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# A Mechanistically Novel, First Oral Therapy for Multiple Sclerosis: The Development of Fingolimod (FTY720, Gilenya)

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## Abstract

Multiple sclerosis (MS) is a chronic autoimmune disorder affecting the central nervous system (CNS) through demyelination and neurodegeneration. Until recently, major therapeutic treatments have relied on agents requiring injection delivery. In September 2010, fingolimod/FTY720 (Gilenya, Novartis) was approved by the FDA as the first oral treatment for relapsing forms of MS. Fingolimod is a novel compound produced by chemical modification of a fungal precursor. Its active metabolite, formed by *in vivo* phosphorylation, modulates sphingosine 1-phosphate (S1P) receptors that are a subset of a larger family of cell-surface, G protein-coupled receptors (GPCRs) mediating the effects of bioactive lipids known as lysophospholipids. Fingolimod's mechanism of action in MS is not completely understood; however, its relevant biology indicates a fundamentally different mechanism compared to all previously approved MS therapies, with evolving research supporting both immunological and nervous system activities. This duality may herald a paradigm shift in the treatment of MS and other neurological disorders.

## Introduction

Multiple sclerosis (MS) is a neurological disorder characterized by demyelination and neurodegeneration within the central nervous system (CNS) with common relapsing forms produced through autoimmune inflammatory mechanisms (Compston and Coles, 2002) involving autoreactive lymphocytes that penetrate the blood-brain barrier to attack the nervous system (Figure 1). MS is a major cause of nervous system disability affecting individuals in the prime of life with a 2–3:1 female to male ratio (Compston and Coles, 2002) and an estimated global prevalence of 2.5 million that is highest in Northern latitudes particularly amongst Caucasians (Noseworthy *et al.*, 2000; Rosati, 2001). The initiating causes of MS remain unknown (Baranzini *et al.*, 2010).

Several MS forms have been identified, the most common of which shows a waxing and waning of signs and symptoms to produce a "relapsing-remitting" clinical presentation. "Relapsing remitting MS" (RRMS) affects ~85% of patients (Compston and Coles, 2002).

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Within a decade, approximately a third of these patients will suffer from "secondary progressive MS" (SPMS) (Weinshenker *et al.*, 1989), a form that shows less inflammation and more neurodegeneration. A distinct form of MS that is not clearly associated with relapses, "primary progressive MS" (PPMS) shows neurodegeneration with a relative lack of inflammatory change. Late stage sequelae from advanced MS include permanent disability and death.

Over the last two decades, therapeutic approaches to MS have emerged that can reduce the relapse frequency and the rate of disability progression. First-line therapies include interferon-β formulations (Avonex, Biogen-Idec and Rebif, Merck-Serono) and glatiramer acetate (Copaxone, Teva) (PRISMS Study Group, 1998; Jacobs *et al.*, 1996; Johnson *et al.*, 1995; The IFNB Multiple Sclerosis Study Group, 1993). Second-line therapies include a humanized antibody natalizumab (Tysabri, Elan/Biogen-Idec) that recognizes the α4 subunit of α4β1 integrin, a cell adhesion molecule expressed on lymphocytes (Polman *et al.*, 2006; Steinman, 2005). All of these therapies target the immune system and require parenteral delivery through injection or infusion, which can be associated with both patient compliance issues as well as deleterious side effects. Moreover, it is debatable whether any of the current therapies can alter neurodegenerative endpoints of MS, a view underscored by the current absence of any approved therapies for PPMS. There is a clear medical need for new therapies, particularly those with new mechanisms of action that might promote direct CNS activities as part of their disease modifying effects.

In addition to efficacy issues for current MS therapies, side effects are a major concern for all currently used agents. Interferons are often associated with injection-site reactions and flu-like symptoms (Patti, 2010), with other less commonly reported events including liver dysfunction and cytopenias (Rice *et al.*, 2001). Of greater concern with the efficacious agent natalizumab is the increasing incidence of progressive multifocal leukoencephalopathy (PML), a rare but serious infection associated with immunosuppression (Berger and Major, 1999; Focosi *et al.*, 2010; Steinman, 2005) that has resulted in multiple fatalities in MS patients receiving this therapy (Weissert, 2011). Another example is the cytostatic agent mitoxantrone that has a cumulative dose-dependent cardiac toxicity with additional risk of leukemia, both of which limit its long-term use (Kingwell *et al.*, 2010). The latter two agents underscore immunosuppressive liabilities of these and other current and future MS therapies that primarily inhibit immune function as their primary mechanism of action. Efforts to develop new MS treatments, as evidenced by those in current development, underscore both limitations and a need for therapies with more convenient, effective, and safe treatment profiles.

Despite their introduction nearly 20 years ago, interferon therapies still represent the most commonly prescribed disease modifying agents for MS. This situation epitomizes deficiencies in the current state of therapeutics using agents that lack ease of delivery, such as those requiring injection, as well as efficacy characteristics, particularly for preventing neurodegeneration. Drugs under development for MS include the monoclonal antibodies named rituximab, ocrelizumab, and ofatumumab (which target CD20 to deplete B cells); alemtuzumab/Campath-1H (which targets CD52 to deplete T and B cells and some monocyte-derived dendritic cells) (Buttmann, 2010); and small molecules including the oral agents. These oral agents include cladribine (a cytotoxic adenosine deaminase-resistant purine nucleoside, recently withdrawn from commercial development), dimethylfumarate BG-12 [an activator of the nuclear factor-E2-related factor 2 (Nrf2) transcriptional pathway that alters glutathione levels (Lin *et al.*, 2011), but is also associated with tumorigenesis (DeNicola *et al.*, 2011) and immunosuppression (Lehmann *et al.*, 2007; Vandermeeren *et al.*, 1997)], laquinimod (unknown cellular target), and teriflunomide (an inhibitor of dihydroorotate dehydrogenase, which catalyzes the rate-limiting step in the *de novo*

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synthesis of pyrimidines). All of these agents target lymphocytes as well as other immune-related cells (Niino and Sasaki, 2010). As of this writing, they also all remain subject to additional clinical and regulatory evaluation before possible entry into the therapeutic armamentarium.

A major step towards achieving the goal of a mechanistically novel, orally bioavailable agent has recently been taken through FTY720/fingolimod (commercial name Gilenya; Novartis) that represents the first oral MS therapy approved by the United States Food and Drug Administration (FDA), the European Union, and several other countries. This compound interacts with a new, until now clinically unassessed molecular target: receptors for the signaling lysophospholipid known as sphingosine 1-phosphate (S1P). Here we review highlights of the discovery of fingolimod, its receptor targets, clinical efficacy and safety profiles, and evolving understanding of its mechanism of action in MS that has revealed effects in not only the immune system, but direct action in the CNS. Additional detailed information on fingolimod can be found elsewhere (Brinkmann *et al.*, 2010; Chun and Hartung, 2010; Cohen *et al.*, 2010; Cohen and Chun, 2011; Kappos *et al.*, 2010; Noguchi and Chun, 2011). The development of fingolimod also epitomizes a convergence of basic and clinical science that points to untapped mechanisms for the treatment of MS, as well as potentially other CNS disorders.

## Origins of Fingolimod: A Folk Medicine from Fungi

Fingolimod emerged from the study of the fungal phylum *Ascomycota* (Cavalier-Smith, 1998; Sung *et al.*, 2007), historically, commonly, and taxonomically referred to as class *Ascomycetes* within the kingdom Fungi. Within this group of "sac fungi" is the species *Cordyceps* of family *Cordycipitaceae,* which constitutes over 400 members characterized prominently by their entomopathogenic activity wherein the fungus infects a range of insect host species at different points in their development, parasitize them to grow out of the corpse to form the stalk and fruiting body of the fungus (Sung *et al.*, 2007). This biological cycle was at least in part recognized in Chinese herbal medicine through the name, "Dong Chong Xia Cao," meaning "winter worm, summer grass" (Zhou *et al.*, 2009) that describes the infected insect larval form followed later by the fungal stalk form of most *Cordyceps* species (Figure 2; shown for *Isaria sinclairii*). Folk medicine recognized an extensive range of health benefits produced by ingesting the fungus (Ng and Wang, 2005), and modern analytical techniques have identified important fungal metabolites that have human biological activities, including Cordycepin (3′-deoxyadenosine) that inhibits tumor growth and was derived from *C. militaris* that parasitizes the silkworm (Ng and Wang, 2005; Paterson, 2008), Cyclosporine, a classical immunosuppressant derived from *C. subsessilis* that infects the scarab beetle (Borel and Kis, 1991; Illana Esteban, 2007), and an immunosuppressant, myriocin (ISP-1) (Fujita *et al.*, 1994). Myriocin is a metabolite of *Isaria sinclairii* (Figures 2 and 3) — the anamorph (asexual)-stage name historically referenced by its presumed telomorph-stage name, *Cordyceps sinclairii* (Fujita *et al.*, 1994; McNeill and International Association for Plant Taxonomy, 2006) — that parasitizes Cicada nymphs (Figure 2) (Chiba *et al.*, 1996; Miyake *et al.*, 1995). Efforts to produce chemically modified compounds derived from myriocin (Adachi *et al.*, 1995; Suzuki *et al.*, 1996a) led, in 1994, to the identification of a novel compound, fingolimod (Adachi *et al.*, 1995; Fujita *et al.*, 1995), whose original name, FTY720 (Figure 3), reflects the discoverers: Fujita and colleagues at Kyoto University, Taito Company (now Mitsui Seito that produces sugar), and Yoshitomi Pharmaceutical Industries (now Mitsubishi Tanabe Pharma). The compound was of interest based upon preclinical data that supported activities that might be relevant to improved organ transplantation (Chiba *et al.*, 1996; Mitsusada *et al.*, 1997; Suzuki *et al.*, 1998; Yuzawa *et al.*, 1998), and this led to interest by the industrial producers of cyclosporine (Cyclosporin A, Cyclosporine), Sandoz (Stahelin, 1996), which merged with Ciba-Geigy in

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1996 to form Novartis. Fingolimod showed activity in models of organ transplantation when combined with Cyclosporin A, which led to in-licensing of fingolimod by Novartis for evaluation as a therapeutic agent in renal transplantation.

The mechanism of fingolimod seemed to be quite different from two other fungal agents isolated from *Cordyceps*: Cyclosporin A that interacts with a protein phosphatase, calcineurin, and myriocin that inhibits serine-palmitoyl-transferase (SPT) (Chen *et al.*, 1999; Miyake *et al.*, 1995). Fingolimod is involved in sphingosine metabolism (Miyake *et al.*, 1995). In particular, chemical modification efforts of the parental myriocin compound (Figure 3) to produce fingolimod might have been expected to maintain some of the myriocin activities; however, fingolimod had no activity against SPT (Chen *et al.*, 1999; Miyake *et al.*, 1995). Consistent with an altered activity profile and contrasting with myriocin, fingolimod did not inhibit activation, proliferation, or memory formation of T cells; moreover, it did not affect the production of antibodies by B cells or cytokines by T cells and, as a consequence, did not impair immune against systemic viral infection (Pinschewer *et al.*, 2000; Brinkmann *et al.*, 2001; 2000). Thus, fingolimod appeared to be acting in a way distinct from classically defined immunosuppressants through an unclear molecular target(s) and mechanism of action.

## Parallel Discovery of Lysophospholipid S1P Receptors, a Molecular Target for Fingolimod

Around the same period of fingolimod's discovery, a completely independent line of research was investigating genes involved in CNS development (Hecht *et al.*, 1996), which led to the identification of the first member for what is now known as the lysophospholipid family of receptors (Choi *et al.*, 2010; Chun, 2007; Fukushima *et al.*, 2001; Ishii *et al.*, 2004; Mutoh *et al.*, 2011). These G protein-coupled receptors (GPCRs) mediate the actions of at least two major classes of signaling lysophospholipids that are known as lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), the latter of which comprises the major receptor target for fingolimod. The first family member recognized was for LPA (Fukushima *et al.*, 1998; Hecht *et al.*, 1996) and this identity aided deorphanization of other LPA receptors, as well as the S1P receptors (An *et al.*, 1997; Lee *et al.*, 1998b) that at one point were hypothesized to interact with both LPA and S1P (Lee *et al.*, 1998a) reflecting their marked amino acid sequence similarities. There are currently 11 receptors, six for LPA and five for S1P. Lysophospholipid receptors were first identified by exploiting knowledge of their expression and activity in the developing CNS, and their other activities and the cell types on which they express have now been identified within the CNS (Choi *et al.*, 2008; Gardell *et al.*, 2006; Noguchi and Chun, 2011; Rivera and Chun, 2008; Yung *et al.*, 2011). These receptors also have prominent expression in the immune system as well as other tissues, and have been implicated in a broad range of biological and pathophysiological processes (Gardell *et al.*, 2006; Herr and Chun, 2007; Noguchi and Chun, 2011; Ye and Chun, 2010).

The structural similarity of fingolimod to sphingosine led to the key identification of S1P receptors as the target for phosphorylated fingolimod (Brinkmann *et al.*, 2002; Mandala *et al.*, 2002). Sphingosine is a metabolite of the cell-membrane-derived sphingolipids such as sphingomyelin and ceramide (Brinkmann *et al.*, 2010). Phosphorylation is produced by the action of endogenous sphingosine kinases (SPHKs) to produce fingolimod-phosphate (fingolimod-P) (Brinkmann *et al.*, 2002; Mandala *et al.*, 2002), primarily involving the enzyme SPHK2 (Figure 3) (Zemann *et al.*, 2006). Receptor studies revealed that fingolimod-P (but not parent fingolimod) is an agonist at $S1P_1$, $S1P_4$, $S1P_5$ ($EC_{50}$ values of ~ 0.3–0.6 nM), and $S1P_3$ ($EC_{50}$ values of ~ 3 nM), with essentially no activity at $S1P_2$ at these concentrations (Brinkmann *et al.*, 2002; Mandala *et al.*, 2002). Stereochemical analyses

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identified (*S*)-fingolimod-P as the biologically active form *in vivo* (Brinkmann *et al.*, 2002), whereas (*R*)-enantiomer was not detected (Albert *et al.*, 2005). Thus, the discoveries of fingolimod and of their targeted lysophospholipid receptors provided a basis for understanding fingolimod's actions. However, the therapeutic indications for fingolimod evolved in unanticipated ways.

## From Renal Transplantation to Multiple Sclerosis

Initial studies driving the discovery and development of fingolimod focused on its utility in preventing organ graft rejection in animal models using high-dose fingolimod (10 mg/kg) in monotherapy (Chiba *et al.*, 1996; Enosawa *et al.*, 1996; Suzuki *et al.*, 1996a), and doses of 0.3–3 mg/kg in combination with classical immunosuppressive agents (Brinkmann *et al.*, 2002; Chiba *et al.*, 1996; Mitsusada *et al.*, 1997; Suzuki *et al.*, 1998; 1996b; Yanagawa *et al.*, 1998; Yuzawa *et al.*, 1998). However, the apparent synergy observed between fingolimod and cyclosporine in some animal models was not substantiated in human Phase III clinical trials for renal transplantation, where fingolimod did not support reduction of the dose of cyclosporine, compared to standard-of-care treatment (Mansoor and Melendez, 2008). This lack of superior efficacy in preventing transplant rejection was likely related to insufficient immunosuppression by fingolimod, but the effects on cell migration/trafficking opened up other possible therapeutic indications including MS and other autoimmune disorders.

In addition, basic studies on lysophospholipid receptors identified expression patterns and functions that were consistent with possible roles in a range of CNS cell types and processes relevant to MS, including receptor expression in myelinating cells (Chun *et al.*, 2000; Weiner and Chun, 1999; Weiner *et al.*, 1998) that are affected by demyelination in MS, and immunological effects that included T lymphocytes that can damage the CNS (Brinkmann *et al.*, 2002; Chun *et al.*, 2002; Goetzl and An, 1999; Huang *et al.*, 2002). Fingolimod efficacy in MS animal models, particularly experimental autoimmune encephalomyelitis (EAE), was observed independently by multiple investigators (Brinkmann *et al.*, 2002; Fujino *et al.*, 2003; Kataoka *et al.*, 2005; Webb *et al.*, 2004), and was supported by findings in other animal models [e.g., dark agouti rat model (Balatoni *et al.*, 2007; Foster *et al.*, 2009)]. These animal studies also identified a range of possible side-effects, including $S1P_1$-dependent transient bradycardia (Forrest *et al.*, 2004; Gergely *et al.*, 2009; Koyrakh *et al.*, 2005; Sanna *et al.*, 2004) and $S1P_1$-dependent vascular permeability (Sanna *et al.*, 2004; Oo *et al.*, 2011). The possible safety concerns have not been borne-out by serious adverse events in human clinical trials (discussed next), underscoring species differences and/or the use of much higher fingolimod concentrations in the animal studies compared to the approved dose in humans.

Initial human studies with fingolimod led to two pivotal Phase III clinical trials focused on a possible reduction in the annualized relapse rate (ARR) as the primary endpoint using fingolimod in relapsing remitting MS (RRMS): a placebo-controlled trial of 1,272 patients (the FREEDOMS trial) and an active comparator trial involving 1,292 patients (the TRANSFORMS trial). In FREEDOMS, fingolimod was investigated in a double blind, 2-year study that involved patients randomized to a reduced dose arm of 0.5 mg or 1.25 mg as compared to a placebo (Kappos *et al.*, 2010). Once again, the ARR was significantly reduced in both experimental arms (0.18 and 0.16, respectively) compared to the placebo (0.40), as was risk of disability progression (17.7% and 16.6%, respectively) compared to placebo (24.1%) and MRI-detected lesions, which included the previously assessed gadolinium-enhancing lesions, and interestingly, a reduction in brain-volume loss (atrophy) (−0.7% in both fingolimod arms compared to −1.0% with placebo). In TRANSFORMS, a 1-year, double blind, double-dummy trial, fingolimod was compared to $IFN^2$-1a (Avonex, Biogen Idec), a major, first-line therapy for MS (Cohen *et al.*, 2010). The same fingolimod

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doses were compared to standard administration of IFNβ-1a. Both fingolimod arms showed an ARR that was significantly lower (0.16 and 0.20, respectively), compared to the IFNβ-1a arm (0.33). Disability progression was similar, albeit difficult to assess during the short, 1-year trial; however, both fingolimod doses showed superior MRI endpoints of fewer new or enlarged lesions on T2-weighted images, gadolinium-enhancing lesions, and reduced brain atrophy at 12 months.

Serious adverse event rates in the 2-year FREEDOMS trial (Kappos *et al.*, 2010) were similar amongst all groups including placebo (10.1–13.4%), whereas in the 1-year TRANSFORMS study (Cohen *et al.*, 2010), the incidence of adverse events was higher in the high dose arm (1.25 mg) but comparable in the 0.5 mg fingolimod vs. IFNβ-1a arms. The incidence of adverse events that resulted in drug discontinuation was similar between the 0.5 mg fingolimod and all control groups, but was higher in the 1.25 mg fingolimod groups. The combined data from both Phase III trials did not suggest an increased incidence of either infections or malignancies associated with fingolimod treatment (Cohen *et al.*, 2010; Kappos *et al.*, 2010). The two lethal herpes infections (one primary varicella zoster infection and one case of herpes simplex encephalitis) that occurred in patients receiving 1.25 mg fingolimod (Cohen *et al.*, 2010) may have involved confounding factors related to the use of high-dose steroids. In view of the comparable efficacy of the lower 0.5-mg fingolimod dose, along with its improved safety profile, the 0.5-mg fingolimod dose was nominated for regulatory approval in providing the best risk-to-benefit profile, and during 2010–2011, fingolimod (commercial name Gilenya) received approvals in the United States, the European Union, and several other countries.

## Fingolimod Immunological Activities

In part reflecting its origins in transplantation research, fingolimod has been extensively studied for its effects on immune system. Early studies raised the possibility that fingolimod might interfere with T cell trafficking rather than function, and that its mode of action may involve Gαi protein-coupled receptors (Brinkmann *et al.*, 2000). It was then found that conversion of fingolimod to its phosphorylated metabolite, fingolimod-P, and the interaction with cognate S1P receptors result in trafficking effects as demonstrated by the sequestration of lymphocytes in secondary lymphoid organs (Brinkmann *et al.*, 2002; Mandala *et al.*, 2002).

These studies provided a link to the biological effects of fingolimod, shown in transfected cell lines, to produce, paradoxically, S1P receptor internalization, removing them from the cell surface (Graler and Goetzl, 2004) to inhibit S1P signaling, despite the initial characterization of fingolimod as a receptor agonist (after phosphorylation) (Brinkmann *et al.*, 2002). Immunological studies of knockout mice for $S1P_1$ — whereby this receptor was removed from lymphocytes — resulted in lymphocyte trafficking defects that were similar to the effects of fingolimod exposure, suggesting that fingolimod was acting as a “functional antagonist” to inhibit S1P receptors (Brinkmann *et al.*, 2004; Matloubian *et al.*, 2004). Interestingly, mutations in the $S1P_1$ receptor that abrogated internalization, but not signaling of the receptor, were sufficient to blunt lymphocyte trafficking effects of fingolimod, confirming that receptor internalization rather than signaling was required, presumably to prevent S1P-directed migration of cells (Oo *et al.*, 2011). In other words, although fingolimod-P initially acts as an S1P receptor agonist, chronic exposure to it results in S1P receptor loss from the surface of the cell (Figure 4) and abrogation of receptor-mediated S1P signaling.

The orchestrated role for S1P signaling that allows lymphocytes to egress from the lymph nodes predominates over another signaling system mediated by chemokines and the receptor

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known as CCR7, which promotes retention of lymphocytes in secondary lymphoid organs with preferential effects on "naïve" and early "central" memory T-cells rather than late, terminally differentiated "effector" memory T-cells (Henning *et al.*, 2001; Pham *et al.*, 2008; Sallusto *et al.*, 1999). Thus, $S1P_1$ signaling appears to predominate over CCR7-mediated retention by promoting lymphocyte egress from lymph nodes, whereas functional antagonism of $S1P_1$ by fingolimod inhibits egress from lymph nodes. By contrast, egress of CCR7-negative effector memory T cells (Sallusto *et al.*, 2004) appears to occur independently of $S1P_1$ receptor signaling and these cells are refractory to the trafficking effects of fingolimod. This latter point is supported by studies in mice (Brinkmann *et al.*, 2004; Metzler *et al.*, 2008; Pham *et al.*, 2008) and humans (Brinkmann, 2009; 2004; Mehling *et al.*, 2008; Metzler *et al.*, 2008).

Two corollaries with particular relevance to MS through this differential effect of fingolimod on CCR7-positive vs. CCR7-negative T cells may contribute to efficacy and safety, respectively. First, fingolimod may produce efficacy by sequestering the CCR7-positive cells, which include naïve and central memory T cells, the latter of which have a key role in immunological memory. Following antigen exposure, central memory T lymphocytes can undergo clonal expansion and differentiation to generate effectors/effector memory T cells which provide adaptive immunity against recognized antigens (Iezzi *et al.*, 2001; Sallusto *et al.*, 2004). Central memory T cell retention by fingolimod could function as a therapy in MS since more than 90% of T cells that are found in the cerebrospinal fluid (CSF) appear to be of the central memory subset (Kivisakk *et al.*, 2004). The contained autoreactive, pathological T cells could therefore be prevented from entering the CNS by fingolimod sequestration, thereby abrogating their differentiation into pathological effectors and effector memory T cells upon interaction with CNS-resident antigen-presenting dendritic cells. In animal models, fingolimod prevented accumulation of pathological Th17 cells in the nervous system (Zhang *et al.*, 2008; 2009), supporting Th17 cell- or Th17 cell precursor sequestration as an efficacy mechanism. Accordingly, phenotypic Th17 cells were reduced in the circulation in fingolimod-treated MS patients (Mehling *et al.*, 2010). In addition to efficacy by CCR7-positive cell sequestration of pathological T cells, fingolimod could provide safety through maintained immunosurveillance. Such functionality would be produced by preferentially *not* affecting CCR7-negative effector memory T cells of any functional phenotypes (Mehling *et al.*, 2008), which may leave lymph nodes, independent of $S1P_1$ signaling (Pham *et al.*, 2008).

In support of the above, another study proposed that Gαi2 null T cells egress independent of S1P-mediated chemotaxis (Zhi *et al.*, 2011), and these cells were also not retained by fingolimod. Intravital imaging of lymph nodes revealed that T cells approach and engage cortical sinusoids in lymph nodes similarly in the presence or absence of fingolimod. However, after engagement of the sinus, most T cells retract and migrate back into the parenchyma in fingolimod-treated animals, due to a failure of the cells to establish adhesion on the sinus, whereas Gαi2-deficient T cells adhere firmly on the sinus, which prevents their retraction, facilitating their transmigration of the lymphatic endothelial barrier. Interestingly, Gαi2-deficient T lymphocytes are hyper-responsive for T cell receptor signaling and cytokine production, with a relaxed costimulatory requirement (Huang *et al.*, 2003) — a phenotype matching effector memory T cells — again supporting sparing of this subset by fingolimod.

Collectively, the data show that activation and proliferation of naïve and central memory T-cells, as well as differentiation and trafficking of effector memory T-cells, may not be significantly affected by fingolimod, thereby preserving this arm of the adaptive immune system that can reduce the risk of infection and cancers common with immunosuppressive agents. Consistent with this mechanism, the combined data from both aforementioned Phase

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III trials did not suggest an increased incidence of either infections or malignancies associated with fingolimod treatment (Cohen *et al.*, 2010; Kappos *et al.*, 2010).

In addition to the immunomodulatory effects, fingolimod also simultaneously accesses a completely different biology: direct CNS actions on cells relevant to MS, which if present, would be an efficacy mechanism independent of immunomodulation. While the exact role that fingolimod's CNS actions might have in MS are not known, a growing body of basic and clinical literature supports direct CNS influences that are discussed next.

## Fingolimod CNS Activities

Based upon its relationship to sphingosine, and its interactions with lysophospholipid S1P receptors, fingolimod was likely to have direct CNS effects. As an analog of the lipid sphingosine, fingolimod could have a range of possible roles within the CNS, since that is where sphingosine and related sphingolipids (e.g., phospholipids that contain sphingosine, like sphingomyelin) were first identified from early studies of the brain during the 1800s (Thudichum, 1884; Vauquelin, 1812). The functions of sphingosine were then as enigmatic as the Sphinx, from which its name was coined (Merrill *et al.*, 1997; Thudichum, 1884). Consistent with CNS actions, fingolimod and fingolimod-P localize to the CNS as revealed by radiolabeling studies (Foster *et al.*, 2007). Independent support for CNS activities came from studies of lysophospholipid receptors that were first identified from the brain (Hecht *et al.*, 1996), with most, including S1P receptors, expressed in CNS lineages where they have a rich neurobiology (Brinkmann, 2009; Choi *et al.*, 2010; Fukushima *et al.*, 2001; Ishii *et al.*, 2004; Kingsbury *et al.*, 2003; Mizugishi *et al.*, 2005; Mullershausen *et al.*, 2007; Trimbuch *et al.*, 2009; Weiner and Chun, 1999; Yung *et al.*, 2011).

Data supporting possible direct CNS effects included the aforementioned fingolimod localization within the CNS (Foster *et al.*, 2007), the rapid onset of therapeutic effects with a "rescue" therapy started 40 days after disease onset (Foster *et al.*, 2009), and a discordance between clinical scores and peripheral lymphocyte levels seen in some EAE animal models (Webb *et al.*, 2004). Receptor-mediated S1P signaling has been documented in CNS cell lineages that have relevance to MS, consistent with broad expression of lysophospholipid receptors in general within the brain (Chun, 1999; Mutoh and Chun, 2008; Noguchi and Chun, 2011). Astrocytes in particular express $S1P_1$ and $S1P_3$ (Choi *et al.*, 2011; Mullershausen *et al.*, 2007; Osinde *et al.*, 2007; Pebay *et al.*, 2001; Rao *et al.*, 2003; Sorensen *et al.*, 2003; Wu *et al.*, 2008), and these two receptors have been reported to be upregulated in MS astrocytes (Van Doorn *et al.*, 2010). Oligodendrocytes and their precursor cells also express S1P receptors (Dev *et al.*, 2008; Jaillard *et al.*, 2005; Miron *et al.*, 2008; Mutoh *et al.*, 2011; Terai *et al.*, 2003) particularly $S1P_5$ in mature oligodendrocytes. Neural progenitor cells, and likely some neurons, can also express $S1P_1$, along with other S1P receptor subtypes (Choi *et al.*, 2011; Kajimoto *et al.*, 2007; Kimura *et al.*, 2007; McGiffert *et al.*, 2002; Mizugishi *et al.*, 2005). In addition, resident non-neural cells like microglia can also express S1P receptors (Durafourt *et al.*, 2011; Schilling *et al.*, 2002; Tham *et al.*, 2003). The diversity of both cell types and S1P receptor subtypes underscore potential effector activities of fingolimod within the CNS in MS. The receptor mechanisms could involve some degree of transient, initial agonism; however, continuous exposure to fingolimod would be expected to produce functional antagonism — a net loss of S1P receptor signaling — at least for $S1P_1$ that has been best characterized in non-neural cells. Pharmacological $S1P_1$ loss through functional antagonism can be rigorously modeled by use of genetics via the production of null mutation knockouts.

This knockout strategy was used to address functional consequences of removing $S1P_1$ from specific cell lineages in the CNS while leaving the immune system intact, combined with

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challenge by EAE (Choi *et al.*, 2011). The resulting mutants were then assessed for 1) effects on fingolimod activity, and 2) effects on clinical disease, independent of fingolimod exposure, combined with other analyses (Choi *et al.*, 2011). $S1P_1$ was conditionally deleted (using *loxP* technologies) from various CNS cell lineages while still maintaining immunological competence as evidenced by normal responses of peripheral blood lymphocytes to fingolimod exposure, and an ability of mutant lymphocytes to produce disease following adoptive transfer from mutant into normal animals. Of the lineages assessed, $S1P_1$ deletion from astrocytes but not neurons produced a dramatic effect, eliminating fingolimod activity in EAE, compared to vehicle controls, and also attenuating MS-like disease. Consistent with these clinical assessments of disease, $S1P_1$ deletion on its own protected against histologically detected damage as compared to control animals challenged by EAE, including a marked reduction in astrogliosis — a reactive state of astrocytes that increases their number and alters their morphology —along with preservation of axons and myelin that would usually be damaged by EAE. Fingolimod exposure during EAE produced a similar histological picture when assayed in normal (non-mutant) animals, and competitive receptor binding assays using membrane preparations from brains of these animals confirmed down-modulation of S1P receptors by the drug, supporting the functional antagonism model of $S1P_1$ loss that had been previously observed in the immune system, a receptor mechanism that was further shown to occur in astrocytes as well.

Overall, these data identify $S1P_1$ signaling in astrocytes as a major influence on models of MS, as well as a necessary component of fingolimod efficacy (Figure 1). The combined effects of the drug on lymphocyte trafficking and astrogliosis may reduce neurodegeneration and favor remyelination after damage, as observed in models of EAE and cuprizone-induced demyelination (Balatoni *et al.*, 2007; Foster *et al.*, 2009; Kim *et al.*, 2011). Other S1P receptor subtypes and/or involved cell lineages may also have related influences (Soliven *et al.*, 2011; Wu *et al.*, 2008), and these remain to be addressed in MS models. One or more of these processes might explain the reduction in brain atrophy observed with fingolimod treatment in MS Phase III trials (Cohen *et al.*, 2010; Kappos *et al.*, 2010), contrasting with distinct and at times increasing atrophy signals, observed with immunologically targeted therapeutics like natalizumab (Miller *et al.*, 2007).

## Immunomodulatory Approaches to MS Therapy

A notable corollary of the dual immunological and CNS fingolimod mechanisms is that fingolimod does not fit the profile of an immunosuppressive agent like those in common use in the transplantation field - e.g., “classical” immunosuppressive agents like calcineurin inhibitors [Cyclosporine, Tacrolimus (Borel and Kis, 1991; Juhasz *et al.*, 2009; Stahelin, 1996)], high dose corticosteroids [e.g., Prednisone (Goetzl, 2008)], and cytotoxic and/or antimitotic agents [azathioprine, mycophenolate, or cladribine (Goetzl, 2008; Neuhaus *et al.*, 2007)] or biologicals, including a growing number of humanized antibodies raised against immune cell targets [CD3, IL-2 receptor, integrins, CD52 (Buttmann, 2010; Nitta *et al.*, 1992; Steinman, 2005; Wolff *et al.*, 2004)]. Early approaches to the treatment of MS utilized classical immunosuppressive strategies, some of which continue to be used today (Neuhaus *et al.*, 2007). However, risk of serious neoplastic and/or infectious adverse events limits their use. This issue has been underscored by the rare occurrence of progressive multifocal leukoencephalopathy (PML) associated with the use of natalizumab or rituximab (Buttmann, 2010). T cell immunosuppression may be involved in both cases; in addition to its effects on T cell trafficking, natalizumab may interfere with the VLA4-VCAM1 costimulatory pathway that is critical to human CD4 T cell proliferation (Weitz-Schmidt *et al.*, 2001), and therapeutic B cell depletion by rituximab was shown to impair B cell antigen-presentation and, as a consequence, CD4 T cell activation and clonal expansion in response to pathogen challenge (Bouaziz *et al.*, 2007). Therefore, the sparing of effector memory T cells in both

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CD4 and CD8 populations by fingolimod could be critical to immunosurveillance; in the meninges of mice, fingolimod preferentially reduced naive and central memory T cells, whereas anti-VLA4 treatment primarily depleted the effector memory population (Derecki *et al.*, 2010) (see also above for the key role of circulating central memory T cells in pathology of MS).

Compared to human CD8 T cells, circulating CD4 T cells contain larger numbers of CCR7-positive naive and central memory T cells, and this could explain the more profound retention of CD4 T cells in lymph nodes by fingolimod (Brinkmann *et al.*, 2010). Importantly, infection-relevant effector memory T cells could still be generated in lymph nodes and would recirculate independent of S1P1 (Pham *et al.*, 2008); thus, the reduced *total* CD4 T cell count in blood may not prove useful as an indicator of immunosuppression in fingolimod-treated patients.

The above data support the notion that fingolimod at its approved dose may not act as a potent immunosuppressant: 1) CNS effects are unrelated to immunosuppression; 2) suboptimal prevention of graft rejection was achieved in renal transplantation studies in combination with cyclosporine, despite being at 10X the approved MS dose; 3) immunological constituents are maintained (cellular and humoral), with reversible effects on cell location of some (but not all) lymphocyte subsets —without inhibition of proliferation, differentiation, and cytotoxicity; 4) immunological surveillance is maintained through relatively unaffected effector memory T-cells; and 5) clinically, the overall incidence of infections as well as of serious and severe infections was not increased over placebo control in the FREEDOMS trial in phase III studies. Overall, the emerging picture identifies S1P receptor pathways in MS that can provide efficacy through mechanisms different from classical immunosuppressants.

## Future Prospects

Fingolimod is the first compound targeting lysophospholipid receptors to receive regulatory approval as a human medicine. Its direct effects on both the immune and nervous systems via a defined class of molecular targets, lysophospholipid S1P receptors, make it unique amongst approved MS therapies. Fingolimod also provides human validation for the efficacy and safety of S1P receptor modulation in MS. Evidence for direct CNS activity raises the possibility that fingolimod could access mechanisms relevant to non-relapsing forms of MS, particularly PPMS or SPMS. There are currently no approved therapies for PPMS, and a Phase III trial has been started to assess the ability of fingolimod to improve disability progression over a 3–5 year period towards assessing its efficacy in this form of MS.

Beyond MS, fingolimod and other S1P receptor modulating compounds could have relevance to both immunological diseases as well as those of the CNS. Autoimmune disorders that may be susceptible to S1P receptor modulators include lupus, psoriasis, arthritis, and diabetes (Brinkmann, 2007; Brinkmann and Lynch, 2002). In particular, the effects of fingolimod on IL-17 cytokine-secreting Th17 lymphocytes that reduce IL-17 mediated inflammatory sequelae (Mehling *et al.*, 2010; Zhang *et al.*, 2008; Zhang *et al.*, 2009) may be of special relevance. Additionally, fingolimod's direct action on neural cells, especially astrocytes (Choi *et al.*, 2011), portend the use of fingolimod or related lysophospholipid receptor-modulatory compounds in a range of other neurological diseases. This in part reflects the important roles for astrocytes in most major neurological disorders that include stroke and neurodegenerative disease (Alzheimer's and Parkinson's disease), which gives rise to the possibility of therapeutically treating disease through the pharmacological modulation of S1P receptors. More broadly, the documented effects of

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lysophospholipid signaling in the CNS, which include cell survival for myelinating (Contos *et al.*, 2000; Weiner and Chun, 1999) and neural progenitor cells (Hecht *et al.*, 1996; Herr *et al.*, 2011; Kingsbury *et al.*, 2003; Yung *et al.*, 2011) as well as modulation of synaptic activity (Trimbuch *et al.*, 2009), suggest new approaches to treat major human diseases through lysophospholipid receptor modulation: a first step along this path has now been taken with the introduction of fingolimod into clinical practice for MS.

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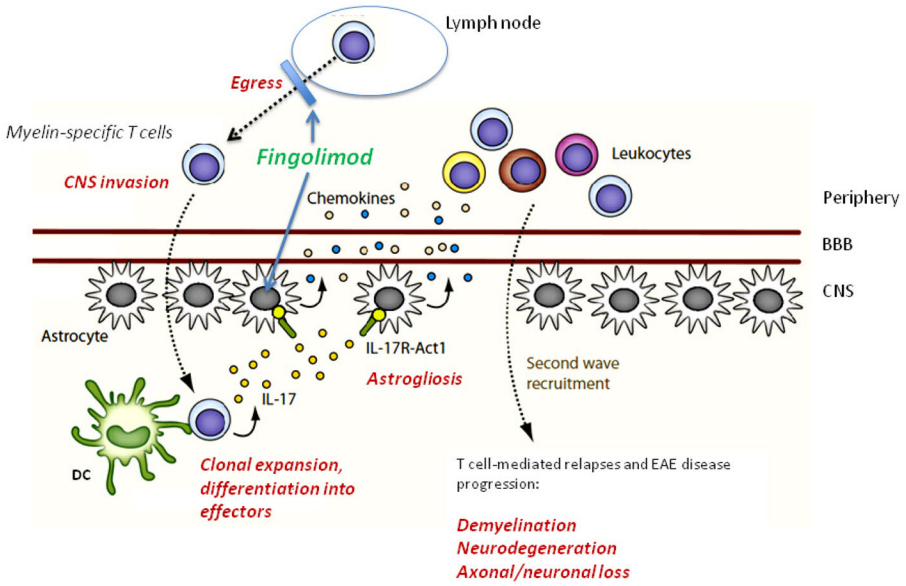


**Figure 1.**

Model for the role of T cells and astrocytes in the pathology of MS. Recent data suggest a necessary role for Th17 cells and astrocytes in EAE pathology. Activated myelin-specific Th17 cells of the central memory phenotype infiltrate the CNS and are restimulated to produce IL-17 by local dendritic cells to undergo clonal expansion and differentiation into effectors/effector memory T cells. Astrocytes respond via expression of IL-17R to T cell-released IL-17 and produce leukocyte-attracting chemokines in an Act1-dependent manner. Astrocyte-derived chemokines then recruit a second wave of peripheral inflammatory cells, which mediate EAE relapses and progression via Th17 cell-mediated bystander demyelination (adapted from Rodgers *et al.*, 2010).

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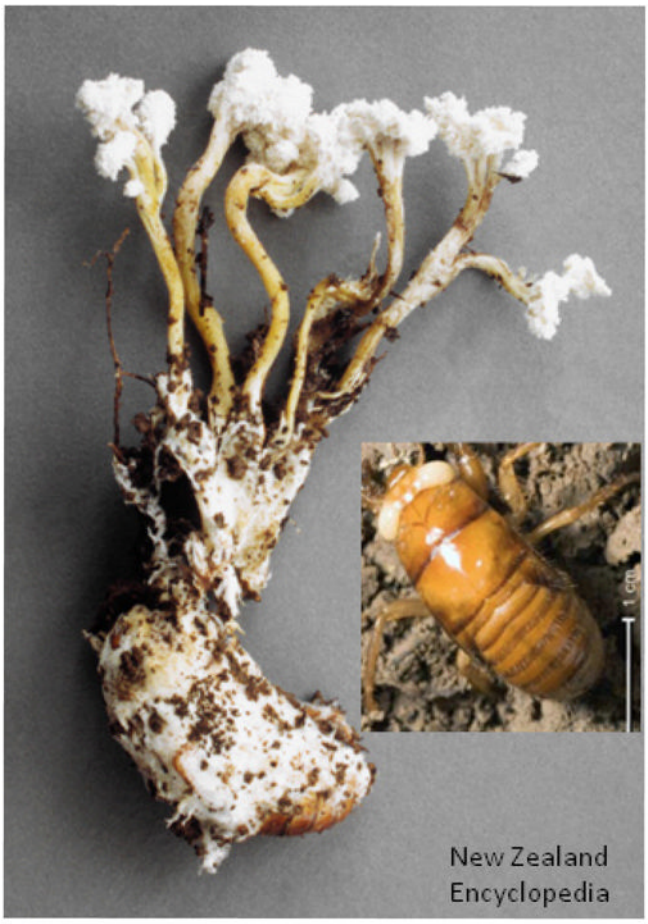


**Figure 2.**
Fungal source of fingolimod precursors compounds. Fingolimod is a chemical derivative of myriocin/ISP-1, a fungal metabolite that was isolated from *Isaria sinclairii*, the anamorph stage of the ascomycete species *Cordyceps*. Along with ginseng and the young antlers of deer, *Cordyceps* fungi were considered as one of the three oriental medicines that give 'eternal youth.' *Cordyceps* fungi enter into living insects, feed on the insides of the host, and eventually grow onto the surface of the cadaver in the summer. Drugs derived from *Cordyceps* are Cordycepin (3′ deoxyadenosine) which inhibits tumor growth, Cyclosporin, the classical immunosuppressant used in transplant medicine, and Myriocin (ISP-1), the immunosuppressant that targets serine palmitoyl transferase (SPT). Fingolimod, despite being a chemical derivative of myriocin, has lost activity on SPT but targets the class of G protein-coupled S1P receptors. *Isaria sinclairii*, photo courtesy of Mr. Clive Shirely; cicada nymph, photo courtesy of Alastair Robertson and Maria Minor; soil bugs, from An Illustrated Guide to New Zealand Soil Invertebrates, http://soilbugs.massey.ac.nz. © Massey University.

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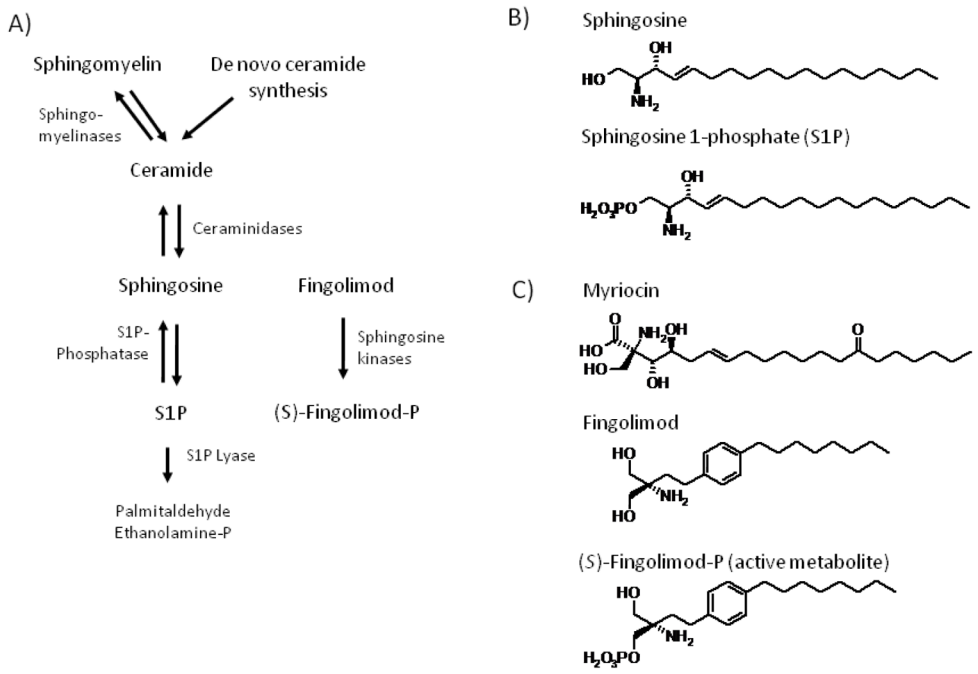


**Figure 3.**

Fingolimod-relevant chemical structures. Fingolimod and fingolimod-phosphate (fingolimod-P) are structural analogues of sphingosine and sphingosine 1-phosphate (S1P), respectively. S1P is generated via the intracellular ceramide pathway, and ceramide is formed through *de novo* biosynthesis or degradation of the cell membrane constituent sphingomyelin. Ceramide is *N*-deacetylated to yield sphingosine, and both sphingosine and fingolimod are phosphorylated by sphingosine kinases to yield S1P and (*S*)-fingolimod-P, respectively, whereas (*R*)-fingolimod-P is not found *in vivo*. The (*S*)-fingolimod-P is the biologically active principle of the drug in animal models of autoimmune disease (Albert *et al.*, 2005; Brinkmann *et al.*, 2002).

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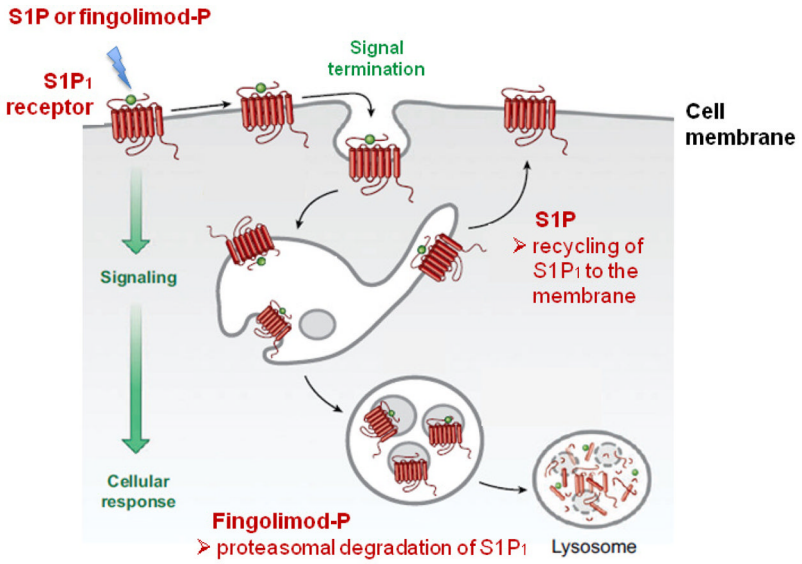


**Figure 4.**

Modulation of $S1P_1$ receptors by S1P and fingolimod. To ensure that extracellular stimuli are translated into intracellular signals of appropriate magnitude and specificity, most signalling cascades are tightly regulated. One of the major mechanisms involved in the regulation of G protein-coupled receptors (GPCRs), including S1P receptors, involves their endocytic trafficking. GPCR endocytic trafficking entails the targeting of receptors to discrete endocytic sites at the plasma membrane, followed by receptor internalization and intracellular sorting to terminate the signal. Shown is the fate of S1P- and fingolimod-P-signalling at $S1P_1$ receptors. S1P produces a signal that is terminated by internalization of the S1P-$S1P_1$ complex. After dissociation of S1P from $S1P_1$, the receptor is recycled to the cell membrane. The higher affinity of fingolimod (compared to S1P) to $S1P_1$ leads to tight binding and less recycling of $S1P_1$ and, as a consequence, to proteasomal degradation of the drug-receptor complex. This results in termination of an initial agonistic signal, and a "functional antagonism" of $S1P_1$ receptors that, in models of MS, terminates T cell inflammation and astrogliosis.