Sphingosine 1-phosphate receptor modulation suppresses pathogenic astrocyte activation and chronic progressive CNS inflammation

Veit Rothhammera, Jessica E. Kenisona, Emily Tjonb, Maisa C. Takenakaa, Kalil Alves de Limaa, Davis M. Boruckia, Chun-Cheih Chaoba, Annabel Wilzb, Manon Blainb, Luke Healyb, Jack Antielb, and Francisco J. Quintanaabc1

aAnn Romney Center for Neurologic Diseases, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115; bNeuroimmunology Unit, Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, QC, Canada QC H3A 2B4; and cBroad Institute of MIT and Harvard, Cambridge, MA 02142

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Multiple sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the CNS that causes disability in young adults as a result of the irreversible accumulation of neurological deficits. Although there are potent disease-modifying agents for its initial relapsing-remitting phase, these therapies show limited efficacy in secondary progressive MS (SPMS). Thus, there is an unmet clinical need for the identification of disease mechanisms and potential therapeutic approaches for SPMS. Here, we show that the sphingosine 1-phosphate receptor (S1PR) modulator fingolimod (FTY720) ameliorated chronic progressive experimental autoimmune encephalomyelitis in nonobese diabetic mice, an experimental model that resembles several aspects of SPMS, including neurodegeneration and disease progression driven by the innate immune response in the CNS. Indeed, S1PR modulation by FTY720 in murine and human astrocytes suppressed neurodegeneration-promoting mechanisms mediated by astrocytes, microglia, and CNS-infiltrating proinflammatory monocytes. Genome-wide studies showed that FTY720 suppresses transcriptional programs associated with the promotion of disease progression by astrocytes. The study of the molecular mechanisms controlling these transcriptional modules may open new avenues for the development of therapeutic strategies for progressive MS.

multiple sclerosis | sphingolipid metabolism | astrocytes | EAE | secondary progression

Multiple sclerosis (MS) is a chronic autoimmune disease of the CNS that, in most patients, initially presents with a relapsing-remitting course. This relapsing-remitting stage is often followed by a secondary progressive phase characterized by the progressive and irreversible accumulation of neurological deficits. The available therapeutic approaches for relapsing-remitting MS (RRMS) show limited efficacy in secondary progressive MS (SPMS), reflecting our insufficient understanding of the pathologic mechanisms that drive disease progression in SPMS and primary progressive MS (1). Recent findings, however, suggest that the innate immune response in the CNS promotes disease progression in MS. Indeed, astrocytes (the most abundant cell population in the mammalian CNS), microglia, and proinflammatory monocytes are thought to promote neurodegeneration, demyelination, and scar formation (1–6). However, therapeutic strategies targeting these cell types remain elusive to date.

Sphingosine 1-phosphate (S1P) is a sphingosine-containing lipid generated from ceramide, which binds G protein-coupled receptors [Sphingosine 1-phosphate receptors (S1PRs) 1–5] and modulates the proliferation and trafficking of several cell types, including immune cells. Consequently, S1PRs are considered candidate therapeutic targets for inflammatory diseases, including MS, psoriasis, asthma, and polyneuropathy, and also for hematologic and solid tumors, ischemic stroke, and wound healing (7–12).

FTY720 (fingolimod) is a modulator of SIP receptors 1, 3, 4, and 5 with therapeutic effects on RRMS (13–18). The therapeutic effects of FTY720 in RRMS are thought to result mainly from the internalization of S1PR1 in T and B cells, blocking lymphocyte egress from lymph nodes and consequently limiting their recruitment to the CNS (19). FTY720 has also been shown to modulate proinflammatory pathways in B and T cells (19–22).

In addition to these effects of FTY720 on the peripheral immune system, phosphorylated FTY720 crosses the blood-brain barrier (BBB) and is thus capable of interacting with CNS-resident cell populations (19, 22, 23).

In vitro observations suggest direct effects of FTY720 on astrocyte biology, neurodegeneration, and remyelination, which impact mechanisms of disease pathogenesis relevant for the progressive stages of MS (20, 22–28). Indeed, animal studies using acute models of RRMS suggest that FTY720 modulates the activity of CNS-resident cell populations (28–31). However, limited information is available on the effects of S1PR modulation on SPMS and its experimental models of CNS chronic inflammation and progressive neurodegeneration. Thus, we investigated the effects of FTY720 on the chronic progressive model of experimental autoimmune encephalomyelitis (EAE) in nonobese diabetic (NOD) mice, which resembles several aspects of SPMS (32). We found that FTY720 ameliorates EAE in NOD mice and decreases the production of proinflammatory and neurotoxic mediators by mouse and human astrocytes. These findings identify potential targets for the modulation of local inflame.
CNS inflammation driven by astrocytes, microglia, and inflammatory monocytes in SPMS.

Results

FTY720 Ameliorates Chronic Progressive EAE in NOD Mice. EAE induced in NOD mice by immunization with myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) peptide recapitulates several features of SPMS, including the progressive accumulation of neurodegeneration and axonal loss and the chronic activation of the innate immune system in the CNS (3, 33, 34). Thus, to assess the therapeutic potential of S1P receptor modulators in SPMS, we evaluated the effects of FTY720 on NOD EAE.

Following immunization with MOG35-55 in complete Freund’s adjuvant (CFA) and pertussis toxin administration, NOD mice initially develop an acute neurological event, which is followed by a chronic progressive phase that starts at approximately day 25 (Fig. 1A). Thus, we initiated treatment with daily doses of FTY720 (0.3 mg/kg body weight) or vehicle 40 d after NOD EAE induction and monitored disease development until day 120. FTY720 ameliorated NOD EAE during the progressive phase, as indicated by the reduction of clinical scores and mortality (Fig. 1A and B). The beneficial effects of FTY720 in NOD EAE were also reflected in decreased demyelination and axonal loss (Fig. 1C). However, the amelioration of NOD EAE by FTY720 was not linked to alterations in the recall T-cell response to MOG35-55, or with changes in peripheral or CNS cell counts (Fig. 1D and Fig. S1). Thus, FTY720 ameliorated progressive NOD EAE without significant effects on the peripheral T-cell response.

FTY720 Reduces Pathogenic CNS Innate Immune Activation. The CNS innate immune response plays a central role in the progressive phase of NOD EAE (1, 3, 5, 6, 35–39). Thus, we analyzed the transcriptional profile of astrocytes, microglia, and proinflammatory monocytes isolated from vehicle- and FTY720-treated mice 120 d after EAE induction using custom-made NanoString nCounter arrays (Table S1) (2). FTY720 administration downregulated the expression of proinflammatory cytokines and chemokines in astrocytes, including Il6, chemokine (C-C motif) ligand 2 (Ccl2), Ccl20, Ifng, Il23a, C-X-C motif chemokine 10 (Cxc10), and Il1b, among others (Fig. 2A). Importantly, neurotoxic mediators such as Tnfa and Nos2 as well as factors governing proinflammatory macrophage polarization, including colony stimulating factor 2 (Csf2) and Il12a, showed diminished expression under FTY720 treatment. Conversely, the expression of anti-inflammatory factors such as Cxcl12 (40) and Il33 (41, 42) was up-regulated by FTY720 (Fig. 2A).

Microglia and CNS-infiltrating proinflammatory monocytes are thought to play an important role in the pathogenesis of SPMS and the progressive phase of NOD EAE (36, 37, 43, 44). We thus analyzed the transcriptional profile of microglia and CNS-infiltrating CD11b+CD45+Ly6C+ proinflammatory monocytes in vehicle- and FTY720-treated mice. We detected decreased expression of proinflammatory gene clusters associated with NOD EAE pathology (2, 3) in microglia and CNS-infiltrating proinflammatory monocytes (Fig. 2B–D). Of note, the down-regulation of proinflammatory gene expression was stronger in microglia than in monocytes. Moreover, microglia displayed strong up-regulation of factors expressed in alternatively activated microglia such as Csf2, Chil3, and Ccl20 in FTY720-treated animals (Fig. 2B). In summary, FTY720 decreased the expression of proinflammatory, chemotactic, and neurotoxic molecules thought to mediate the pathogenic role of astrocytes, microglia, and CNS-infiltrating monocytes in MS and NOD EAE.

Astrocytic S1P1 Controls Astrocyte Neurotoxicity and Monocyte Recruitment and Activation. S1PR1 expression in astrocytes contributes to the protective effects of FTY720 in acute EAE (22), but the mechanisms involved are not completely understood. We detected decreased expression of proinflammatory chemokines (Ccl2), cytokines (Csf2, Il6, Tnfa), neurotoxic (Nos2, Tnfa), and macrophage polarizing factors (Csf2) in highly purified primary astrocyte cultures (Fig. S2) activated in the presence of FTY720 in vitro. Conversely, FTY720 up-regulated the expression of neuroprotective Il10 (Fig. 3A). Thus, S1PR modulation in astrocytes affects the expression of mediators thought to promote disease progression in MS and NOD EAE.

Astrocytes display neurotoxic activities in the context of chronic CNS inflammation and also induce and amplify pathogenic activities in microglia and monocytes recruited to the CNS (40, 45–51). Thus, to evaluate the relevance to disease pathogenesis of the effects of FTY720 on the transcriptional program of astrocytes, we analyzed the effects of FTY720 on the neurotoxic potential of astrocytes and on their ability to control migration and monocyte polarization (2).

Astrocytes promote neuronal death through the production of TNF-α, glutamate, lactate, and reactive oxygen species, among

Fig. 1. FTY720 ameliorates chronic progressive EAE in NOD mice. EAE was induced in NOD/ShiIL2−/− mice, which were treated with daily i.p. injections of FTY720 or vehicle in the secondary progressive phase of the disease starting from day 40 after disease induction. (A) Clinical scores (left) and linear regression analysis (right) of mice under treatment with FTY720 or vehicle (n = 10 mice per group; two-way ANOVA). (B) Kaplan-Meier survival analysis of mice in the experiment described in A by two-way ANOVA. (C) Histologic examination of transversal lumbar spinal cord sections isolated from FTY720- or vehicle-treated mice at day 120. (Left) representative sections stained for Luxol fast blue (LFB) for demyelination or Bielschowsky’s Silver stain (silver) for axonal loss. Representative of three sections of three mice. (Right) Quantification of demyelination and axonal loss in FTY720- or vehicle-treated mice (Student’s t test). (D) Proliferation assay from splenocytes isolated on day 120 of the experiment (n = 5; two-way ANOVA). Throughout, data are mean ± SEM and representative of two independent experiments (**P < 0.05 and ***P < 0.01; ns, not significant).
monocytes (Fig. 3D). Mouse and human activated microglia, however, did not exhibit significant changes in the expression of proinflammatory cytokines or neurotoxic mediators upon exposure to FTY720 (Fig. S3 A and B). Also, neurotoxicity and induction of migration was not altered in supernatants of activated microglia upon treatment with FTY720 (Fig. S3 C and D).

FTY720 treatment led to a significant suppression of NF-κB p65 nuclear translocation in activated astrocytes (Fig. S4A), concomitant with decreased production of the proinflammatory and neurotoxic mediators IL-6, TNF-α, GM-CSF, CCL2, and nitric oxide (NO; Fig. S4 B and C). Indeed, in blocking experiments, we identified CCL2 and IL-6 as the active components in ACM driving monocyte migration in vitro, whereas TNF-α, GM-CSF, and IL-6 mediated astrocyte neurotoxic activity (Fig. S4 D and E).

Collectively, these studies suggest that S1PR modulation in astrocytes by FTY720 contributes to the amelioration of chronic progressive NOD EAE.

**FTY720 Modulates Activation of Human Astrocytes.** To further investigate the relevance of S1PR modulation in astrocytes to its potential effects on MS, we analyzed the genome-wide transcriptional response of human astrocytes activated in vitro in the presence of FTY720. We identified 1,221 transcripts that were differentially regulated under FTY720 treatment (Fig. 4 A and B). Ingenuity pathway analysis determined that FTY720 modulates the expression of transcriptional modules associated with migratory pathways, antigen presentation, inflammasome activation, axonal guidance, and fatty acid α-oxidation (Fig. 4C). Interestingly, NF-κB signaling was significantly altered by FTY720 in human astrocytes (Fig. 4C), recapitulating our findings of decreased NF-κB others (1, 6, 49). Thus, we used a cell-based assay to evaluate the effects of FTY720 on astrocyte-driven neurotoxicity. In this assay, we activated astrocytes in vitro in the presence of FTY720 or vehicle for 24 h and then added new culture medium after extensive washings. Following culture for additional 48 h, astrocyte-conditioned medium (ACM) was collected. To evaluate the neurotoxic activity of ACMs, we exposed neuronal cells to the ACMs and monitored neuronal death by quantifying lactate dehydrogenase (LDH) release. FTY720 treatment led to a small but significant reduction of ACM neurotoxic activity (Fig. 3B).

During MS and EAE, proinflammatory monocytes are recruited to the CNS, where they play a central role in promoting neurodegeneration (2, 36, 37, 39). Thus, chemotactic factors produced by astrocytes play an important role in promoting disease progression in MS (40, 52, 53). To address the effects of FTY720 on the recruitment of Ly6C<sup>hi</sup> proinflammatory monocytes by astrocytes we used an in vitro transwell migration assay. We found that FTY720 decreased Ly6C<sup>hi</sup> inflammatory monocyte recruitment by ACM (Fig. 3C).

Astrocytes also modulate myeloid cell activation and polarization in the CNS (1, 54). To test the effects of FTY720 on the control of monocytes by astrocytes, we activated astrocytes in the presence of FTY720 or vehicle for 24 h, washed them extensively, and established cocultures with sorted Ly6C<sup>hi</sup> inflammatory monocytes. Following coculture for 24 h, the monocytes were reisolated and their transcriptional profile was analyzed. Treatment of astrocytes with FTY720 decreased the expression of proinflammatory cytokines, chemokines, and neurotoxic molecules in
Collectively, these findings reveal an overall neuroprotective effect of FTY720 on human astrocytes mediated, at least in part, by the suppression of NF-κB signaling. In addition, these findings highlight targets of FTY720 on astrocytes, which may guide novel potential therapeutic interventions for the modulation of astrocyte function during progressive MS.

Discussion

The immunosuppressive and disease-ameliorating effects of FTY720 in RRMS are mostly attributed to the blockade of inflammatory cell migration into the CNS (19, 28). Phosphorylated FTY720, however, crosses the BBB and acts on CNS-resident cells, including astrocytes (22, 24, 26). The effects of FTY720 in astrocytes, neurons, and oligodendrocytes have been previously documented in vitro (26, 27, 55, 56).

p65 activation in mouse astrocytes in vitro (Fig. S4A). NF-κB plays a central role in the control of astrocyte activities that promote inflammation and neurodegeneration (2, 40). Thus, we performed an in-depth computational analysis of the NF-κB pathway, which confirmed that FTY720 down-modulates NF-κB signaling in human astrocytes (activation z-score, −1.89; Fig. 4D).

To evaluate the functional relevance of our transcriptional analyses, we validated the expression of selected transcripts on independent samples and examined the functional effects of FTY720 on human astrocytes. FTY720 decreased the expression of proinflammatory and neurotoxic mediators, including cytokines, chemokines, and degenerative mediators, while increasing anti-inflammatory IL10 in human astrocytes, as determined in quantitative PCR (qPCR) analyses from separate sets of samples (Fig. 5A). Moreover, ACMs from FTY720-treated human astrocytes exhibited a marked reduction in neurotoxic and chemotactic properties, concomitant with an increased production of neuroprotective mediators (Fig. 5B–D). Human microglia, however, did not show a significant response to exposure to FTY720 (Fig. S3B).
Given the beneficial effects of FTY720 on organotypic cultures in vitro and its BBB permeability (27, 56), FTY720 has the potential to modulate the local innate immune response in the CNS, thought to contribute to disease progression in MS (1, 6, 49). However, the effects of FTY720 on chronic CNS-innate immune responses in vivo, as well as their relevance to the pathogenesis of disease progression in MS, are still unknown.

Our studies highlight the anti-inflammatory and neuroprotective effects of SIPR modulation on mouse and human astrocytes at transcriptional and functional levels. Moreover, we evaluated the biological relevance of these observations in the NOD EAE model (32). In the chronic phase of this EAE model, disease progression is driven by the pathogenic activity of astrocytes, microglia, and inflammatory monocytes, without a major contribution of the adaptive immune system (e.g., T cells), thus recapitulating several aspects of SPMS (3, 32, 57, 58). Our data show that FTY720 ameliorates progressive CNS inflammation and neurodegeneration, most likely as a result of its CNS-intrinsic effects on astrocytes, microglia, and proinflammatory monocytes. Of note, our in vitro data on microglia point to an indirect effect of FTY720 on microglia mediated by the modulation of astrocyte-derived factors that influence microglia polarization and pathogenic activities.

Five isoforms of S1P receptors are expressed by immune and nonimmune cells relevant to autoimmune diseases, stroke, and cancer (11, 12, 59–62). Thus, S1P receptors constitute attractive targets for therapeutic intervention. Indeed, SIPR modulation by FTY720 is efficacious in the treatment of RRMS (13, 14). SIPR modulation did not reach the primary endpoints in the INFORMS (oral fingolimod in primary progressive multiple sclerosis) trial in primary progressive MS (63), but ameliorated MRI parameters of disease activity (63). BAFF312 (siponimod) is a BBB-permeable modulator of SIP1 and SIP5 receptors effective in slowing down demyelination in vitro (64–66). The safety and therapeutic efficacy of BAFF312 on RRMS has been recently demonstrated in terms of clinical and MRI parameters in the phase II BOLD (BAF312 on MRI lesion given once daily) study (67). The therapeutic effects of BAFF312 on SPMS are currently being investigated in the EXPAND (exploring the efficacy and safety of siponimod in patients with secondary progressive multiple sclerosis) trial. Based on the effects of FTY720 on the CNS-innate immune response during NOD EAE, SIP receptor modulation by BAFF312 is likely to modulate local CNS inflammation in SPMS. Indeed, preliminary results of the EXPAND trial suggest a protective effect of BAFF312 on 3-mo confirmed disease progression in SPMS (68). However, side-by-side comparisons of the effects of FTY720 and BAFF312 on adaptive and innate immune cells are needed to determine whether the structural differences existing between these compounds affect their biologic activities on specific cell populations relevant to SPMS. Indeed, it is worth noting that, whereas FTY720 modulates SIP1, SIP3, SIP4, and SIP5 receptors, BAFF312 modulates SIP1 and SIP5 receptors only.

FTY720 is also reported to modulate sphingolipid metabolism, which is thought to promote disease progression in NOD EAE and MS (3, 19, 23, 69, 70). Indeed, we showed that inhibitors of B4GALT6 and lactosyl ceramide production suppress disease progression in NOD EAE by arresting local CNS-innate immunity and neurodegeneration (3). The arrest of NOD EAE obtained with B4GALT6 inhibitors is stronger than the amelioration obtained with FTY720, thus calling for caution as to the therapeutic potency of FTY720 and its analog BAFF312 in SPMS. Moreover, these findings suggest that the beneficial effects of FTY720 on NOD EAE may reflect its moderate inhibitory effects on sphingolipid metabolism.

Finally, our unbiased genome-wide analyses identify additional potential targets for the therapeutic modulation of astrocyte function and the CNS-innate immune response, which may guide the development of novel therapeutics for the treatment of progressive MS.

Materials and Methods

Mice. Female NOD/ShiLtJ mice and 1–3-d-old pups from C57BL/6J mice were obtained from the Jackson Laboratory and were kept in a pathogen-free facility at the Harvard Institutes of Medicine. All experiments were carried out in accordance with guidelines prescribed by the institutional animal care and use committee at Harvard Medical School.

EAE Induction and Treatment. EAE was induced in 8-wk-old mice by s.c. immunization with 150 μg MOG<sub>35-55</sub> peptide emulsified in CFA (Difco Laboratories) per mouse, followed by administration of 200 ng pertussis toxin (List Biological Laboratories) on days 0 and 2 as described previously (2, 3). Starting from day 40 after disease induction, mice were treated daily with i.p. injections of FTY720 0.3 mg/kg body weight or vehicle, respectively.

Generation of Astrocyte- and Microglia-Conditioned Medium for Migration and Neurotoxicity Assays. In vitro astrocyte and microglia cultures from WT pups were treated with LPS (100 ng/mL) in the presence of FTY720 (1 μg/mL) or vehicle for 24 h, extensively washed, and supplemented with fresh culture medium. Forty-eight hours later, supernatants were spun down and kept for migration and neurotoxicity assays at ~80°C.

Monocyte Migration Assay. Splenic monocytes were purified from WT mice by CD11b beads (Miltenyi) and sorted for Ly6C<sup>hi</sup>Ly6C<sup>th</sup> (SSC, sideways scatter). These monocytes were seeded in the upper chamber of a 24-well cell culture insert, with a 5-μm pore size (Corning), containing astrocyte- or microglia-conditioned medium (as detailed earlier). Migrating monocytes in the lower chamber were quantified after 3 h by FACS.

Neurotoxicity Assay. N2A neuronal cells (CCL-131, American Type Culture Collection) were grown in 96-well plates and preactivated with mouse IFN-γ (100 ng/mL, R&D Systems) for 24 h. Thereafter, medium was replaced, after extensive washing with PBS solution, with astrocyte- or microglia-conditioned medium. Cytotoxicity was measured by using LDH release (Cytotox 96 Nonradioactive Cytotoxicity Assay; Promega) after 24 h as suggested by the manufacturer’s protocol.

Monocyte Polarization Assays. Primary astrocyte cultures were activated in the presence of FTY720 or vehicle for 24 h. Thereafter, activation medium was removed and primed astrocytes were washed extensively. CD11b<sup>+</sup>CD45<sup>+</sup>Ly6C<sup>hi</sup> proinflammatory monocytes were FACs-sorted from spleens of naive WT mice and cocultured with primed astrocytes. After 24 h, monocytes were reisolated and RNA was isolated, transcribed, and subjected to qPCR analysis.

EAE clinical scoring, mouse and human primary astrocyte and microglia cultures, isolation of cells from adult mouse CNS, subcellular fractionation and immunoblot analysis, T-cell proliferation, flow cytometry staining and acquisition, ELISA, detection of NO, histology, nCounter gene expression, qPCR, Affymetrix gene assay, heat-map generation, and Ingenuity pathway and statistical analysis are provided in SI Materials and Methods.

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