE resulted in a statistically significant reduction of IL-17–, IFN-γ–, and TNF-α–producing cells in the CNS. When pDCs were pre-depleted prior to AUY954 treatment, a statistically significant reduction in IL-17– and IFN-γ–producing CD4+ T cells was no longer observed. In contrast, pDC depletion does not alter the effects of AUY954 on reducing the number of TNF-α–producing CD4+ T cells in the CNS (Fig. 6). Therefore, the presence of pDCs may be required to prevent the accumulation of IFN-γ– and IL-17–producing CD4+ T cells. Consistent with this notion, depletion of pDCs during the acute phase of EAE in mice that were not treated with AUY954 results in exacerbated clinical disease accompanied by increased numbers of IFN-γ– and IL-17–producing CD4+ T cells (Fig. 7), implying that pDCs either control T cell polarization within the CNS or migration of Th1/Th17 cells to the CNS.

**Discussion**

In this study we show that a specific agonist directed at the S1P1 receptor is an effective treatment for pre-established EAE in SJL/J mice. By treating mice at a time point when lymphocyte entry had already been initiated, we were able to decrease the amplitude of...
the acute phase of disease, and this correlated with a reduction in lymphocyte numbers in the CNS. We also found that AUY954 treatment could reduce the severity of EAE relapses (Supplemental Fig. 3), although because of the heterogeneity of the inflammatory response during this phase of disease, it was difficult to correlate therapeutic efficacy with modulations of lymphocyte numbers in the CNS. Nevertheless, the efficacy we observe with AUY954 treatment parallels what is observed with FTY720 treatment (13), where reductions in lymphocytes in the CNS have also been observed. It is difficult to predict the comparable efficacy of pan-agonists for S1P receptors (i.e., FTY720) versus small molecules that only target S1P1 owing to their differential bioavailability. Nevertheless, in agreement with a recent report examining the effect of S1P1 activity on astrogliosis in a C57BL/6 model of neuroinflammation (13), one can conclude that targeting S1P1 is sufficient for disease attenuation.

Concomitant with AUY954 treatment we observed a reduction in the number of cytokine-secreting CD4+ T cells in the CNS. This may simply reflect the overall reduction in the numbers of CD4+ T cells that accumulate in the CNS; however, the observation that pDC depletion partially restored numbers of IL-17– and IFN-γ– secreting CD4+ T cells (Fig. 6) suggests a more complex mechanism of action for AUY954. Thus, other mechanisms beyond modulation of lymphocyte trafficking may be relevant to the efficacy of AUY954. Indeed, astrogliosis, which precedes clinical signs of EAE (20), was found to be dependent on S1P1 (13). Further examination of the effect of AUY954 in animal models where S1P1 is conditionally deleted in specific leukocyte subsets and various glial cells would test this possibility.

We observed that although AUY954 induces a dramatic reduction of absolute lymphocyte counts in the blood and reduced accumulation of lymphocytes in the CNS, pDC numbers in the blood remained stable, consistent with the finding that pDCs do not express high levels of S1P1 (10). Accordingly, AUY954 treatment does not result in a reduction of pDCs in the CNS. In cases of moderate EAE (average clinical score at the peak of disease <10), we observed a net increase in absolute pDC numbers in the spinal cord (Supplemental Fig. 4). At this time we do not know why pDC numbers would increase during AUY954 treatment, but we speculate that perhaps there may be limited resources within the perivascular niche of the CNS that would allow pDC expansion to occur in the absence of significant lymphocyte accumulation. In agreement with this possibility, pDCs exhibit very rapid turnover (21), and their homeostatic proliferation is inversely related to the homeostatic proliferation of lymphocytes (22). Such “homeostatic reciprocity” may be at play between lymphocytes and pDCs within the CNS. Alternatively, pDC expansion in the CNS could be actively prevented by factors/cytokines secreted by lymphocytes.

Because pDC accumulation in the CNS correlated with the clinical efficacy of AUY954, we examined whether pDCs were necessary for disease relief. In agreement with this hypothesis, we observed that the clinical efficacy of AUY954 was blunted in the absence of pDCs. Furthermore, the corresponding reduction in IFN-γ– and IL-17–producing cytokines observed with AUY954 treatment was not fully achieved in dual-treatment animals. This suggests that pDCs may influence either the polarization of Th1 and Th1 CD4+ T cells within the CNS, or the migration of Th1 and Th1 cells into the CNS, and our data examining the effect of anti-PDCA1 treatment alone (Fig. 7) support these possibilities. Interestingly, the impaired secretion of TNF-α by CD4+ T cells in AUY954-treated EAE mice was unaffected by pDC depletion. Because TNF-α plays a complex role in both inflammation and repair in the CNS, and blockade of TNF-α is harmful in MS (23), the relative sparing of TNF-α production by Th cells in anti-pDCA1–treated mice may be an additional harmful outcome.

Our results agree with other studies where pDC depletion has been previously shown to reduce the number of Th1 and Th17 CD4+ T cells in the CNS during EAE (4), and CNS-resident pDCs have been shown to be suboptimal APCs for priming encephalogenic CD4+ T cells (24). Deliberate targeting of myelin oligodendrocyte glycoprotein peptides to pDCs impairs the Th1/Th17 polarization and reduces the severity of EAE (25). Although the timing of pDC depletion may have differential effects on EAE outcome (5), our data and those of others point to an anti-inflammatory function of pDCs. Indeed, pDCs that have the capacity to migrate in response to CCL25 can directly suppress and/or tolerize graft-specific T cells (26). It is also possible that the anti-inflammatory capacity of pDCs may be exerted outside of the CNS in the draining LN (27). How pDCs negatively modulate T cell priming in EAE remains unclear, but could be tied to the unique array of cytokines secreted by these cells, which include type I IFNs (28).

In conclusion, we have shown that agents that singularly target the S1P1 receptor are an effective means for treating established EAE. Our findings that correlate the efficacy of AUY954 and pDC accumulation in the CNS suggest that agents that target specific S1P receptors may be an effective means for reducing the clinical severity of pre-established EAE by modulating the trafficking of selected leukocyte subsets across the BBB. A full assessment of S1P1 receptor expression on human pDCs, as well as their responsiveness to different S1P receptor agonists in vitro and in the clinical context, may illuminate the role of pDCs in regulating neuroinflammation.

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Disclosures
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References


