6 Druggable Sphingolipid Pathways: Experimental Models and Clinical Opportunities

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Abstract

Intensive research in the field of sphingolipids has revealed diverse roles in cell biological responses and human health and disease. This immense molecular family is primarily represented by the bioactive molecules ceramide, sphingosine, and sphingosine 1-phosphate (S1P). The flux of sphingolipid metabolism at both the subcellular and extracellular levels provides multiple opportunities for pharmacological intervention. The caveat is that perturbation of any single node of this highly regulated flux may have effects that propagate throughout the metabolic network in a dramatic and sometimes unexpected manner. Beginning with S1P, the receptors for which have thus far been the most clinically tractable pharmacological targets, this review will describe recent advances in therapeutic modulators targeting sphingolipids, their chaperones, transporters, and metabolic enzymes.

Keywords

Sphingolipid · S1P · Fingolimod · Siponimod · Ozanimod

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6.1 Introduction

The family of bioactive sphingolipid molecules is immense, but all are characterized by the same core component, the sphingoid backbone, which is simply an amino alcohol with a long carbon chain [1]. The canonical sphingolipid, sphingosine, can be enzymatically modified to add fatty acids, phosphorous-containing head groups (e.g., phosphocholine), sugar moieties, and changes in acyl chain saturation [1–3]. Flux through the sphingolipid pathway has far-ranging effects, from cellular architecture to multi-organ system coordination [4, 5]. The ubiquity of sphingolipids presents both challenges to and opportunities for their manipulation. Unlike other bioactive lipids with proposed shunting to different enzymatic pathways, the flow of sphingolipids rarely has an alternative for degradation or synthesis other than reversal [6–11].

Subsequently, while inhibition of a particular enzyme stops generation of a specific product, the biological outcome could be the result of increased concentrations of upstream precursors and not necessarily the most immediate parent molecule. For instance, a great deal of effort has focused on the inhibition of two enzymes, the sphingosine kinases (Sphk1/2), for the treatment of cancer. Although the desired decrease in product may be achieved, an increase in the parent molecule of the Sphk substrate is commonly credited with affecting the biological outcome.

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Like a malfunctioning traffic light, a blockage at one sphingolipid node can often have repercussions throughout the metabolic pathway (Fig. 6.1). This review will present known mechanisms for therapies that target sphingolipid signaling pathways, including our current understanding of sphingosine 1-phosphate (S1P) receptor modulators, S1P chaperones and transporters, and sphingolipid metabolic enzymes [12–14].

6.1.1 Sphingosine 1-Phosphate (S1P) and Its Receptors, $S1P_{1-5}$

Unlike the on-demand production of other bioactive lipids, the signaling molecule sphingosine 1-phosphate (S1P) is omnipresent in blood and lymph circulation, with both human and murine concentrations of 18:1 S1P in the mid-nanomolar to low micromolar range [15–18]. Blood plasma concentrations of other powerful bioactive lipids, such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), are approximately 10 times less than S1P [19]. Despite high plasma concentrations, tissue concentrations of S1P are far lower than circulating levels, setting up an "S1P gradient" in which small alterations in local S1P concentrations are sensed by finely-tuned mechanisms within specific cell types, potentially triggering dramatic effects [20–22].

The five S1P receptors (S1PR), $S1P_{1-5}$, are members of the rhodopsin class of G protein-coupled receptors (GPCR). This family of proteins constitutes 40% of drug targets [23–27]. The first S1PR, $S1P_1$, was cloned from endothelial cells (EC) owing to its critical role in endothelial cell and vascular biology [28, 29]. Lack of *S1pr1* results in embryonic lethality due to hemorrhage from immature vasculature [30]. Induced $S1pr1^{-/-}$ in endothelial cells (EC) causes increased vascular leakage and inflammatory molecule expression, and $S1pr1^{-/-}$ tumor vessels have disordered architecture and poor perfusion [31–33]. Conversely, overexpression of EC $S1P_1$ reinforces the vascular barrier, increasing perfusion of tumor vessels [31, 33]. As one of the genes regulated by the transcription factor KLF2, a hemodynamic responsive protein, EC *S1pr1* also signals in response to fluid shear stress [31, 32, 34].

In the vasculature, as well as in other cell and tissues types, $S1P_1$ and $S1P_2$ signaling counteract each other, with $S1P_3$ signaling acting as a modifier [35–37]. Effects on the vasculature are of great importance in understanding the mechanisms and potential side effects of S1PR modulating drugs. The literature regarding S1PR and the vascular system is vast, and the reader is referred to several recent excellent reviews on the topic for more detailed descriptions [33, 38, 39].

Although the first S1PR was cloned from EC, S1PR modulating drugs work primarily through immune cell modification [23, 28]. There are currently three FDA-approved S1PR modulating drugs: the first-in-class FTY720 (fingolimod/Gilenya), BAF312 (siponimod/Mayzent), and RPC1063 (ozanimod/Zeposia) are approved for treatment of the relapsing-remitting form of multiple sclerosis (RRMS) (Fig. 6.2) [12, 40–42]. A New Drug Application (NDA) has been submitted for a fourth compound, ACT128800 (ponesimod), also for the treatment of RRMS [43, 44]. Siponimod is also approved for secondary progressive MS (SPMS), the stage of MS disease progression after RRMS. While numerous S1PR modulators are utilized as pharmacological tools in the laboratory, most were not tested in the clinical setting due to poor solubility, *in vivo* stability, half-life, or specificity. The side effects of fingolimod and siponimod illustrate why increasing target specificity is a driving factor behind the continued development of S1PR modulators.

FTY720 is a sphingosine analogue derivative of a fungal metabolite and must be phosphorylated (FTY720P) for recognition by S1PRs [45, 46]. Although FTY720P activates all S1PRs except $S1P_2$, $S1P_1$ binding FTY720P causes its polyubiquitination and degradation, resulting in "functional antagonism" [47]. In humans, FTY720 has a long *in vivo* half-life of greater than 100 hours, combined with low oral clearance and a high volume of distribution that is likely due to absorbance into lipid-rich tissues and cell membranes [48–51]. Lymphopenia is the most striking effect of FTY720 treatment and the

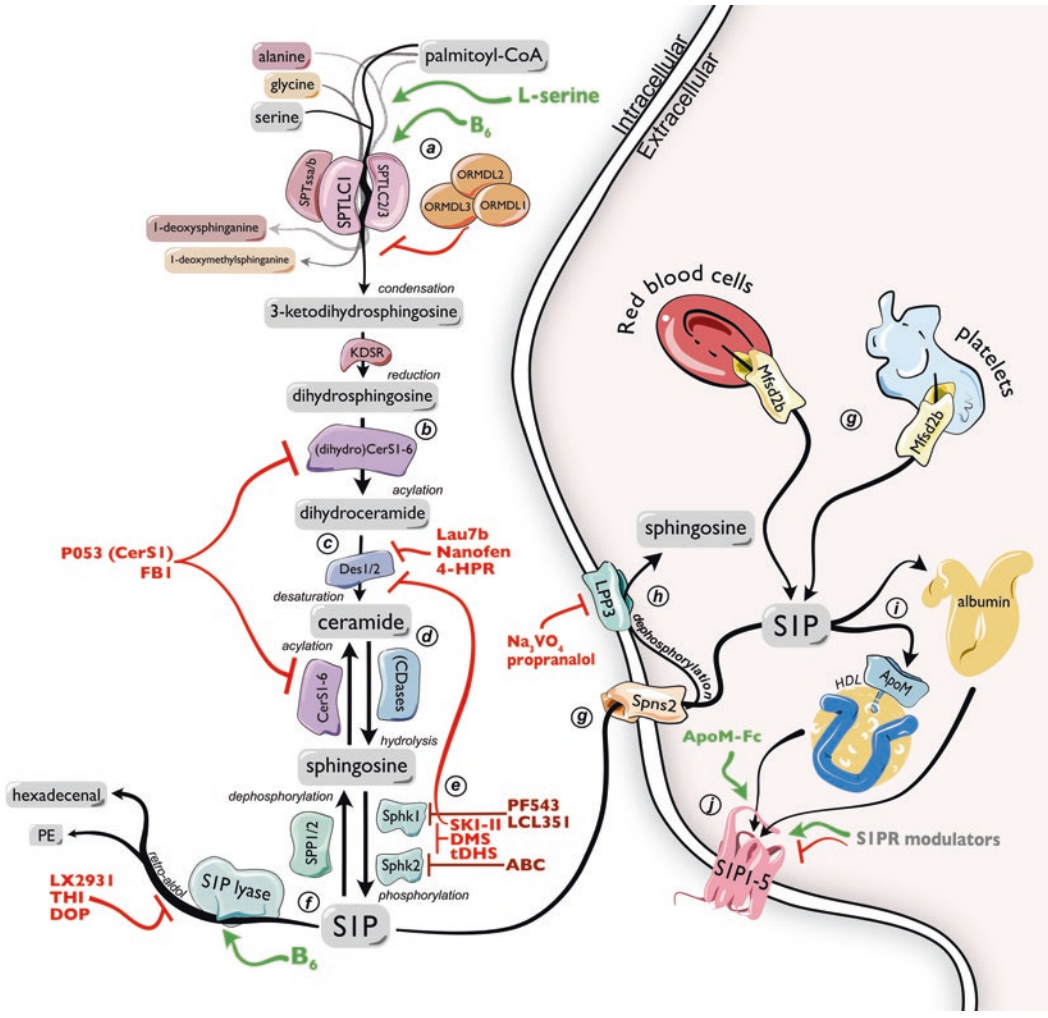

Fig. 6.1 Sphingolipid metabolism, signaling pathways, and pharmacological interventions

(**a**) *De novo* sphingolipid synthesis begins in the endoplasmic reticulum (ER) membrane with the condensation of the fatty acyl-CoA palmitoyl-CoA and the amino acid serine to form 3-ketodihydrosphingosine. The reaction is catalyzed by the pyridoxal 5′-phosphate (PLP)-dependent heterodimeric enzyme serine palmitoyl-CoA transferase (SPT), which consists of the SPTLC1 subunit and either SPTLC2 or SPTLC3. SPT activity is enhanced by SPTssa or SPTssb and additional vitamin B_6 (B_6) and is homeostatically inhibited by the ORMDL1-3 proteins. If alanine or glycine are utilized instead of serine, toxic 1-deoxysphinganine or 1-deoxymethylsphinganine, respectively, are produced. Generation of these toxic deoxysphingolipids can be reduced by supplementation with L-serine. 3-ketodigydrosphingosine is then reduced by 3-ketodihydrosphingosine reductase (KDSR) to dihydrosphingosine. (**b**) Acylation of dihydrosphingosine by the (dihydro) ceramide synthases (CerS) 1-6 generates dihydroceramide. All CerS can be inhibited by fumonisin B1 (FB1) and P053 specifically inhibits CerS1. (**c**) Dihydroceramide is then desaturated by dihydroceramide desaturase (Des1/2) to ceramide. This reaction can be inhibited by Lau7b, Nanofen, or fenretinide (4-HPR). (**d**) Ceramide is hydrolyzed to sphingosine by ceramidases (CDases) according to subcellular location. Sphingosine can be converted back to ceramide by CerS1-6 or (**e**) phosphorylated by sphingosine kinases (Sphk1/2) to sphingsosine 1-phosphate (S1P). Sphk1 and 2 are inhibited by SKI-II, dimethylsphingosine (DMS) or *D,L*-threo-dihydrosphingosine (tDHS). Sphk1 is specifically inhibited by PF543 or LCL351 and Sphk2 is specifically inhibited by ABC294640 (ABC). (**f**) Intracellular S1P can be dephosphorylated by S1P phosphatases (SPP1/2) or irreversibly degraded by retro-aldol cleavage of the C2-C3 bond by S1P lyase to form (2E)-hexadecenal and phosphoethanolamine (PE). S1P lyase is a PLP-dependent enzyme whose activity can be increased by B_6 supplementation or inhibited by LC2951, 2-acetyl-4-tetrahydroxybutylimidazole (THI), or 4-deoxypyridoxine (DOP). (**g**) S1P can be actively transported out of cells by Spns2 or from red blood cells and platelets by Mfsd2b. (**h**) Extracellular S1P can be dephosphorylated by lipid phosphate phosphatase 3 (LPP), which is non-specifically inhibited by sodium orthovanadate (Na_3VO_4) or propranolol. (**i**) S1P in circulation is carried by albumin or the HDL-bound S1P-specific chaperone apolipoprotein M (ApoM). (**j**) Albumin-S1P, ApoM-S1P, or synthetic ApoM-Fc-S1P can activate cell surface S1P receptors, $S1P_{1–5}$. The multiple S1PR agonists and antagonists in clinical use or testing are described in more detail in Fig. 6.2

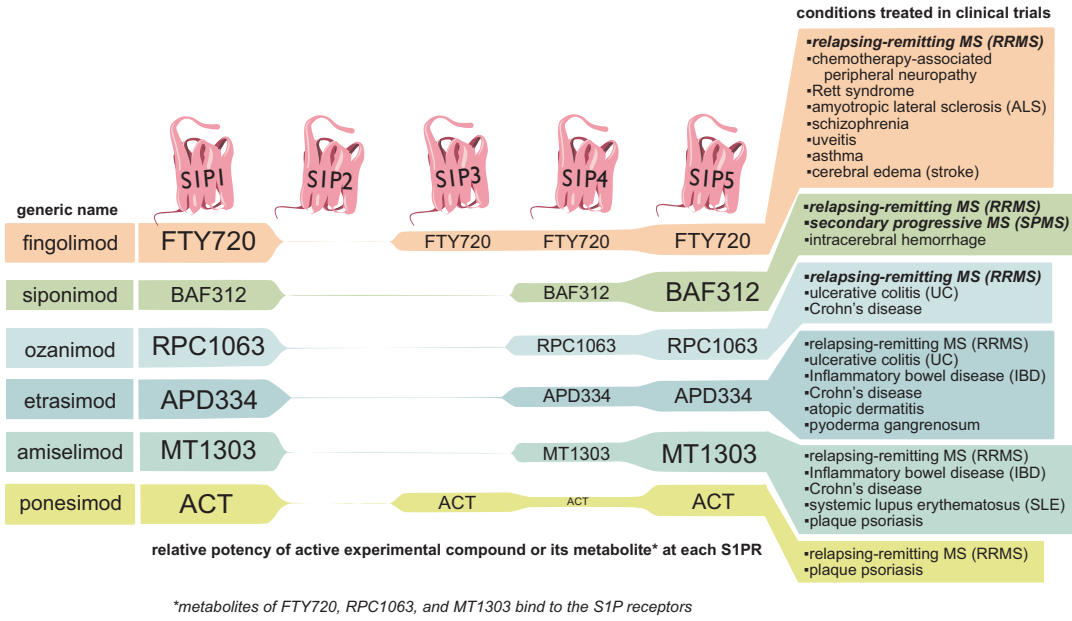

Fig. 6.2 S1P receptor modulators and their clinical uses There are six drugs that target S1P receptors (S1PRs) and are FDA-approved for clinical use or currently undergoing clinical testing: fingolimod (FTY720), siponimod (BAF312), ozanimod (RPC1063), etrasimod (APD334), amiselimod (MT1303), and ponesimod (ACT128800; abbreviated "ACT"). Generic drug names are given on the left. FTY720, BAF312, etc. are the experimental compounds actually administered, but FTY720, RPC1063, and MT1303 must be metabolized to an active form in order to bind to the S1PR. For each compound, the relative potency of it or its active metabolite is shown below each receptor. For instance, the active metabolite of FTY720, FTY720P, has the greatest effect on $S1P_1$, the second strongest effect on $S1P_5$, relatively similar potency at $S1P_3$ and $S1P_4$, and no binding to $S1P_2$. Conditions for which each drug has undergone clinical trials are listed on the right. Listed in **bold** are conditions for which that drug is a currently FDA-approved treatment

greatest contributor to its mechanism of action: it restricts lymphocytes from exiting the lymphoid organs, preventing them from trafficking to attack other organs [15, 52–55]. Lymphopenia is largely the result of lymphocyte-expressed $S1P_1$ functional antagonism, since mature B and T cells use $S1P_1$ to sense and migrate toward the high circulating S1P concentrations [56]. In the animal model of MS, experimental autoimmune encephalomyelitis (EAE), and in MS patients treated with FTY720, autoreactive lymphocytes are prevented from entering circulation and reaching the central nervous system (CNS) [45, 57].

A phase III trial comparing FTY720 to cyclosporine in the prevention of renal transplantation rejection found that although higher doses are generally well-tolerated compared to high doses of other classical immunosuppressants, kidney function was consistently lower in FTY720-treated cohorts and small increases in pulmonary resistance were also documented [58]. The most frequently observed adverse effect in clinical studies of FTY720/fingolimod was macular edema, which, along with the other adverse events, directly correlated with dose, whereas efficacy did not [50, 59, 60]. This indicates that the escalating dose results in increased engagement of the S1PR in order of their affinity for FTY720P: $S1P_1 > S1P_3 > S1P_4 \geq S1P_5$ [61, 62]. With increasing S1PR engagement comes greater signaling complexity, since cells are likely to express multiple S1PR [23].

S1PR modulators may also find use in the treatment of neurological disorders other than MS. In the $mSOD1^{G93A}$ model of amyotrophic lateral sclerosis (ALS), treatment of mice with 0.1 mg/kg FTY720 significantly delayed progression of neurological deterioration and

extended survival [63]. In brains of Alzheimer's disease patients (AD), *S1PR1* mRNA was significantly decreased, but *S1PR3* was increased [64]. An increase in brain $S1P_3$ may partially explain why FTY720 treatment could effectively reduce brain Aβ plaques and neuroinflammation and improve neurological function in multiple mouse models of AD [65–67]. In a mouse model of AD, FTY720 treatment reduced the numbers of activated microglia and astrocytes [67]. Astrocyte-intrinsic $S1P_1$ may be key to FTY720's efficacy in neurological disorders, since a subset of astrocytes are also activated during EAE and reduced with FTY720 treatment [68].

The possibility of using FTY720 as an anticancer therapy has been enthusiastically pursued since early reports of *in vitro* and *in vivo* pro-apoptotic activity [69–71]. The immunosuppressive effect of FTY720 was originally attributed to induction of T cell apoptosis [69, 70, 72, 73]. Subsequently, FTY720 has been tested for efficacy in cancer cells derived from, and cancer models affecting, almost every organ system including leukemia, prostate, glioma, breast, mesothelioma, non-small cell lung carcinoma, pancreatic, and colorectal [70, 71, 73–81]. Rather than affecting S1PR signaling, the proposed anti-tumoral/pro-apoptotic effect of FTY720 is inhibition of sphingosine kinases and activation of protein phosphatase 2A (PP2A) [81–84]. A recent study used NMR spectroscopy to characterize the direct interaction of FTY720 with SET, a PP2A inhibitor [85].

The recommended dosage for Gilenya (FTY720, fingolimod) is 0.5 mg once daily, and multiple clinical trials examining the long-term effects of FTY720 administration in patients have been completed or are ongoing (NCT01201356, NCT02720107, NCT00662649, NCT01281657, NCT02307877, NCT03216915, NCT02232061, NCT01442194). Clinical trials have confirmed that adverse events, both common (acute bradycardia, macular edema, renal dysfunction) and uncommon (fatal infection, seizure, lymphoma) are dose-dependent while efficacy and effectiveness are not [59, 60, 86–88]. Adverse events are not commonly recapitulated in mice, and mouse studies investigating the use of FTY720 in cancer models have frequently (although not always) used extended dosing of 10 mg/kg. However, the approximate conversion of a 10 mg/kg dose for a mouse reveals that this is equivalent to a single oral dose of 56 mg in a human – greater than 100 times the approved dose and 10 times the highest dose administered in clinical trials [89, 90]. Currently, two clinical trials are investigating the use of FTY720 treatment during cancer, but with the goal of reducing chemotherapy-induced neuropathy (NCT03943498, NCT03941743). No other S1PR modulators are undergoing clinical trials for cancer as the targeted condition.

There is potential for modulating S1PR signaling for the treatment of viral infections, particularly HIV. T cells from the lymph nodes (LN) of HIV patients showed impaired migratory activity, including toward S1P, possibly explaining HIV lymphadenopathy [91]. CD69 expression usually inversely correlates with that of $S1P_1$ because their physical interaction down-regulates surface $S1P_1$ and $S1P_1$ expression suppresses that of CD69, yet both CD69 and $S1P_1$ were decreased in cells from viremic HIV patients compared to control cells [91, 92]. KLF2, the primary transcription factor responsible for T cell *S1PR1* gene activation, was also down regulated in LN, but surprisingly, mRNAs for *S1PR1* and *KLF2* from purified T cells were *not* decreased and antiretroviral therapy (ART) improved responsiveness to S1P [91, 93]. *In vitro*, cells expressing $S1P_1$ allowed greater HIV replication and treatment with the $S1P_1$ agonist SEW2871 resulted in reversal of latency and reactivation of viral replication in peripheral blood cells and LN, whereas FTY720P decreased *in vitro* virus production by monocyte-derived dendritic cells [94]. An early study found that FTY720 administration to simian immunodeficiency virus (SIV)-infected macaques did not affect viremia and proviral DNA [95]. However, a more recent study reported that FTY720 treatment of SIV-infected rhesus macaques initiated after at least 4 months of combination ART (cART) both increased LN numbers of cytotoxic T cells and decreased the infection of LN T follicular helper (Tfh) cells, as determined by level of proviral DNA [96]. The

authors suggested that FTY720 treatment could be started concurrently with cART or cytotoxic T cell activating IL-2 or IL-15 therapies, and by decreasing the number of circulating cells with viral RNA, the viral reservoir allowed into circulation would be decreased. FTY720 may also protect from HIV-associated dementia in the context of inflammation. Human neural progenitor cells (hNP1) exposed to HIV and treated with FTY720P had decreased expression of immune and inflammatory response-related genes [97]. Thus, FTY720 treatment at doses relevant to human patients, particularly in the context of cART, has potential for increasing immune responses that would be beneficial for controlling HIV replication and suppressing inflammatory responses that would be neurotoxic.

$S1P_2$ expression is also necessary for regulating migration of many cell types and coordinates S1P responsiveness with $S1P_1$ for proper positioning of various B and T cell subsets within the lymphoid organs, including germinal center B cell (GCB) confinement, and suppression of their over-proliferation and apoptosis [98]. Tonsil-derived T central memory (TCM) and T resident memory (TRM) cells are chemorepulsed *in vitro* by S1P via $S1P_2$ signaling, which counters pro-migratory CXCL12 responses [99]. Both TCM and TRM are defined by markers known to counter-regulate $S1P_1$ responses: TCM are $CCR7^+$ and TRM are $CD69^+$ [100, 101]. Immunohistochemically, expression of $S1P_1$ and $S1P_2$ are mutually exclusive and vary between different diffuse large B cell lymphoma (DLBCL) types [102]. *S1PR2* mutations were present in more than 25% of diffuse large B cell lymphoma (DLBCL) patients, and the *S1PR2* mutations present in GCB-DLBCL resulted in altered protein expression or an inability to bind the G protein $G\alpha_{13}$, leading to loss of GCB confinement [103, 104]. Expression of a negative regulator of *S1PR2*, the transcription factor FOXP1, correlates with poor survival in activated B cell (ABC)-DLBCL patients [105]. A subset of Tfh with high PD-1 expression coordinates $S1P_2$ and CXCR5 signaling to localize to the GC, indicating that $S1P_2$ mutations could impair antibody production and lymphomagenesis through altered T-B cell interactions [106].

Follicular B cells (FBC) utilize $S1P_1$ to migrate to the marginal zone (MZ) from the follicle, whereas MZ B cells (MZB) use $S1P_1$ signals to shuttle between the MZ and follicle [107, 108]. MZB $S1P_1$ phosphorylation by G protein-coupled receptor kinase 2 (GRK2) results in their desensitization to S1P, permitting them to respond to other chemotactic signals [108]. GRK2 also inhibits MALT1, a protease and scaffold protein positive regulator of NF-κB, impairing the survival of DLBCL cells [109, 110]. Since ABC-DLBCL expression of GRK2 positively correlated with patient survival, altered S1P-directed migratory patterns may be related to anti-apoptotic signaling [110].

In vitro studies repeatedly indicated that $S1P_3$ would be the primary regulator of bone marrow (BM) B cell egress; however, *in vivo* studies determined that agonism and subsequent down-regulation of $S1P_1$ allowed $S1P_3$ signals to be misinterpreted as the dominant BM egress signal [111, 112]. In a mouse model of autoantibody production, immature B cells in the BM use $S1P_3$ to migrate from the parenchyma to the sinusoids, but only if they are not autoreactive [112]. Subsequently, $S1P_1$ signaling draws them from BM into circulation [111]. Mature B cells also use $S1P_3$ to position themselves within the MZ, in addition to using $S1P_1$ as their cue to migrate to the MZ [113].

In human leukemia cells (CLL (chronic lymphocytic leukemia), pre-B-ALL (pre-B cell acute lymphoblastic leukemia), and CLL (chronic lymphocytic leukemia)), $S1P_1$ was down regulated by 10-fold and had impaired *in vitro* chemotaxis toward S1P as compared to control cells [114]. *S1PR4* mRNA was co-expressed with that of *S1PR1* and its over-expression appeared to modulate $S1P_1$-directed chemotaxis [114]. Highly expressed by lymphocytes, $S1P_4$ appears to have only modulatory effects on $S1P_1$-induced migration in most lymphocyte types, but may be critical for the regulation of lymphocyte proliferation and activation. T cell $S1P_4$ can suppress proliferation and IL-2 and IL-4 production initiated by

anti-CD3/CD28 activation and signaling, providing a partial explanation for the skewing of *S1pr4*$^{-/-}$ immune response to Th2 and away from Th17 [115, 116]. $S1P_4$ is not a critical regulator of B cell migration or activation in the spleen, but some peritoneal B cell subsets rely on $S1P_4$ signaling, rather than $S1P_1$. Peritoneal B1a, B1b, and B2 B cells all expressed *S1pr4* mRNA, although only B1a and B1b cells appeared to use either $S1P_1$ or $S1P_4$ for migratory cues [117]. Although *S1pr4*$^{-/-}$ animals had normal numbers of B cells in circulation, the numbers of B1a and B1b peritoneal cells were significantly reduced, as was the level of secretory IgA in the small intestine [116, 117].

$S1P_4$ signaling may play a greater role in the innate immune system by regulating inflammatory responses. A missense variant of the human *S1PR4* was discovered that correlated with decreased numbers of neutrophils in circulation [118]. Dendritic cells (DC) utilize $S1P_4$ for their migration to lymph nodes in cooperation with CCL21 [116]. High concentrations of S1P *in vitro* (>3 μM) inhibited neutrophil and macrophage 5-lipoxygenase (5-LO), a leukotriene biosynthetic enzyme critical for innate immune function and activated in multiple inflammatory and autoimmune disorders, including arthritis and asthma [119]. Whole blood cell mRNA profiling of patients after aneurysmal subarachnoid hemorrhage found higher *S1PR4* transcript levels correlated with a greater risk of vasospasm, a major cause of severe cognitive defects and mortality, the pathogenesis of which may be linked to neutrophil recruitment and activation [120, 121]. Neutrophils are also of interest in psoriasis because of their induction of Th17 responses as well as direct pro-inflammatory activities [122]. *S1pr4*$^{-/-}$ mice had less inflammation in an imiquimod model of psoriasis as a result of decreased production of the macrophage and neutrophil chemokines CCL2 (MCP-1) and CXCL1 (KC), possibly because of decreased NF-κB activation [123].

S1pr4$^{-/-}$ mice had decreased pathology in the dextran sulfate sodium (DSS) colitis model, which correlated with decreased IL-6 production and a skewing from Th17 to increased Th2 differentiation, although their $CD4^+$ and $CD8^+$ cells had increased *in vitro* migration toward an intermediate S1P concentration (0.1 μM), and their DC had increased migration toward draining lymph nodes [116]. The S1PR agonist etrasimod activates $S1P_1 > S1P_5 > S1P_4$ with no activity at $S1P_2$ or $S1P_3$ and decreased inflammation in a T cell transfer model of colitis [124]. Etrasimod has also shown efficacy at a dose of 2 mg in clinical trials for treatment of moderate to severe active ulcerative colitis (UC), including histologic remission [125].

6.2 S1P Chaperone: Apolipoprotein M (APOM)

An ideal pharmacological target is one that has minimal impact on other components of the pathway. With this qualifier, the S1PR present the best option, since agonism or antagonism of a single receptor could be anticipated to impact only the signaling of the remaining S1PR, rather than changing flux within the entre sphingolipid metabolic pathway. The target with the second lowest possibility of large pathway perturbation is the primary S1P chaperone, apolipoprotein M (ApoM), the other chaperone being the non-specific lipid transporter albumin [126, 127].

High concentrations of S1P can be found in plasmas of both blood and lymph, known to be bound to protein and lipoprotein fractions [128]. Although ApoM is present in the lymph, *Apom*$^{-/-}$ mice do not have significantly altered lymph S1P concentrations compared to the 60–70% drop in blood S1P, indicating that either all lymph S1P is bound to albumin or there is another lymph-specific S1P chaperone [16]. In the blood of mice constitutively lacking both albumin and ApoM, S1P is bound to ApoA4 [129]. A recent study of human males described a third pool of ApoM, neither lipoprotein nor protein associated, in blood, although it is unclear what the purpose is of this ApoM population [130].

In mice, the 60–70% ApoM-S1P bound to lipoprotein is usually associated with HDL; how-

ever, human lipoprotein studies have found that while S1P is usually highly correlated to ApoM, the lipoprotein class that ApoM is found on may vary based on several factors, including sex, race, age, and disease. The correlation of HDL anti-inflammatory activity with S1P content has driven the search for diseases in which ApoM could be outcome predictive, including coronary artery disease (CAD), type 1 (TI) and type 2 diabetes (T2D), metabolic syndrome (MetS), lupus, IgA nephropathy, and insulin resistance [131–139].

There are diseases where altered ApoM or ApoM-HDL concentrations appear to correlate with increased disease severity or mortality. ApoM and HDL-S1P were both decreased in T2D and mortality of African Americans with T2D inversely correlated with ApoM or S1P levels [135, 140]. MetS patients without diabetes have both higher TG and lower S1P [130, 133]. Although the ratio of ApoM to ApoA1, an HDL-specific protein, was unchanged in MetS patients, the molar ratio of S1P:ApoM was 30% decreased compared to controls [132]. CAD patients also have normal HDL but decreased plasma S1P [141, 142]. In post-menopausal women, although plasma S1P concentrations are the same as in pre-menopausal women, they have increased ApoM, resulting in a greater than 25% decrease in the S1P:ApoM ratio, a characteristic accompanied by endothelial dysfunction and metabolic syndrome [143]. Interestingly, women with very low TG and low LDL had an increased risk of hemorrhagic stroke, but there was no significant correlation between total cholesterol or HDL and hemorrhagic stroke risk [144].

Alternatively, although the absolute concentration of ApoM may be the same, a shift in the lipoprotein particle that it associates with can be indicative of a loss of anti-inflammatory or atheroprotective quality. T1D patients can have normal or above normal HDL concentrations, but are still at increased risk of cardiovascular disease (CVD) [145, 146]. Female T1D patients had ApoM on less dense HDL particles which are believed to be less atheroprotective and in some cases, pro-inflammatory [147, 148]. While still capable of activating the $S1P_1$-ERK pathway and inducing $S1P_1$ internalization in endothelial cells, T1D HDL was ineffective at activating AKT, a major inducer of endothelial nitric oxide synthase (eNOS), the enzyme responsible for production of homeostatic nitric oxide production induced by $S1P_1$ signaling [149]. MetS patient ApoM was more frequently found on LDL, having been transferred from the HDL particles [150]. A study investigating the impact of a hypercholesterolemic diet on HDL content in a porcine model of ischemia reperfusion found that less ApoM was present in HDL particles from hypercholesterolemic pigs versus controls [151].

MetS patient HDL, which has lower S1P in addition to other lipidome alterations, was also less effective at activating eNOS [150, 152, 153]. Once S1P was loaded exogenously onto MetS HDL, the ability to activate eNOS was restored [132]. Loading S1P onto S1P-poor HDL of CAD patients also restored S1PR signaling to levels achieved with HDL from control patients [154]. Another group found that recombinant HDL without sphingomyelin (SM), a metabolic S1P precursor present in high concentrations in the HDL particle, is not sufficient to activate eNOS, but the phosphorylation of eNOS is not directly proportional to HDL SM content, since too much SM will decrease eNOS phosphorylation [155]. These effects may be due to providing the S1P precursor as well as the biophysical effect SM has on the HDL particle itself. High SM content in a lipid layer reduces fluidity, which would alter the flexibility of the HDL particle and subsequently cell membranes to which it transferred lipid cargo to [156, 157]. In control patients, S1P and ApoM are usually enriched on the smaller HDL3 particles, in which the ratio of S1P to SM is over 30 times greater than that of HDL2 [158, 159].

The link between ApoM and metabolism has recently become even more complex than attempting to correlate plasma ApoM concentrations with S1P and lipoproteins. The *Ldlr*$^{-/-}$ (LDL receptor) mouse is a key mouse model of atherosclerosis, and the impact of ApoM-S1P on vascular integrity and endothelial cell health would imply that *Apom*$^{-/-}$ mice would be more prone to atherosclerotic disease, but

Apom$^{-/-}$*Ldlr*$^{-/-}$ double knockout mice are protected from atherosclerosis [160]. Our current understanding of the roles ApoM may play in lipoprotein metabolism indicate that defective LDL regulation, as in the setting of LDLR deficiency, lead to a compensatory increase in plasma ApoM, which in turn, resulted in increased circulating LDL, promoting a pro-atherogenic phenotype that was mitigated by concomitant ApoM deficiency [161]. The impact of ApoM expression in other models of atherosclerosis, such as *Apoe* or LDLR-related protein 1 (*Lrp1*) knockouts, is dependent on the LDL metabolism in each model [160–162].

Soon after its discovery, *APOM* was identified as a leptin-modulated gene [163], the expression of which positively correlated with leptin measurements in human plasma and was suppressed in the obesity model, leptin receptor-deficient (*ob/ob*) mice [164]. More recently, it was reported that *Apom*$^{-/-}$ mice had increased brown adipose tissue (BAT) and were protected from diet-induced obesity, a phenotype reversed by $S1P_1$ agonist administration [165]. A study of tissue from almost 500 human patients found adipose ApoM was produced by adipocytes and secreted to plasma, with ApoM levels inversely correlating with obesity, metabolic syndrome, and T2D [166]; however, it is unclear if S1P is involved in this adipocyte-derived ApoM signaling. In a mouse model of diabetes, insulin administration reversed the decrease in ApoM levels [167]. It is likely that ApoM and insulin cross regulate each other, since *APOM*^{Tg} mice have increased circulating insulin, which can be reduced by treatment with the $S1P_{1/3}$ antagonist VPC23019 [168].

In systemic lupus erythmatosus (SLE), low plasma ApoM correlated with the presence of disease activity markers, including nephritis, leukopenia, and anti-double stranded DNA antibodies (anti-dsDNA) [138, 169]. In an *in vivo* model of immune complex deposition, the reverse Arthus reaction (RAR), mice lacking endothelial cell (EC) $S1P_1$ developed a stronger response [170]. Surprisingly, *Apom* knockout alone did not impact the magnitude of the RAR generated, but when treated with a low dose of $S1P_1$ antagonist the response was significantly greater. Conversely, patients with IgA vasculitis had increased serum ApoM but those with nephritis as a complication had lower ApoM levels than those without [171]. The authors suggest that renal tubular epithelial cell destruction triggered by renal inflammation may have led to decreased ApoM production.

The vascular role of ApoM impacts the development of inflammation and responses to infection in addition to direct signaling on immune cells. In sepsis patients, ApoM produced in the liver drastically drops within 12 h and S1P and ApoM in plasma drop at 6–12 h in both human patients and a baboon model of lethal sepsis [172, 173]. The most severe cases of infection tend to have the lowest ApoM levels, and this drop in ApoM-S1P may contribute to the defects in vascular barrier function that occur in sepsis. ApoM deficiency does not result in gross vascular permeability in the same way that loss of EC $S1P_1$ does [16, 31, 174]. While larger molecules cannot diffuse freely across the BBB of *Apom*$^{-/-}$, paracellular transport of much smaller molecules (<0.07 kDa) was increased in specific vessel types, as was transcytosis; however, not all of the vessels were responsive to $S1P_1$ agonist rescue [175]. The differences in regulation of vascular bed permeability are dramatically illustrated by the pulmonary vascular leakage seen in $S1P_1$ EC-specific knockout (ECKO) animals as compared to the small effect on the BBB of the same animals [16, 174, 176]. In sepsis, effects on barrier integrity within organs such as those in $S1P_1$ ECKO versus ApoM KO mice highlight the necessity for more detailed characterization of the effects of both the S1PR and its chaperones [177].

APOM mRNA has been identified in EC but protein, if produced, is below the limit of detection and the inflammatory stimulus TNFα does not change this mRNA production [178]. The authors put forth the hypothesis that this distinctive expression may imply a purpose for endothelial versus hepatic ApoM, particularly in an inflammatory context such as sepsis, where the plasma levels of ApoM and S1P decrease, as does HDL [173, 179]. The ability of EC to make and retain their own ApoM while producing and secreting their own S1P would allow for some

tissue-intrinsic control in response to the drastic systemic decreases in ApoM-S1P seen in sepsis.

6.2.1 ApoM-Fc

Disease modulation through targeting of ApoM signaling pathways has recently been demonstrated by administration of a recombinant ApoM fused to a modified immunoglobulin Fc domain (ApoM-Fc) [180]. ApoM-Fc has improved *in vivo* stability versus a traditional recombinant ApoM protein and has similar *in vitro* properties as ApoM-HDL. The effects of ApoM-Fc appear to be endothelium-centric: it reduced infarct size in the middle cerebral artery occlusion (MCAO) model of stroke, preserved cardiac function in a model of myocardial ischemia/reperfusion, and reduced pulmonary inflammation in the RAR model of immune complex injury [170, 180]. Since immune cell numbers were not affected, particularly lymphocytes, it is possible that ApoM-Fc cannot access the hematopoietic compartment and therefore may provide a tool for differential delivery of ApoM-S1P to EC, sparing the immune system. Subsequent iterations of ApoM-Fc may aim to target endothelial subtypes or the specific S1P receptors they express. However, since ApoM prevents excessive bone marrow lymphopoiesis and HDL is known to affect survival of mature T cells, T regulatory cell (Treg) differentiation, and antigen presentation to T cells, modification of ApoM-Fc or development of a novel ApoM mimetic that targets lymphocytes and/or their progenitors, could be beneficial for direct immunosuppression [16, 181–183].

6.2.2 ApoM Receptor Megalin

To date, the lipocalin receptor megalin is the only known ApoM receptor but it does not appear to be involved in recognition of ApoM outside of the kidney [184]. In this context, megalin is believed to rescue only locally synthesized ApoM from secretion in the urine, but it is also involved in the resorption of albumin from urine, a role that can be modified by a high glucose diet that reduces megalin expression [184–187]. Reported competitive inhibitors of megalin include cilastatin and receptor-associated protein (RAP) [188–190]. Although modulation of megalin expression or activity may provide an indirect mechanism for altering the S1P signaling axis, since this could impact both albumin and ApoM metabolism, without identifying ways of creating specificity for these two proteins the impact on the resorption of other megalin binding partners makes this a less attractive therapeutic target.

6.2.3 Megalin and Vitamin D_3

Vitamin D_3 is either ingested or synthesized in the skin from 7-dehydrocholesterol, then undergoes sequential metabolism to 25(OH)D_3 (calcidiol) in the liver and is then converted to the active metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$; calcitriol) in the kidney [191, 192]. Megalin, also known as LDL receptor-related protein 2 (LRP2), is involved in renal uptake of 25(OH)D_3 for conversion to $1,25(OH)_2D_3$ through binding vitamin D binding protein (DBP) and may be expressed in other tissues, allowing their vitamin D metabolism [188, 193, 194]. Low D_3 has been correlated to glucose intolerance and increased risk of diabetes and CVD, although the effect of $1,25(OH)_2D_3$ or D_3 on circulating lipid profiles is unclear [195–197]. A recent meta-analysis of 41 randomized controlled trials found that D_3 supplementation lowered LDL and TG, but in many studies D_3 had no effect on circulating HDL, in others it raised HDL; however, the trend was actually toward decreased HDL in response to D_3 supplementation [198]. One group reported that increased deoxysphingolipids, particularly deoxysphinganine (deoxydihydrosphingosine) are predictive of T2D development in non-obese individuals and correlate with increased TG and glucose, whereas another found that total dihydroceramides (dhCer), particularly C18:0, were elevated at least five years before T2D onset [199, 200]. In another T2D patient cohort, after 6 months of D_3

supplementation plasma $25(OH)D_3$ was increased, as were C18 dhCer (d18:0/18:0) and C18 Cer (d18:1/18:0), but there was no effect on plasma S1P or dihydrosphingosine 1-phosphate (dhS1P, dihydrosphinganene) [201]. Yet monocytes from T2D patients stimulated *ex vivo* in the presence of $1,25(OH)_2D_3$ secreted less S1P, had decreased mRNA *S1PR1* and S1PR2 mRNA and increased *S1PR3* and *S1PR4* mRNA [202]. A study of overweight and obese Asian-Australians found that individuals with low D_3 may benefit from D_3 supplementation by developing increased glucose tolerance; however, supplementation does not offer added protection in patients that are at risk of developing diabetes but already have levels of circulating D_3 that are within the normal range [203].

As the chaperone for S1P, ApoM has emerged as not only a critical component of the signals triggered by S1P binding to its receptors, but also as a potential target for pharmaceutical manipulation. GPCRs are the most popular pharmacologic targets but the inability to selectively bind a single S1PR or a group of S1PR expressed by a specific tissue or cell type has led to a search for other points of modification within the S1P-S1PR signaling pathway. Manipulating the S1P chaperone is an unconventional approach that may provide the opportunity to target subpopulations of cells that are more likely to be exposed to blood or lymph plasma, such as endothelial or immune cells [129, 170, 180].

6.3 S1P Transporters

6.3.1 Spinster 2 (Spns2)

Although the ABC transporters may be involved in the subcellular localization of S1P or its secretion by a limited subset of cells, multiple studies have determined that they are not involved in efflux from the major cell sources of S1P: EC, red blood cells (RBC), and platelets [176, 204–206]. There are now two confirmed S1P transporters, spinster 2 (Spns2) and Mfsd2b. Spns2 is the EC S1P transporter and the secreted S1P modulates specific biological effects of blood and lymph [21, 207]. *Spns2*$^{-/-}$ are lymphopenic, although not to the same extent as mice lacking the S1P biosynthetic enzymes, Sphk1/2. S1P produced by Sphk and secreted by lymphatic EC Spns2 binds naïve T cell $S1P_1$ and promotes their migration and survival through maintenance of mitochondrial numbers [22, 208]. High endothelial venules (HEV) are blood vessels specialized in regulating lymphocyte trafficking into secondary lymphoid organs at homeostasis and tertiary lymphoid organs under inflammatory and disease conditions, including cancer [209]. DC recruited to HEV by CCL21 produce lymphotoxin-β receptor (LTβR) ligands to activate HEV LTβR, which in turn promotes HEV function and EC survival [209]. HEV EC require Spns2 to secrete S1P, which then acts in an autocrine fashion, activating $S1P_1$ signaling and increasing CCL21 production to recruit DC [18].

6.3.2 Major Facilitator Superfamily Domain Containing 2b (Mfsd2b)

Two groups recently reported the characterization of major facilitator superfamily domain containing 2b (Mfsd2b) as the S1P transporter in RBC and platelets [210, 211]. Unlike *Spns2*$^{-/-}$, *Mfsd2b*$^{-/-}$ mice were not lymphopenic, despite a 50% drop in blood plasma S1P, approximately the same decrease as seen in *Spns2*$^{-/-}$. Intriguingly, *Apom*$^{-/-}$ mice had a 65% decrease in blood plasma S1P and albumin/ApoM double knockout mice had a 75% decease in blood plasma S1P and both have significantly more lymphocytes in circulation, further emphasizing the need to better understand the microenvironmental regulation of S1P concentrations by key cell types through production, secretion, and degradation [16, 129]. Mfsd2b also transports docasahexanoic acid (DHA) in the form of lysophosphatidic acid (LPA) precursor molecule lysophosphatidylcholine (LPC), the binding of which is dependent upon the phosphocholine head group and transports LPC from the plasma into brain parenchyma [212]. Mice lacking Mfsd2b also have increased transcytosis by EC

involved in the BBB, resulting in leaky CNS vasculature without a breakdown in tight junction (TJ), a phenotype also reported in *Apom*$^{-/-}$ mice [175, 213]. Mfsd2b also suppressed endocytic vesicle formation without affecting TJ by altering the lipid composition of the cell membranes themselves, making assembly of caveolae domains less favorable for vesicle formation [214].

6.4 S1P Metabolism

6.4.1 S1P Lyase (SPL)

Terminal metabolism of intracellular S1P occurs through cleavage of the C2–3 bond by the ER membrane-bound S1P lyase (SPL), yielding phosphoethanolamide (PE) and (2E)-hexadecenal [215]. Almost all mammalian cells express some level of SPL. For example, the brain, kidneys, and splenic and thymic stromal cells have high expression, whereas splenic and thymic immune cells have low expression [216, 217]. Platelets lack SPL and RBC appear to have little to no SPL activity, allowing these two cell types to carry S1P cargo without the danger of degradation [218, 219].

Unlike most of the S1PR, mutations in the SPL gene *SGPL1* are known to cause human disease, such as a form of Charcot-Marie-Tooth disease, the most common hereditary peripheral neuropathy, with the earliest manifestations including weakness in the feet and lower legs [220–222]. More commonly, *SGPL1* mutations manifest as nephrotic syndromes, such as congenital nephrotic syndrome with adrenal calcification and steroid-resistant nephrotic syndrome (SRNS) and adrenal insufficiency, often present with comorbidities of icthyosis, immunodeficiency, gastrointestinal disorders, and neurological deterioration, recently named nephrotic syndrome type 14 (NPHS14) or SPL Insufficiency Syndrome (SPLIS) [223–227].

SPL inhibitors targeted for the clinic have been developed based on the chemical structure of 2-acetyl-4-(tetrahydroxybutyl)imidazole (THI), an SPL inhibitor commonly used in experimental settings and a component of caramel food coloring [228, 229]. Most known SPL inhibitors, including parental compounds THI and 4-deoxypyridoxine (DOP), act by blocking the binding site of cofactor pyridoxal 5′ phosphate (PLP), the active form of vitamin B_6 [228, 230]. Both DOP and THI must be metabolized to a form compatible with the PLP site of SPL. Ohtoyo et al. hypothesized that THI is metabolized by the gut microbiota to an intermediate form before phosphorylation and SPL binding, providing an explanation for why THI will not block SPL activity *in vitro* or under B_6-rich conditions [231, 232]. There are over 100 PLP-dependent enzymes in eukaryotes, one of which is serine palmitoyltransferase (SPT), a complication for data interpretation and the design of SPL inhibitors because SPT is the initiating enzyme in the *de novo* biosynthesis of sphingolipids [232, 233]. Most recently, creation of the compound RBM10-8 was reported as an SPL inhibitor structurally based on S1P that acts as an enzyme substrate, irreversibly binding in the active site by forming a covalent bond [234]. Although RBM10-8 would not require metabolism for activity, its *in vivo* utility and specificity have not yet been studied.

SPL activity is critical for normal and pathological development of the nervous system. Neuron-specific knockdown of the *Drosophila* SPL, *sply,* caused progressive axonal degradation similar to that seen with *SMN* deletion, the gene responsible for spinal muscular atrophy (SMA) in humans [220, 235]. Mice with *Sgpl1* deleted in neuronal progenitors, ependymal cells, and oligodendrocytes with *Nestin-Cre* (*Sgpl*$^{Nes\text{-}Cre}$) had accumulation of brain S1P, decreased PE, and behavioral abnormalities concomitant with alterations in the hippocampus, increased microglial activation, and decreased neuronal autophagosome formation [236–239]. Microglia from these mice also had decreased expression of beclin-1, ATG7, and LC3-II, rendering them defective in autophagy induction [239, 240]. Similarly, autophagic flux in neurons from *Sgpl1*$^{Nes\text{-}Cre}$ mice could be restored *ex vivo* by incubation with exogenous PE [237].

The effect of SPL on neuronal autophagy is notable in the context of several neurodegenera-

tive diseases, since inducers of autophagy are being investigated as possible therapeutics to degrade toxic protein plaques or aggregates [241]. Mice transgenic for mutant human FUS protein (*FUS (1-359)*), an RNA/DNA-binding protein responsible for altered splicing and cytoplasmic aggregation of target mRNAs in amyotrophic lateral sclerosis (ALS), had significantly increased *Sgpl1* mRNA and decreased *Sphk2* [242, 243]. Sph was significantly increased in brains and spinal cords of *FUS (1-359)* mice, and although S1P concentrations were not significantly different, this may have been due to a dramatic increase in SPL activity [243]. Loss-of-function mutations in a FUS target gene, *MECP2*, are the genetic cause of the X-linked neuroregressive disorder Rett syndrome [242, 244]. Plasma from Rett syndrome patients had significantly higher S1P and dhS1P, as well as Sph and dhSph [245]. Rett syndrome patient fibroblasts had defective autophagosome formation and mice lacking *Mecp2* (*Mecp2*$^{-/y}$) developed cerebellar intracellular aggregates as they aged, concurrent with clinical phenotype development [246]. When incubated *in vitro* with THI, mouse primary neurons expressing exon 1 of mutant *huntingtin* (mHTT), the same abnormal splice product seen in brains of Huntington's disease patients, had increased autophagy and consequently, longer survival compared to control-treated cells [247]. In brain tissue from AD patients, Sphk1 was decreased and SPL1 was increased, potentially resulting in a net decrease in S1P concentrations [248]. SPL expression correlated histologically with focal amyloid β deposits in the entorhinal cortex and changes in *SGPL1* mRNA were already significantly increased in brains of patients with the lowest clinical dementia ratings [248, 249].

Model organisms have helped to clarify how the loss of SPL activity can manifest in various organ systems besides the nervous system. *Drosophila* lacking functional Sply are flightless and have a decreased number of dorsal longitudinal flight muscles (DLM) [250]. In a mouse model of post-menopausal osteoporosis, administration of LX2931 (LX3305) restored bone volume by increasing osteoblast activity, subsequently increasing cortical bone thickness and mechanical bone strength [251]. In a collagen-induced arthritis mouse model, LX2931 prevented development of clinical disease and had a small ameliorative effect on joint swelling and inflammation without affecting anti-collagen antibody titers [252]. Phase I and II clinical trials were conducted where rheumatoid arthritis patients received oral LX3305, but results have not published (NCT00847886, NCT00903383, NCT01417052).

SPL also has roles in immune homeostasis because of its control over S1P concentrations. Global *Sgpl1*$^{-/-}$ mice have increased circulating and tissue S1P as well as pro-inflammatory cytokine production [253]. *Drosophila* lacking methyltransferase 2 (Mt2) activity had reduced Sply activity as they aged, resulting in increased total S1P and Cer concentrations, altered hematopoiesis and immune cell morphology, and defective antibacterial immune responses [254]. Although present in very low numbers, thymic parenchymal CD11c^{+} DC with SPL activity were found to be major regulators of the S1P gradient required for T cell egress [255]. While mature T cell-intrinsic SPL also affected thymic S1P concentrations and had a moderate effeon egress, thymic stromal epithelial cells expressing large amounts of SPL protein were not involved in maintaining the S1P gradient required for T cell egress. Treatment of mice with SPL inhibitor for only three days showed a trend toward decreased numbers of double positive thymocytes (CD4^{+}CD8^{+}), and prolonged SPL inhibitor administration resulted in significant depletion of CD4^{+}CD8^{+} cells in the thymus [20, 256].

Subcutaneous administration of an SPL inhibitor in an imiquimod-induced mouse model of psoriasis was as effective as cyclosporine at decreasing redness and epidermal thickness [257]. In the DSS/azoxymethane (DSS/AOM) mouse model of colitis-associated cancer, gut epithelium-specific knockout of *Sgpl1* resulted in increased disease severity and tumor formation accompanied by increased numbers of macrophages and Th17 T cells [258]. Loss of SPL in bone marrow immune cells also resulted in increased colonic inflammatory lesions com-

posed of infiltrating myeloid cells and T cells and loss of crypt architecture; however, *Sgpl1*$^{-/-}$ mice that received wild-type bone marrow cells developed more severe colitis, more tumors, and had increased mortality [259]. Conversely, administration of DOP or THI decreased inflammation, T cell recruitment, and Crohn's disease-like pathology in mice that overexpress TNFα in intestinal epithelium (*TNF*$^{i\Delta ARE}$) [256, 260]. Results from studies of SPL in colitis models corroborate the hypothesis of a key role for S1P in the numerous clinical studies assessing the efficacy of S1PR modulating drugs etrasimod (APD334), ozanimod (RPC-1063), and amiselimod (MT-1303) in ulcerative colitis (UC) and Crohn's disease [125, 261–263].

The vascular nature of the lung and its role as an interface between the host and microorganisms indicates that regulation of S1P would be crucial in this organ. For instance, severity of cystic fibrosis (CF) is potentially influenced by altered regulation of S1P. CF varies in the number of organ systems it affects, but the greatest morbidity and mortality are due to progressive lung inflammation and dysfunction arising from defective cystic fibrosis transmembrane conductance regulator (CFTR) protein [264]. 352 of the greater than two thousand mutations in the *CFTR* gene are causative for CF, although severity of symptoms is typically based on the mutation, the most common of which is ΔF508 (https://cftr2.org/) [265]. Although bacterial infections are usually associated with CF, viral infections can also capitalize on defective mucus clearance and host immunity [266]. Mice expressing ΔF508 showed increased lethality when infected with an enterovirus (coxsackievirus B3 Nancy) despite viremia levels similar to controls [267]. Yet ΔF508 mice had increased viral titers in lymphoid tissues, decreased IFNα production, and decreased virus-specific IgM and IgG titers.

Defects in antiviral activation could be linked to SPL activity. IKKε-mediated inhibition of influenza A (IVA) virus replication was regulated by SPL, which enhanced type I interferon (IFN) responses *in vitro* in response to viral RNA [268]. However, the authors stated that enzymatic activity was not necessary for the antiviral effect. Another group found ΔF508 mice had lungs with decreased S1P but high basal innate and adaptive immune cell infiltration [269]. Administration of LX2931 reduced immune cell numbers in the lungs of ΔF508 mice, including inducible NOS (iNOS)$^{+}$ granulocytes, reducing the cellular infiltrate to wild-type levels. Work from a different group contradicted the implication that SPL inhibition would have beneficial anti-inflammatory effects in CF, reporting that S1P was a negative regulator of CFTR cell surface expression and activity, making SPL inhibition particularly counterproductive in CF patients [270]. A study of patients with community-acquired pneumonia (CAP) found elevated circulating S1P compared to patients without pneumonia [271]. This was not a general response to lung dysfunction, because chronic obstructive pulmonary disease (COPD) patients only had increased S1P concentrations if they developed pneumonia. A clinical trial (NCT03473119) is currently underway to determine if plasma S1P concentrations could serve as a reliable biomarker in CAP cases.

Infection with the malaria parasite *Plasmodium spp.* may also engage the sphingolipid pathway. In a malaria model using infection with *Plasmodium berghei*, humanized mice with decreased SPL activity (h*SGPL1*$^{-/-}$) had approximately the same level of parasitemia but almost no mortality. Curiously, administration of LX2931 did not have a significant effect on survival in experimental cerebral malaria, but FTY720 treatment of h*SGPL*$^{-/-}$ mice had a small survival benefit, particularly when co-administered with the anti-malarial drug artesamate [272]. Compared to children with uncomplicated malaria, children with cerebral malaria have decreased plasma S1P, possibly due to a drop in platelet numbers [272].

The potential for therapeutic manipulation of SPL-derived disorders is dependent upon how the lyase deficiency manifests. For instance, administration of the SPT inhibitor L-cycloserine decreases the amount of substrate entering the S1P biosynthetic pathway, reducing the amount of SPL substrate available, but reportedly inhibits SPL in addition to SPT [227]. However, patients with non-functional SPL could potentially bene-

fit from such a compound. Conversely, as in the autophagy studies cited earlier, if a lack of SPL products is causing disease, it may be possible to administer those molecules to patients. A recent retrospective study of SPLIS patients supplemented with vitamin B_6 found 30% of patients responded positively with increased SPL activity and decreased sphingolipids, emphasizing that SPL variant should determine treatment regimen [273].

6.4.2 Sphingosine 1-Phosphate Phosphatases (SPP1/2)

There are other, nondegradative enzymes that metabolize S1P by dephosphorylation: the S1P phosphohydrolases, SPP1 and SPP2, are specific for long chain sphingoid bases, and LPP3 (discussed below), which is nonspecific and better characterized with regard to LPA metabolism [274]. Both SPP1 and SPP2 localize to the ER but are expressed in different cell types [275–277]. For instance, *SGPP1* mRNA is highly expressed in human placenta and has moderate expression in liver and skeletal muscle, whereas *SGPP2* mRNA is highest in heart [277]. Both *SGPP1* and *SGPP2* mRNAs are highly expressed in human kidney. In mice, northern blotting showed *Sgpp1* to be highly expressed in liver with little expression in skeletal muscle [275]. Differential expression was evident in the endometrium of women with endometriosis, where *SGPP1* was increased and *SGPP2*, along with *SGPL1*, were decreased in endometriosis [278]. SPP1 over-expression *in vitro* led to Cer accumulation and apoptosis, whereas knockdown of *SGPP1* in MCF7 cells caused S1P and dhS1P accumulation and induced an ER stress response, leading to autophagosome formation [276].

Mice lacking *Sgpp1* on a pure C57Bl/6 genetic background are viable, but have severely stunted growth and detachment of an abnormally thin stratum corneum from thickened subcorneal layers [279]. Loss of SPP1 activity caused almost no significant changes in the epidermal sphingolipidome profile, with only C26 Cer significantly decreased and trends toward decreased C24 Cer and increased S1P and dhS1P. Keratinocytes were hyperproliferative and differentiated abnormally in response to increased epidermal Ca2+ concentrations. *Sgpp1*$^{-/-}$ mice maintained on a mixed C57/129sv genetic background reportedly did not have the same epidermal defects [280]. In studies utilizing these *Sgpp1*$^{-/-}$ and *Sgpp2*$^{-/-}$ mice on a mixed background, compared to wild-type controls, *Sgpp1*$^{-/-}$ animals developed increased DSS-induced colitis and their colons had increased proinflammatory cytokines TNF, IL-6, and IL-1b. Conversely, cytokines in colons of *Sgpp2*$^{-/-}$ animals did not increase with DSS administration, resulting in decreased colitis severity. Both *Sgpp1* and *Sgpl1* were downregulated in bone marrow-derived DC stimulated with lipopolysaccharide (LPS) [281]. While naïve DC showed nuclear SPP1 staining, LPS stimulation caused its translocation from the nucleus to the cytosolic compartment. There are currently no drugs that can specifically target either SPP1 or SPP2.

6.4.3 Lipid Phosphate Phosphatase 3 (LPP3)

Lipid phosphate phosphatase (LPP3) also dephosphorylates S1P, but shows substrate promiscuity, metabolizing various extracellular phospholipids [282]. LPP3 has a cytoplasmic motif targeting it to the basolateral membrane [283]. Global loss of *Plpp3*, the LPP3 gene, is embryonic lethal because of abnormal Wnt/β--catenin signaling generating vascular defects [284]. Constitutive endothelial/hematopoietic-specific deletion using the *Tie2-Cre* yields a similar phenotype [285]. Transcriptional regulation of *PLPP3* by NF-κB can be induced by inflammation in the monocyte-like cell line THP-1 [274]. However, a SNP in a regulatory element for endothelial *PLPP3* was identified as protective against coronary artery disease (CAD) and ischemia/stroke [286]. The protective allele sequence created a binding site for KLF2, the same transcription factor regulating *S1pr1*. Modulation of LPP3 expression in primary human aortic EC *in vitro* altered extracellular and

intracellular S1P concentrations [287]. In the thymus, LPP3 produced by both endothelial and epithelial cells is necessary for maintaining the S1P gradient utilized by T cells for egress into circulation [285]. Despite difficulties in characterizing the physiological roles of LPP3-mediated S1P metabolism, it is believed to be the primary dephosphorylating enzyme for FTY720P, although SPP1 is also able to do so [288]. There are no published LPP3-specific inhibitors and compounds that have been used, such as sodium orthovanadate and propranolol, are too nonspecific for clinical use [289].

6.5 Biosynthetic Enzymes

6.5.1 Sphingosine Kinases 1 and 2 (Sphk1/2)

The sphingosine kinases, Sphk1 and Sphk2, are differentially expressed by cell and tissue type and although both generate S1P from sphingosine, they show different subcellular localization patterns [5, 290, 291]. The topic of Sphk is quite broad, so this review will address biology directly relevant to common inhibitors and the development of new compounds [14, 292–295]. The Sphks have long been targets for inhibition but isoform specificity, which is particularly important, has been lacking. Double null *Sphk1/2*$^{-/-}$ is embryonic lethal because of massive hemorrhage [296]. When *Sphk1* alone is deleted, circulating S1P concentrations go down, although compensatory activity by Sphk2 prevents tissue S1P concentrations from changing substantially [297]. *Sphk1*$^{-/-}$ animals also develop lymphopenia when administered FTY720, demonstrating that Sphk2 is the kinase responsible for the majority of FTY720 phosphorylation *in vivo* [297]. Contrary to expectation, deletion of *Sphk2* resulted in increased, rather than decreased, circulating S1P concentrations [298]. The proposed mechanism for this increase is a regulatory pathway where S1P is dephosphorylated by LPP3 and some of the Sph is subsequently taken up by cells and rephosphorylated by Sphk2 [298, 299]. The Sphks can also display a differential substrate preference: Sphk1 will preferentially metabolize dhSph over Sph [300]. When cells overexpressing Sphk1 were incubated with FTY720, intracellular dhS1P and S1P increased, but dhSph and Sph concentrations remained the same [300]. This occurred because Sphk2 is 30 times more efficient at phosphorylating FTY720 and FTY720 itself can act as a Sphk inhibitor at micromolar concentrations [46]. Of note is that Spns2 expression is high in the same tissues where Sphk2 is expressed and where FTY720P is secreted, illustrating the interconnectedness of the entire sphingolipid metabolic system [300].

While the search for S1PR inhibitors tends to focus on vascular and autoimmune diseases, the development of Sphk inhibitors is driven by cancer research [301]. The primary anti-tumoral mechanism of action of Sphk inhibition is believed to be an increase in intracellular Cer, triggering cancer cell apoptosis. *D,L*-threo-dihydrosphingosine (tDHS) or *N,N*, dimethylsphingosine (DMS) act as competitive nonspecific inhibitors [290, 302]. The most frequently used inhibitor is SKi (also referred to as SKI-II), which inhibits both Sphk1 and 2 [303]. Like many Sphk inhibitors subsequently developed, it must be present in micromolar concentrations for full potency and since it inhibits both Sphks there are unwanted and unexpected effects [304]. The block on catalytic activity by Sphk1 inhibitors correlates with its degradation, a unique mechanism where inhibitor binding induces polyubiquitnylation, targeting Sphk1 to the proteasomal degradation pathway [305–307]. The binding of inhibitors induces a conformational change in Sphk1, allowing ubiquitnylation of Lys183 and subsequent binding of the Kelch-like protein 5 (KLHL5)-cullin 3 ubiquitin ligase complex [308]. While *Klhl5* knockdown reduced Sphk1 degradation, KLHL5 expression was correlated with decreased chemotherapy sensitivity [309]. SKi also inhibits another enzyme in the sphingolipid metabolic pathway, Des1, in a noncompetitive manner at submicromolar concentrations, which may account for the cellular accumulation of dhCer with the use of this inhibitor [310].

Several Sphk1-specific inhibitors with improved specificity and/or potency have been

described. LCL351 is a sphingosine analogue that has 10 times greater inhibitory activity for Sphk1 versus Sphk2, but still requires low micromolar concentrations for inhibition [311]. Although not clinically viable, LCL351 is a useful Sphk1-specific tool. Patients with UC have increased Sphk1 expression and *Sphk1*$^{-/-}$ mice develop less severe DSS-induced colitis [312]. LCL351 administration prevented the development of DSS-induced colitis, reducing colonic neutrophil recruitment in combination with a decrease in colon S1P concentrations, although circulating S1P concentrations were slightly elevated [311].

PF543 is a Sph competitive Sphk1 inhibitor at low nanomolar concentrations and was used in the crystallization of Sphk1 [313, 314]. Incubation of 1483 (squamous cell carcinoma) cells with nanomolar concentrations of PF543 did not increase Cer concentrations but did increase Sph in direct correlation with the S1P decrease [314]. Since PF543 did not induce apoptosis in cancer cell lines A549 (lung adenocarcinoma), Jurkat (T cell acute lymphoblastic leukemia), LN229 (glioblastoma), MCF7 (invasive ductal carcinoma), or U937 (acute monocytic leukemia) but did inhibit S1P generation, this illustrated that the pro-apoptotic effect of Sphk inhibition was not a result of lost S1P, but the increase in Cer [314]. PF543 also decreased the severity of DSS-induced colitis, concurrent with the decrease in S1P concentrations [315, 316]. EC in isolated rat aortic and coronary arteries transiently exposed to hypoxic conditions upregulated Sphk1, increasing S1P and vasodilation that was blocked by PF543 [317]. *In vivo*, PF543 blocked S1P production and increased cardiac Sph concentrations in the angiotensin II (AngII)-dependent model of arterial hypertension and cardiac remodeling [318]. At a dose of 1 mg/kg, PF543 blocked development of cardiac hypertrophy and decreased $S1P_1$ protein in the heart with a subsequent decrease in activated STAT3 and ERK1/2 [318]. Despite the intense interest in Sphk1 inhibitors, none have made it to clinical trials.

However, ABC294640 (ABC), an Sphk2 inhibitor, has been in multiple clinical trials since it was first reported in 2010 [319]. The first clinical trial, in patients with solid tumors (cholangiosarcoma, colon, pancreatic), found a dose of 500 mg twice a day was well tolerated [320]. An important finding was that after trial initiation, a protocol amendment was needed requiring fasting blood glucose below 160 mg/dL because of dose-limiting hyperglycemia [320]. Experimentally, ABC suppressed the development of cancer in the DSS/AOM colon cancer model, decreased chemoresistance in breast and ovarian cancer models, and suppressed inflammation in models of arthritis and lupus [257, 321–327]. Some ABC efficacy may be due to its accumulation in tumor tissues, since the half-life in human plasma is only 5.5 h [319]. Currently, ABC (Opaganib/Yeliva) is being investigated in two clinical trials: ABC plus androgen antagonist in metastatic castration-resistant prostate cancer (NCT04207255) and alone or in combination with hydroxychloroquine sulfate in advanced cholangiosarcoma (NCT03377179). So far, no changes have been posted for the second trial with regard to possible hydroxychloroquine shortages during the COVID-19 pandemic [328].

Sphks could also be targets for inhibition during some viral infections, although efficacy is likely to be pathogen- and manifestation-specific. Influenza A virus (IAV) infection increased Sphk2 expression and activation *in vitro* and treatment with ABC during IAV infection increased survival and decreased lung viral titers [329]. Inhibition with non-specific DMS had the same effect, and although SKi treatment did not result in the same magnitude of survival increase, it did significantly decrease viral titers better than ABC, indicating that Sphk1 and Sphk2 may be responsible for different aspects of IAV viral reproduction and host response [329, 330]. Dengue virus type 2 (DENV2), a positive-sense single-stranded RNA virus, actively downregulated *Sphk1* transcription, decreasing the activation of IFN-responsive genes [331]. *In vitro*, DENV2 replicates less efficiently in *Sphk2*$^{-/-}$ mouse embryonic fibroblasts, which did not produce IFNβ in response to viral infection, but lack of Sphk2 did not impact viral replication *in vivo* or survival [332]. IAV and DENV2 have

different modes of transmission, different cycles, and are not related, but similar responses involving Sphks *in vitro* implicate a more general role in the anti-viral immune response [333].

6.5.2 Ceramide Synthases (CerS)

Production of Cer can occur through two pathways, one of which is the salvage pathway: reacylation of Sph by Ceramide synthases (CerS) [334, 335]. Alternatively, Cer is produced *de novo* from dhCer, which will be covered in the following section. There are six CerS, which are ER membrane-bound enzymes that catalyze the *N*-acylation of sphingoid bases and require phosphorylation of C-terminal residues for catalytic activity [336–338]. Each of the CerS exhibit different cellular expression patterns and acyl chain preferences, and inhibition or deletion of one CerS typically results in up-regulation of another and production of different Cer species [336, 339]. CerS2 is the most ubiquitously expressed and produces C20–C26 Cer [334, 340]. Knockout or knockdown in MCF7 cells resulted in accumulation of dhSph and Sph, increases in *CERS4*, *5*, and *6*, and decreased very long chain (VLC) Cer [336, 341]. CerS2 overexpression increases VLC Cer production, causing insulin resistance and oxidative stress in cardiomyocytes [342]. Conversely, CerS1 has the most restricted expression and is highest in the CNS, skeletal muscle (SkM), and testis and generates only C18 Cer, which decreases with CerS1 knockdown [334, 340, 343]. Mutations in *CERS1* have been linked to progressive myoclonus epilepsy and CerS1 interactions with mutant heat shock protein (Hsp27) result in decreased mitochondrial Cer, leading to neurodegeneration in Charcot-Marie-Tooth variant 2F disease [344–346].

There are two inhibitors of CerS, the most specific of which is P053, an FTY720 derivative and selective noncompetitive inhibitor of CerS1 [347]. P053 selectively decreased C18 Cer in SkM while liver and adipose Cer concentrations were not affected. P053 also decreased triacylglycerol (TAG) by 50% in SkM of HFD-fed mice but did not affect TAG in SkM of normal chow-fed mice [347]. P053 may not effectively cross the BBB since it is found in much lower concentrations in brain tissue versus SkM and has less of an effect on brain C18 Cer production. CerS1-specific inhibition also increased mitochondrial capacity and enhanced fatty acid oxidation in SkM while decreasing whole body fat mass, despite HFD consumption and no effect on insulin resistance [347]. However, genetic deletion of *Cers1* in SkM did show increased insulin and glucose tolerance with HFD feeding, in addition to reduced adiposity [348].

The other CerS inhibitor is fumonisin B1 (FB1), a fungal toxin with a deoxysphingoid base structure and known carcinogenic activity [349–351]. FB1 inhibits all six CerS, leading to increased S1P, dhS1P, Sph, and dhSph, and decreased Cer and dhCer [11, 352]. It also causes accumulation of 1-deoxysphinganine, possibly compounding neurological sequelae of CerS inhibition, similar to production of deoxysphingolipids associated with hereditary sensory and autonomic neuropathy type 1 (HSAN1) [351, 353].

6.5.3 Dihydroceramide Desaturase (Des1 and 2)

De novo Cer synthesis occurs by insertion of a 4,5 *trans* double bond into the sphingoid backbone of dihydroceramide (dhCer) by the dihydroceramide desaturases, Des1 and Des2 [354, 355]. CerS are responsible for the production of the dhCer substrate, so their inhibition affects both salvage and *de novo* pathways [356]. Compared to Des1, far less is known about Des2, which in addition to desaturase activity can also exhibit C4-hydroxylase activity and synthesize phytoceramides (phytoCer) [357]. Membrane-bound cytochrome b_5 affinity and complex formation may determine which of these enzymatic activities Des2 engages in [358]. Des2 is highly expressed in the digestive tract, kidneys, and skin, where phytoCer are critical [355, 358]. *DEGS2*, the Des2 gene, is also expressed in the adult brain and was significantly upregulated in brains of schizophrenia patients and downregu-

lated in major depressive disorder patients [359]. A *DEGS2* missense mutation also correlated with cognitive deficits in schizophrenia patients [360].

Des1 is ubiquitously expressed and its activity is most associated with insulin resistance and cancer [361]. Palmitate upregulates *Degs1* mRNA in SkM myoblasts, increasing Cer and subsequently inducing insulin resistance, which was reversible by oleate incubation [362]. Cells and mice lacking *Degs1* have increased dihydroxysphingolipids and uncoupled nutrient and apoptosis signaling [363]. *Degs1*$^{-/-}$ mice crossed with the obesity model *ob/ob* mice had significantly increased dhCer and decreased Cer in liver, white adipose tissue, and serum [364]. *ob/ob Degs1*$^{-/-}$ animals subsequently had lower fat mass, blood glucose, and improved liver function. Accumulation of dhCer in plasma had previously been proposed as a biomarker for diabetes progression [199].

Des1 is the target of the synthetic retinoid chemotherapeutic, fenretinide (4-hydroxyphenyl retinamide (4-HPR)) [365–367]. 4-HPR also increases activity of SPT, leading to the accumulation of cytotoxic dhCer [14, 368, 369]. In HEK293 cells, 4-HPR induced polyubiqutinylation of Des1, increasing enzymatic activity but targeting it for degradation, making Des1 activity dependent upon the rate of degradation induced by polyUb [310, 370]. Metabolites of 4-HPR differentially affect the 4-HPR target enzymes [371]. The 3-keto-HPR metabolite inhibits all targets, stearoyl CoA desaturase (SCD1), β-carotene oxyegnase (BCO1), and Des1, but the metabolite N-[4-methoxyphenyl]retinamide (MPR) specifically affects BCO1. SCD1 converts saturated fatty acids (FA) to monounsaturated FA, particularly palmitic acid or stearic acid to palmitoleic or oleic acid, respectively, but was also reported to decrease Cer in cardiomyocytes [372–374]. However, SCD1 deficiency decreased Cer and mRNA for SPT components in SkM, so its inhibition by 4-HPR may contribute to effects attributed to Des1 inhibition *in vivo* [373, 375].

This target combination may also explain results of 4-HPR treatment in CF. 4-HPR decreased inflammation and corrected the FA imbalance and Cer deficiencies seen in *CFTR*$^{-/-}$ mouse models and in CF patients [376–378]. A new oral formulation of 4-HPR by Laurent Pharmaceuticals, Lau-7b, is currently in phase II trials for CF (APPLAUD, NCT03265288) and reportedly normalized blood and lung polyunsaturated FA (PUFA) and Cer concentrations in mouse models of asthma [379]. Similar to the *Degs1*$^{-/-}$ mice, 4-HPR has been shown to prevent or partially reverse obesity, insulin resistance, and hepatic steatosis by blocking Cer synthesis [380–382]. The effects of 4-HPR in obese patients have been investigated in a clinical trial, with results under review by the FDA in January 2020 (NCT00546455).

The most extensive clinical testing of 4-HPR has been in clinical trials for a wide range of cancers: neuroblastoma, glioblastoma, lymphomas, leukemias, recurrent ovarian and prostate, lung, bladder, head and neck, and breast (Clinicaltrials.gov search "fenretinide"). A new formulation of 4-HPR complexed with 2 hydroxypropyl-β-cyclodextrin (Nanofen) was recently described [383]. Nanofen had improved bioavailability and efficacy in lung tumor xenograft models, having led to C18 dhCer accumulation and inducing tumor apoptosis.

6.5.4 Ceramidases (CDases)

The enzymes that convert dhCer to dhSph or Cer to Sph are ceramidases (CDases), which hydrolyze the N acyl linkage between the FA and the sphingoid base [14, 384]. The CDases are categorized based on their optimal catalytic pH: neutral ceramidase (NCDase), alkaline ceramidases (Acer), and acid ceramidase (ACDase). Like the CerS, the CDases have substrate specificity and subcellular localization. Sph can be converted to Cer by ACDase, NCDase, and Acer1-3, whereas dhSph and phytoSph are converted from their dhCer and phytoCer precursors by Acer2 & 3 [384, 385].

Defects in ACDase lead to Faber disease and SMA with progressive myoclonic epilepsy [386]. mRNA for two CDases, *Asah1* (ACDase) and *Asah2* (NCDase), are altered in the brains of ALS model *FUS (1-359)* mice [243]. Knockout

of ACDase (*Asah1*$^{-/-}$) is embryonic lethal, and conditional *Asah1*$^{-/-}$ mice have elevated ovarian Cer levels, leading to decreased fertility [387, 388].

NCDases are present in humans, mice, and pathogens, including *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* [389, 390]. Mice lacking NCDase (*Asah2*$^{-/-}$) were relatively normal and demonstrated the critical role of NCDase in the intestines [391]. *Asah2*$^{-/-}$ have high circulating endotoxin and inflammation of the gut epithelium in the DSS colitis model [327].

Loss of each of the alkaline CDases (*Acer1–3*) manifests in different tissues. Acer1 is critical for epidermal Cer regulation, and *Acer1*$^{-/-}$ mice have progressive alopecia due to altered hair follicle cycling and have increased energy expenditure with decreased body fat [392, 393]. *Acer2*$^{-/-}$ mice have significantly decreased circulating Sph, dhSPh, S1P, and dhS1P [394]. *In vitro*, Acer2 displays broad substrate specificity and is upregulated by serum deprivation [395]. Acer3 preferentially hydrolyzes C18:1 Cer and is highly expressed in brain, increasing with age [396]. *Acer3*$^{-/-}$ mice appear mostly normal until they age beyond 8 months, at which point C18:1 Cer is significantly decreased in addition to Sph and S1P. As their brain sphingolipid composition changes, *Acer3*$^{-/-}$ mice develop impaired balance, motor coordination, and grip strength due to Purkinje cell degeneration [396]. In humans, *ACER3* deficiency manifests in childhood as progressive leukodytrophy [397]. Although CDase inhibitors exist for laboratory use, none have been advanced to the clinic [398–400].

6.5.5 Serine Palmitoyl-CoA Transferase (SPT)

The initial reaction in the *de novo* sphingolipid biosynthetic pathway is the condensation of the amino acid serine and palmitoyl-CoA by serine palmitoyltransferase (SPT) to 3-ketodihydrosphingosine [401, 402]. The essential components of SPT are the protein subunits SPTLC1 and SPTLC2 or 3, with SPTLC1 being ubiquitously expressed and SPTLC2 and 3 showing some tissue specificity [403, 404]. SPTLC2 and 3 contain the PLP consensus motif for cofactor binding and whichever is included in the heterodimer (SPTLC1 plus SPTLC2 versus 3) determines whether longer fatty acyl-CoAs (palmitoyl and larger) are incorporated (SPTLC2) or shorter myristoyl or lauroyl (SPTLC3) are selected [405, 406]. Other components of the enzyme complex are the proteins SPT small subunit a and b (SPTssa/b), which bind to the SPTLC complex conferring optimal catalytic activity [407]. A mutation in *SPTssb* results in increased SPT activity and over-production of C20 long chain bases, resulting in abnormal membranes and vacuoles in the brain, leading to ataxia and early death [408]. SPT activity is negatively regulated by the ORMDL proteins, which are always complexed with the SPT holoenzyme and act through conformational changes in response to sphingolipid concentrations, particularly D-erythro Cer [409–411].

ORMDL deficiency in mice is not lethal and null animals appear normal at weaning [412]. *Ormdl3*$^{-/-}$ mice have significantly increased brain sphingolipids, particularly Cer and Sph, but dhCer is increased only in *Ormdl1/3*$^{-/-}$ double-null mice. These mice have smaller body weights, exhibit neurological defects, and their sciatic nerves contain significantly greater concentrations of dhSph, dhCer, Cer, and Sph. Altered sphingolipid concentrations manifest as abnormal sciatic nerve morphology with excessive (redundant) myelination, a phenotype recapitulated in mice with an inducible constitutive SPT [412]. SNPs in *ORMDL3* and a *cis* gene, *GSDML*, are linked to non-allergic childhood asthma [413]. DNA methylation sites in *ORMDL3* were also independently correlated with childhood asthma and DNA methylation regions in the 5′ UTR of *ORMDL3* were significantly less methylated in CD8^{+} T cells and children with asthma [414].

Mutations in *SPTLC1* or *2* are responsible for hereditary sensory and autonomic neuropathy (HSAN1) types 1A and 1C, respectively, character-

ized by damage to peripheral neurons leading to progressive neuropathy, ulcerations, and weakness [415–417]. The most prevalent mutations change SPT substrate amino acid preference, but others change affinity for acyl-CoAs of different chain lengths and increase basal activity [405, 418–420]. Mutations causing substrate preference to change from serine to alanine or glycine result in accumulation of neurotoxic 1-deoxysphingolipids, such as 1-deoxysphinganineand1-deoxymethylsphinganine [418–421]. HSAN1C patients also have decreased $CD8^+$ T cell sphingolipid synthesis upon activation and impaired proliferation and survival [422].

Dietary supplementation of 10% L-serine decreased plasma deoxysphingolipids and improvement in motor and coordination testing in mice with the C133W HSAN1 mutation, whereas L-alanine supplementation led to accumulation of deoxysphingolipids and motor function deterioration [423]. In a randomized controlled trial, 400 mg/kg/day of L-serine resulted in improvement of disease scores concomitant with dramatic, significant decreases in plasma deoxysphingolipids [423, 424]. Surprisingly, despite improvement in neuropathy, L-serine-treated patients had no decrease in ulcers and a higher frequency of skin infections and osteomyelitis, which the authors suggested could be due to permanent nerve damage occurring before supplementation [424]. L-serine supplementation has been shown to increase D-serine in plasma and CSF in a mouse model of *GRIN2B* encephalopathy, a Rett-like syndrome [425]. Considering the frequent overlap of substrate specificity for various sphingolipid enzyme inhibitors, a relatively simple diet modification that is efficacious in severe diseases related to SPT activity would be a preferred treatment.

6.6 Glycosphingolipids

The glycosphingolipids (GSL) are a large subfamily of sphingolipid molecules created by attachment of glycans to a ceramide moiety that anchors them in the lipid bilayer, primarily in the plasma membrane [1, 426]. Galactosylceramides (GalCer) and glucosylceramides (GlcCer) are synthesized in the Golgi by **β**-linkage of the respective galactose or glucose sugar moiety to the primary hydroxyl of a ceramide [427, 428]. GlcCer can then be metabolized to lactosylceramide (LacCer), which serves as the base molecule for the more complex GSL: globo/isoglobo-, ganglio/isoganglio-, and lacto/neolacto-series [428, 429]. Clinically, the GSL are most widely recognized for their roles in lipid storage diseases: Fabry, Tay-Sachs, Sandhoff, Gaucher, Krabbe, Niemann-Pick C, and GM1 and GM2 gangliosidosis [430, 431]. The complexities of the glycosphingolipid metabolic pathways are such that interested readers are directed to the detailed reviews referenced in this section.

6.7 Summary

The known contributions of sphingolipids to all aspects of biological homeostasis and pathogenesis have continuously expanded since their first description almost a century and a half earlier [432]. The generation of animal models and the increasing depth of genetic sequencing have allowed researchers and clinicians to discover unexpected phenotypes and rare mutations in the sphingolipid metabolic and signaling pathways responsible for diseases in every biological system. Although the greatest successes in sphingolipid pharmaceutical targeting have been S1PR modulating drugs, the development of new compounds and modification of old ones have generated promising results. Complex rules governing sphingolipid flux combine with cellular and subcellular specialization to create a network that is, unfortunately, at times irreducible. However, the restricted utilization of sphingolipid biosynthetic and signaling pathways also provides opportunities for targeted therapeutic exploitation.

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