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Sphingosine-1-Phosphate Signaling in Immunology and Infectious Diseases

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Preface

For convenience, the early immune response occurring during the first few days after exposure to a foreign agent is termed innate, while the later response, after day 5, is termed adoptive. Both immune responses have defined cellular components although they vary according to different types of cells and the degree of their participation. Further, there is a balance between a necessary immune response required to protect the host verses an exaggerated response that possess unwanted and severe injury and difficulties. In both instances, the immune response causes immune-mediated tissue injury by limiting the infection through its ability to destroy infected cells, such cells are factories for manufacturing increasing infectious progeny or by reacting against self antigenic material. Thus, there needs a controlling rheostat or servo-mechanisms that can maximize the beneficial aspect as well as minimize the excessive immune-mediated injury (immunopathology) caused by cells of the immune system.

Sphingosine-1-phosphate (S1P) is a signaling lipid present at a concentration of 1–3 μM in plasma and roughly 100 nM in lymph. The majority of S1P in plasma is bound to high density lipoprotein but a small portion, approximately 15–45 nM, is unbound in the blood. Physiologically, S1P levels are under tight homeostatic control. S1P signals through specific G-coupled S1P receptors of which there are five. These receptors regulate a wide variety of signaling pathways that are specific for different cells, tissues, and organs. The purpose of this CTMI volume is to focus on S1P and its analogs in the induced sequestration of lymphocytes in secondary lymphoid organs or in microenvironment of tissues involved in infection or autoimmune disease. By this means, first, trafficking and lymphoid organization are, in part, controlled; second, migration of effect or lymphocytes, NK cells, and macrophages to distal areas where such cells might mediate immunopathologic injury leading to disease can also be restrained and; third, cytokines and chemokines regulated in the microenvironment of selected tissues. To achieve such desired therapeutics, a series of agonists and antagonists to S1P receptors have been synthesized to evaluate and control normal lymphocyte trafficking thereby employed in modulating acute infections and autoimmune disorders.

This CTMI volume illuminates this rapidly expanding field of basic and translational clinical research. Initial chapters define the pathways to understand S1P signaling from the organization of the signaling systems to the structural biology of the $S1P_1$ receptor to the chemical and genetic tools available and useful

to explore this area of research and therapeutics. The later chapters focus on the biology covering S1P and endothelial integrity, lymphocyte migration in the spleen, and S1P agonist in controlling immunopathologic manifestations in the lung of acute respiratory influenza virus infection and its accompanying cytokine storm as well as immunopathologic disease of the central nervous system including beginning treatments in multiple sclerosis. Also included is a chapter revealing other lipid molecules that can play a role and their use for better understanding lipid signaling and its potential in the modulation of immune responses.

La Jolla, CA 2014

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Contents

The Organization of the Sphingosine 1-Phosphate Signaling System

Hugh Rosen, M. Germana Sanna, Pedro J. Gonzalez-Cabrera and Edward Roberts

Abstract The understanding of the role of the sphingosine 1-phosphate signaling system in immunology and host defense has deepened exponentially over the past 12 years since the discovery that lymphocyte egress was reversibly modulated by sphingosine 1-phosphate receptors, and with the development of fingolimod, a prodrug of a nonselective S1P receptor agonist, for therapeutic use in the treatment of relapsing, remitting multiple sclerosis. Innovative genetic and chemical approaches, together with structural biology, now provide a more detailed molecular understanding of a regulated lysophospholipid ligand with a variety of autocrine, paracrine, and systemic effects in physiology and pathology, based upon selective interactions with a high affinity and selective evolutionary cluster of G-protein-coupled receptors.

Contents

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1 Sphingosine 1-Phosphate: A Signaling Lysophospholipid

Sphingosine 1-phosphate (S1P) is a lysophospholipid synthesized by phosphorylation of the long chain base sphingosine by one of two intracellular sphingosine kinases in both mouse and man, or through the extracellular hydrolysis of sphingosine phosphoryl choline by the enzyme autotaxin. The long chain base sphingosine is a small component of the mass balance within the pathway, because it is not a result of denovo biosynthesis, rather a product of ceramide metabolism, where the proapoptotic affects of ceramide intracellularly are regulated by its degradation by ceramidase N-deacylation, generating sphingosine (Fig. 1) (Maceyka et al. 2012; Spiegel and Milstien 2011).

Thus, the most abundant pools of sphingosine are to be found in sphingomyelin within plasma membrane lipid rafts, and the reservoir for ceramide generation following cleavage by various sphingomyelinases, and most abundantly, within the circulating pool of S1P partitioned within the HDL component of plasma (Theilmeier et al. 2006; Murata et al. 2000).

Sphingosine kinase 1 is the major source of circulating S1P and is a ubiquitously expressed cytosolic enzyme with a significant pool within erythrocytes, that provides the bulk of S1P synthesis (Allende et al. 2004; Mizugishi et al. 2005; Don and Rosen 2009; Don et al. 2007). Sphingosine kinase 2 has a nuclear localization signal and may be more responsible for strategic intracellular stores of S1P, that mediate effects through molecular interactions with as yet undefined or incompletely defined targets, that do not require the presence of extracellular G-protein-coupled receptors. Intracellular S1P is controlled by three mechanisms that may act in concert. First, S1P may be dephosphorylated by phosphohydrolases in the reverse reaction to the long chain base (Mandala et al. 2000; Nanjundan and Possmayer 2001, 2003; Humtsoe et al. 2005; Wary and Humtsoe 2005; Garcia-Murillas et al. 2006; Tomsig et al. 2009). Second, it can be removed entirely from signaling pathways through the action of S1P lyase, which cleaves S1P to hexadecanal and ethanolamine-phosphate (Allende et al. 2011; Bektas et al. 2010; Bandhuvula et al. 2005; Oskouian et al. 2005; Zhou and Saba 1998). Third, intracellular S1P can be exported to the extracellular environment. The mechanisms of S1P secretion to the extracellular environment are understood in part through genetic evidence for the role of the Spinster2 channel, where deletion of this protein in both zebra fish and mice, leads to embryonic lethality at day 13.5, replicating the defects seen with both the dual sphingosine kinase deletion, or S1PR1 deletion (Rong et al. 2011; Osborne et al. 2008; Nakano et al. 2001; Mendoza et al. 2012; Fukuhara et al. 2012).

These data highlight the critical roles of S1P production, secretion, and receptor action in embryogenesis and early development. Proia and colleagues, in a series of seminal contributions, showed that disruption of S1P signaling was lethal at day 13 of embryonic development as a result of the failure to formation of the arterial media, and major vessel rupture into the brain and yolk sac (Allende et al. 2004, 2008; Mizugishi et al. 2005; Chae et al. 2004; Matloubian et al. 2004; Allende and Proia 2002; Liu et al. 2000). A confluence of mutually supportive genetic

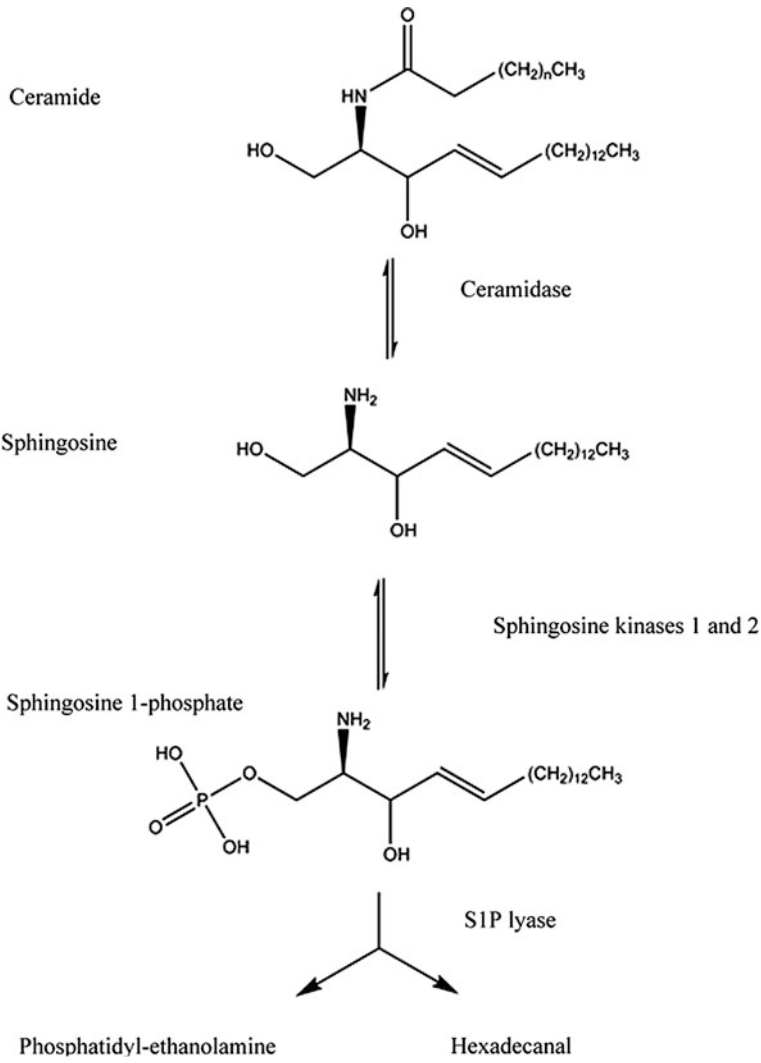


Fig. 1 *The Sphingolipid Biosynthetic Pathway. Schematic representation of the metabolism and catabolism of S1P. S1P, sphingosine 1-phosphate*. Degradation of ceramide to sphingosine by ceramidase and subsequent phosphorylation by sphingosine kinase *1* or sphingosine kinase *2* produces S1P. S1P can be reversibly degraded to sphingosine by S1P phosphatase and lipid phosphate phosphatases or irreversibly degraded by S1P lyase to *2–trans* hexadecenal and phosphoethanolamine

evidence in mouse and zebra fish provided significant insights into mechanism where the phenotype of arterial media disruption and large arterial rupture has been replicated by deletion of s1pr (Allende and Proia 2002; Mendelson et al. 2013) or the zebra fish *mls* allele, deletion of the endothelial-expressed s1pr1 showing that the PDGF-dependent migration of the arterial media from its ventral starting point

dorsally to form the intact media was dependent upon an endothelial S1P signaling event (Allende and Proia 2002), that the identical phenotype was replicated by the double sphingosine kinase knockout (Mizugishi et al. 2005), and by the deletion of Spinster2 in both zebra fish (Osborne et al. 2008) and mouse (Fukuhara et al. 2012).

Evidence has accumulated for rate-limiting signaling events in physiology and pathology that are mediated by S1P as a critical extracellular mediator with autocrine, paracrine together with events within the circulatory compartment that impact upon embryonic development, the development and maintenance of circulatory integrity (Sanna et al. 2006; Rosen et al. 2009), the maintenance of cardiac rhythm (Sanna et al. 2004), an intracellular rheostat with ceramide regulating the balance between survival and apoptosis (Spiegel and Milstein 2011) together with regulating key events in evolutionarily more recent integrated physiologies such as lymphocyte recirculation (Rosen et al. 2003; Mandala et al. 2002) and the modulation of host defenses (Oldstone et al. 2013; Walsh et al. 2010, 2011a, b; Teijaro et al. 2011; Marsolais et al. 2008, 2009). These are shown schematically in Fig. 2.

The concentration-response curve to circulating S1P provides key insights into the biology of this lysophospholipid ligand, and the exquisitely tight requirements around its control at the interface with the cell surface. S1P is buffered within the plasma compartment by partitioning into HDL (Murata et al. 2000) with 98.5 % in the bound fraction and perhaps 15–45 nM in the free compartment. Extracellular S1P may be locally regulated at the plasma membrane by catabolism. A family of nonselective lipid phosphohydrolases *lpp1*, *lpp2*, and *lpp3* are GPI-linked extracellular enzymes that effectively limit the action of S1P in the environs of the plasma membrane hydrolyzing it to sphingosine (Billich and Baumruker 2008; Mechtcheriakova et al. 2007). *Lpp3*, in particular, is a key enzyme in the degradation of fingolimod-phosphate to its amino-alcohol prodrug. These failsafe mechanisms are essential to attenuate the effects of free ligand prior to receptor ligation. Rapid infusion of S1P, sufficient to elevate free plasma concentrations in the three-to-five fold range is sufficient to cause circulatory collapse in rodents, largely a result of alterations in cardiac rhythm. This bell-shaped curve for S1P activity is seen in cardiac function, in the regulation of blood pressure and in the control of lymphocyte recirculation (Rosen et al. 2013). These data suggest that early events selecting for fitness in populations may well have been driven by the role of S1P in the maintenance of vascular integrity and early vascular development, as these functions are essential for survival, while high concentration toxicities select against survival (Rosen et al. 2008).

The mysterious, sphinx-like phenotypes of this very hydrophobic yet strongly zwitterionic ligand, thus reflect the unique advantages conferred upon this ligand system by its remarkable physical properties (Roberts et al. 2013). Sphingosine has a cLogP of 5.94 (tPSA 66.48) while S1P has a CLogP of 4.91 and a Total polar surface area of 114.63. S1P does not cross membranes, is sparingly soluble in methanol and almost insoluble in water, and inserts in the outer leaflet of plasma membranes. Sparing aqueous solubility drives partitioning into HDL and helps maintains low free levels of ligand. Significant ligand hydrophobicity though, while manifested in simple ligand stickiness to surfaces driven by dielectric

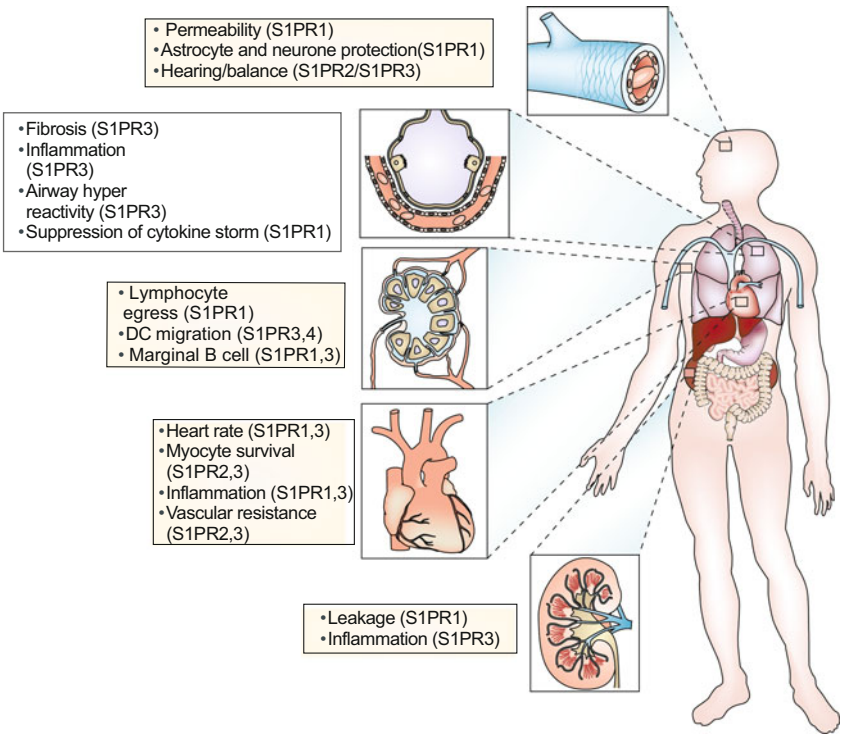


Fig. 2 *Schematic representation of the involvement of sphingosine-1-phosphate (S1P) receptors in the regulation of physiological and pathophysiological phenomena*. S1P receptors (S1PRs) regulate many events in health and disease including hearing, vasodilation/vasoconstriction, heart rate, airway hyperresponsiveness, and lymphoid tissue function. S1PR activation enhances pulmonary barrier integrity, endothelial integrity in kidneys and myocardium after ischemia reperfusion stress, and blood–brain barrier protection. S1P receptors are involved in the regulation of various physiological and pathophysiological phenomena, including hearing, vasodilation, and vasoconstriction, heart rate, airway hyperresponsiveness, and lymphoid tissue function. Accumulating literature supports the use of small molecules that target the S1P immunoregulatory pathway to modulate barrier activity in different organs. For example, S1P receptor activation was shown to favor pulmonary barrier integrity in models of acute lung injury and acute respiratory distress syndrome and in the kidneys and myocardium after ischaemia–reperfusion stresses and to favor blood–brain barrier protection during experimental autoimmune encephalomyelitis. Moreover, the S1P pathway was shown to enhance the lymphatic endothelial barrier integrity in lymph nodes, leading to sequestration of T cells in the lymph nodes

solvent forces in aqueous solution, does not mean that the structure of the long acyl chain is disordered or agglomerated in biological systems (Rosen et al. 2013; Hanson et al. 2012). In fact, the structure of the ligand binding pocket for S1P in its receptors has cleverly co-evolved to effectively utilize these nuances of hydrophobic packing forces and headgroup to signal with exquisite control, and the details of the high resolution crystal structure of the liganded S1PR1 is discussed in greater detail in the chapter by Hanson and Peach (this issue).

2 High Affinity G-Protein-Coupled Receptors for S1P

Five high affinity G-protein-coupled receptors are described for S1P. These are designated *s1pr1* through *s1pr5* for the gene nomenclature, and S1PR1 through S1PR5 for the protein nomenclature. These five receptors share a branch of the class A GPCR dendrogram with three related receptors for the lysophospholipid lysophosphatidic acid, a hydrophobic anionic lysophospholipid ligand that makes interactions through an acyl chain as well as with its phosphate headgroup, but lacks the quaternary amine of sphingosines. The nearest neighbors are the cannabinoid receptors, which like the S1P receptors show many similarities in the maintenance of receptor tone as a key event in useful signaling (Howlett et al. 2011).

Table 1 provides the chromosomal locations, relatedness, coupling and genetic and chemical tools for the S1P receptors. As seen in Table 2, S1PR1, S1PR3, S1PR4, and S1PR5 are expressed within cells of the lymphoid, myeloid, and endothelial lineages and thus impact upon the function of these various cells in important elements of immune surveillance and host defense.

S1P receptors have a relatively capacious binding pocket that reaches from the residues at the top of transmembrane helix 3 (E120 and R121) that anchor the amino-phosphate headgroup electrostatic interactions, to the deep hydrophobic pockets on either side of the tryptophan residue (W269 in S1PR1) that is highly conserved in all rhodopsin-family Group A GPCRs. The extent of these interactions are known through a combination of structural biology (the 2.8a resolution S1PR1 structure) (Rosen et al. 2013; Hanson et al. 2012) together with an extensive molecular pharmacology mutagenesis of the receptor (Parrill et al. 2000a, b; Wang et al. 2001; Sardar et al. 2002; Fujiwara et al. 2005, 2007; Inagaki et al. 2005; Jo et al. 2005) and binding and functional data derived (Gonzalez-Cabrera et al. 2008) from the extensive compound libraries designed during the drug discovery process. Taken together, these lines of evidence define a receptor pocket for S1P receptors that has the capacity for binding the orthosteric (natural) ligand and for the identification and discovery of allosteric agonists that do not compete for S1P biding, as well as of bitopic ligands where a single ligand competes for both orthosteric and allosteric interactions (Hanson et al. 2012; Jo et al. 2005; Jo et al. 2012). The pocket is thus large enough to allow for functional plasticity, where agonist and antagonist interactions can be delineated and interconverted, shedding light onto mechanisms of receptor triggering (Hanson et al. 2012). Initial predictions of S1PR as volume triggered receptors were insightful and important (Parrill et al. 2000a; Fujiwara et al. 2007).

A meaningful understanding of the gatekeeper residues within the S1P receptor binding pockets depend upon understanding the crystal structure of S1PR1 together with the significant gain and loss of function point mutations. A significant point mutation can best be defined as one that retains normal receptor surface expression and N-linked oligosaccharide maturation, and where the interactions of a subset of ligands are altered (either gain or loss), while some ligands remain unaltered in both binding and signaling. The resulting changes signify either a

Table 1 The S1P receptors

Receptor	Coupling	Knockout phenotypes (fluorescence-tagged knock-in)	Agonists	Antagonists
S1PR1 1p21[a] 381[b] 0.47–0.67[c]	Gi/o	Embryonic lethal (E12.5-E14.5) Vascular maturation defects Failure of null thymocyte to egress in irradiated chimeras S1PR1-eGFP knock-in viable, physiologically and pharmacologically normal	FTY720-P, KRP-203, AUY954 SEW2871 CYM-5442 Arylpropionic acids	VPC-23019 W123 (R)-W146[e] VPC44116
S1PR2 19p13.2[a] 353[b] 0.30–0.35[c]	Gs, Gi/o, Gq, G12/13	Slightly reduced viability[d] Seizures in certain genetic backgrounds Deafness	CYM-5520 (allosteric)	JTE-013
S1PR3 9q22.2[a] 378[b] 0.17–0.26[c]	Gi/o, Gq, G12/13	Worsens sepsis outcome Slightly reduced viability S1PR3-mCherry knock-in viable, physiologically and pharmacologically normal	FTY720-P KRP-203 CYM-5541 (allosteric)	VPC-23019 SPM-202 SPM-354
S1PR4 19q22.1[a] 384[b] 34–95[c]	Gi/o, G12/13	N/A	FTY720-P KRP-203 CYM-50308 (selective)	CYM-50374
S1PR5 19p13.2[a] 398[b] 0.50–0.61[c]	Gi/o, G12/13	No obvious phenotype	FTY720-P KRP-203	N/A

[a] Chromosome location
[b] Protein length
[c] Measured by inhibition of [^{33} P]-S1P binding to stably expressed human $S1P_{1\text{-}5}$ in CHO-K1 cell membranes
[d] Null $S1P_2$–$S1P_2$ mice have marked perinatal lethality
[e] (S) version is the chiral inactive isomer *N/A* Not available

direct interaction between small molecule and the altered amino acid side-chain (and should then be amenable potentially to gain of function transfer of binding and signaling activity to other family members, or that the side-chain provides a steric impediment to the access of discrete chemical classes to the biding pocket. These "gatekeeper" mutations may differentiate between nonselective ligands such as S1P from subtype selective ligands that bind S1PR1 for example and lack activity on S1PR3.

All such examples have been demonstrated for the S1PR family and the implication of the gatekeeper residues for understanding which receptors most readily support the identification of true allosteric compounds may be important. For example, the earliest synthetic agonist for the S1PR family was FTY720-phosphate, which had activity on all S1PR except for S1PR2, with a notable difference (F274 in S1PR2) (Fig. 3). Selectivity studies on S1PR1 agonists that had improved tolerability in rodents by selecting against S1PR3 activity, led Parent and colleagues

Table 2 Expression of S1P receptor mRNA based upon Northern Blotting or In-situ hybridization

Subtype	Distribution (mRNA)	Cellular functional expression and consequences
s1pr1	[Widespread] Brain, Heart, Spleen, Liver, Lung, Thymus, Kidney, Skeletal muscle, Lymphoid	Astrocyte—Migration B cell—Blockade of egress; Chemotaxis Cardiomyocyte—Increased β-AR positive inotropy Endothelial cell—Early vascular system development; Adherens junction assembly; APC mediated increased barrier integrity Neural stem cell—Increased migration Pericyte—Early vascular system development T-cell—Blockade of egress; Chemotaxis ; Decreased late-stage maturation VSMC (early vascular system development)
s1pr2	[Widespread] Brain, Heart, Spleen, Liver, Lung, Thymus, Kidney, Skeletal muscle	Cardiomyocyte—Survival to ischemia-reperfusion Epithelial cell (stria vascularis)—Integrity/development Epithelial hair cells (cochlea)—Integrity/development Endothelial cell (retina)—Pathological angiogenesis; Adherens junction disruption Hepatocyte—Proliferation/matrix remodeling Fibroblast (MEF) Mast cell—Degranulation VSMC—Decreased PDGF induced migration
s1pr3	[Widespread] Brain, Heart, Spleen, Liver, Lung, Thymus, Kidney, Testis, Skeletal muscle	Cardiomyocyte—Survival to ischemia-reperfusion Dendritic cell (hematopoietic)—Worsening experimental sepsis lethality/inflammation/coagulation
s1pr3	[Restricted] Lymphoid, Lung	T cell—Migration/cytokine secretion
s1pr4	[Restricted] Brain, Skin, Spleen	NK cell—Trafficking Oligodendrocyte—Survival OPC—Glial process retraction; Inhibition of migration)

(Deng et al. 2007) to define the key gatekeeper residue L276 in S1PR1, which is replaced by phenylalanine 263 in S1PR3. The aromatic sidechain changes the shape of the receptor binding pocket and sterically interferes with the ability of molecules like SEW2871 to bind to S1PR3, while the natural ligand S1P and structurally similar sphingosine analogs like FTY720 which share a long acyl-chain (Fig. 3) and a requirement to bind to sphingosine kinase 2 for phosphorylation, have unimpaired access to the S1PR3 binding pocket. Substitution of this gatekeeper phenylalanine with the leucine from S1PR1, caused S1PR3 to lose its selectivity for S1PR1-selective ligands and provided a gain of function mutation. Mutation of L276F in S1PR1 induced loss of function for S1PR1-selective ligands, while S1P binding and agonism remained fully intact. F263 serves as both a gatekeeper residue for the exclusion of S1PR1-selective compounds such as SEW2871 or CYM-5442, as well as being a critical point of interaction for S1PR3-selective ligands discovered from

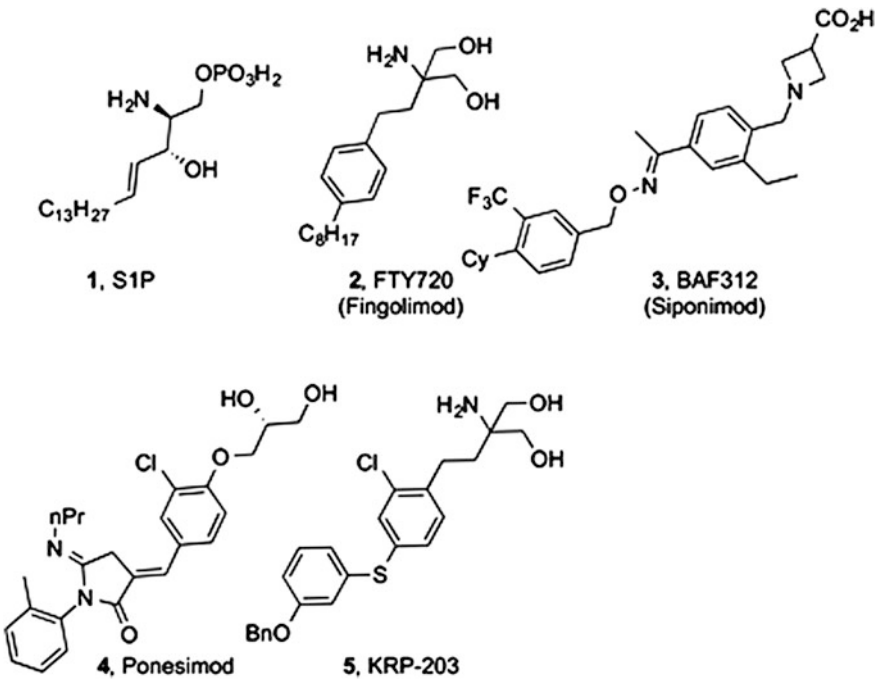


Fig. 3 *Synthetic chemical modulators of sphingosine-1-phosphate (S1P) receptors.* The natural ligand S1P and structurally similar synthetic sphingosine analogs like FTY720 (*Fingolimod*), BAF312, *Ponesimod* and the prodrug KRP-203 are shown

high-throughput screening. It is notable that the efficiency of small molecule ligand discovery for S1PR1, which lacks the key steric hindrances of the gatekeeper residues such as the phenylalanines described here, is approximately two orders-of-magnitude more efficient than that seen for S1PR2 or S1PR3 (Fig. 4). This was measured by screening the same chemical diversity set from the NIH Molecular Library of 85,000 compounds for activity on all three receptors. The confirmed actives were then arrayed computationally in chemical diversity space in six dimensions, and then the number of confirmed hits per cell of chemical space calculated (Jo et al. 2012; Schurer et al. 2008). Chemical libraries, because of their richness in linked heterocycles, are much less flexible than the physiological ligand S1P, where the length acyl chain has more flexibility in conforming around steric obstacles deep within the receptor binding pocket. Thus, the critical importance of bulky aromatic amino side-chains, is that by altering access of more rigid structures, these ligands may bias the output of screens toward receptor-selective ligands. Only in the details of the molecular pharmacology, where receptor mutations are combined with multiple chemical probes, can the anatomy of the binding pocket be best understood.

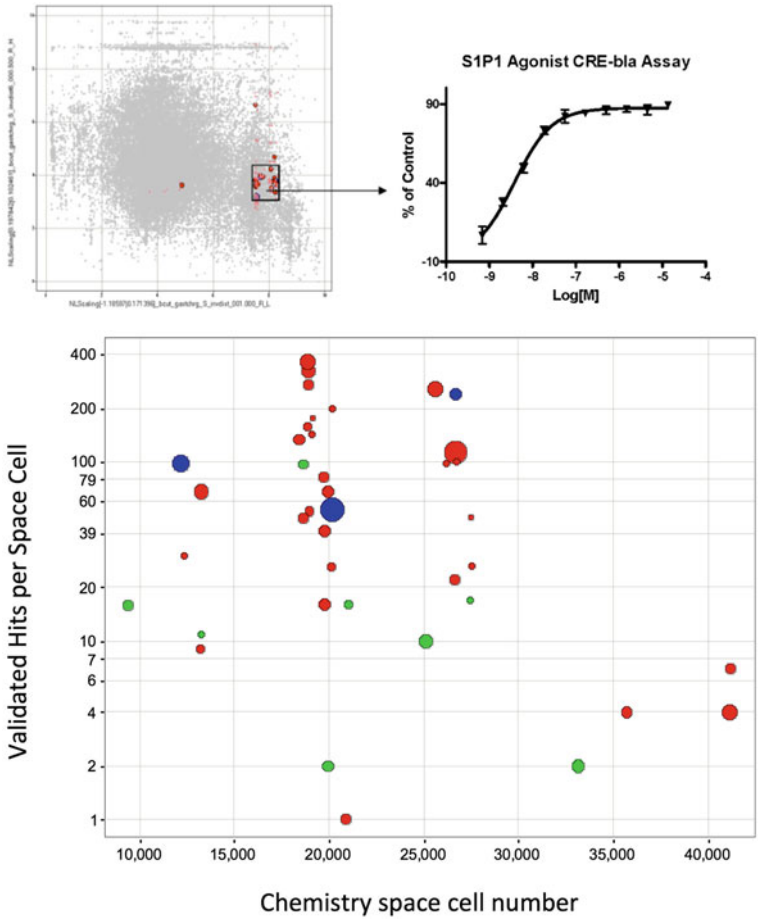


Fig. 4 *Representative S1P1 agonists screening and validation assay. Left Panel Library screening panel* S1PR1 agonist screening from the Molecular Libraries Small Molecule Repository (MLSMR) and the Maybridge HitFinder (MBHF) collection using a chemistry-space- and fingerprint-based method; The MBHF and MLSMR libraries were screened in 384 and 1536 well formats, respectively. With the S1P1 cell line, two closely related screens were conducted, one for agonists and the second designed to identify potentiators of S1P1 agonists. Thus we used the data from both assays to identify compounds that reproducibly act as S1PR1 agonists. Active wells in the primary screening assay were confirmed and counterscreened against the parental cell line to eliminate compounds. *Right panel* Dose response curve of an identified S1PR1 agonist using a CRE-bla assay. Cell containing the human S1PR1 receptor as well as the beta-lactamase (BLA) reporter-gene under control of the cyclic AMP response element (CRE) promoter was used to measure S1PR1 activation. *Bottom Panel* Population density (occupancy) of the regions of chemistry space that include identified S1PR1 or S1PR3 agonists. The optimized 6D BCUTS chemistry space characterizing the MLSMR and MBHF libraries is split into 46,656 cells (6 bins per dimension), 6,438 of which are occupied. *Red* Cells with S1P1 agonists, *green*, cells with S1P3 agonists, *blue*, cells with both S1P1 and S1P3 agonists, size by pEC50 of the most active agonist in the cell

Given the relative capaciousness of S1PR1 compared to S1PR2 or S1PR3 through the lack of an aromatic gatekeeper, it may be unsurprising that S1PR1 ligands discovered have always had some overlap with the orthosteric pocket, as defined by competition for labeled S1P in binding assays (Gonzalez-Cabrera et al. 2008; Schurer et al. 2008). True orthosteric ligands show no differences on receptor interactions compared to S1P, and FTY720-phosphate (Mandala et al. 2002), SEW2871 (Sanna et al. 2004; Jo et al. 2005) and a variety of headgroup-containing ligands including the orthosteric agonist W146 (ML056) (Sanna et al. 2006) all show that pattern. All require critical head-group interactions, e.g., E120 and R121, and the critical hydrogen-binding and hydrophobic interactions made by W146 have been resolved to 2.8A by X-ray crystallography, where the position all of the backbone atoms in W146 within the receptor was solved (Hanson et al. 2012). Insights into volume triggering of the receptors have been inferred from W146. This orthosteric neutral antagonist can be converted into a full agonist by extending the acyl chain with a further four methylenes. Molecular dynamic simulation studies revealed that if the extended acyl chain is anchored within the limited hydrophobic spaces, the head-group interactions are no longer accommodated, and vice versa when the head-group interactions are anchored. The antagonist crystal structure simply cannot accommodate the extended chain and some alteration in binding pocket is required to accommodate the agonist. The smallest change in the pocket that can achieve this agonist accommodation, and is compatible with the known physical possibilities of limiting side-chains within the binding pocket, is the cis-trans isomerization of the indole ring of W269 (Hanson et al. 2012), a binding pocket change compatible with those seen in agonist crystal structures of the Rhodopsin GPCR family, including beta-2 adrenergic and adenosine A2 receptor structures (Rasmussen et al. 2007, 2011a, b; Rosenbaum et al. 2007, 2011; Warne et al. 2011; Doré Andrew et al. 2011; Xu et al. 2011).

In contrast, ligands from screening that lacked any evidence for head-group interactions with S1PR1 have different profiles in receptor interaction studies. Such ligands, exemplified by CYM-5442 (Hanson et al. 2012; Gonzalez-Cabrera et al. 2008), have been referred to as Class II agonists, and the body of evidence supports the identification of these as bitopic ligands, with some overlap in orthosteric space, yet making discrete molecular interactions with receptor residues not required for orthosteric ligand binding and receptor activation. Specifically, CYM-5442 was identified as an agonist in an allosteric modulator screen. A counterscreen, examining receptor activation by CYM-5442 in competition with the orthosteric antagonist W146, showed that CYM-5442 was noncompetitively antagonized by W146, in contrast to its competitive inhibition of signaling by S1P, FTY-P or SEW2871. A binding pocket hypothesis based upon the model developed by Schuerer et al. (2008) led to the postulate that CYM-5442 might be making a discrete stacking interaction with a critical aromatic reside within the receptor pocket. Point mutagenesis of all of the aromatic residues within the binding pocket, perhaps counterintuitively including the conserved tryptophan W269 provided an unexpected clue. The W269L mutation was well tolerated by the receptor, which is surface expressed, has normally processed oligosaccharides, and binds and signals for the orthosteric ligands

S1P, SEW2871, and FTY720-P. In contrast, CYM5442 no longer binds nor activates the receptor, suggesting a direct interaction with W269. Interestingly, mutation of the tryptophan with the more conservative aromatic substitutions of F or Y, provide intermediate activity of CYM-5442, suggesting that the aromatic pi stacking interaction alone is sufficient for some biological activity, but the unique electronics of the tryptophan indole ring provides some additional and quantitatively significant binding interactions and thus energy (Hanson et al. 2012). The alternative binding mode in this series can be switched from bitopic to true orthosteric when the dominance or the head-group interactions is introduced. Addition of a headgroup switches these ligands to the orthosteric space by picking up ~ -4 kcal.mol of binding energy from the electrostatic head-group interactions and thus no longer reaching down deep into the base of the binding pocket for an aromatic interaction with the tryptophan. The sort-acting picomolar agonist RP-001 exemplifies this shift from bitopic to orthosteric (Cahalan et al. 2011).

While not understood well initially, the outcomes of agonist screens for both S1PR3 and S1PR2 provided very different outcomes. Selective agonists for S1PR3 inactive of S1PR1 were readily discovered, and these required the presence of the gatekeeper phenylalanine (Schurer et al. 2008; Guerrero et al. 2013), because the activity could be demonstrated in gain-of-function experiments comparing the L276 wild-type to the L276F mutant of S1PR1. Subsequent binding studies (Jo et al. 2012) confirmed that CYM-5541 was a true allosteric agonist for S1PR3, activating the receptor but not competing for the binding of the natural ligand S1PR3. Fortunately, Kohno and colleagues based upon structure-activity studies around the S1PR1 agonist KRP-203, discovered a series of S1PR3-selective competitive antagonists, exemplified by SPM242 (Jo et al. 2012). SPM242 is competitive for S1P biding to S1PR3, but is also a full, competitive antagonist for the allosteric agonist CYM-5541. These data show that SPM242 is a true bitopic antagonist, making fully competitive orthosteric interactions yet in addition making interactions that compete with the true allosteric agonist. These data show the proximity between the orthosteric pocket and the allosteric pocket occupied by CYM5541, and it may be the additional interactions competing with the selective allosteric site, that provide the selectivity for SPM242 in its interactions with S1PR3 as compared to other members of the receptor subfamily (Fig. 5).

Sequence alignment of the binding pocket residues across the S1P receptor family (Fig. 6) revealed that Ballesteros-Weinstein residue 7.40 in the pocket is a leucine in S1PR1 and a phenylalanine in S1PR2, and functions as the analogous gatekeeper residue for S1PR2. The results in chemical discovery suggest this. Agonist screening on S1PR2 defined a series of allosteric agonists that were non-competitive with both S1P and the true orthosteric antagonist JTE-013 (Satsu et al. 2013). Signaling by CYM-5520 in S1PR2 cell lines required the presence of the receptor, whether wild-type or headgroup-binding mutant receptor, demonstrating once again that the identification of ligands that lack any head-group interactions likely biases the output to agonists that make fully or partly noncompetitive interactions with orthosteric space, because of the strong binding energy derived from the strongly zwitterionic S1P amino-phosphate headgroup.

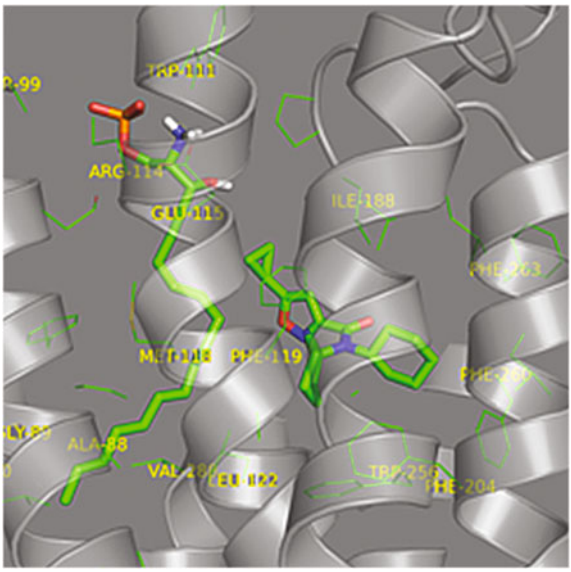
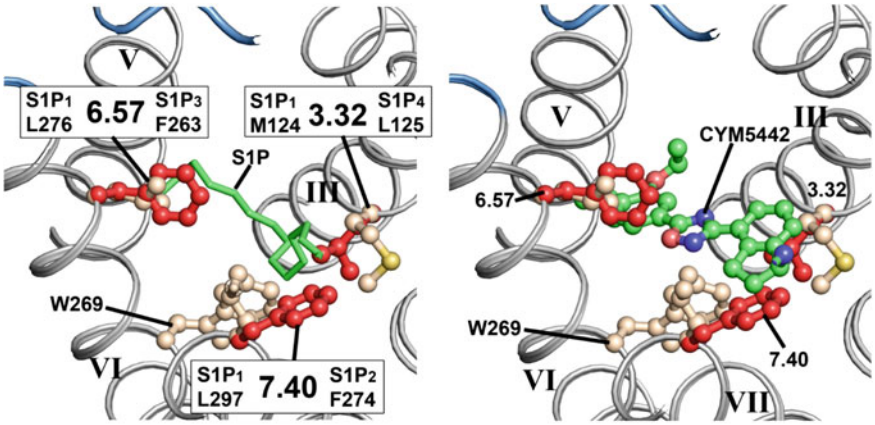


Fig. 5 *Visualization of the receptor binding pocket by homology modeling and docking*. Three-dimensional plot of S1P3 binding to S1P and CYM-5541. S1P and CYM-5541 codocked to S1P3. Codocking of S1P and CYM-5541 suggested that the receptor pocket could spatially expand in the lower region of the hydrophobic pocket to accommodate CYM-5541 in addition to S1P. In the presence of S1P, the pocket opens up in the lower hydrophobic region adjusting CYM-5541

In contrast, no aromatic gatekeeper residues were identified for S1PR4 and S1PR5. While S1PR5 has not been the subject of detailed screening, largely because the structure-activity relationships between S1PR1 and S1PR5 are so significantly overlapping, high throughput screening and chemical optimization have resulted in agonist and antagonist ligands for S1PR4 (Guerrero et al. 2010, 2011, 2012; Urbano et al. 2011a, b), and these are likely orthosteric, with no evidence thus far for the definition of an allosteric interaction, though a significant effort is required to prove such alternate interactions with multiple, orthogonal lines of evidence. It appears though, at first approximation, that S1P receptor subtypes with aromatic gatekeepers predispose to the discovery of allosteric agonists, while those that are less spatially constrained predispose to the discovery of orthosteric or bitopic ligands with a substantial presence in the orthosteric space.

The presence or absence of interactions outside the orthosteric pocket has not as yet, for this family of receptors, been predictive of any element of quantitative signaling bias. For example, S1P and FTY-phosphate have subnanomolar Kd on S1PR1, are both orthosteric and competitive for each other, yet differ by three logs in their ability to down-modulate the receptor. This difference stems from the ability to drive receptor polyubiquitination, lysosomal sorting, and degradation (Gonzalez-Cabrera et al. 2007, 2012; Oo et al. 2007, 2011), events occurring in the large vesicular body (late endosome) and that reflect receptor phosphorylation as evident from the constitutively active S1PR1 knock-in (Oo et al. 2011; Thangada et al. 2010), and most especially in lymphocytes, the activation of GRK2 phosphorylation and beta-arrestin signaling (Lo et al. 2005; Pham et al. 2008; Arnon et al. 2011; Cyster and Schwab 2012). Ligands that achieve sub-nM potencies require a slow off-rate, and that the duration of the endosomal signaling by S1PR1



BW Index	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$	LPA_1	LPA_2	LPA_3	Subtype variability
2.57	Y98	F86	Y92	Y99	Y89	Y102	Y85	Y83	13%
2.60	N101	N89	N95	N102	N92	L105	L88	L86	38%
3.25	W117	W105	W111	W118	W108	W121	W104	W102	0%
3.28	R120	R108	R114	R121	R111	R124	R107	R105	0%
3.29	E121	E109	E115	E122	E112	Q125	Q108	Q106	38%
3.32	M124	A112	M118	L125	V115	I128	L111	L109	75%
3.33	F125	F113	F119	F126	F116	D129	D112	D110	38%
3.36	L128	L116	L122	L129	L119	L132	L115	L113	0%
5.39	Y202	Y190	Y196	Y204	Y193	Y206	Y189	Y187	0%
5.42	F205	C193	F199	F207	F196	F209	V192	F190	25%
5.43	C206	V194	C200	C208	C197	W210	W193	W191	50%
5.46	V209	I197	I203	I211	A200	F213	S196	S194	88%
5.47	F210	F198	F204	F212	F201	N214	S197	N195	38%
6.48	W269	W246	W256	W264	W264	W271	W254	W252	0%
6.53	L272	A249	L259	L267	L267	G274	G257	G255	50%
6.54	F273	F250	F260	F268	F268	L275	Q258	L256	38%
6.57	L276	L253	F263	L271	L271	L278	L261	L259	13%
7.35	R292	H269	Q279	D288	D287	K292	K276	R274	88%
7.37	E294	K271	K281	G290	Q289	Y294	V278	V276	88%
7.40	L297	F274	L284	L293	L292	L297	L281	L279	13%
7.41	V298	A275	V285	A294	G293	L298	L282	L280	75%
7.43	A300	S277	A287	A296	A295	A300	A284	A282	13%
7.44	V301	V278	V288	V297	M296	E301	E285	L283	50%
BP identity	100%	52%	83%	78%	74%	43%	39%	43%	

Fig. 6 Mining the S1P pocket for allosteric agonists (*Top left*). Docking of S1P ligand (*green*) in the orthosteric pocket of S1P-R1 and threading of additional S1P-Rs, define subtle differences within the principal, core-transmitting and highly conserved W2.69 rotamer "switch" in transmembrane helices III, V and VI, which have been exploited to define allosteric pockets of selectivity. High throughput screening, receptor binding, mutagenesis and functional studies have highlighted M124, L276, and L297 in S1P-R1, F274 in S1P-R2, F263 in S1P-R3 and L125 in S1P-R4 as structural determinants of agonist hit selectivity

is demonstrated to be especially prolonged (Gonzalez-Cabrera et al. 2012; Mullershausen et al. 2009; Cahalan et al. 2013), the differences between S1P and other ligands either reflect a conformational change in receptor (for which no strong evidence has yet been shown) or a difference in the stability of the signaling complex between S1P and FTY-phosphate. A relatively faster off-rate for S1P may be sufficient to alter the duration of the endosomal signaling complex and the recruitment of beta-arrestin and an E3 ligase (Oo et al. 2011). The biochemical basis for these differences must still be worked out by a more detailed analysis of the cell biology of the signaling complexes and the influence on receptor fate. It

can be noted that for S1PR1 at least, Gi-coupled assays have been the most efficient for the discovery of immunomodulators, while beta-arrestin format assays have proved to be better at differentiating between antagonists than agonists. The importance of long-term endosomal signaling on the biological events that impact on therapeutics in multiple sclerosis for example, are very imperfectly understood, and a great deal of higher resolution biochemistry is required to unravel precise mechanisms.

One approach to studying both physiological and perturbed events at better resolution has been to introduce receptor knock-in mice. Such mouse models are discussed in detail by Cahalan in this volume, and together with deletional models, now provide the possibility of isolation of signaling receptor complexes from primary tissues under resting and perturbed conditions in quantities sufficient for proteomic analyses. Much can be learnt about integrated receptor function when genetic and chemical approaches can be combined in vivo, with quantitative pharmacological outputs.

Study of S1P receptors in these models has given insights into receptor expression across and within tissues, and the malleability of receptor modulation by ligand in vivo, and how that may differ in tissue specific ways (Teijaro et al. 2011; Cahalan et al. 2011; Gonzalez-Cabrera et al. 2012; Cahalan et al. 2013; Sarkisyan et al. 2012).

2.1 Boundary Conditions for Therapeutic Efficacy: The Challenge of Translation from Mouse to Man

A useful way to look at S1P signaling in immunomodulation is to look through the mirror of therapeutics, for it is through the principles of prospective translation that mechanisms of successful immunomodulation can be distinguished from the boundary conditions discernible on acute manipulation of the system in model systems. Fingolimod is approved for the treatment of relapsing remitting MS. Strikingly, clinical trials showed no dose response for efficacy, with all levels tested including 0.5 mg/d providing similar efficacies (Kappos et al. 2006, 2010), though significant adverse effects on heart rate, liver enzymes, and blood pressure, while present at the 0.5 mg dose, have an increased prevalence at higher doses. Though fingolimod was identified as a suppressor of skin allograft rejection in rats, and then postulated to work through the inhibition of lymphocyte recirculation by sequestration (Mandala et al. 2002; Forrest et al. 2004) upon lymphocyte S1PR1 signaling attenuation (Matloubian et al. 2004; Pham et al. 2008), statistical analyses by both sponsor and FDA showed no correlation with the degree of lymphopenia, and all doses trending toward a 50 % reduction in circulating lymphocytes were sufficient for efficacy in man. Lymphopenia is thus a predictor of efficacy, but the absolute correlation between degree of lymphopenia in choice of dose is not complete (Kappos et al. 2006; Kappos et al. 2010; Khatri et al. 2011;

Devonshire et al. 2012; Radue et al. 2012). Other agents in clinical development have also shown efficacy in the reduction of gadolinium-enhanced scans in Phase II clinical studies with only 50 % reduction in circulating lymphocytes (Selmaj et al. 2013; Reyes et al. 2014; You et al. 2013; Rey et al. 2013; Fernandez et al. 2013; Brossard et al. 2013; Sobel et al. 2013; Piali et al. 2011; Bolli et al. 2010). Clearly, immunomodulation by S1PR1 signaling has multiple points of engagement in the autoimmune inflammatory cascade and the tissue response to injury.

These multiple points of engagement have been clearly demonstrated in animal model studies including extrinsic allergic encephalitis (EAE) (Cahalan et al. 2013; Choi et al. 2011). Studies of S1PR1 expression and efficacy in the S1PR1-eGFP knock-in mice have demonstrated that S1PR1 protein is expressed in lymphocytes, endothelia, astrocytes, and neurons (Cahalan et al. 2011; Cahalan et al. 2013). The use of short-acting S1PR1 agonists that produce lymphopenia for only one-third of the 24-h dosing interval and restore blood lymphocyte numbers to normal are sufficient for complete efficacy in EAE, which correlates better with prolonged exposures to agonist within brain rather than blood (Gonzalez-Cabrera et al. 2012; Cahalan et al. 2013). The kinetics of down-modulation of CNS receptor in neurons and astrocytes by CYM-5442 is prolonged, with evidence for receptor degradation in these cells. Of note in this study was the diminution of lymphocyte infiltration of the brain parenchyma even with the full restoration of blood lymphocytes in each dosing interval. Lymphocytes tended to remain in perivascular cuffs and the infiltration of white matter and demyelination was inhibited. Direct evidence for the role of the astrocytic receptor is provided by an important genetic study where the deletion of astrocyte S1PR1 reduced the severity of EAE and altered sensitivity to fingolimod (Choi et al. 2011). Differences in receptor fate in different tissues also contribute to the basis of potential adverse effects. Fingolimod-induced receptor degradation by recruitment of the WW2 E3 ligase (Oo et al. 2011) was essential for the induction of vascular leakage though not for the induction of lymphopenia. Therefore, multiple tissue specific mechanisms are evident. The bell-shaped curve of S1PR1 signaling tone and lymphocyte egress from thymus, lymph nodes, and Peyer's patch into blood is clear, with lymphocyte egress requiring physiological S1PR1 signaling on lymphocytes. Deletion of S1PR1 in lymphocytes (Matloubian et al. 2004; Pham et al. 2008) inhibits egress irretrievably, while complete antagonist occupancy of S1PR1 produces a very transient lymphopnia (Cahalan et al. 2013) that is rapidly restored when receptor occupancy falls to even 90 %. In contrast, low doses of agonist reversibly disrupt lymphocyte egress (Sanna et al. 2006; Rosen et al. 2007) and allow titration of blood lymphocyte number by agonist-antagonist balance (Wei et al. 2005), suggesting that pharmacological agonists work by the disruption of S1PR1 ligand sensing, and that for lymphocytes at least, agonists cause directional confusion (rounding on intravital imaging) that can rapidly be restored by balancing the attenuating signal with antagonist titration (Lo et al. 2005; Cyster and Schwab 2012; Schwab et al. 2005; Pappu et al. 2007; Grigorova et al. 2010; Pham et al. 2010).

It is likely then that motile lymphocytes utilize S1PR1 for the critical egress signal from microenvironments of low S1P concentrations to higher, hence their

specific microanatomic arrest at the level of traversing the lymphatic endothelium or medullary egress from thymus, and are highly sensitive to alterations in spatial agonist signals (Cyster and Schwab 2012). Sessile cells whether endothelia, neurons, or astrocytes utilize tonic S1PR1 signaling, and alteration of cellular function occurs with, for example, enhanced endothelial integrity in the presence of ligand (Sanna et al. 2006; Rosen et al. 2007) to significant vascular leakage in the absence of receptor signaling, whether by antagonist occupancy (Cahalan et al. 2013) or receptor degradation (Oo et al. 2007, 2011).

2.2 Choosing the Right Chemical Tool

The biomedical importance of these receptor subtypes has spurred the development of many excellent pharmacological tools to probe elements of the S1PR signaling system in vitro and in vivo. Because much of this work has been reviewed elsewhere (Rosen et al. 2013; Roberts et al. 2013; Urbano et al. 2013), the objective here is to draw attention to data that show that different compounds with different properties may work best for certain experimental settings. Fingolimod has a very long half-life, exists in equilibrium between the phosphorylated form and the amino-alcohol pro-drug, is water soluble, can be dosed by almost any route including the drinking water, and is most useful when a nonselective agonist with a very long duration of action and infrequent dosing is required in long-term experiments (Brinkmann et al. 2002). The chiral FTY720 analog pro-drug AAL(R), is useful for pulmonary delivery in water when a nonselective agonist is required (Walsh et al. 2010; Marsolais et al. 2008, 2009, 2011). An advantage is the availability of the inactive (S)—enanatiomer which serves as an excellent negative control (Mandala et al. 2002; Brinkmann et al. 2002). Selective agents like CYM-5442 are shorter acting, may require twice daily dosing, but provide stable, long-term levels within the CNS (Cahalan et al. 2011; Gonzalez-Cabrera et al. 2011, 2012). Very potent short-acting agonists such as RP-001 have utility in settings where rapid induction and reversal of effects is desired or in pulse-chase time courses (Cahalan et al. 2011). Orally active compounds with excellent penetration of lung such as RP-002 have been useful in both mouse and ferret experiments examining the role of S1PR1 in the modulation of cytokine storm to influenza (Oldstone et al. 2013; Walsh et al. 2011a, b; Teijaro et al. 2011). Well-characterized antagonists for S1PR1 exist, with W146 providing stabilization of the receptor to enable its crystal structure determination (Hanson et al. 2012), while its inactive enantiomer W140 is a useful control compound. The potent Novartis antagonist Ex26 (Cahalan et al. 2013) has also proved to be an outstanding pharmacological tool. Care should be taken in the selection of the best compound for the experiment, and for the confirmation of compound presence and target engagement in the appropriate compartment during the experimental time course to establish causality in pharmacokinetic-pharmacodynamic terms (Rosen et al. 2009). This has usually been achieved by liquid chromatography mass spectrometric analysis and was instrumental in the initial description of fingolimod-phosphate as

the metabolic product that was an S1P receptor ligand altering lymphocyte egress (Mandala et al. 2002). The caveats of small molecule use and misuse mean that investigators should choose well-characterized molecules, where the on-target and off-target activities are known, where activities on the desired target have been demonstrated in multiple assays with orthogonal methodologies, and use them after appropriate solubilization and delivery, at peak plasma concentrations that are usually under 10 μM. In addition, having systems in place (knock-out, knock-down, knock-in) to validate chemical findings genetically remains very important in the analysis of signaling pathways.

2.3 Future Directions

With fingolimod in clinical use, and a series of S1PR1-selective agonists in advanced clinical trials (phase 2/3) for multiple sclerosis, ulcerative colitis, and psoriasis, the tools are now available to assess the role of sphingosine 1-phosphate signaling pathways is a variety of human pathologies in which the S1PR1 plays a direct or an indirect role. The broad modulation of the receptor on both lymphoid, endothelial, neuronal, and astrocytic cells amongst others, in ways that reflect differential signaling and receptor fate, are providing new insights into autoimmune diseases as well as into the collateral immune damage that contributes so much to the morbidity of acute viral infections such as influenza. Both the basic pathways and the translational elements of this biology promise to be a fertile ground for continued rapid progress.

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Structural Biology of the $S1P_1$ Receptor

Michael A. Hanson and Robert Peach

Abstract The sphingosine 1 phosphate receptor family has been studied widely since the initial discovery of its first member, endothelium differentiation gene 1. Since this initial discovery, the family has been renamed and the primary member of the family, the $S1P_1$ receptor, has been targeted for a variety of disease indications and successfully drugged for the treatment of patients with relapsing multiple sclerosis. Recently, the three-dimensional structure of the $S1P_1$ receptor has been determined by X-ray crystallography and the specifics of the sphingosine 1 phosphate ligand binding pocket mapped. Key structural features for the $S1P_1$ receptor will be reviewed and the potential binding modes of additional pharmacologically active agents against the receptor will be analyzed in an effort to better understand the structural basis of important receptor–ligand interactions.

Contents

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1 Introduction

Signal transduction is a fundamental process at the center of cellular activity and organismal function. The ability of cells to respond to signals in their environment allows adaptive responses central to survival. Cells are by nature isolated from their environments by means of a plasma membrane barrier which also facilitates the specific signals that impact the internal environment and behavior of the cell. This selectivity in signal transduction is achieved by means of a set of membrane proteins which control entry of reagents and information into the cytoplasm. One of the most important members of signal transduction set of membrane proteins are the G protein-coupled receptors (GPCRs).

GPCRs function through transmission of signals from the extracellular milieu to the cytoplasm of the cell where they are amplified by a variety of second messenger systems initiated by direct interaction with various G proteins or arrestins. As a family, the GPCRs recognize a wide spectrum of extracellular ligands including photons, ions, small organic molecules, peptides, proteins, and bioactive lipids. The GPCR family is one of the largest and most diverse membrane protein families consisting of more than 800 genes in the human genome. Each receptor is capable of recognizing specific ligands and transmitting the binding event to a wide variety of cytosolic signaling networks by means of conformational changes triggered by the specific ligand–receptor interactions (Kenakin and Onaran 2002). These receptor conformational changes are traditionally associated with three general pharmacological effects: inverse agonist, neutral antagonists, and agonist. Inverse agonists are ligands that alter the conformational landscape of the receptor so that it does not trigger any downstream signaling events. Inverse agonists are often classified as antagonists based on their ability to reduce agonist binding or signaling in a dose-dependent manner. Neutral antagonists are also often classified as antagonists based on their ability to reduce agonist binding or signaling, however, they do not alter the conformational landscape of the receptor. Agonists are compounds that alter the conformational landscape of the receptor to trigger a signaling event. It is often the case that the classification of a compound as an agonist depends on the assay used to measure signaling. Many compounds are capable of altering the conformational landscape of a receptor to signal along one pathway (i.e., arrestin) but not others (i.e., G protein) (Kenakin 2012). This phenomenon is known as ligand-biased signaling with the end result being that ultimate pharmacological classification of many compounds depends on the type of assay employed. Once triggered the downstream signaling networks result in a multitude of cellular responses that are dependent not only on the receptor and extracellular signal but also on the tissue type and cellular environment in which the signaling takes place.

Structural characterization of the GPCR superfamily has recently come of age with the solution of over 20 members (Cherezov et al. 2007; Warne et al. 2008; Jaakola et al. 2008; Chien et al. 2010; Wu et al. 2010; Granier et al. 2012; Hanson et al. 2012; Haga et al. 2012; Kruse et al. 2012; Manglik et al. 2012; Thompson

et al. 2012; Wu et al. 2012; White et al. 2012; Zhang et al. 2012; Wacker et al. 2013; Wang et al. 2013a, b; Hollenstein et al. 2013; Siu et al. 2013; Tan et al. 2013), with the β_2 adrenergic receptor (β_2AR) having structural snapshots of multiple pharmacologically relevant signaling conformations (Cherezov et al. 2007; Rasmussen et al. 2011a, b). This wealth of structural information represents a relatively new addition to the corpus of knowledge developed around this family and will inevitably aid the multitude of ongoing efforts to develop pharmaceutics targeting the GPCR receptors.

From a global perspective, all of the structurally analyzed members of the GPCR family consist of a seven-helix bundle with the N-terminus oriented to the extracellular space of the receptor and the C-terminus the intracellular space. Adjacent helices are separated by loops termed extracellular loop (ECL) 1–3 and intracellular loops (ICL) 1–3. The extracellular loops are of variant lengths across the family and are highly divergent in terms of their primary, secondary, and tertiary structural characteristics. Intracellular loops one and two are similar in length and topology across the GPCR superfamily, while ICL3 is highly divergent and is probably unstructured in most GPCRs in the absence of G protein interactions.

From a more detailed perspective, GPCRs can be divided into four regions commonly referred to as domains (Fig. 1). The extracellular region controls access of the receptors' ligand to the binding pocket. In some receptors the extracellular region also provides important contacts within the ligand binding pocket as well. The transmembrane region is responsible for the core functionality of the receptor family, namely signal transduction through ligand binding and conformational rearrangement. The details of the roles that these helices play for each receptor are now being determined with the benefit of the structural information being generated. The intracellular region forms the canonical allosteric interaction site for the GPCRs where the cytosolic signaling partners such as the G proteins or arrestin couple to the receptor. The fourth, ligand binding region is composed of specific residues within the transmembrane and extracellular domains and as its name implies is responsible for recognition of each receptor's endogenous ligand. Each of these regions will be characterized in more detail in the context of the $S1P_1$ receptor.

One can further stratify the function of the transmembrane region of GPCRs into ligand binding and signal transduction modules (Katritch et al. 2012). Comparing the structural deviation for the ligand binding module to the signal transduction module reveals greater structural diversity within the ligand binding region among the disparate GPCR family members. This of course is not surprising given that they each recognize distinct ligands but signal along similar pathways. Interestingly, when analyzing the β_2AR structural representatives with an inverse agonist bound compared to an agonist and agonist plus G protein complex, the structural changes in the intracellular region associated with G protein binding are apparent, whereas conformational changes in the ligand binding module associated with agonist interactions are not significantly different (Cherezov et al. 2007; Rasmussen et al. 2011b).

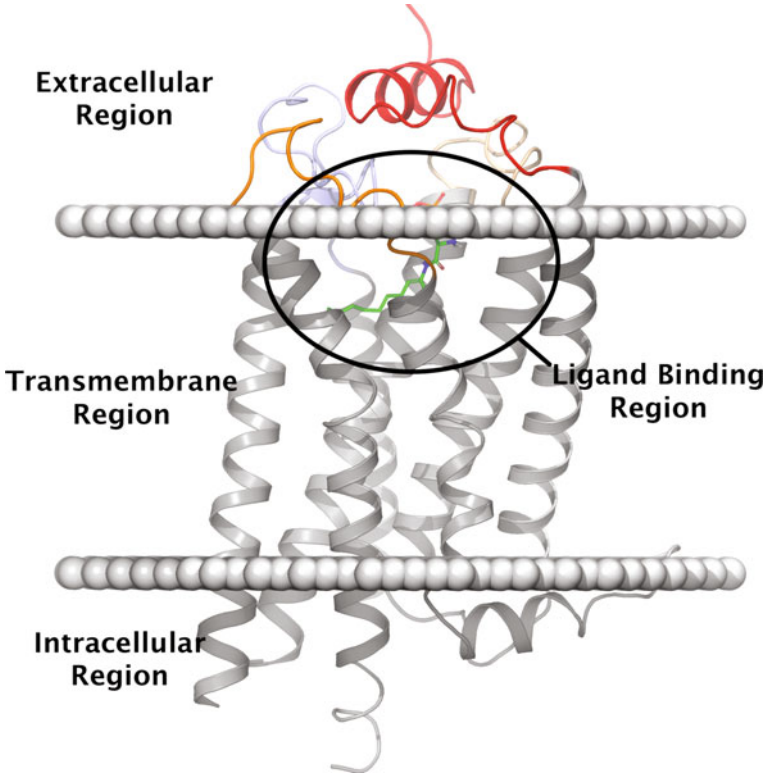

Fig. 1 Structural overview of the $S1P_1$ receptor and its four main regions. The position of the membrane is shown with *white spheres*

The idea that the conformation of the ligand binding region of GPCRs need not undergo significant structural rearrangement during G protein signaling lends credence to the idea that structures with agonists bound to the conformationally inactive state of the receptor are valid starting points for analysis of agonist structure activity relationship (SAR) programs. Indeed, it should also be within range of modern molecular modeling techniques to predict agonist binding poses using an antagonist structure as an initial template. Thus, the utility of each new GPCR structure will extend well outside of the immediate pharmacological state it was solved in and support multiple efforts in pharmacology research and drug development.

The superfamily of GPCRs can be divided into six major classes based on sequence homology, termed A–F, with the class A family being the most prevalent and well represented among the structurally known GPCRs (Fredriksson et al. 2003). The class A family recognizes a diverse set of ligands from photons to peptides and proteins and is the grouping in which the sphingosine 1 phosphate (S1P) family of lipid binding receptors is classified.

Prior to deorphanization, the S1P and lysophosphatidic acid (LPA) family of receptors were collectively termed the endothelial differentiation gene (Edg) family of receptors. This naming was based on the first discovered member of the family, Edg1 which is involved in a set of immediate early response gene products cloned from human umbilical vein endothelial cells (Hla and Maciag 1990). Subsequent Edg family receptors were discovered and classified based on sequence similarity, however, they have no involvement in endothelial differentiation. The classification was formalized in 2002 to follow standard International Union of Pure and Applied Chemistry (IUPAC) naming conventions and Edg-1 became the $S1P_1$ receptor based on its highest affinity endogenous ligand S1P and the chronological order of discovery within the family (Chun et al. 2002). At the time, there were five members of the S1P receptor family and three members of the closely related LPA receptor family. Currently, two additional LPA receptors have been characterized (Choi et al. 2010).

The $S1P_1$ receptor plays a crucial role in lymphocyte trafficking and is expressed on both the lymphocytes themselves and the sinus-lining endothelium (Cahalan et al. 2011). $S1P_1$ receptors are differentially regulated in different cell types with lymphocyte populations coupling to $G\alpha_{2i}$ and exhibiting rapid loss of cell surface-expressed receptor in response to agonist, in contrast to the endothelium-expressed receptor which has a ten-fold higher expression level and significant signaling reserve (Pham et al. 2008b; Cahalan et al. 2011; Arnon et al. 2011). Disruption of $S1P_1$ receptor signaling can either result in an excursion of lymphocytes under low or transient agonist occupancy or a profound lymphopenia in the presence of high-affinity agonists which cause internalization of the receptor resulting in functional antagonism (Rosen et al. 2013). Similarly, it has been shown that high-affinity antagonists can induce a similar lymphopenia but with a significant increase in capillary leakage relative to agonists which may restrict the utility of antagonists for the $S1P_1$ receptor in clinical settings (Sanna et al. 2006; Oo et al. 2011).

The ability of $S1P_1$ receptor agonists to modulate immune responses by selectively arresting the trafficking of naïve and central memory T and B lymphocytes in peripheral lymphoid organs without affecting the trafficking of effector memory populations has prompted the development of agonists for a variety of autoimmune disorders with multiple sclerosis being the flagship indication for this class of compounds.

The high interest in $S1P_1$ receptor pharmacological agents combined with a rapidly increasing confidence in the crystallizability of the GPCR superfamily in general (Hanson and Stevens 2009) ultimately led to the successful structure determination of the $S1P_1$ receptor in its antagonist-bound state (Hanson et al. 2012). The details of this structure along with models of interactions for other ligands of various classes that bind to the $S1P_1$ receptor will be discussed in this chapter.

2 Structural Analysis of the $S1P_1$ Receptor

2.1 Structure Determination Process

The structural characterization of the $S1P_1$ receptor was initiated after early success in the GPCR family on the β_2AR (Cherezov et al. 2007) and adenosine A_{2a} receptor (Jaakola et al. 2008). Both of these receptors employed the strategy of replacing one of the intracellular loops (ICL3) with a small soluble protein domain derived from T4-lysozyme (T4L) to facilitate crystal contact formation (Rosenbaum et al. 2007). A similar strategy was employed for the $S1P_1$ receptor.

Full-length wild-type and T4L-fused receptor each with an N-terminal Flag epitope tag (to assess receptor expression levels) and a C-terminal 10x histidine tag (to facilitate receptor purification) proved intractable to further structural studies due to formation of higher-order oligomers. A serial deletion of the C-terminus of the receptor in four residue increments resolved this issue (Fig. 2a). Small-scale expression studies of this family of constructs revealed a dramatic improvement in reducing the oligomeric state of the receptor after extraction and purification as the C-terminus was shortened.

After preliminary crystallization trials failed in lipidic cubic phase (LCP), further rounds of construct optimization were initiated. A series of combinatorial adjustments of the insertion point for the T4L fusion partner were tested for expression and stability in the presence and absence of ligand (Fig. 2b). Extraction and purification of each construct with and without the small molecule antagonist W146 (Sanna et al. 2006) (later renamed ML056 to avoid confusion with amino acid abbreviations) followed by analysis with size exclusion chromatography (SEC) (the position and width of an SEC peak gives a good indication of oligomeric state and sample homogeneity) showed a significant effect on stability as a function of fusion insertion point. The optimal construct was selected based on its SEC profile after heating (which should remain unchanged relative to unheated samples) in the absence of ligand and subsequently scaled up for crystallization trials.

The final crystallization construct was incorporated into the baculovirus genome which was then amplified to obtain a high-titer stock of recombinant baculovirus for infection of large-scale expression batches of Sf9 insect cells, to ultimately generate sufficient protein for crystallographic studies after purification. Extraction of the receptor from the insect plasma membrane was performed using high concentrations of dodecyl maltoside detergent. Purification of the recombinant $S1P_1$ receptor was facilitated by the presence of the 10x histidine tag on the C-terminus of the receptor which was utilized for binding to immobilized metal affinity resin in a single-step chromatography protocol. After purification, the protein was concentrated to approximately 50 mg/mL. Receptor extraction, purification, and concentration were performed in the presence of saturating amounts of ML056.

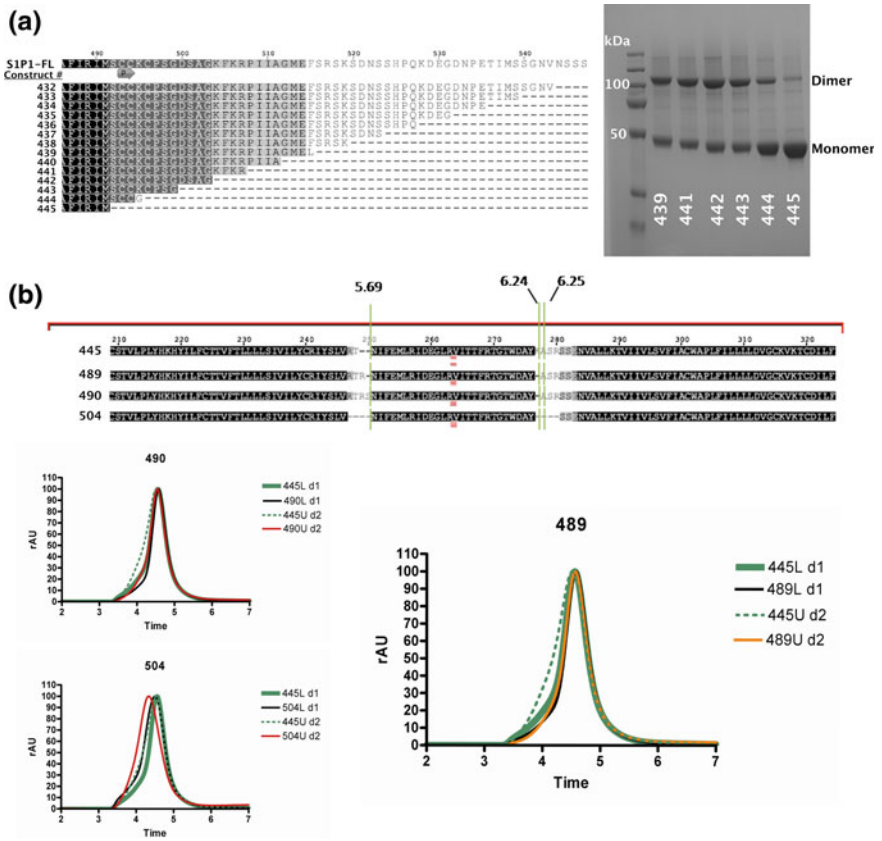

Fig. 2 Select data from the protein chemistry and construct design effort for the $S1P_1$ receptor. **a** A series of C-terminal truncations serially removing four amino acids at a time. The presence of the dimer in the SDS-PAGE gel was reduced and then disappeared for the optimal constructs 444, 445. **b** SEC stability analysis of junction adjustment mutations both with ligand (L) and without ligand (U). Each construct was compared to 445 and the quality was assessed based on peak profile without ligand after two days at 4 °C

Crystallization of the receptor was attempted using the LCP approach where protein samples of $S1P_1$ receptor in complex with ML056 were reconstituted into monoolein which when hydrated forms a cubic mesophase capable of supporting crystallization of a wide variety of membrane proteins (Landau and Rosenbusch 1996; Caffrey 2000; Cherezov 2011). Resulting crystals were harvested directly from LCP matrix and flash frozen in liquid nitrogen for diffraction analysis.

X-ray diffraction data for approximately 400 crystal samples were collected and ultimately used in the final dataset. Due to the rapid onset of radiation damage, data collection was limited to a maximum of 6° oscillation per crystal of which only the first 1–2 frames diffracted to the maximum resolution. Data were processed using a novel method (Hanson et al. 2012) to extract individual reflections

from decayed images and the structure was solved and refined by standard methods to 2.8Å. The final coordinates were deposited in the protein data bank under accession number 3V2Y. The supplemental material associated with the original publication of the $S1P_1$ receptor structure contains complete details on the structure solution process (Hanson et al. 2012) which has since been used effectively in one other published GPCR structure determination effort (Hollenstein et al. 2013).

2.2 Structural Characterization

We will discuss in detail the extracellular, transmembrane, and ligand binding regions of the $S1P_1$ receptor in this section. The lack of resolved intracellular loops with the exception of ICL1 combined with the presence of the T4L fusion protein inserted in ICL3 precludes any in-depth analysis of the intracellular region of the receptor.

2.2.1 Transmembrane Region

From a global perspective, the $S1P_1$-T4L receptor structure (referred to as the $S1P_1$ receptor for the remainder of the chapter) shares many common features with previous and subsequently determined receptors, including seven-transmembrane helices arranged in a structurally conserved bundle, and similar length and orientation of intracellular loop one. However, there are some important differences associated with the $S1P_1$ receptor structure and presumably the family that lends itself to binding of its endogenous lipid ligand. We will examine these differences in more detail beginning with an analysis of the transmembrane region core.

In a recent review, Venkatakrishnan and colleagues examined conserved contact points among all of the GPCR structures determined to date (Venkatakrishnan et al. 2013). These contact points serve as a scaffold from which the diversity of the GPCR family is built and are used here to facilitate the comparison between the $S1P_1$ receptor and other human class A GPCR structures. We use the core residues as a template for overlaying each of the human class A inverse agonist structures with the $S1P_1$ receptor. The set was limited to restrict the interpretation to a single species and pharmacological state. The root mean square deviation (RMSD) for just the Cα atoms after aligning with the $S1P_1$ receptor shows a significantly improved RMSD for the core residues compared to the entire transmembrane region (Fig. 3b). This type of overlay provides a superior template for visually assessing structural differences between the receptors' transmembrane regions (Fig. 3a). Calculation of the root mean square fluctuation allows mapping of the structural deviations across the receptor set in order to understand the regions responsible for conveying the structural changes associated with recognition of ligand diversity across the family (Fig. 3c).

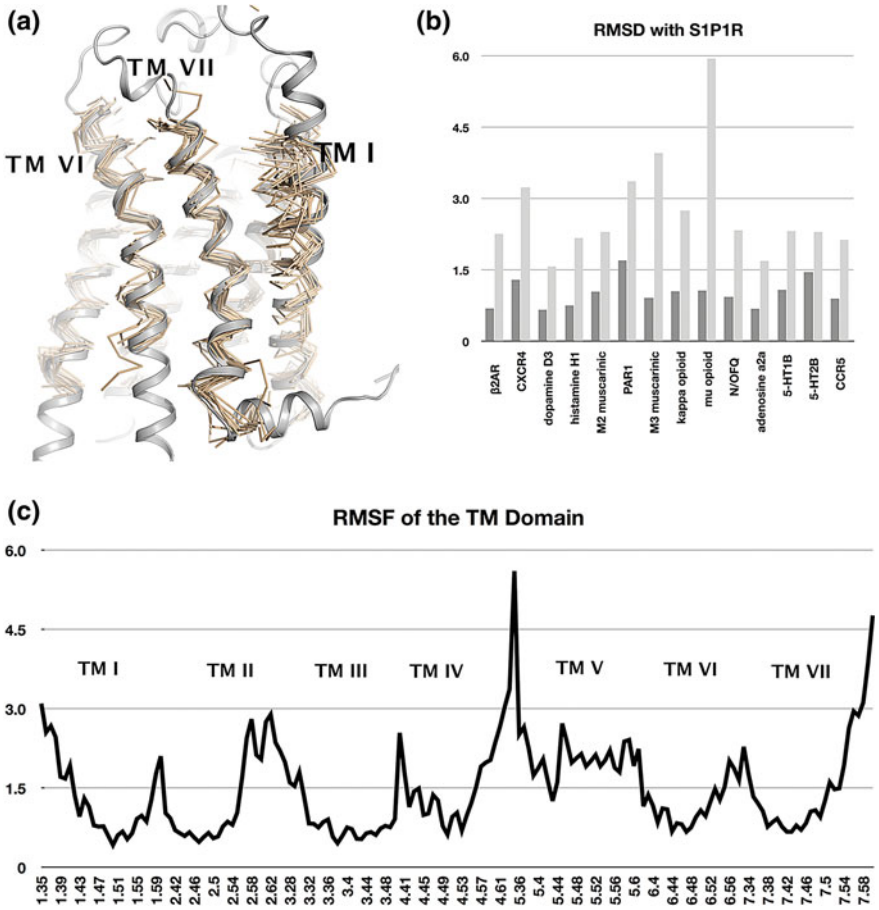

Fig. 3 Analysis of the structural differences in the transmembrane (*TM*) region over the entire set of class A human GPCRs in the inverse agonist state. **a** Structural overlay focusing on *TM VI*, *TM VII*, and *TM I*. The shift in *TM I* for the $S1P_1$ receptor could result in a larger gap between *TM I* and *TM VII* and is the largest helical divergence for the receptor. **b** Plot of the RMSD values of the receptor family in comparison with $S1P_1$ receptor. The *dark gray* values are core RMSD, and the *light gray values* are transmembrane domain RMSD. Both are comparing only Cα atoms. **c** Graph of the root mean square fluctuation over the set of GPCRs

The position of the core contact residues are listed in Table 1 in their Ballesteros–Weinstein notation as well as residue number associated with the $S1P_1$ receptor (Vroling et al. 2011). These contact positions are conserved throughout the determined class A structures and serve as an anchor point for our analysis of the bioactive lipid binding receptors as well as other class A GPCRs although the sequence identity at some of the positions can vary significantly (Table 1). These core contact residues can be further grouped based on spatial proximity to each other and the helices that are constrained by them. For instance, one cluster of core

Table 1 Core and binding pocket residues within the Edg family including LPA 1–3

BW	Group	Cluster	S1P1R Residue	S1P1R	S1P2R	S1P3R	S1P4R	S1P5R	LPA1R	LPA2R	LPA3R	Edg Entropy	Dass A entropy
1.46	1	1	59	I	I	I	V	I	I	V	I	0.8	2.4
1.49	2	1	62	E	E	E	E	E	A	T	S	1.5	1.8
1.50	3	1	63	N	N	N	N	N	N	N	N	0.0	0.1
1.53	4	1	66	V	V	V	V	V	V	V	V	0.0	2.0
1.57	5	1	70	I	V	I	I	L	I	I	V	1.3	3.0
2.42	6	2	83	F	F	F	C	L	L	L	L	1.4	2.6
2.43	7	1	84	I	L	I	L	L	M	L	L	1.3	2.5
2.44	5	1	85	G	G	G	V	G	A	G	A	1.3	3.2
2.47	3 | 4	1	88	A	A	A	T	T	A	A	A	0.8	1.9
2.50	3	1	91	D	D	D	D	D	D	D	D	0.0	1.0
2.57	NA	BP	98	Y	F	Y	Y	Y	Y	Y	Y	0.5	3.1
2.60	NA	BP	101	N	N	N	N	N	L	L	L	1.0	4.0
3.25	NA	BP	117	W	W	W	W	W	W	W	W	0.0	0.9
3.28	NA	BP	120	R	R	R	R	R	R	R	R	0.0	3.8
3.29	NA	BP	121	E	E	E	E	E	Q	Q	Q	1.0	3.9
3.32	NA	BP	124	M	A	M	L	V	I	L	L	2.2	3.7
3.33	NA	BP	125	F	F	F	F	F	D	D	D	1.0	3.8
3.36	9	3 |BP	128	L	L	L	L	L	L	L	L	0.0	3.6
3.38	10	2	130	A	A	A	A	A	A	A	A	0.0	2.8
3.40	11	3	132	V	V	T	T	V	V	V	L	1.3	2.6
3.44	12	4	136	L	L	L	L	L	L	L	L	0.0	2.9
3.46	6	2	138	I	I	I	T	I	I	I	I	0.5	2.0
3.47	13	4	139	A	A	A	A	A	A	A	A	0.0	1.7
3.51	14	4	143	Y	H	H	F	S	H	H	H	1.5	1.8
4.50	10	2	168	W	W	W	W	W	W	W	W	0.0	0.3

(continued)

Table 1 (continued)

BW	Group	Cluster	S1P1R Residue	S1P1R	S1P2R	S1P3R	S1P4R	S1P5R	LPA1R	LPA2R	LPA3R	Edg Entropy	Dass A entropy
4.53	8 110	2	171	s	S	A	A	s	A	A	A	1.0	2.6
4.57	8	2	175	G	G	G	G	G	G	G	G	0.0	3.2
5.40	NA	BP	203	I	V	I	I	V	L	L	L	1.6	3.9
5.43	NA	BP	206	C	V	C	C	C	W	W	W	1.4	3.9
5.44	NA	BP	207	T	V	I	L	V	A	A	T	2.3	3.6
5.47	NA	BP	210	F	F	F	F	F	N	S	N	1.3	1.9
5.54	12 l 15	4	217	I	I	I	I	I	M	M	M	1.0	2.7
5.57	13 l 14	4	220	L	L	L	L	L	L	V	V	0.8	2.8
5.60	14	4	223	R	R	R	A	R	H	R	R	1.1	3.6
6.41	15	4	262	L	L	V	L	L	L	L	L	0.5	2.8
6.44	11	3	265	F	F	F	F	F	F	F	F	0.0	2.0
6.47	16	3	268	C	C	C	C	C	C	C	C	0.0	1.6
6.48	9	3 l BP	269	w	w	w	W	w	w	w	w	0.0	1.9
6.51	17	3 l BP	272	L	A	L	L	L	G	G	G	1.4	2.7
6.55	NA	BP	276	L	L	F	L	L	L	L	L	0.5	4.0
7.38	17	3	296	F	F	F	I	F	F	F	F	0.5	3.4
7.39	17	3l BP	297	L	F	I	L	L	L	L	L	1.1	3.8
7.42	NA	BP	300	A	S	A	A	A	A	A	A	0.5	2.9
7.43	NA	BP	301	V	T	V	V	M	E	E	L	2.2	3.4
7.45	16	3	303	N	N	N	N	N	N	N	N	0.0	2.2
7.46	3	1	304	S	S	S	S	S	S	S	S	0.0	2.2
7.47	1	1	305	G	L	A	A	L	A	L	V	1.8	3.0
7.50	2	1	308	P	P	P	P	P	P	A	P	0.5	0.6
7.53	7	1	311	Y	Y	Y	Y	Y	Y	Y	Y	0.0	0.8

The positions are classified according to the contact groups (1–4 and BP), and the groups are further clustered to discrete areas of the 7-TM helical domain. Sequence entropy is shown across the set of residues for the family indicating the highly conserved core residues in the family as well as for the class A overall

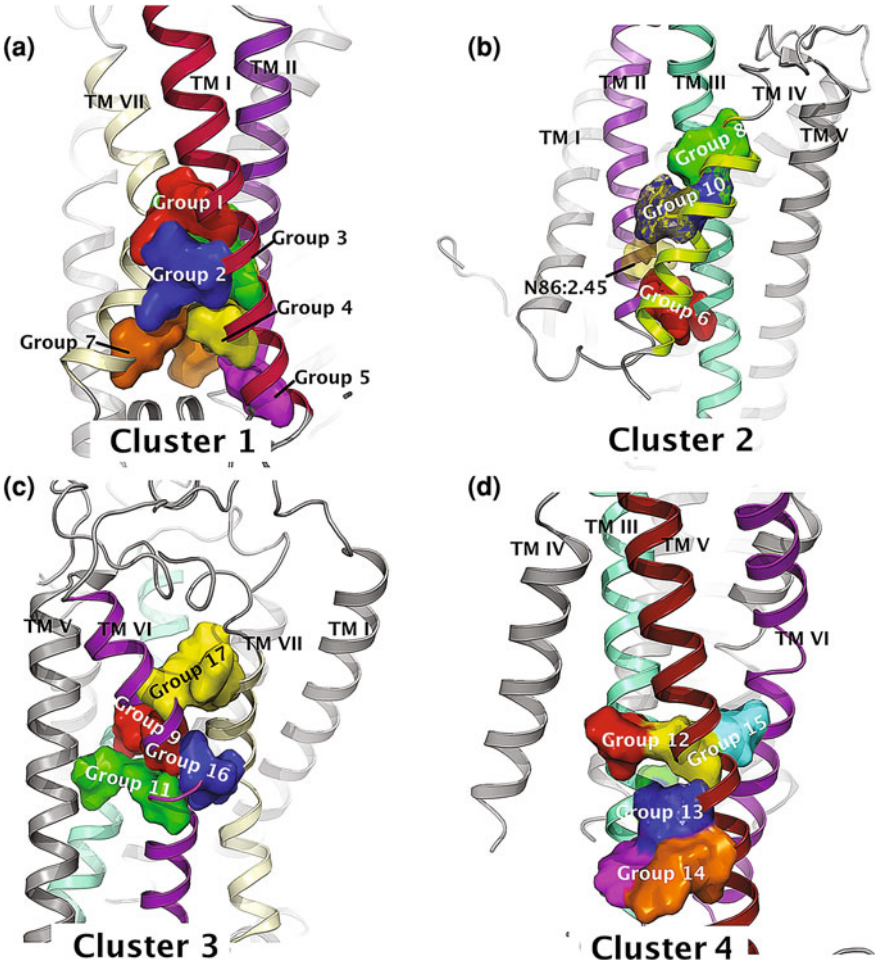

Fig. 4 Structural view of the clusters of core residues as defined in Table 1. Each cluster comprises a number of interacting groups that fit together to form a rigid scaffold. These clusters are distributed throughout the receptor helical bundle and help define the structural characteristics of the GPCR family

contacts links transmembrane (TM) I, II, and VII (Fig. 4a), cluster 1 consists of individual interaction groups 1, 2, 3, 4, 5, and 7. The second cluster of interactions links TM II, III, and IV through a series of four interaction groups 6, 8, 10 and a frequently observed interaction between position 2.45 and 4.50. These two positions interact through a hydrogen bond between an Asn (found in 60 % of class A GPCRs) or Ser (30 %) at 2.45 and the indole nitrogen of a Trp (found in 94 % of class A GPCRs) at position 4.50 (Fig. 4b).

A third cluster of conserved contacts links together TM helices III, VI, and VII in the vicinity of the $S1P_1$ receptor ligand binding pocket (Fig. 4c). These

positions maintain important contacts between TM VI and TM III through two side chain-mediated interactions. The sequence conservation across the lipid binding receptors is shown in Table 1. Position 3.36 which is a Leu in the $S1P_1$ receptor as well as 94 % of Edg family receptors interacts with the well-characterized Trp toggle switch at 6.48. In the Edg family, this location is 100 % conserved over all orthologs and homologs. Both these amino acids are adjacent to the canonical class A orthosteric binding pocket and as such contribute significant interactions to ligand binding. In contrast to the conservation of position 6.48 across class A GPCRs, position 3.36 is quite variable and has one of the highest sequence entropies of all of the core positions (Table 1). Importantly, the contrast between sequence entropy within the Edg family and overall entropy is the highest implicating this position as a major determinant of ligand specificity. It is reasonable based on the proximity of this cluster to the ligand binding pocket that ligand interactions also affect the strength of interaction between TM III and VI creating a potential linchpin for pharmacological activity at this position. Immediately below this important interaction is a second pair of residues whose conformation is often linked to receptor pharmacology (Fig. 4c). At the 3.40 and 6.44 positions are Val132 and Phe265; Val is conserved at 3.40 in the Edg family almost 70 % of the time, whereas overall class A receptors Val is found only 19 % of the time with Ile being the dominant amino acid. Phe is 100 % conserved at 6.44 in the Edg family and 77 % overall for class A receptors. It is likely based on this analysis that the interactions provided by this cluster serve an important role in maintaining the appropriate conformation of TM VI and that van der Waals interactions are the primary means by which this is achieved. This creates an environment sensitive to the shape and character of the binding pocket itself and by extension the nature of the bound ligand. During conformational switching, these contacts are maintained forcing a translation of small perturbations in the ligand binding pocket to the intracellular region by a series of compensatory movements. In $S1P_1$ receptor SAR, it is known that the conformational switch between the antagonist state of the receptor and agonist state is driven by volume changes within the binding pocket (Davis et al. 2005). On the other side of TM VI, there are two groups of interactions that hold the top of TM VI and VII together (Fig. 4c). The first group consists of three interacting residues, Leu272:6.51, Phe296:7.38, and Leu297:7.39 which form a tight van der Waals cluster that is mediated mainly through mainchain and Cβ atoms and therefore relatively independent of the identity of the residue. The second group consists of Cys268:6.47 and Asn303:7.45. This interaction is dependent on side chain proximity and probably serves an important role in positioning the Asn residue for interacting through a hydrogen bond with the indole nitrogen of Trp269(6.48) (Fig. 4c). Asn is conserved in this position in 51 % of class A GPCRs and as an Asn, His, or Ser in 83 % of class A GPCRs, indicating that the ability of this position to serve as a hydrogen bond acceptor or donor is important for $S1P_1$ receptor and GPCR function in general. This position also hydrogen bonds with a conserved network of tightly bound water molecules that play a role in the activation mechanism of GPCRs. However, the water network was not observed in the $S1P_1$ receptor

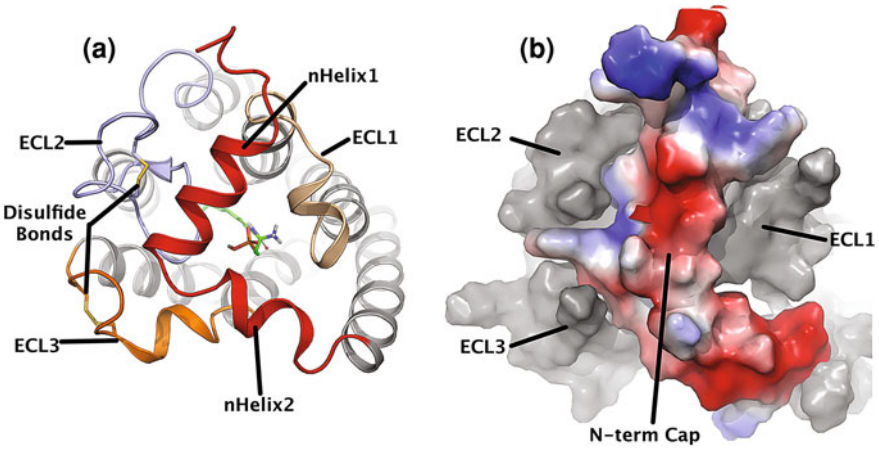

Fig. 5 Overview of the extracellular region of the $S1P_1$ receptor as viewed from the extracellular space looking down on the plane of the membrane. **a** Ribbon diagram representation of the extracellular region with the N-terminal capping helices and loops marked along with the disulfide bonds in ECL2 and ECL3. **b** Surface representation of the same view. The N-terminal capping helix packs tightly with the extracellular loops and prevents access of the ligand into the binding pocket directly from the extracellular space

structure, probably due to resolution limitation. Finally, cluster number four consists of three interactions that constrain TM V relative to TM III and one interaction between TM V and TM VI (Fig. 4d). At the bottom of the TM III/TM V interface there are a series of interactions mediated by three residues. Arg223:5.60 forms a hydrogen bonding interaction with Tyr143:3.51 (Fig. 4d). An Arg residue at position 5.60 is highly conserved in the Edg family and somewhat conserved throughout the GPCR class A superfamily at 72 and 26 %, respectively. The lone interaction connecting TM V to TM VI occurs in this cluster and is mediated by a van der Waals contacts from the side chain of the amino acid at position 6.41 to the Cβ and mainchain atoms of position 5.54. Position 6.41 is a Leu in the case of $S1P_1$ receptor and 90 % of Edg receptors, whereas it is a conserved as a Leu in 14 % of class A GPCRs. The most common residues in this position across the class A family are similar in hydrophobic side chain bulk with Val, Met, Leu, and Ile representing 82 % of class A GPCRs. The sequence conservation as function of Ballesteros–Weinstein position are determined over all species using the 7-TM explorer website http://gris.ulb.ac.be/cgi-bin2/xplor.py (Van Durme et al. 2006).

2.2.2 Extracellular Region

The extracellular region for all GPCRs consists of three loops: ECL1 between TM helices II and III, ECL2 between TM helices IV and V, and ECL3 between TM

helices VI and VII. Optionally, there is a structured N-terminus that interacts with the ECLs such as with rhodopsin, CXCR4, CCR5, and $S1P_1$ receptors (Fig. 5) (Palczewski et al. 2000; Wu et al. 2010; Hanson et al. 2012; Tan et al. 2013). In the case of the chemokine receptors CXCR4 and CCR5, this structured N-terminus participates in important interactions with the chemokine ligands. In the case of rhodopsin and the $S1P_1$ receptor the structured N-terminus occludes the binding pocket, in the antagonist-bound state, cutting off access to the extracellular milieu (Fig. 5b). One possible role for this structured N-terminus is that it is a feature of the $S1P_1$ receptor structure in general and its presence implies the ligand does not access the binding pocket from the extracellular space directly. Instead, it is possible that the ligand gains access to the binding pocket through the lipid membrane where there is an enlarged gap between TM I and TM VI. This gap is larger in the $S1P_1$ receptor than other class A GPCRs largely due to a shift in the position of the extracellular end of TM I away from TM VII in the $S1P_1$ receptor (Fig. 2a). This certainly makes sense in that the endogenous ligand is a lipid, more at home in a plasma membrane than the aqueous environment of the extracellular space. It is likely that access to the binding pocket is achieved by initial partitioning of the ligand into the plasma membrane where it then enters through an access channel formed by gaps in the apical section of the transmembrane bundle (Filipek et al. 2003; Schadel et al. 2003; Hurst et al. 2010, 2013). In addition, the limited access to the ligand binding site from the extracellular region is also supported from ligand binding pharmacology where $S1P_1$ receptor ligands, including S1P itself, show slow saturation of receptor binding in the presence of excess ligand as well as slow off-rates (Rosen et al. 2009).

2.2.3 Ligand Binding Region

The general region of the orthosteric binding pocket is roughly the same across the GPCR class. However, the details of binding within this region diverge considerably for different receptors and ligands (Katritch et al. 2012). Residue positions involved in binding pocket interactions are largely preserved but with each receptor class interacting specifically with a subset of approximately half of the potential contacts in the ligand binding cradle (Venkatakrishnan et al. 2013). The $S1P_1$ receptor provides 18 residues from the transmembrane region for interactions with the ML056 antagonist along with three additional residues from ECL2 and two from the N-terminus, which are not factored into the referenced analysis (Table 1, Fig. 6).

ML056 lies in an amphipathic pocket where the head group phosphonate interactions are largely polar in nature and the alkyl chain tail interactions are largely hydrophobic as would be expected (Fig. 6b and c). The polar interactions observed for ML056 largely confirm mutagenesis data establishing the importance of Arg120:3.28 and Glu121:3.29 which were identified as important residues for supplying interactions with the zwitterionic sphingosine head group (Parrill et al. 2000b; Jo et al. 2005). In addition, the phosphonate head group of ML056 is

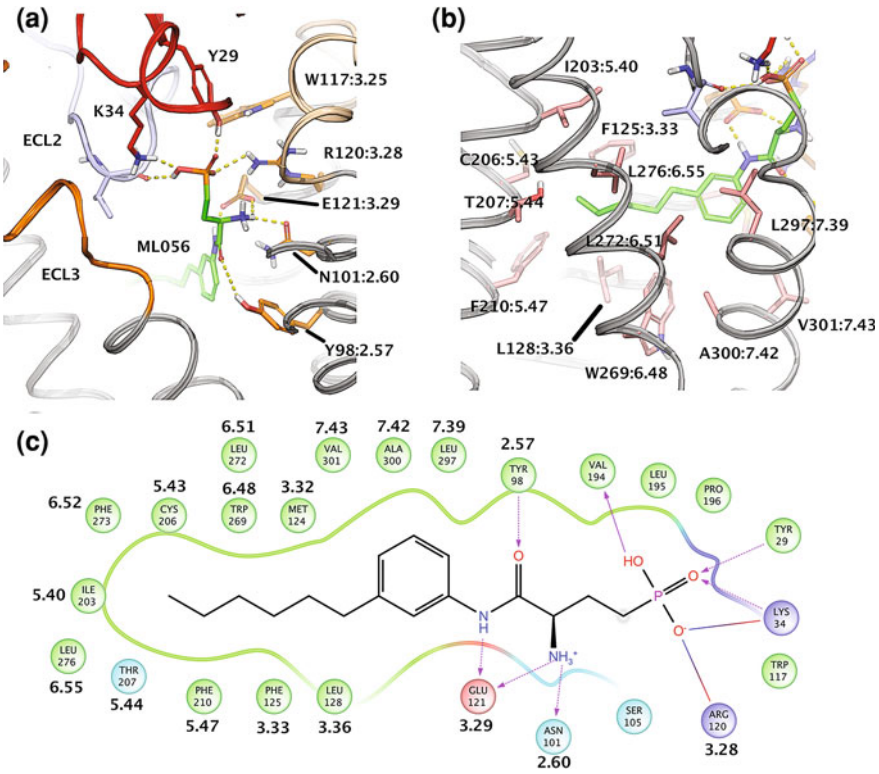

Fig. 6 Detailed $S1P_1$ receptor ligand binding pocket interactions. ML056 is colored with *green carbons*. Interacting residues are rendered as sticks and colored according to region and binding pocket location. **a** Polar binding interactions are shown as *orange carbons* from the transmembrane region and *blue* or *red carbons* from ECL2 and N-terminus, respectively. **b** Hydrophobic interactions are shown as *pink carbons*. Residue labels designate the amino acid, $S1P_1$ receptor number and Ballesteros–Weinstein index after the colon. **c** ML056 ligand interaction diagram showing all of the residues that are within 4Å of the ligand position in the structure

surrounded by a ring of positively charged and polar residues contributed by TM helices III and VII, ECL2, and the N-terminal capping helix. Together these residues form a pocket that provides charge complementarity and high-affinity interactions to the phosphate group of the sphingolipids (Fig. 6a). A feature of ML056 is a primary amine located in the beta position relative to the phosphonate group. This primary amine is likely protonated and charged at physiological pH, thus enhancing its interactions with Glu121:3.29 through salt bridge formation. In addition to Glu121, Asn101:2.60 and Tyr98:2.57 provide hydrogen bonding interactions with the primary amine and amide linkage of ML056, respectively (Fig. 6a). The phenyl aryl tail of ML056 inserts into a hydrophobic pocket consisting of residues from TM helices III, V, VI, and VII, as well as ECL2 (Fig. 6b).

The pocket is lined with short aliphatic residues that define the shape and hydrophobicity of the pocket and four aromatic residues that provide the potential for specific interactions (Fig. 6c). The importance of some of these residues in the binding and signaling of the $S1P_1$ receptor was determined previously through molecular modeling and mutagenesis; however, it is important to keep in mind that the antagonist interactions may differ from agonist interactions, and indeed, it has postulated that the antagonist aryl chain occupies a discrete pocket compared to the endogenous ligand (Parrill and Tigyi 2013).

3 Analyses of $S1P_1$ Receptor Ligands

The $S1P_1$ receptor represents the first example of a lipid binding GPCR being structurally determined. A good deal of biochemical and biophysical characterization of this receptor predated the actual structure solution. This type of information is critical for understanding and interpreting the experimental electron density maps and together they provide an important framework for analyzing additional compounds that bind to the $S1P_1$ receptor. We review here an analysis of $S1P_1$ receptor ligand space where we utilize the coordinates of the $S1P_1$ receptor structure bound to ML056 to predict the binding poses of naturally occurring and synthetic antagonists and agonists.

3.1 $S1P_1$ Receptor Antagonists

Perhaps the most straightforward extrapolation of the structural information is in using the receptor to analyze the binding mode of pharmacologically similar agents. In the case of the $S1P_1$ receptor, we will analyze the antagonist space first as it requires little additional manipulation of the model to facilitate the discussion. The antagonist space has received little attention for the $S1P_1$ receptor until quite recently due in part to the demonstrable success of agonists for immunomodulation as well as early reports of antagonists causing significant capillary leakage in vivo along with a dose competitive reversal of lymphopenia (Sanna et al. 2006). Antagonists were developed mainly as tool compounds, however, there were numerous reports on the application of certain antagonists for the $S1P_1$ receptor typically for inhibition of angiogenesis and recent findings suggest that sufficiently potent antagonists have the same pharmacological effect on lymphocytes as agonists, a finding which may renew interest in developing pharmaceuticals that block the $S1P_1$ receptor for immunomodulation in autoimmune indications such as rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis.

3.1.1 Alkyl Phenyl Amide Phosphonates and Structural Analogs of FTY720-P

Initial efforts at developing antagonists for the S1P receptor family focused on generating structural analogs of the agonist FTY720-P, a phosphorylated and pharmacologically active metabolite of FTY720 (fingolimod) (see Sect. 3.2.2 for a description of FTY720-P) (Davis et al. 2005). These efforts resulted in antagonists with a very steep SAR profile and specificity for the $S1P_1$ and $S1P_3$ receptors. It was found during the course of this development effort that substitution around the central phenyl ring dictated the pharmacology observed for the compounds. For instance, para-substitution around the phenyl ring generated agonists for the $S1P_1$ receptor, whereas meta-substitution coupled with progressive shortening of the aryl chain generated antagonists with varying degrees of receptor subtype specificity. Interestingly, meta-substituted 10 carbon aryl chain compound (VPC23069) possessed agonist pharmacology, whereas the 8 (VPC23019) and 6 (VPC23031) carbon aryl chains did not. Thus increasing hydrophobic volume on a meta-substituted compound or employing para-substitution could convert antagonist compounds into agonist (Davis et al. 2005). This family of compounds is similar to the ML056 antagonist and the SAR for the VPC series can be used to generate receptor models capable of docking agonist molecules for analysis. Indeed, this series of compounds was used to generate an agonist model of compound binding through induced fit docking protocols as reported in the initial structural analysis of the $S1P_1$ receptor (Sherman et al. 2006; Hanson et al. 2012).

Analysis of the molecular interactions for VPC23019 after docking into the ML056-$S1P_1$ receptor binding pocket shows a similar orientation compared to ML056 itself (Fig. 6). This is not unexpected as the compounds share a high degree of similarity, the main difference being a two-carbon extension of the meta-substituted aryl chain of VPC23019 compared to ML056, which is easily accommodated in the antagonist binding pocket.

More recently, the SAR around the hydrophobic region of the $S1P_1$ receptor binding pocket, head group vinyl phosphonate analogs of FTY720-P showed pan antagonism for the S1P receptors on calcium mobilization assays, indicating that the modifications around the head group region can also have an effect on the exhibited pharmacology of the compounds. Interestingly, these compounds were still active on an extracellular signal-regulated kinase (ERK) phosphorylation assay raising the possibility of pathway bias (Valentine et al. 2010).

3.1.2 Non-lipid Antagonists

Recently, three lead-like non-lipidic compounds have been characterized as antagonists of the $S1P_1$ receptor (Urbano et al. 2013). The first to be reported is a series of dipeptide, proline, triazole compounds that were optimized from a screening effort at Exelixis (Ibrahim et al. 2012). These compounds were shown to have in vivo efficacy against tumor growth with oral administration of mice

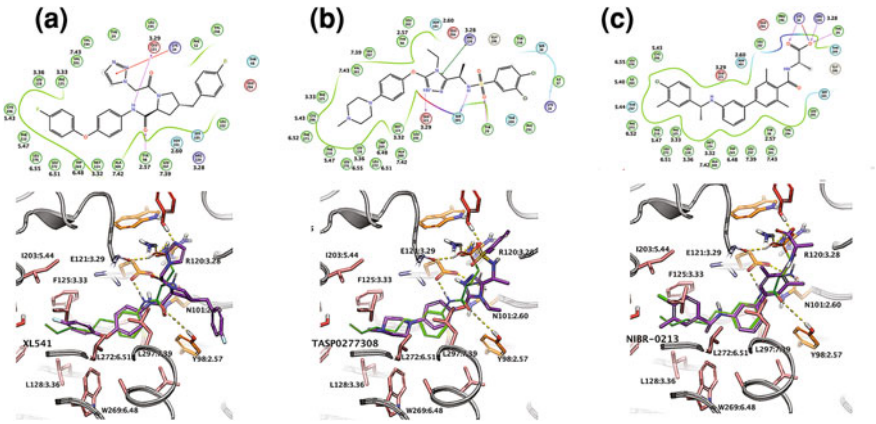

Fig. 7 Proposed molecular interactions for three antagonists docked into the ML056 binding pocket. Each antagonist is represented as a ligand interaction diagram showing all of the residues in the $S1P_1$ receptor binding pocket within 4Å of the docked pose of the ligand (*top*) and structural interactions of each ligand (*purple carbons*) compared to ML056 (*green carbons*) (*bottom*). **a** Docking analysis of XL541. **b** Docking analysis of TASP0277308. **c** Docking analysis of NIBR-0213

implanted with MBA- MB-231T breast adenocarcinoma xenografts. Evaluation in higher order species demonstrated promising pharmacokinetic profiles in rat, dog, and cynomolgus monkey (Ibrahim et al. 2012).

The primary example of this compound series XL541 features a central tri-substituted proline ring with a 1,2,3-triazole substituent extending into the anionic binding region of the polar pocket (Fig. 7a). This triazole moiety provides the main ionic interactions driving compound binding while two additional substituents, a fluorophenyl and an oxydibenzene moiety, fill the putative access channel and hydrophobic tail region, respectively. An amide linkage to the oxydibenzene group presents the amide carbonyl functionality as a hydrogen bond acceptor for Tyr98:2.57 and the amide nitrogen as a hydrogen bond donor for Glu121:3.29 (Fig. 7a). The first ring of the oxydibenzene system is positioned similarly to the ML056 phenyl ring, whereas the terminal phenyl ring is positioned to interact with Phe125:3.33 (Fig. 7a).

The second series of antagonists under this category was discovered from a screening deck without optimization and reported to bind to the $S1P_1$ receptor with a low nM IC50. This compound, TASP0277308, showed efficacy in a mouse collagen-induced arthritis model (Fujii et al. 2012a) and induces lymphopenia while suppressing swelling leukocyte infiltration and hyperplasia in a mouse contact hypersensitivity model (Fujii et al. 2012b). Finally, this antagonist has been tested and has shown efficacy for inhibition of VEGF-induced endothelial tube formation in vitro and suppressed tumor cell-induced angiogenesis in vivo (Fujii et al. 2012c).

The molecular interactions for TASP0277308 follow a similar pattern as other antagonists in the presentation of a polar anion to the phosphonate binding region of the ML056 pocket. In the case of TASP0277308, a sulfonamide moiety linked to a dichlorophenyl ring interacts at the top of the binding pocket. In addition, a 1,2,4-triazole acts as a protonated positively charged central ring which interacts through charge coupling with Glu121:3.29 (Fig. 7b). Finally, a para-substituted phenylpiperazine ring system fills the hydrophobic binding pocket with the phenyl ring lining up roughly with the ML056 phenyl and the piperazine ring interacting with Phe125:3.33 (Fig. 7b).

Finally, a series of N-biaryl(hetero) arylsulfonamide compounds originally reported in the patent literature and later optimized for pharmaceutics properties to produce NIBR-0213 (Berst et al. 2007; Quancard et al. 2012) have shown comparable efficacy to FTY720-P in mouse experimental autoimmune encephalomyelitus) models of human multiple sclerosis.

The molecular interactions for a docked NIBR-0213 which presents a carboxyl group as an anion for interacting with Arg120:3.28, Lys34 and Tyr29 (Fig. 7c). There are no predicted polar interactions with Glu121:3.29 or Asn101:2.60 which may be an important route for improving the potency of this compound series. This series of compounds features a meta-substituted phenyl ring that is predicted to bind in a similar position as the phenyl ring of ML056 (Fig. 7c) and a terminal 1-chloro-2-methylbenzene ring that interacts with Phe125:3.33 (Fig. 7c).

Together these data suggest that antagonists targeting the $S1P_1$ receptor may have some benefit for indications involving immunomodulation, angiogenesis, and pain modulation (Welch et al. 2012), without the cardiac side effects observed with all of the $S1P_1$ receptor agonists to varying degrees (Gergely et al. 2009; Schmouder et al. 2012; Fernandez et al. 2012; Zipp et al. 2012). However, it does appear that the antagonist class carries a greater burden of increased vascular permeability which may manifest as lung or macular edema (Sanna et al. 2006; Cahalan et al. 2013). The molecular interactions predicted for each of these antagonist compounds tracks closely with those of ML056 itself. Particularly important is the presence of a central aromatic ring with the correct orientation of substituents to mimic the meta-substitution pattern of ML056. This ensures adequate spacing between the ligand and Trp269:6.48 while maintaining hydrophobic or aromatic interactions with the rest of the lipid tail binding pocket. The final component is the correct presentation of polar or charged groups for interacting with the polar binding region of the binding pocket. Although occupation of all of these polar sites is not necessary, provided good van der Waals interactions are achieved in the lipid binding region.

3.2 $S1P_1$ Receptor Agonists

The initial discovery of the Edg family of receptors and subsequent characterization of their endogenous ligands and role in the immune response, triggered

development efforts for $S1P_1$ receptor agonists in the treatment of immune-mediated pathologies beginning with solid organ transplant rejection through the approval of the non-selective S1P receptor prodrug agonist fingolimod for treatment of relapsing multiple sclerosis. Recently, a number of second-generation compounds with enhanced selectivity and pharmaceutics properties relative to fingolimod have entered the development and clinical trial pipeline (O'Sullivan and Dev 2013). These compounds can be roughly categorized into two types depending on their reliance on sphingosine-like head group interactions (class 1) or their independence on such interactions (class 2) (Hanson et al. 2012). We will examine the putative modeled binding mode for three agonist compounds, including the natural ligand S1P, using the antagonist-bound structural coordinates along with available mutagenesis data as a starting point.

3.2.1 Sphingosine 1-Phosphate

The endogenous signaling molecule for the S1P family of receptors, the zwitterionic lipid S1P is an important component of biological membranes and has evolved as a highly versatile signaling molecule regulating many cell responses such as proliferation (Zhang et al. 1991), apoptosis (Cuvillier et al. 1996), differentiation, migration (Hobson et al. 2001; Paik et al. 2001) and also immunological responses (Huwiler et al. 2000; Spiegel and Milstien 2003). Dysregulation of S1P itself has been implicated in a multitude of disease states including Alzheimers (Takasugi et al. 2011), pain (Coste et al. 2008; Welch et al. 2012), multiple sclerosis (Kułakowska et al. 2010), diabetes (Whetzel et al. 2006), and cancer (Xia et al. 2000; Ogretmen and Hannun 2004; LaMontagne et al. 2006; Visentin et al. 2006; Pyne and Pyne 2010) among others (O'Sullivan and Dev 2013). The lipid is generated from sphingomyelin as part of the sphingomyelin cycle which involves generation of ceramide, sphingosine, and finally sphingosine 1- phosphate (Fyrst and Saba 2010). S1P elicits its effect primarily through its actions on five S1P receptors (S1P1,2,3,4,5) (Huwiler and Pfeilschifter 2008). Analysis of the potential binding mode of S1P in the $S1P_1$ receptor binding pocket will serve as a useful entry point for discussion of the potential differences and similarities between antagonist and agonist binding.

Modeling efforts to predict the binding mode of the endogenous ligand for $S1P_1$ receptor have been ongoing prior to the solution of the receptor structure with varying degrees of success (Parrill et al. 2000a, b; Wang et al. 2001; Lim et al. 2004; Holdsworth et al. 2004; Inagaki et al. 2005; Deng et al. 2007; Pham et al. 2008a; Schürer et al. 2008; van Loenen et al. 2011). These modeling efforts have recently been reviewed in comparison with the antagonist-bound $S1P_1$ receptor structure (Parrill and Tigyi 2013).

Using the structurally derived binding pocket as a starting point coupled with induced fit docking, there are essentially two possibilities for the orientation of the S1P molecule within the $S1P_1$ receptor. The first possibility is that binding of long acyl chains is accommodated within the antagonist binding pocket or agonist

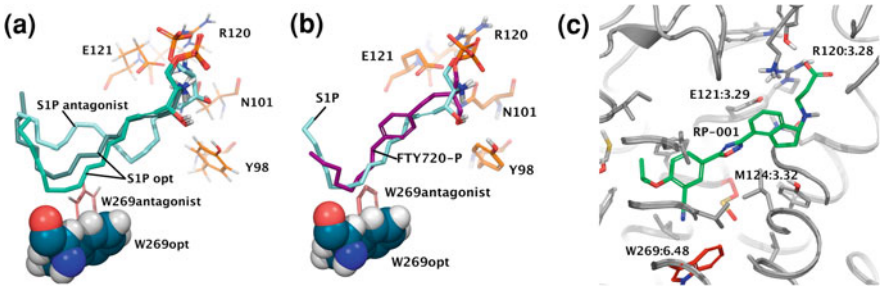

Fig. 8 Proposed binding poses for three agonist compounds. **a** S1P docks into the antagonist binding pocket while maintaining polar head group interactions, but with considerable strain in the aryl chain. The strain in the aryl chain of S1P (S1P antagonist) is mainly the result of the position of the W269 side chain which rotates out of the path of the extended aryl chain (S1P opt) during the course of molecular dynamics simulations to relieve the strain. **b** FTY720-P docks into the S1P-induced binding pocket with a similar pose compared to S1P itself. **c** RP-001 has increased rigidity compared to both S1P and FTY720-P but still docks within the S1P-induced binding pocket forming van der Waals interactions with the agonist position of W269 and polar interactions with E121 and R120

induced expanded version. The hydrophobic volume of the long chain agonists trigger a conformational change associated with $S1P_1$ receptor agonism. This is consistent with the SAR around the VPC antagonist compounds where sequential lengthening of the aryl chain resulted in a switch from agonist to antagonist pharmacology (Davis et al. 2005).

Docking of the S1P ligand into the antagonist binding pocket is straightforward with structurally derived coordinates able to accommodate S1P aryl chain while maintaining polar head group interactions (Fig. 8a). This is somewhat surprising given the finding that an agonist binding pocket requires an increased volume to accommodate agonist compounds. One explanation may be that the S1P aryl chain is by its nature very flexible and able to conform to many different binding pocket shapes albeit with varying degrees of associated conformational strain. Although the chain can be accommodated by the antagonist binding pocket of ML056, it is not optimal and is in a partially strained conformation based on the observed aryl chain dihedral angles measured after docking and minimization (Fig. 8a). The strain associated with nonoptimal torsional angles can be resolved by subtly changing the shape of the binding pocket through short-term molecular dynamics simulations. The entire system was first equilibrated in a phospholipid bilayer placed by alignment with the adenosine A2a receptor, water was added to the solvent accessible regions and ions were added to generate a charge neutral system. The simulation was carried out for 1.2 ns after equilibration to observe if the ligand would attain a more energetically favorable conformation and if there are any significant changes to the binding pocket in response (Shivakumar et al. 2010). Interestingly, much of the strain around the aryl chain torsion angles was resolved along with subtle changes in side chain positions of residues lining the binding

cavity (Fig. 8a). Notably, the $\chi 2$ angle of Trp269:6.48 side chain changed from a value of 95° in the antagonist structure to an average angle of 124° in the molecular dynamics simulation (Fig. 8a).

Based on this limited analysis the endogenous ligand could bind in roughly the same pocket as antagonist compounds. The conformational strain associated with this binding, however, will eventually be relieved by subtle conformational changes of the residues lining the pocket which will not only change the shape and characteristics of the cavity but also trigger a substantial receptor conformational change on the intracellular region associated with agonist signaling. The idea that subtle changes in the binding pocket can trigger the antagonist to agonist conformational switch has been validated with the recent structure of class A GPCRs with agonist bound and coupled to G protein (Rasmussen et al. 2011b).

3.2.2 Fty720-P

FTY720 (fingolimod) was synthesized in an effort to minimize the toxic effects of ISP-1, a fungal metabolite with immunosuppressive properties, which has been used in traditional Chinese herbal medicine as an eternal youth elixir (FUJITA et al. 1994; Napoli 2000). FTY720 was subsequently found to be effective in a variety of autoimmune and transplant models (Brinkmann et al. 2001). It is now known that FTY720 acts as a prodrug becoming phosphorylated in vivo through the action of sphingosine kinase 1 and 2. The phosphorylated active metabolite of FTY720 is termed FTY720-P(S) which is a non-selective agonist for S1P1,3,4, and 5 receptors (Mandala et al. 2002; Brinkmann 2002) and can function both as a receptor agonist and pharmacological functional antagonist in vivo (Gräler and Goetzl 2004; Oo et al. 2007; Ishii et al. 2009). The phenomenon of functional antagonism occurs when receptors are internalized and targeted to the polyubiquitination pathway destined for degradation as opposed to recycling back to the cell surface. It is thought that functional antagonism is a necessary property for $S1P_1$ receptors to be effective in their role as immunomodulators. Since its discovery and characterization, FTY720 demonstrated efficacy in humans against multiple sclerosis, a neurodegenerative autoimmune disorder characterized by inflammation and demyelination in the central nervous system (Cohen et al. 2010; Kappos et al. 2010). FTY720 has completed a rigorous clinical trial program and is now indicated to treat patients with relapsing multiple sclerosis.

We examine here the potential binding mode of FTY720-P in the context of the $S1P_1$ receptor in its modeled agonist-induced conformation. Because FTY720-P is a sphingolipid mimic the polar head group interactions are well characterized and should be similar to the ML056 crystal structure (Hanson et al. 2012). Both ML056 and FTY720-P have phenyl rings proximal to their polar head group, however, the substitution pattern around the phenyl ring of ML056 is meta relative to a 6-carbon aryl chain, whereas it is para for FTY720-P relative to its 8-carbon aryl chain. It has been shown with the VPC series of compounds that this substitution pattern can steer the pharmacology for the sphingolipid-like compounds where a meta-substituted

8-carbon aryl chain (VPC23019) is a potent antagonist on the $S1P_1$ and $S1P_3$ receptors, whereas the equivalent para-substituted compound (VPC24191) is an agonist (Davis et al. 2005; Welch et al. 2012). The molecular determinants of this pharmacology switch are speculative, however, it is interesting that the para-substitution pattern positions a significant increase in volume adjacent to W269:6.48 (Fig. 8b), similar to S1P. In the case of FTY720-P, resolution of this ligand strain through simple molecular dynamics simulations was not possible due to the large conformational shifts and a more thorough modeling effort is out of the scope of this chapter.

Interestingly, it appears that as rigidifying elements are added to the agonist compounds, fitting into the nonoptimally shaped antagonist binding pocket while maintaining polar head group interactions, necessitates placing more strain on the rotatable bonds. The conformational changes associated with releasing that strain through shifting to the agonist binding pocket become increasingly favorable energetically.

3.2.3 RP-001

A number of non-sphingolipid mimic compounds have been discovered as agonists for the $S1P_1$ receptor (Sanna et al. 2004; Li et al. 2005; Vachal et al. 2006; Yan et al. 2006, 2007; Gonzalez-Cabrera et al. 2008; Saha et al. 2010) and a handful of these have been developed for testing in a clinical setting exemplified by RPC1063 (Hartung et al. 2013; Urbano et al. 2013). RPC1063 builds on the findings of CYM-5442 in that interactions mimicking the phosphate anion salt bridges are not necessary for generating picomolar agonist compounds at the $S1P_1$ receptor. During the course of development of RPC1063, a number of compounds were created that added this salt bridge interaction for proof of concept purposes. One such compound, designated RP-001 was tested in vivo in a $S1P_1$-eGFP knock-in mouse for its effects on the expression of the $S1P_1$ receptor on both lymphocyte and endothelial populations of receptors (Cahalan et al. 2011).

The number of rotatable bonds for RP-001 is reduced compared to the FTY720-P and S1P which increases the rigidity and places an extra burden on the binding pocket geometry, such that the compound is not able to dock effectively in the ML056 binding pocket. This necessitates an agonist associated conformational change being modeled prior to docking and analysis of this compound. We use the S1P-induced binding pocket modeled above through molecular dynamics simulations, which allows placement of the compound with good polar and hydrophobic contacts that align well with mutagenesis for this series (Hanson et al. 2012). The carboxylic acid of RP-001 forms ionic and polar interactions with Y29 and R120:3.28, while the secondary amine which is likely protonated at physiological pH participates in hydrogen bonding interactions with N101:2.60 and charge pairs with E121:3.29. The central ring oxadiazole is positioned to interact indirectly (perhaps through water bridged hydrogen bonds) with E121:3.29 and Y98:2.57.

The third cyano-isopropoxy benzene ring fits tightly between F125:3.33 and W269:6.48 with the cyano group appearing to pin W269 in the S1P-induced conformation (Fig. 8c).

4 Conclusions

The elucidation of the $S1P_1$ receptor structure provided a framework for understanding the molecular interactions of not only the crystallized antagonist ligand ML056, but also a variety of antagonist and agonist sphingolipid and non-sphingolipid-like compounds. Compound development efforts and mutagenesis studies suggest the trigger for agonism on the $S1P_1$ receptor is associated with an increase in binding pocket volume. This combined with the expectation that the polar interactions with the sphingosine zwitterion will be consistent across pharmacologies has allowed us to model an agonist binding pocket without the benefit of an agonist structure. This agonist binding pocket is capable of docking a variety of compounds including the Receptos 1063 series which, due to its lack of requirement for sphingosine-like interactions, was optimized to produce superior selectivity and pharmaceutics properties compared to compounds such as FTY720-P. We continue to use this structure and the models derived from it to advance our understanding of the molecular interactions employed by the $S1P_1$ receptor agonists and potentially to design further improved compounds. Recently, it has been established that the $S1P_1$ receptor agonists act, at least partially, through a functional antagonist mechanism. This finding introduces the possibility of designing agonist compounds that preferentially induce internalization of the receptor over other signaling pathways, a phenomenon known as biased ligand signaling (Xu et al. 2013; Healy et al. 2013). This concept could be an important mechanism for further improvements to the safety profile of immunomodulatory $S1P_1$ receptor agonists, and indeed, many other classes of small molecule therapeutics designed to modulate G protein-coupled receptor pharmacology. Discovering the structural basis for biased ligand signaling could signify the next breakthrough in our understanding of GPCR pharmacology.

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Chemical and Genetic Tools to Explore S1P Biology

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Abstract The zwitterionic lysophospholipid Sphingosine 1-Phosphate (S1P) is a pleiotropic mediator of physiology and pathology. The synthesis, transport, and degradation of S1P are tightly regulated to ensure that S1P is present in the proper concentrations in the proper location. The binding of S1P to five G protein-coupled S1P receptors regulates many physiological systems, particularly the immune and vascular systems. Our understanding of the functions of S1P has been aided by the tractability of the system to both chemical and genetic manipulation. Chemical modulators have been generated to affect most of the known components of S1P biology, including agonists of S1P receptors and inhibitors of enzymes regulating S1P production and degradation. Genetic knockouts and manipulations have been similarly engineered to disrupt the functions of individual S1P receptors or enzymes involved in S1P metabolism. This chapter will focus on the development and utilization of these chemical and genetic tools to explore the complex biology surrounding S1P and its receptors, with particular attention paid to the in vivo findings that these tools have allowed for.

Contents

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1 Chemical Tools to Explore S1P Biology

Despite the relatively recent identification of S1P receptors pinpointing S1P as an important player in many physiological systems, a wide variety of chemical tools have been developed to understand the biology of S1P and its receptors. The S1P axis continues to be an area of significant drug discovery efforts. Chemical tools possess several benefits, including the ability to examine acute effects following treatment as opposed to genetic models where precise temporal control is not possible. This section will discuss three broad categories of chemical tools that have been generated and used to explore S1P biology: First, those that affect the normal production or degradation of S1P produced. Second, chemical agonists that activate S1P receptors. Third, chemical antagonists that inactivate S1P receptors. Special attention will be paid to the in vivo effects that these compounds have, and the relation of these compounds to treating human disease.

1.1 Chemical Modulators of Physiological S1P Levels

S1P levels are precisely controlled both in circulation, where S1P is present in high-nanomolar concentrations (Hla 2004), and in peripheral tissues, where S1P levels are significantly lower (Schwab et al. 2005) through the coordinated actions of sphingosine kinases, which produce S1P, S1P transporters, which export S1P into the extracellular environment, and S1P phosphatases and lyase, which degrade S1P. Two intracellular sphingosine kinases Sphingosine kinases, SphK1 and SphK2, act to phosphorylate the hydroxyl group of sphingosine to produce S1P. While S1P can act upon intracellular targets, its actions on S1P receptors requires transport to the extracellular environment by one or more S1P transporters, including Spns2 (Kawahara et al. 2009; Fukuhara et al. 2012; Mendoza et al. 2012; Kohama et al. 1998; Liu et al. 2000). S1P is degraded by either reversible dephosphorylation by two S1P-specific phosphatases and three nonspecific lipit phosphate phosphatases (Kai et al. 1997; Roberts et al. 1998; Mandala 2001) or by irreversible cleavage at the C_{2-3} carbon bond by S1P lyase (Zhou and Saba 1998). Chemical tools have been generated to affect several components of this pathway, and others remain possible targets for development.

Chemical modulation of sphingosine kinases began with the discovery that the sphingosine analogs D-, L-, and DL-threo-dihydrosphingosine and N, N-dimethylsphingosine inhibited the activity of sphingosine kinase in human platelets (Buehrer and Bell 1992; Yatomi et al. 1995). These sphingosine derivatives, though

potent, exhibit significant nonselectivity, particularly the inhibition of protein kinase C (Merrill et al. 1989; Khan et al. 1990). Numerous other sphingosine analogs, including FTY720, a S1P receptor prodrug used clinically for the treatment of relapsing-remitting multiple sclerosis, also inhibit sphingosine kinases (Tonelli et al. 2010). In addition to binding competitively, several sphingosine kinase inhibitors also induce proteasomal degradation after binding, providing additional inhibition of the generation of S1P (Tonelli et al. 2010; Lim et al. 2011). Continued efforts have generated nanomolar potency, isoform-selective antagonists of both SphK1 (Paugh et al. 2008; Kennedy et al. 2011) and SphK2 (French et al. 2010).

Sphingosine kinase inhibitors have been investigated as potential treatments for a variety of diseases, particularly inflammatory disorders (Snider et al. 2010) and cancer (Maceyka et al. 2012). The non-S1P-like sphingosine kinase inhibitor ABC747080 was found to reduce inflammation and tissue S1P concentrations in an acute model of inflammatory bowel disease (Maines et al. 2008), while the selective SphK1 inhibitor SK1-i inhibited a mouse model of allergic asthma (Price et al. 2012). Selective antagonism of SphK2 by the antagonist ABC294640 has also demonstrated efficacy in mouse models of inflammatory Crohn's disease (Maines et al. 2010) and osteoarthritis (Fitzpatrick et al. 2011). Sphingosine kinase inhibitors can also have antitumor effects, as SK1-i was originally found to inhibit leukemia cell proliferation in vitro and inhibit xenograft tumor growth in vivo (Paugh et al. 2008). While efforts have largely focused on the ability of sphingosine kinase inhibitors to reduce proliferation of tumor cells, SK1-i also has been found to inhibit breast cancer tumor growth and metastasis in mice by affecting angiogenesis and lymphangiogenesis (Nagahashi et al. 2012). Inhibition of SphK2 by ABC294640 has demonstrated a similar ability to inhibit tumor progression in hepatocellular carcinoma xenografts (Beljanski et al. 2011). The precise mechanisms by which chemical inhibition of sphingosine kinases can lead to either suppression of inflammation or tumor growth and/or metastasis remain unclear. Lowering S1P levels by inhibiting sphingosine kinases may have many effects, both extracellular through reducing signaling through S1P receptors, and intracellular through undetermined mechanisms (Strub et al. 2010), that may act in coordination to alleviate pathology (Table 1).

While inhibitors of sphingosine kinases effectively reduce the abundance of S1P, inhibitors of the enzymes that normally degrade S1P have also been designed to raise the concentrations of S1P present in both tissues and circulation. One such inhibitor is tetrahydroxybutylimidazole, or THI, a caramel food coloring that at high concentrations acts as a S1P lyase inhibitor, elevating the concentration of S1P 100-fold in lymphoid tissues and leading to the sequestration of lymphocytes from blood and lymph, similar to treatment with either $S1P_1$ agonists or antagonists (Schwab et al. 2005). THI recently has also been shown to acutely alleviate cardiac ischemia-reperfusion injury concomitant with an increase in both tissue and plasma concentrations of S1P (Bandhuvula et al. 2011). S1P lyase inhibition has also been pursued clinically for the treatment of rheumatoid arthritis, presumably in part by sequestering lymphocytes from circulation (Bagdanoff et al. 2010).

Table 1 Summary of chemical and genetic tools for studying S1P biology

		Chemical				Genetic		
		In vivo agonist		In vivo antagonist		Knockout		Other
		Selective	Nonselective	Selective	Nonselective	Constitutive	Conditional	
Genes affecting S1P synthesis, degradation, and transport	Sphingosine kinase 1			✔	✔	✔	✔	Transgenic overexpression
	Sphingosine kinase 2			✔	✔	✔	✔	
	S1P Lyase				✔			
	LPP3					✔	✔	
	SPNS2					✔	✔	
S1P Receptors	S1P1	✔	✔	✔	✔	✔	✔	Fluorescent and non-phosphorylable knockins, transgenic overexpression
	S1P2			✔	✔	✔		
	S1P3		✔	✔	✔	✔		Fluorescent knockin
	S1P4		✔			✔		
	S1P5	✔	✔			✔		

While not a small molecule, one additional tool that has been developed to modulate the availability of S1P is sphingomab, a monoclonal antibody directed against S1P itself which is being developed as an anticancer drug. In animal models, treating mice with sphingomab can inhibit both growth and metastasis of tumors (Visentin et al. 2006; Ponnusamy et al. 2012). Additionally, treatment of mice with sphingomab can cause lymphopenia despite increasing blood and lymphatic concentrations of S1P, much of which is bound to sphingomab (Sensken et al. 2011). The continued development of tools that can increase, decrease, or bind S1P that is normally produced will help dissect out the many roles that S1P can play in physiology and may eventually lead to effective treatments for human diseases.

1.2 S1P Receptor Agonists

S1P receptor agonists have been heavily investigated following the discovery that FTY720 (fingolimod, Gilenya), an immunosuppressive derivative of the fungal metabolite myriocin, was phosphorylated in vivo to generate a highly potent, nonselective agonist of four of five S1P receptors (Mandala et al. 2002). In the past decade a remarkably wide array of compounds that activate one or more S1P receptors have been developed for use as chemical tools to investigate S1P receptor function, with FTY720 already progressing to clinical use.

1.2.1 Nonselective S1P Receptor Agonists

As previously mentioned, the phosphorylated form of FTY720, FTY720-P, is a highly potent yet nonselective agonist of $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ with EC_{50} values in the low nanomolar range (Brinkmann et al. 2002). FTY720 is an analog of sphingosine and is phosphorylated in vivo exclusively by Sphk2 to generate FTY720-P (Paugh et al. 2003; Kharel et al. 2005), which can be exported into the extracellular environment by Spns2 (Hisano et al. 2011). AAL-R, a close relative of FTY720 differing only in the lack of a hydroxyl group, is also efficiently phosphorylated by Sphk2 to generate AFD-R, a nonselective agonist of S1P receptors (Jary et al. 2010). Treatment with nonselective S1P receptor agonists leads to rapid, sustained sequestration of both T and B cells from the blood and the lymph and causes short-lasting bradycardia in both mice and humans (Luo et al. 1999; Budde et al. 2002; Sanna et al. 2004). The immunosuppressive properties of FTY720 and other nonselective S1P receptor agonists led to investigation into their potential as a therapy for both transplant rejection (Suzuki et al. 1996; Brinkmann et al. 2001; Shimizu et al. 2005) and autoimmune disorders, particularly multiple sclerosis (Fujino et al. 2003; Thomson 2006). While it has not progressed to clinical use for transplant rejection, FTY720 has been approved as a treatment for relapsing-remitting multiple sclerosis in humans.

While it may contribute to its clinical efficacy, the lack of selectivity of FTY720 makes it a less than ideal tool for dissecting the roles of individual S1P receptors play in physiology, FTY720 has been very useful in helping elucidate the differences in downstream signaling and receptor fate across S1P receptors. Treatment of cells expressing different S1P receptors with FTY720 leads to rapid and sustained internalization and degradation of $S1P_1$, even after washout, but does not lead to the degradation of $S1P_3$ or $S1P_4$ (Graler and Goetzl 2004; Mullershausen et al. 2009). Given the high potency of FTY720-P across S1P receptors, most of the focus of generating new chemical tools to study S1P receptors has focused on improving selectivity, which has allowed for elucidating the role that individual S1P receptors can play in physiology and pathology.

1.2.2 Selective $S1P_1$ Agonists

$S1P_1$ has been by far the most studied S1P receptor both chemically and genetically due to its critical role in lymphocyte recirculation. $S1P_1$-selective agonists, even from direct screening hits such as SEW2871, have generally been able to achieve strong potency, with EC_{50} values at least in the nanomolar range, and have been generated with a wide variety of chemical scaffolds (Zhang et al. 2009; Meng et al. 2012; Nakamura et al. 2012; Reed et al. 2012), of which several shown in Fig. 1. It is readily apparent that many $S1P_1$ agonists exhibit long hydrophobic cores along with a polar or charged headgroup. These structures resemble the amphipathic nature of S1P, which consists of a long acyl chain and a zwitterionic headgroup consisting of a positively charged amine and a negatively charged phosphate. These groups in S1P interact with charged arginine and glutamate residues (Arg120 and Glu121) that are positioned within the binding pocket close to the extracellular side (Parrill et al. 2000). Despite the lack of a charged headgroup, SEW2871 still relies on interaction with Arg120 to activate $S1P_1$ (Jo et al. 2005). One relatively unique agonist is CYM-5442, which does not possess a charged or highly polar headgroup, instead having a hydroxyl group. CYM-5442 has been shown to not require interactions with Arg120 or Glu121 of $S1P_1$, and in fact binds $S1P_1$ in a different manner, instead relying on interaction with a critical tryptophan residue (W269) deep in the $S1P_1$ pocket for its potency (Gonzalez-Cabrera et al. 2008; Hanson et al. 2012). Modifications of CYM-5442 have led to a class of arylpropionic acids that activate $S1P_1$ with picomolar EC_{50} values (Cahalan et al. 2011; Teijaro et al. 2011). These $S1P_1$ agonists can vary significantly in their potency, pharmacokinetic profiles, and their effects on $S1P_1$ following binding. Some $S1P_1$ agonists, like SEW2871 or the native ligand S1P, cause $S1P_1$ to be internalized from the cell surface but then recycled back to the surface, whereas others, such as the FTY720-P homolog AFD-R and CYM-5442, cause internalization, polyubiquitination, and degradation via the proteasome (Gonzalez-Cabrera et al. 2007).

The ability of FTY720 to sequester lymphocytes from the blood and the lymph relies on its activity on $S1P_1$, as $S1P_1$-selective agonists lead to similar

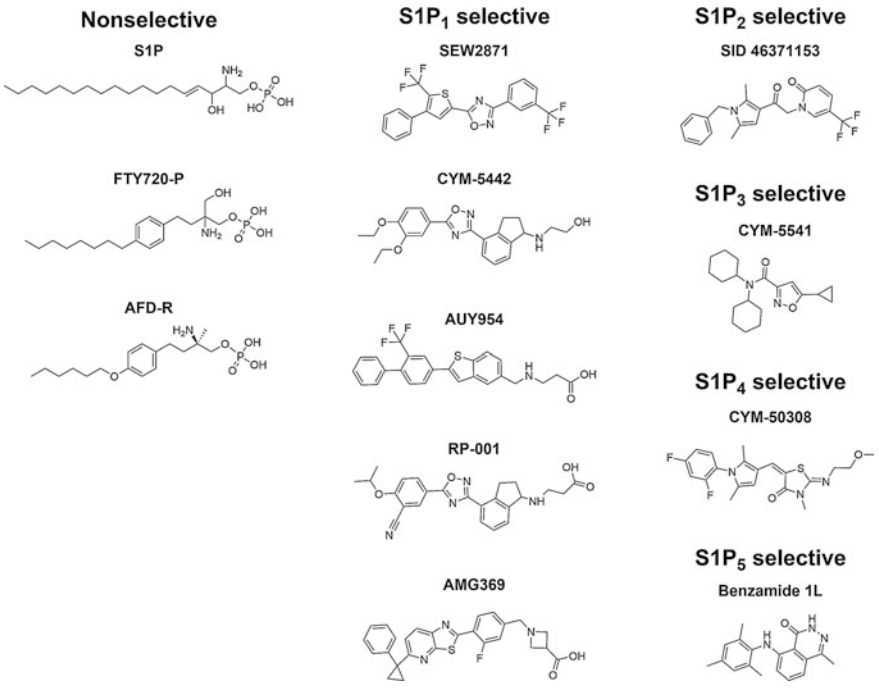

Fig. 1 Chemical structures of S1P receptor agonists

sequestration of lymphocytes both in mice (Sanna et al. 2004) and in humans. Treatment of mice with SEW2871 causes the arrest of lymphocytes close to the lymph node medullary sinus but does not affect the velocity of lymphocytes found in the cortex (Wei et al. 2005; Sanna et al. 2006). While the receptor mediating lymphocyte sequestration induced by FTY720, $S1P_1$, is the same between mice and humans, the receptors mediating bradycardia elicited by FTY720 differ significantly. Bradycardia caused by FTY720 in mice relies on its actions on $S1P_3$ (Sanna et al. 2004), with $S1P_1$-selective agonists causing negligible bradycardia, whereas $S1P_1$-selective agonists can cause significant bradycardia in humans by activating the G protein-coupled inwardly rectifying potassium (GIRK) channel expressed in myocytes (Gergely et al. 2012).

Since the activation of $S1P_1$ inhibits lymphocyte recirculation, selective $S1P_1$ agonists have been investigated as treatments for autoimmune diseases as potential alternatives to nonselective agonists like FTY720-P. $S1P_1$-selective agonists have demonstrated efficacy in several animal models, including multiple sclerosis (Galicia-Rosas et al. 2012; Gergely et al. 2012; Gonzalez-Cabrera et al. 2012), autoimmune arthritis (Piali et al. 2011), and ulcerative colitis (Sanada et al. 2011). Several $S1P_1$ agonists have progressed into clinical trials focused on multiple sclerosis, with the goal of limiting the off-target effects resulting from treatment with FTY720. However, the possibility exists that the actions of FTY720 on other

S1P receptors or other targets such as sphingosine kinases may aid in its efficacy. Whether these selective drugs can improve outcomes or minimize adverse events when compared to FTY720 remains to be seen.

Recent studies have also found that $S1P_1$ agonists can have compelling effects in inhibiting cytokine production following infection by influenza virus. Local treatment with the nonselective S1P receptor proagonist AAL-R in the lungs of mice prior to infection with influenza virus leads to the suppression of viral specific T cell response and the suppression of cytokine production within the lungs (Marsolais et al. 2008; Marsolais et al. 2009). The suppression of cytokines by AAL-R is of particular interest, as excessive cytokine production has been implicated in the pathogenicity of several "pandemic" influenza strains, including H5N1 "avian" flu and H1N1 strains from both the 1918 pandemic and the more recent "swine" flu outbreak in 2009 (Arankalle et al. 2010; Lee et al. 2011). Indeed, mice treated with AAL-R exhibited significantly less mortality than untreated mice following infection with a 2009 H1N1 influenza strain, and this reduction in lethality was synergized by the administration of the clinically used neuraminidase inhibitor oseltamivir (Walsh et al. 2011). While the mechanisms by which AAL-R inhibits cytokine production aren't fully understood, it appears that $S1P_1$ expressed on endothelial cells plays a critical role in this suppression of cytokine production as well as inhibiting the infiltration of innate immune cells into the lung (Teijaro et al. 2011). The ability of $S1P_1$ agonists to suppress cytokine production may be of much broader significance, as excessive cytokine production has also been implicated in other viral infections such as SARS (Huang et al. 2005; Nagata et al. 2008) or bacterial infections such as pneumococcal pneumonia (Fernandez-Serrano et al. 2003).

1.2.3 Selective $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ Agonists

Unlike $S1P_1$, very few selective agonists have been developed against $S1P_2$ (Rosen 2007), $S1P_3$ (Jo et al. 2012), $S1P_4$ (Urbano et al. 2011) or $S1P_5$ (Mattes et al. 2010). In fact, no in vivo activity of any selective agonist of these receptors has been established to date, and only for a selective agonist of $S1P_5$, which increased the number of mature oligodendrocytes obtained in vitro from neonatal rat cortices, has any physiological effect been determined in vitro (Mattes et al. 2010). While the abundance of $S1P_1$-selective agonists in comparison to the dearth of selective agonists of other S1P receptors is largely due to the high-clinical relevance of $S1P_1$, there may be additional factors at play, including the difficulty in actually discovering and generating compounds that fit specifically into the binding pocket of those receptors. For instance, $S1P_3$ is thought to have a significantly smaller binding pocket than $S1P_1$, which may constrain the amount of interaction between the agonist and the receptor; additionally compounds found in high-throughput screening libraries are more similar to identified $S1P_1$ agonists than they are to $S1P_3$ agonists (Schurer et al. 2008). The only published $S1P_3$-selective agonist to date is in fact an allosteric agonist, CYM-5541, that does not

compete with S1P binding. Such allosteric agonists may provide an alternative route to discovering selective agonists to the S1P receptors, relying predominantly on interactions deep within the binding pocket rather than interactions with charged residues located near the top of the binding pocket.

1.3 S1P Receptor Antagonists

While FTY720-P was initially characterized as a potent agonist of S1P receptors, it was soon determined that FTY720-P and other agonists could cause the internalization of $S1P_1$ from the surface of the cell. This ability of FTY720-P led many to develop antagonists against S1P receptors in part to determine what the effect of in vivo blockade of normal S1P receptor signaling could have, and also to determine whether treatment with S1P receptor antagonists could replicate the effects seen following treatment with FTY720 or other S1P receptor agonists.

1.3.1 $S1P_1$ Antagonists

The first published $S1P_1$ antagonists were discovered following the finding that moving the substituents around the phenyl ring in FTY720-P from para- to *ortho*- caused a switch from agonism to antagonism on $S1P_1$ (Davis et al. 2005), as shown in Fig. 2. The resulting compound, VPC23019, was a dual $S1P_1/S1P_3$ antagonist, though it exhibits significantly greater inhibition of $S1P_1$ compare to $S1P_3$. Another early $S1P_1$ antagonist exhibiting a similar *ortho*- substitution, W123, was found to be selective for $S1P_1$, with no activity on $S1P_3$, and could reverse the arrest of lymphocytes in the medulla of explanted lymph nodes (Wei et al. 2005). However, both of these initial antagonists were unsuitable for in vivo work due to their instability when administered to mice.

Optimization of these *ortho*-substituted FTY720-P analogs eventually led to the replacement of phosphate groups for a phosphonate group, yielding a chiral antagonist, W146, or ML056, that could act in vivo. While the *R*- enantiomer was found to be an antagonist, the *S*- enantiomer exhibited no activity on $S1P_1$. When administered in vivo, W146 did not appear to cause lymphocyte sequestration and could, in fact, reverse lymphocyte sequestration induced by the $S1P_1$ selective agonist SEW2871 when examined 4 h after treatment (Sanna et al. 2006). VPC44116, which is the equivalent phosphonate of VPC23019, had much the same effect in vivo, not causing lymphocyte sequestration in vivo but reversing the sequestration induced by another $S1P_1$ agonist, VPC44152 (Foss et al. 2007). W146 also induce significant vascular leakage in the lung and exacerbated VEGF-induced leakage in the skin, and recently was shown to stabilize the binding pocket of $S1P_1$ allowing for the crystallization and structural determination of $S1P_1$ joined to T4 lysozyme (Hanson et al. 2012). The lack of lymphocyte sequestration and the reversal of agonist-induced sequestration originally observed with $S1P_1$

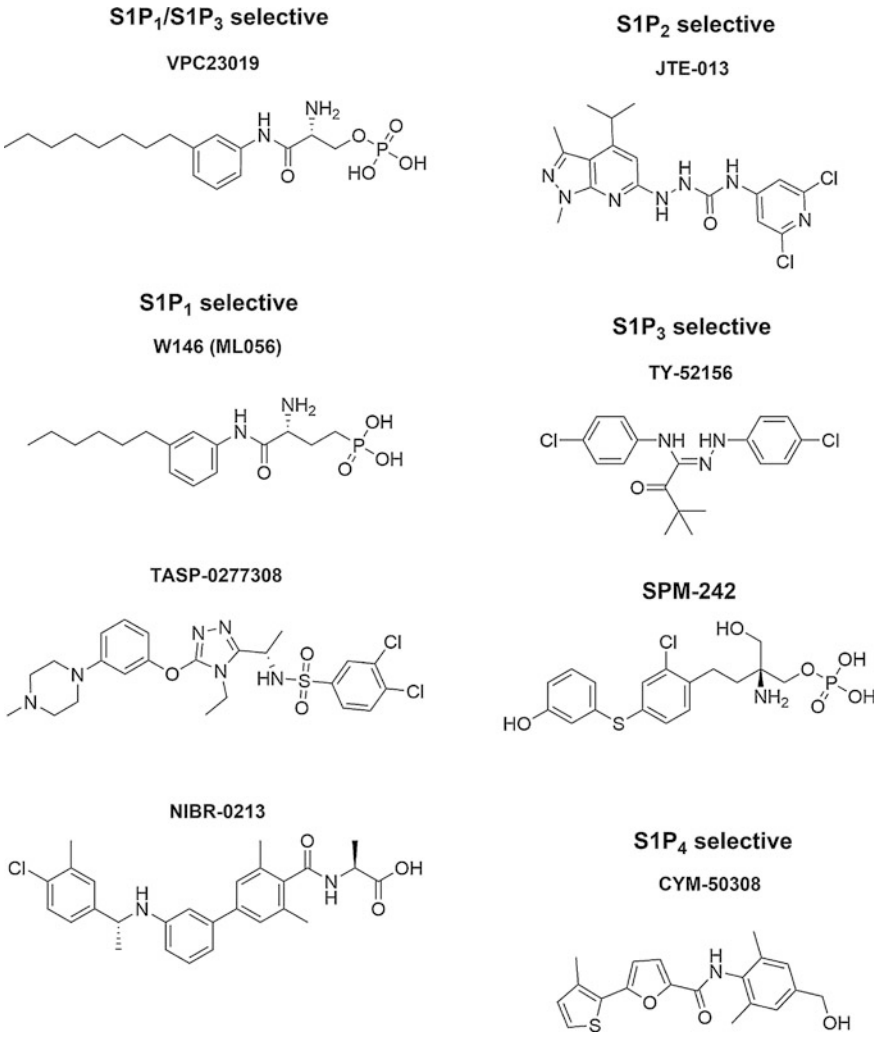

Fig. 2 Chemical structures of S1P receptor antagonists

antagonists led to the hypothesis that agonists acted not as functional antagonists of $S1P_1$ expressed on lymphocytes, but as direct agonists of $S1P_1$ expressed on endothelial cells leading to the tightening of cell–cell junctions and the inhibition of lymphocyte egress.

More recent work has demonstrated that selective $S1P_1$ antagonists, including W146 if examined at earlier timepoints, can in fact cause lymphocyte sequestration by themselves (Tarrason et al. 2011). Additional newer antagonists exhibiting higher potency or improved pharmacokinetic properties can induce lymphocyte sequestration that can be sustained for many hours, including TASP0277308

(Fujii et al. 2012) and a series of biaryl benzylamine derivatives (Angst et al. 2010). These antagonists are structurally different from the previous S1P-like antagonists like W146 and VPC44116 and can upregulate $S1P_1$ on lymphocytes (Cahalan et al. 2013; Fujii et al. 2012), opposite of the effect seen with $S1P_1$ agonists. Furthermore, the ability to sequester lymphocytes by these antagonists has been accompanied by efficacy in animal models of allograft rejection (Angst et al. 2012), arthritis (Fujii et al. 2012), and multiple sclerosis(Quancard et al. 2012; Cahalan et al. 2013).

The ability of S1P-like antagonists such as W146 to reverse lymphocyte sequestration by $S1P_1$ agonists at some timepoints while causing lymphocyte sequestration at earlier timepoints raises some interesting possibilities regarding the competition within the binding pocket and downstream signaling, which may be an area for further study. Future work should also determine whether antagonists may be useful as potential therapeutics for autoimmune disease, or whether they elicit significantly worse adverse events, such as pulmonary or peripheral edema, compared to $S1P_1$ agonists. Additionally, the development of longer-lasting antagonists may allow for extended studies of other physiological and pathological conditions.

1.3.2 $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ Antagonists

Similar to what has occurred with S1P receptor agonists, studies using S1P receptor antagonists have predominantly focused on $S1P_1$, with fewer antagonists against $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$. A specific antagonist to $S1P_2$, JTE-013, has been widely used to study the role of $S1P_2$ both in vitro and in vivo, particularly the role that $S1P_2$ can play in vascular physiology. Treatment of vascular endothelial cells with JTE-013 can improve their barrier integrity in vitro (Sanchez et al. 2007) and inhibit S1P-induced vasoconstriction in excised arteries (Kono et al. 2007). Some questions have been raised about the specificity of JTE-013, as it may have effects independent of $S1P_2$ (Salomone and Waeber 2011; Li et al. 2012).

Two selective $S1P_3$ antagonists have been described: TY-52156, which could inhibit S1P-dependent coronary flow within isolated rat hearts in vitro and inhibit FTY720-induced bradycardia in vivo (Murakami et al. 2010), and SPM-242, which is able to compete both with the native agonist S1P and with an allosteric agonist to $S1P_3$, thus making it a "bitopic" antagonist, although no in vivo actions of this antagonist have been established to date (Jo et al. 2012). Additionally an anti-$S1P_3$ antibody has been described that blocks the activation of $S1P_3$ (Harris et al. 2012). This antibody could block lethality in mice caused by systemic LPS treatment and inhibit the growth of breast cancer xenografts, providing potential avenues for further examination of the roles of $S1P_3$ in two distinct pathological conditions. $S1P_4$ antagonists have been described in vitro (Guerrero et al. 2011), but with no relevant physiological data published to date. With regard to $S1P_5$, no

data has been published describing the inhibition of $S1P_5$ by selective antagonists, though a patent application was filed describing scaffolds that may act as either agonists or antagonists of $S1P_5$ (Harris et al. 2010).

2 Genetic Tools to Explore S1P Biology

In addition to the numerous chemical tools that have been generated to modulate the levels of S1P produced and the activity of the five S1P receptors, genetically engineered mice have been generated to examine the complex physiological processes controlled by S1P. Genetic knockouts have the benefit of completely eliminating the gene of interest, with potentially fewer off-target effects, though the potential for compensation by other genes remain possible. Additionally, cell-type specific deletion or overexpression can be achieved by genetic modification, something not able to be done by chemical treatment. Some significant drawbacks exist, such as the difficulty to control precisely when the gene of interest is inhibited or reversing such inhibition. This section will review briefly three general types of mouse models that have been generated; significantly more detail on several of these mouse models will be discussed in later chapters. First, mice in which the generation, degradation, or transport of S1P has been modified. Second, mice where the expression of S1P receptors have been deleted, either globally or conditionally. Lastly, mice where a tagged or mutated S1P receptor replaces the endogenous S1P receptor.

2.1 Genetic Modification of Physiological S1P Production, Degradation, and Transport

Similar to the variety of chemical modulators of S1P levels in vivo, a number of genetic mouse models have been generated lacking or overexpressing the genes that are involved in regulating the physiological levels of S1P.

2.1.1 Sphingosine Kinase Deficient Mice

Genetic knockouts to both the sphingosine kinases, SphK1 and SphK2, alone and in combination have been generated and have revealed many important roles for the generation of S1P in physiology. While they play many unique roles, they can also act somewhat redundantly, as either SphK1-deficient or SphK2-deficient mice are viable and fertile (Allende et al. 2004; Kharel et al. 2005; Mizugishi et al. 2005). SphK1-deficient mice display significantly reduced SphK activity in some tissues, particularly the spleen, but only in the serum is a significant reduction in S1P observed. SphK2-deficient mice also display only a partial reduction in circulating

S1P, but display a complete inability to phosphorylate FTY720. However, mice that are deficient in both SphK1 and SphK2 are rendered completely unable to produce S1P and begin to exhibit severe vascular hemorrhage in utero, with no embryos surviving past E13.5 (Mizugishi et al. 2005).

The lethality of SphK1/SphK2 double knockout mice has been circumvented using mice where SphK2 is constitutively deleted while SphK1 is deleted shortly after birth in many tissues by using inducible Mx1-Cre driven excision of SphK1 (Pappu et al. 2007). Such "S1P-less" mice are viable and lack detectable amounts of circulating S1P in either the blood or the lymph. The sources of the S1P found in the blood and in the lymph are distinct, with S1P in the blood being produced by hematopoieitic cells, largely erythrocytes, while S1P in the lymph is produced in large part by lymphatic endothelial cells (Pham et al. 2010). S1P-less mice also display significant basal vascular leakage in the lung, similar to what is observed in mice treated with $S1P_1$ antagonists, and also display greatly increased sensitivity to passive systemic anaphylaxis, a sensitivity that could be largely reversed by agonism of $S1P_1$ (Camerer et al. 2009).

Despite playing somewhat redundant roles in the production of circulating S1P, deletion of either of the sphingosine kinases in mice can have effects by themselves. SphK1 can play an important role in inflammation induced by TNF. TNF leads to the activation SphK1, which can eventually lead to production of prostaglandin E2 by COX2 (Pettus et al. 2003). Mice deficient in SphK1 display significantly reduced pathology in several experimental inflammatory pathologies, including dextran sulfate sodium-induced colitis (Snider et al. 2009) and arthritis induced by transgenic expression of TNF (Baker et al. 2010), in keeping with the findings mentioned earlier utilizing the selective sphingosine kinase inhibitor SK1-i. These findings would suggest that inhibiting SphK1 might be a good treatment for these diseases, but this is complicated by the finding that $SphK1^{-/-}$ mice display significantly increased pulmonary edema in response to either inflammatory LPS or PAR-1 agonist peptide treatment, potentially by lowering the amount of S1P that is able to activate $S1P_1$, which normally acts to tighten vessel permeability.

Recent work utilizing SphK2-deficient mice has also suggested a protective role of SphK2 following ischemia. In a model of cerebral ischemia, mice deficient in SphK2 exhibited significantly larger ischemic lesion sizes 24 h following a 2 h occlusion of the middle cerebral artery (Pfeilschifter et al. 2011). FTY720 treatment was found to have a protective effect following ischemic injury, but this protection was abolished in SphK2-deficient mice, pointing toward this protection as a function of FTY720-P. Hearts from SphK2-deficient mice are also sensitized to ischemia/reoxygenation injury within the heart, exhibiting significantly larger infarct sizes (Vessey et al. 2011). Hearts from SphK2-deficient mice were also resistant to protection usually offered by ischemic preconditioning, but the addition of exogenous S1P to SphK2-deficient hearts could offer protection.

2.1.2 S1P Lyase and Phosphatase Deficient Mice

On the opposing side of S1P metabolism, mice deficient in some of the enzymes that normally degrade S1P have been generated to study what role the proper degradation of S1P can play. Mice deficient in S1P lyase, encoded by the gene *Sgpl1*, display significantly reduced viability, size, and weight, and all mice were dead by 8 weeks of age (Schmahl et al. 2007). When examined at early ages, sphingolipid levels of not only S1P, but of ceramide and sphingomyelin, were elevated in plasma, and levels of dihydrosphingosine, ceramide, sphingosine, dihydro-S1P, and S1P were elevated within the liver (Bektas et al. 2010). These mice also display higher levels of cholesterol in the serum and exhibited significant differences in the metabolism of many other lipids in the liver, pointing toward an important role for S1P degradation in total lipid metabolism. The broad elevation of several other sphingolipids points to a potential increase in the recycling of S1P by S1P phosphatases, which may provide more sphingosine that can act as a substrate for ceramide synthase to in turn produce more ceramide (Hagen-Euteneuer et al. 2012). Mice deficient in S1P lyase have significant changes in immune cell development and function, as they have reduced T and B cell numbers in the blood and in the spleen while exhibiting elevated numbers of neutrophils and monocytes in the blood and elevated proinflammatory cytokines in the serum (Allende et al. 2011). The elevated numbers of neutrophils and increase in proinflammatory cytokines could be partially rescued by also deleting $S1P_4$ but not $S1P_1$.

As opposed to the irreversible cleavage of S1P caused by S1P lyase, several phosphatases, either S1P-specific S1P phosphatases (SPPs) or nonspecific lipid phosphate phosphatases (LPPs), can reversibly dephosphorylate S1P to yield sphingosine. While mice lacking either of the specific SPPs have not been described to date, mice lacking each of the LPPs have been generated. Mice deficient in LPP1 have been described to have deficient clearance of lysophosphatidic acid, but were otherwise phenotypically normal (Tomsig et al. 2009). Likewise, mice deficient in LPP2 were found to be viable and did not display any significant phenotype to date (Zhang et al. 2000). Mice deficient in LPP3, in contrast, die in utero prior to E10.5 due to both gastrulation deficits and inefficient vascularization of the yolk sac (Escalante-Alcalde et al. 2003). Conditional deletion of LPP3 in specialized astrocytes known as Bergmann glia can lead to alterations of S1P metabolism within the cerebellum, resulting in motor coordination deficits (Lopez-Juarez et al. 2011). In keeping with the important role for S1P in lymphocyte development, inducible deletion of LPP3 leads to inefficient egress of mature thymocytes into the periphery (Breart et al. 2011). Deletion of LPP3 on either endothelial cells or on epithelial cells leads to a similar inefficiency of egress, concomitant with a downregulation of CD69 that would be expected to be a result of exposure of mature thymocytes to higher concentrations of S1P. LPP3 deletion also has effects in smooth muscle cells, as mice where LPP3 has been deleted in smooth muscle exhibit higher inflammation after vascular injury (Panchatcharam et al. 2013).

2.1.3 S1P Transporter Deficient Mice

Since S1P is produced largely inside the cell, it must be transported to the outside of the cell in order to act on extracellular targets such as S1P receptors. S1P is thought to be transported in part by ABC type transporters such as ABCA1 and ABCC1 (Mitra et al. 2006; Sato et al. 2007), but mice deficient in these transporters display no changes in circulating S1P. Recently, a separate S1P transporter, Spns2, was identified in a zebrafish mutant that displayed impaired migration of myocardial precursors, similar to the deletion of the zebrafish homolog of $S1P_2$ (Kawahara et al. 2009). Several groups have generated mice lacking Spns2, identifying that it is indeed an S1P transporter that when deleted can lead to deficiencies in thymocyte egress and B cell development (Fukuhara et al. 2012; Hisano et al. 2012; Mendoza et al. 2012; Nagahashi et al. 2012; Nijnik et al. 2012). Spns2 expressed on endothelial cells, not erythrocytes, is critical for the efficient egress of mature thymocytes. The presence of significant concentrations of circulating S1P even in the absence of Spns2 points toward some level of redundancy, either through the functions of the ABC type transporters or another S1P transporter that has yet to be determined.

2.2 Mice Deficient in S1P Receptors

2.2.1 $S1P_1$ Deficient Mice

$S1P_1$ was originally identified as an endothelial differentiation gene, and thus was given the gene name *Edg1*. Mice deficient in $S1P_1$ die between E12.5 and E14.5 due to vascular hemorrhage and an inability of smooth muscles to migrate to enclose blood vessels (Liu et al. 2000). Mice that lack $S1P_1$ solely on endothelial cells exhibit a similar phenotype, pointing to the critical role that $S1P_1$ plays in vascular development (Allende et al. 2003). $S1P_1$ expressed on endothelial cells can also play an important role in the development of a mature vascular network, particularly the inhibition of excess sprouting. Abnormal sprouting of vascular networks is seen in the embryos where $S1P_1$ has been deleted only in endothelial cells (Shoham et al. 2012), and a similar increase in branching is seen in mice where $S1P_1$ is deleted inducibly shortly after birth, either in all tissues, or solely in endothelial cells (Jung et al. 2012). Interestingly, $S1P_1$ expression seems to be highest in areas of vasculature with fluid flow, and $S1P_1$ may function as a sensor of shear flow, providing another way that $S1P_1$ can regulate vascular function (Jung et al. 2012). The roles of $S1P_1$ specifically within the vascular system will be discussed in significantly more detail in a later chapter.

Following the finding that FTY720-P acted as an agonist to S1P receptors, many became interested in elucidating the functions of different S1P receptors in lymphocyte function. Deletion of $S1P_1$ in developing T cells, either by conditional knockout or bone marrow chimeric approaches, leads to the retention of mature $CD4^+$ and $CD8^+$ T cells within the thymus, leaving the blood and lymph depleted

of lymphocytes (Allende et al. 2004; Matloubian et al. 2004). Mice in which B cells are deficient $S1P_1$ have also been generated, and such mice display impaired egress of immature B cells from the bone marrow into the blood as well as a decrease in splenic B cell numbers (Allende et al. 2010). A more detailed examination of the roles of lymphocytic $S1P_1$, particularly the functions $S1P_1$ can have in lymphocyte recirculation and the positioning of lymphocytes within the lymphoid tissues, will also follow in a later chapter.

$S1P_1$, in addition to its critical roles in vascular and lymphocyte physiology, plays important roles within the central nervous system. $S1P_1$ is highly expressed within the brain and the spinal cord, across multiple cell types including neurons and astrocytes, and FTY720-P and other $S1P_1$ agonists can penetrate efficiently into the CNS, giving rise to the potential for actions of these agonists directly on CNS tissue. Mice in which $S1P_1$ has been deleted in astrocytes are refractory to developing experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, whereas mice in which $S1P_1$ was deleted only in neurons develop EAE similar to wild-type mice (Choi et al. 2011). Interestingly, whereas wild-type mice with EAE displayed improvements in clinical scores following treatment with FTY720, mice with EAE that lacked $S1P_1$ on astrocytes did not display any additional improvement in their symptoms following FTY720 treatment. $S1P_1$ may also play a role in inflammatory pain, as S1P and the S1P-selective agonist SEW2871 can cause thermal hyperalgesia following intraplanar injection. This hyperalgesia is dependent in part on $S1P_1$ expressed on nociceptive neurons, as mice where $S1P_1$ has been deleted in $Na_v1.8$-expressing neurons have reduced hyperalgesia following S1P administration (Mair et al. 2011).

2.2.2 $S1P_2$ Deficient Mice

Like $S1P_1$, $S1P_2$ has a critical role in vascular physiology. Unlike mice lacking $S1P_1$, mice lacking $S1P_2$ are viable (MacLennan et al. 2001; Ishii et al. 2002; Kono et al. 2004); however, $S1P_2$ deficient mice are deaf, potentially as a result of malformed stria vascularis in the inner ear or defects in hair cells (Herr et al. 2007; Kono et al. 2007). $S1P_2$ deficient mice in some genetic backgrounds can also exhibit spontaneous seizures (MacLennan et al. 2001). $S1P_2$ may play somewhat overlapping roles with both $S1P_1$ and $S1P_3$, as mice lacking both $S1P_1$ and $S1P_2$ display a more severe embryonic lethal phenotype than mice lacking $S1P_1$ alone while mice lacking $S1P_2$ and $S1P_3$ display significant perinatal lethality (Ishii et al. 2002; Kono et al. 2004). Mice deficient in both $S1P_2$ and $S1P_3$ also display significantly larger myocardial infarct sizes following ischemia/reperfusion injury, but mice lacking either display no difference, once again pointing to the significant overlap in function between the two receptors (Means et al. 2007). Another role of $S1P_2$ within the vascular system is seen when examining neovascularization within the eye, as $S1P_2$ deficient mice displayed significantly less intravitreal angiogenesis following ischemia (Skoura et al. 2007). Conditional knockout mouse lines for

$S1P_2$ have yet to be reported, but could potentially determine which cell types are responsible for the phenotypes seen in mice completely lacking $S1P_2$.

2.2.3 $S1P_3$ Deficient Mice

Viable $S1P_3$ deficient mice have also been generated which display no significant abnormalities, but display marginal deficits in reproduction when deficient mice intercrossed (Ishii et al. 2001). $S1P_3$ deficient mice have been used to show roles for $S1P_3$ in cardiac physiology, as $S1P_3$ deficient mice display resistance to bradycardia induced by the nonselective proagonist AAL-R (Sanna et al. 2004) and are resistant to developing cardiac fibrosis observed when SphK1 is transgenically overexpressed (Takuwa et al. 2010). $S1P_3$ deficient mice additionally exhibit a lack of vasorelaxation caused by S1P and show diminished vasorelaxation induced by injection of high-density lipoprotein (HDL), presumably as a result of the high amounts of S1P found within HDL (Nofer et al. 2004).

$S1P_3$ has also been studied for its role in the immune system, particularly marginal zone (MZ) B cells and dendritic cells. While no conditional knockout mouse strain has been described, reciprocal bone marrow chimera experiments and adoptive transfer experiments have been used with great success between wild-type and $S1P_3$ deficient mice. Such approaches have suggested that $S1P_3$ expressed on MZ B cells helps properly position them within the white pulp, and that migration of MZ B cells to S1P is mediated in large part by $S1P_3$ (Cinamon et al. 2004; Cinamon et al. 2008). $S1P_3$ expressed on dendritic cells can regulate their migration to S1P (Maeda et al. 2007) and mediates inflammation induced through various pathways. First, $S1P_3$ deficient mice display significantly less systemic inflammatory cytokine production following LPS treatment than do wild-type mice, a reduction that was reversed by adoptively transferring wild-type dendritic cells (Niessen et al. 2008). $S1P_3$ deficient mice also displayed improved survival compared to wild-type mice following LPS treatment and delayed lethality in a model of cecal ligation and puncture that could be reversed by adding in exogenous wild-type dendritic cells. $S1P_3$ deficient mice are also resistant to kidney ischemia reperfusion injury, and this resistance is dependent on the deletion of $S1P_3$ on dendritic cells, as injection of wild-type dendritic cells into $S1P_3$ deficient mice prior to injury could lead to a reversal in the protection granted by $S1P_3$ deficient mice (Bajwa et al. 2012).

2.2.4 $S1P_4$ Deficient Mice

$S1P_4$ was the last S1P receptor to be described and likewise was the last to have any in vivo phenotype ascribed to its deletion. Like $S1P_1$ and $S1P_3$, $S1P_4$ plays an important role in several different cell types in the immune system. $S1P_4$ knockout mice display impaired terminal differentiation of megakaryocytes, which leads to a deficit in platelet repopulation following administration of an antiplatelet antibody

but not any observable differences in platelet counts under normal conditions (Golfier et al. 2010). $S1P_4$ deficient mice also may help regulate neutrophil inflammation that can be caused by high levels of S1P and other sphingolipids in S1P lyase deficient mice (Allende et al. 2011). $S1P_4$ is also expressed on dendritic cells and may affect both their migration and their activation of T cells, as dendritic cells in mice lacking $S1P_4$ display accelerated in vivo migration to draining lymph nodes while $S1P_4$ deficient dendritic cells may have a reduced ability to cause T cells to secrete IL-17 (Schulze et al. 2011). No conditional $S1P_4$ mice have been described to date.

2.2.5 $S1P_5$ Deficient Mice

$S1P_5$ deficient mice, like mice lacking either $S1P_3$ or $S1P_4$, are viable and fertile. $S1P_5$ knockout mice were first described in the context of the function of $S1P_5$ on oligodendrocytes, as $S1P_5$ deficient oligodendrocytes did not exhibit process retraction following treatment with S1P (Jaillard et al. 2005). The most noted function of $S1P_5$ has also been found in the immune system, particularly its functions in natural killer (NK) cell migration, similar to the way other S1P receptors can regulate the migration of other types of immune cells. $S1P_5$ deficient mice display significant reductions in NK cells found within the blood and the spleen, but increased numbers in the bone marrow and lymph nodes, and NK cells lacking $S1P_5$ do not migrate to S1P as do wild-type NK cells (Walzer et al. 2007; Jenne et al. 2009; Mayol et al. 2011). Like all the other S1P receptors other than $S1P_1$, no $S1P_5$ conditional knockout mice have yet been described.

2.3 Knockin Mice Expressing Tagged or Mutated S1P Receptors

2.3.1 Fluorescence-Tagged Knockin Mice

While many genetically modified mice have been generated to delete S1P receptors in cells of interest, recent work has generated mice in which a fluorescence-tagged version of an individual S1P receptor replaces that S1P receptor. These mice allow for the clarification of the expression of S1P receptors in vivo by both flow cytometry and classic biochemistry, and can also be used in intravital two-photon microscopy to directly visualize changes in the subcellular localization of S1P receptors in real-time. Mice in which $S1P_1$ fused to the fluorescent protein eGFP have been used to study how treatment with different S1P receptor agonists and antagonists can affect the expression of $S1P_1$ on lymphocytes (Cahalan et al. 2011), endothelial cells, and within the central nervous system (Gonzalez-Cabrera et al. 2012), have been used to visualize the differential labeling of distinct vascular types with fluorescent particles (Sarkisyan et al. 2012), and have been critical

in the identification of endothelial cells as important regulators of cytokine production following influenza infection (Teijaro et al. 2011). A similar approach has also been taken in generating viable mice that express $S1P_3$ fused to the fluorescent protein mCherry (Hugh Rosen, personal communication). While many potential avenues of research may derive from native fluorescence of the fusion proteins, many may also come from the ability to immunoprecipitate the receptors using antibodies against these fluorescent proteins in order to determine posttranslational modifications such as phosphorylation and polyubiquitination of the receptors, as well as potentially determining associated proteins.

2.3.2 $S1P_1$ Mutant Knockin Mice

As mentioned previously, $S1P_1$ becomes rapidly internalized following the binding of agonists. As lymphocyte sequestration induced by $S1P_1$ agonists is thought to occur due to this internalization, the mechanisms by which this internalization occurs have been studied in detail. The internalization of $S1P_1$ is mediated in part by the phosphorylation of a number of serine and threonine residues located in C-terminal tail by G protein-coupled receptor kinases, or GRKs, leading to β-arrestin binding and clathrin-mediated endocytosis (Oo et al. 2007). Recently, knockin mice have been generated where native $S1P_1$ has been replaced by $S1P_1$ in which five of these C-terminal serines have been mutated to alanines ($S1P_1$-S5A), thus preventing the internalization of $S1P_1$ (Thangada et al. 2010). While $S1P_1$-S5A expressed on lymphocytes could still be internalized following either agonist treatment or exposure to blood, $S1P_1$-S5A exhibited delayed internalization in response to $S1P_1$ agonist treatment compared to wild-type $S1P_1$. This resistance to internalization paralleled a delay in lymphocyte sequestration in response to either $S1P_1$ agonism or S1P lyase inhibition. $S1P_1$-S5A expressing lymphocytes show increased migration toward high concentrations of S1P, suggesting that higher concentrations of S1P may normally internalize S1P, preventing migration. A similar knockin mouse in which a threonine and two serines in the C-terminal tail have been replaced by alanines ($S1P_1$-TSS) has also been generated that displays similar resistance to internalization and an increase in migration to S1P but not to other chemokines (Arnon et al. 2011). These two knockin mice, which appear to cause similar downstream signaling to wild-type mice, can help separate out which events following $S1P_1$ agonist treatment are a result of $S1P_1$ signaling, and which events may be affected by the internalization of the receptor.

3 Conclusions

The pleiotropic functions and complex biology of S1P make it a delicate system to study. Studying the actions of S1P on its receptors has yielded significant insights into a broad range of physiological systems. S1P will continue to be an exciting

area of study, particularly because of its relevance in human disease. Despite the relatively recent discovery of S1P receptors, modulation of S1P biology has already progressed to clinical treatment of disease. This progression has been aided significantly by the generation of an incredible diversity of both chemical and genetic tools that affect all aspects of S1P biology. While significant progress has been made, several areas of development of these tools remain, particularly the generation of selective in vivo chemical modulators and conditional knockouts. A summary of Additionally, it is likely that other genes regulating S1P function have yet to be found Truly, the combined use of chemical and the genetic tools within a single experiment provides the most useful and conclusive findings into the functions of S1P and its receptors. The continued development and refinement of both will hopefully continue to aid in our understanding of physiology and in the treatment of human disease.

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S1P Control of Endothelial Integrity

Yuquan Xiong and Timothy Hla

Abstract Sphingosine 1-phosphate (S1P), a lipid mediator produced by sphingolipid metabolism, promotes endothelial cell spreading, vascular maturation/stabilization, and barrier function. S1P is present at high concentrations in the circulatory system, whereas in tissues its levels are low. This so-called vascular S1P gradient is essential for S1P to regulate much physiological and pathophysiological progress such as the modulation of vascular permeability. Cellular sources of S1P in blood has only recently begun to be identified. In this review, we summarize the current understanding of S1P in regulating vascular integrity. In particular, we discuss the recent discovery of the endothelium-protective functions of HDL-bound S1P which is chaperoned by apolipoprotein M.

Abbreviations

AJ	Adherens junctions
AKI	Acute kidney injury
ALI	Acute lung injury
ApoM	Apolipoprotein M
BAL	Bronchoalveolar lavage
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
GEFs	Guanine nucleotide exchange factors
GJ	Gap junctions
HDL	High-density lipoprotein
HUVEC	Human umbilical vein endothelial cells
I/R	Ischemia-reperfusion
JAM	Junctional adhesion molecules
LDL	Low-density lipoprotein

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LPP3	Lysophospholipid phosphatase 3
LPs	Lysophospholipids
LPS	Lipopolysaccharide
MLC	Myosin light chain
MLCK	Myosin light chain kinase
PAF	Platelet-activating factor
PE	Phosphatidylethanolamine
PECAM-1	Platelet-endothelial cell adhesion molecule-1
RBC	Red blood cells
S1P	Sphingosine 1-phosphate
SM	Sphingomyelin
Spns2	Spinster 2
SPP	S1P phosphatases
TER	Transmonolayer electrical resistance
TJ	Tight junctions
VE-cadherin	Vascular endothelial cadherin
VLDL	Very low-density lipoprotein
ZO	Zona occludens proteins

Contents

1 Sphingosine 1-Phosphate Metabolism

Lysophospholipids (LPs) are minor lipid components compared to the major membrane phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM). The LPs were originally presumed to be

simple metabolic intermediates in the de novo biosynthesis of phospholipids. However, subsequent research demonstrated that the LPs exhibited significant biological activity by acting as extracellular growth factors or intercellular signaling molecules (Moolenaar and Hla 2012; Chun et al. 2010). As one of the most biologically significant LPs, Sphingosine 1-phosphate (S1P) has drawn considerable attention since the discovery that S1P is a signaling molecule that regulates multiple cell functions such as cell proliferation, differentiation, and migration (Olivera and Spiegel 1993; Lee et al. 1998; Lee et al. 1999; Hla et al. 2001; Paik et al. 2001).

The degradation of SM, a eukaryotic-specific phospholipid essential for the formation of membrane rafts and caveolae, is a major pathway involved in producing S1P. SM is metabolized by the sphingomyelinase pathway to produce progressively polar molecules: ceramide, sphingosine, and S1P (Hannun and Obeid 2008). Ceramide is catalyzed by ceramidase into sphingosine, which is phosphorylated by sphingosine kinase (Sphk) enzymes into S1P (Hait et al. 2006). There are two forms of Sphk, Sphk1 and Sphk2. Sphk1 is generally localized in the cytoplasm and translocates to the plasma membrane upon activation, while Sphk2 is primarily but not exclusively localized in nuclei (Ogawa et al. 2003; Igarashi et al. 2003; Venkataraman et al. 2006). S1P levels in cells are tightly regulated by the balance between its synthesis and degradation. Degradation of S1P occurs through the action of S1P lyase or by the specific S1P phosphatases (SPP1 and SPP2) as well as lysophospholipid phosphatase 3 (LPP3) (Le Stunff et al. 2002; Ogawa et al. 2003). The different S1P phosphatases regenerate sphingosine that can re-enter the sphingolipid metabolic pathway. S1P can also be irreversibly degraded by S1P lyase to yield hexadecenal and phosphoethanolamine, intermediates which are used as a substrate for phospholipid synthesis (Bandhuvula and Saba 2007). The degradation of S1P by the S1P lyase pathway serves as an important pathway for the conversion of sphingolipids into glycerolipids.

Although originally thought to be an intracellular second messenger (Olivera and Spiegel 1993), most of the biological effects of S1P were attributed to the signaling of its five ubiquitously expressed cell surface receptors, designated $S1P_{1\text{-}5}$, all of which bind to the ligand with nM affinity (Chun et al. 2002; Min et al. 2002; Blaho and Hla 2011). Although all S1PRs are G protein-coupled, each receptor subtype exhibits differential coupling efficacy to G protein alpha subunits. Although widely expressed, S1P receptors also display tissue-selective expression patterns as only three of the five S1P receptor subtypes ($S1P_1$, $S1P_2$, and $S1P_3$) are expressed in vascular tissues, whereas expression of the $S1P_4$ and $S1P_5$ receptors are largely confined to cells of the hematopoietic and nervous systems, respectively (Waeber 2013).

2 Sphingosine 1-Phosphate in the Blood

S1P is a pleiotropic lipid mediator capable of modulating the functions of many cell types. However, in mammalian systems S1P is found mainly in the blood and lymph in homeostasis (Pappu et al. 2007; Venkataraman et al. 2008; Hla et al. 2008; Yatomi et al. 2001). Thus, the functions of S1P in these two organ systems have been characterized most extensively. However, in the extravascular compartment, S1P can be produced in an inducible manner (Venkataraman et al. 2008; Hla et al. 2008). Interestingly, a significant concentration gradient of S1P exists between plasma and interstitial fluids: the concentration of S1P in plasma varies from 0.1 to 0.6 μM (Yatomi et al. 1997b; Yatomi et al. 1997a; Caligan et al. 2000; Berdyshev et al. 2005), while tissue S1P levels are generally low (0.5–75 pmol/mg) (Edsall and Spiegel 1999; Allende et al. 2004; Le Stunff et al. 2002; Ancellin et al. 2002; Ogawa et al. 2003; Chun et al. 2002; Venkiteswaran et al. 2002; Min et al. 2002). This concentration gradient, termed the vascular S1P gradient appears to form as a result of substrate availability and the action of metabolic enzymes. The physiological significance of this S1P gradient is now becoming clear but how it is maintained is an active area of investigation.

Serum SIP levels are always higher than those in plasma, which was explained by the abundance of S1P in platelets and its extracellular release upon stimulation by platelet activators such as thrombin. Platelets were assumed to be the major source of S1P in plasma as they express a high activity of SphK1, lack the S1P lyase that irreversibly degrades S1P, and their activation causes the release of S1P (English et al. 2000; Yatomi et al. 1997b; Yatomi et al. 1997a; Tani et al. 2005). However, whether platelets contribute to plasma S1P in vivo was not examined critically until recently. The transcription factor *NF-E2*-deficient had normal plasma S1P concentrations (Pappu et al. 2007), despite having virtually no circulating platelets (Shivdasani et al. 1995). In addition, circulating platelet depletion by infusion of an antibody against platelet glycoprotein GPIba (CD42b) also did not decrease plasma S1P levels (Venkataraman et al. 2008; Hla et al. 2008). These data suggest that platelets are unlikely to be the significant source of plasma S1P under physiological conditions, while recent reports suggest that red blood cells (RBC) and vascular endothelial cells (EC) may be the sources of S1P in plasma (Jessup 2008). However, a recent report suggested that during immune cell trafficking into high endothelial venules of lymph nodes, platelets extravasate into the basolateral surface and interact with fibroblast reticular cells via podoplanin/CLEC-2 interaction, resulting in stimulated formation of S1P and preservation of endothelial barrier function of this specialized endothelium (Herzog et al. 2013).

Significant progress toward identifying the cellular source(s) of S1P were made by using a novel *Sphk1/2* double gene-specific knockout mouse model. With this system, Pappu et al. showed that plasma S1P levels were reduced to undetectable levels in the mouse of *Sphk1/2* deleted by poly(I:C)-induced Mx1-Cre expression (pS1P less mice) (Pappu et al. 2007), although it is difficult to pinpoint the precise cellular source in this system since the Mx1-Cre transgene is responsive to IFN-α/β

and is rapidly induced in multiple tissues including liver, hematopoietic cells, and variably in other cell types (Kuhn et al. 1995). However, by conducting adoptive transfer experiments, they demonstrated that wild-type red blood cells alone have the capacity to restore plasma S1P to normal levels in conditional *Sphk1/2*-double knockout mice after lethal whole-body irradiation, suggesting that red cells are capable of being a major source of S1P in plasma. Erythrocytes possess much weaker Sphk activity compared to platelets but lack the S1P-degrading activities of either S1P lyase or S1P phosphohydrolase and erythrocytes are much more abundant in blood than platelets. This combination suggests that the S1P provided by erythrocytes is much greater (Sanchez et al. 2007; Ito et al. 2007; Xu et al. 2007). The studies from group of Gräler also suggested that erythrocytes are the main blood cell population that is capable of incorporating, protecting, storing, and releasing S1P in vitro or ex vivo (Hanel et al. 2007; Bode et al. 2010).

However, our own findings suggest that hematopoietic cells are not the only cellular source that is capable of producing S1P to maintain high plasma levels (Venkataraman et al. 2008; Hla et al. 2008). Hematopoietic cell depletion experiments showed that mice that are severely anemic (>50 % reduction in hematocrit), thrombocytopenic (>90 % suppression of platelets), and irradiated (leukopenic, thrombocytopenic, and anemic) possessed wild-type S1P levels in plasma. We also found that plasma S1P bound to albumin turns over rapidly with a half-life of $\approx$15 min, which suggests that very active synthetic and degradative pathways of S1P metabolism exist in vivo, and that vascular endothelium might be a major contributor to plasma S1P (Venkataraman et al. 2008; Hla et al. 2008). Spinster 2 (Spns2), a member of the major facilitator superfamily of nonATP-dependent transporters, has been shown to be an S1P transporter (Kawahara et al. 2009). The plasma S1P levels of *Spns2*-deficient mice was reduced to approximately 60 % of wild-type, while analysis of the cells isolated from *Spns2*-deficient mice demonstrated that mammalian Spns2 is the S1P transporter in vascular ECs but not in erythrocytes and platelets (Hisano et al. 2012; Fukuhara et al. 2012). Indeed, in Spns2 EC-specific knockout mice, plasma S1P concentration was decreased to the level observed in $Spns2^{-/-}$ mice (Fukuhara et al. 2012; Mendoza et al. 2012).

Sphk enzyme activity is expressed in various cell types (Yatomi et al. 1997b; Yatomi et al. 1997a), suggesting there could be many cellular sources of blood S1P in addition to erythrocytes and ECs. Recently, Kurano et al. also reported that plasma S1P levels were decreased by hepatectomy, suggesting that liver may contribute to plasma S1P levels in vivo (Kurano et al. 2013). The brain is the organ containing the largest amount of S1P (4–40 nmol/g wet weight, corresponding to about 4–40 μM) (Jiang and Han 2006; Murata et al. 2000b; Murata et al. 2000a), suggesting that neurons and astrocytes may represent a significant source of S1P, able to activate vascular S1P receptors. Indeed, high levels of *Sphk2* mRNA were detected by real-time reverse transcriptase polymerase chain reaction in glial cells and cortical neurons (Blondeau et al. 2007) and S1P has been shown to be released from cultured neural cells, such as astrocytes and granule cells, in response to stimuli (Anelli et al. 2005; Bassi et al. 2006). Whether S1P crosses the blood–brain barrier is not known.

3 Maintenance of Vascular Barrier Integrity by Sphingosine 1-Phosphate

The integrity of blood vessels is critical to vascular homeostasis, as failure of this system results in serious consequences such as hemorrhage, edema, inflammation, and tissue ischemia. Vascular integrity is tightly regulated by a number of factors that ensure proper functions of various components of the blood vessel wall, while S1P has been identified as a robust barrier-enhancing factor with great potential to serve as a novel and specific therapy for EC barrier dysfunction (Lee et al. 1999; Garcia et al. 2001; Sanchez et al. 2003; Jung et al. 2012; Gaengel et al. 2012). In general, the transport of fluids and solutes through the endothelial barrier of "generic" continuous endothelium is determined by two separate pathways: an active transcellular pathway for macromolecules larger than 3 nm, such as albumin, and a passive paracellular pathway for smaller molecules (Predescu et al. 2007; Michel and Curry 1999). This paracellular pathway is regulated by a complex balance of intracellular contractile forces generated by actin-myosin and tethering forces between adjacent ECs and between cells and the extracellular matrix. In various vascular beds, specific mechanisms such as the blood–brain barrier and fenestrae exist to either further increase or decrease the barrier functions, respectively.

3.1 S1P Signaling to Endothelial Cytoskeleton

Cytoskeleton, a complex network of actin microfilaments, microtubules, and intermediate filaments, which combine to regulate shape change and transduce signals within and between neighboring cells. Cytoskeletal rearrangement is a critical cellular event mediating endothelial barrier function (Bogatcheva and Verin 2008). Morphologic studies demonstrate that S1P at 1 μM produces rapid and dramatic enhancement of polymerized F-actin and myosin light chain phosphorylation at the cell periphery, meanwhile, maximal barrier enhancement is observed with 1 μM S1P that peaks after 10–20 min and is sustained for hours. Consistent with the conceptual framework that barrier regulation is intimately linked to the cytoskeleton, changes in the actin cytoskeleton were essential for S1P-mediated barrier enhancement as cytochalasin B, an actin depolymerizing agent, and latrunculin B, which inhibits actin polymerization, both prevent the barrier-enhancing effects of S1P (Garcia et al. 2001). Recently, Arce et al. characterized the structural and mechanical changes in the cytoskeleton of cultured human pulmonary artery ECs in response to S1P, and they found the elastic modulus, an indicator of underlying structural force, is significantly elevated at the peripheral region of the cell by S1P treatment (Arce et al. 2008). These studies suggest a critical role of dynamic actin assembly/disassembly and subsequent cortical redistribution in mediating S1P-induced barrier enhancement.

The actin-associated cytoskeletal proteins, cortactin and myosin light chain kinase (MLCK), also play a role in the augmentation of EC barrier function induced by S1P. Cortactin is involved in stimulating actin polymerization and cortical actin rearrangement, and tyrosine phosphorylation of cortactin is seen after stimuli that cause cytoskeletal rearrangement (Owen et al. 2007; Belvitch and Dudek 2012). MLCK are a family of soluble protein kinases that function principally to phosphorylate myosin light chain (MLC). MLC phosphorylation enables actin-myosin interaction, leading to formation of stress fibers and cell contraction (Takashima 2009). Exposure of ECs to S1P produces rapid and significant translocation of cortactin from the cytoplasm to a peripheral cortical distribution. In ECs, cortactin depletion by antisense oligonucleotide techniques results in a 50 % inhibition of peak S1P barrier enhancement, whereas overexpressing wild-type cortactin showed enhanced transmonolayer electrical resistance (TER) after S1P treatment (Dudek et al. 2004). Similar to cortactin, when exposed to physiologic levels of S1P, MLCK is rapidly redistributed to areas of active membrane ruffling and directly bind to the cortactin Src homology 3 domain. The interaction of cortactin and MLCK appears to be necessary for optimal S1P-induced barrier enhancement since cortactin blocking peptide inhibits S1P-induced MLC phosphorylation and peak S1P-induced TER values.

The effect of S1P on these actin-dependent processes is mediated, in large part, by the Rho family of small GTPases. The Rho family of GTPases (Rho, Rac, and Cdc42) is a group of regulatory molecules that link surface receptors to downstream effectors regulating actin cytoskeletal structure (Spiering and Hodgson 2011). Rho, Rac, and Cdc42 function as molecular switches and promote the formation of stress fibers, lamellipodia, and filopodia, respectively, by catalyzing specific types of actin polymerization. The primary S1P receptors expressed in EC are $S1P_1$, $S1P_2$ and $S1P_3$ (Ancellin et al. 2002), which exhibit distinct coupling to Rho family GTPases. $S1P_1$ activates Rac through a mechanism that requires Gαi-dependent activation of PI3-Kinase. In contrast, $S1P_2$ activates Rho through Gα12/13 and RGS family of Rho-specific guanine nucleotide exchange factors (GEFs) that interact with this G protein. $S1P_3$ can activate Rac through Gαi, but also couples to Gαq/11, and activates Rho through the Trio family of Rho-GEFs (Oo et al. 2011; Blaho and Hla 2011; Sanchez et al. 2003; Hla 2003). Rac activity is required for S1P-induced adherens junction assembly and cytoskeleton rearrangement (Lee et al. 1999). S1P preferentially activates Rac via $S1P_1$ in a pertussis toxin-sensitive fashion and enhances barrier integrity. Overexpression of constitutively active Rac enhances peripheral actin polymerization in the cortical ring, and Rac activation induces lamellipodia formation, membrane ruffling, the formation of cortical actin filaments, and the spreading of ECs (Garcia et al. 2001; Dudek et al. 2004). While inhibition of Rac GTPase leads to increased monolayer permeability and enhances the thrombin-mediated barrier dysfunction response through a variety of signaling proteins (Wojciak-Stothard et al. 2001). Microinjection of dominative negative Rac into ECs dramatically diminishes S1P-induced VE-cadherin and β-catenin enrichment at cell–cell junctions, while overexpression of active Rac reproduces changes in the cortical actin similar to those evoked by

S1P (Lee et al. 1999). Moreover, reduction of Rac expression by siRNA significantly attenuates the S1P TER response (Dudek et al. 2007). The S1P-stimulated cytoskeletal rearrangement and barrier protection are highly dependent on the exact S1P concentrations utilized. Rac GTPases are rapidly activated by physiologic concentrations of S1P (10 nM–2 μM) producing barrier enhancement, whereas higher concentrations of S1P result in $S1P_3$-dependent RhoA-mediated signaling and increased barrier permeability (Shikata et al. 2003).

3.2 S1P Signaling to Endothelial Junctions

In ECs, among the three types of intercellular junctions are adherens junctions (AJ or zonula adherens), tight junctions (TJ or zonula occludens), and gap junctions (GJ). In general, AJ and TJ contribute to the structural integrity of the endothelium (Dejana 2004; Bazzoni and Dejana 2004). Vascular endothelial cadherin (VE-cadherin) is the major structural protein of adherens junctions. Stability of VE-cadherin at adherens junctions, which is controlled by binding to catenins, especially to p120-catenin, is critical to the maintenance of endothelial permeability and integrity (Venkiteswaran et al. 2002). Targeted disruption of VE-cadherin gene or truncation of β-catenin binding domain of VE-cadherin in mice causes lethality at E9.5 days of gestation due to immature vascular development (Carmeliet et al. 1999). In a mouse model, injection of anti-VE-cadherin antibodies induces a marked increase in pulmonary vascular permeability, but a similar effect is not observed in the brain vasculature (Corada et al. 1999). Furthermore, endothelial-specific deletion of β-catenin is embryonic lethal starting at E11.5, presenting with vascular insufficiency, including reduced numbers of endothelial junctions, hemorrhage, and fluid extravasation (Cattelino et al. 2003).

Our studies showed that in confluent human umbilical vein endothelial cells (HUVEC), S1P significantly increases the abundance of VE-cadherin and β-catenin at the cell–cell contact regions and enhances AJ assembly. While the translocation of VE-cadherin to cell–cell contact regions in HUVEC after treatment of S1P was attenuated by microinjection of oligonucleotides designed to interrupt $S1P_1$ and $S1P_3$ receptor expression (Lee et al. 1999). Overexpression of $S1P_1$ in HEK293 cells markedly increases the expression level of P-cadherin and E-cadherin, but not α-catenin and β-catenin, and induces formation of well-developed adherens junctions in a manner dependent on S1P and the small guanine nucleotide binding protein Rho (Lee et al. 1998). Furthermore, $S1P_1$ silencing leads to a reduction in expression of both VE-cadherin and platelet-endothelial cell adhesion molecule-1 (PECAM-1), and the degree of $S1P_1$ knockdown was correlated with the extent of suppression of VE-cadherin and PECAM-1 (Krump-Konvalinkova et al. 2005). Recently, our studies demonstrate that in vivo $S1P_1$ function is required for adherens junction stability in the developing retinal vasculature. Lack of $S1P_1$ promoted junctional destabilization, as evidenced by increased tyrosine phosphorylation, trypsin sensitivity of the extracellular domain

of VE-cadherin (Jung et al. 2012). Gaengel et al. also reported that $S1P_1$ signaling positively and directly regulate the level of VE-cadherin at endothelial junctions, an effect that overrides the negative effect of VEGF on junctional VE-cadherin concentrations (Gaengel et al. 2012). However, the direct functional role of VE-cadherin in mediating S1P-induced endothelial barrier enhancement is complex and still poorly characterized. A recent study indicates that VE-cadherin may not be involved in the rapid and immediate effects of S1P on the barrier regulation as shown through TER studies but may play a role in the delayed onset-sustained effects of S1P on barrier enhancement (Pappu et al. 2007; Xu et al. 2007).

In addition to enhancing adherens junction assembly, there is also evidence that S1P assists in the formation of endothelial tight junctions. The TJ are positioned on the outer leaflets of the lateral membranes between adjacent cells. The TJ anchors into the actin cytoskeleton through the interaction and binding of the occludins, claudins, and junctional adhesion molecules (JAM) with the zona occludens proteins (ZO-1, ZO-2, or ZO-3) (Bazzoni and Dejana 2004). Following stimulation by S1P, ZO-1 is reassigned to the lamellipodia and to the cell–cell junctions via the $S1P_1$/Gi/Akt/Rac pathway, while the enhanced barrier function induced by S1P is attenuated by siRNA downregulation of ZO-1 expression (Lee et al. 2006). Thus, like the AJ, the TJ also plays an important role in S1P-mediated barrier regulation. Whether this mechanism is important in tissues with high vascular barrier, i.e., CNS, testis, retina is not known.

4 Sphingosine 1-Phosphate Regulates Vascular Leak in Disease Models

One of the main pathophysiological mechanisms involved in the genesis of various vascular disease conditions is endothelial dysfunction. Conditions ranging from inflammation, atherosclerosis, anaphylaxis, Ischemia-reperfusion injury, and cerebrovascular disease have a basis in endothelial barrier imbalance and dysregulation (Mullin et al. 2005). As presenting at high concentrations in plasma, S1P is important to maintain vascular integrity and regulate vascular leak. Indeed, mice selectively lacking S1P in plasma (pS1Pless mice) and mice with degradation of $S1P_1$ in vivo exhibit basal vascular leak and increased local response to leak-inducing agents (Camerer et al. 2009; Oo et al. 2011; Karuna et al. 2011; Argraves et al. 2011; Lee et al. 2011).

4.1 Acute Lung Injury

Acute lung injury (ALI) is a devastating inflammatory lung disease characterized by a marked increase in vascular permeability which is often exacerbated by the mechanical ventilation (Matthay et al. 2012). Intratracheal administration of

lipopolysaccharide (LPS) is a well-characterized experimental model to mimic the clinical presentation of ALI. In an isolated perfused murine lung model, intravenous administration of S1P significantly reduces the inflammatory histological changes produced by LPS and attenuates neutrophil infiltration in lung parenchyma. Similarly, intraperitoneal injection of FTY720, the S1P analog, significantly decreases LPS-induced pulmonary microvascular leakage (Peng et al. 2004; McVerry et al. 2004). Use of a large animal canine model allowed investigation of regional lung changes in ALI and the effect of S1P on these changes. In a canine model of ALI, Intravenously delivered S1P also significantly attenuated both alveolar and vascular barrier dysfunction (Peng et al. 2004; McVerry et al. 2004). Moreover, S1P also protected against intrabronchial LPS-induced ALI in a canine model. S1P attenuated the formation of shunt fraction and both the presence of protein and neutrophils in bronchoalveolar lavage (BAL) fluid compared to vehicle controls (Szczepaniak et al. 2008). Recently, Zhao et al. also reported that inhibition of S1P lyase resulted in increased S1P levels in lung tissue and bronchoalveolar lavage fluids and reduced lung injury and inflammation (Zhao et al. 2011). However, there are differential effects for S1P receptors on airway and vascular barrier function in the murine model of ALI. At physiologically relevant concentrations, S1P is barrier protective via ligation of $S1P_1$, regardless of delivery via intratracheal or intravenous routes. The activation of $S1P_2$ and $S1P_3$ receptors, however, contributes to alveolar and vascular barrier disruption, whereas the targeted deletion or silencing of $S1P_2$ and $S1P_3$ was found to be beneficial (Sammani et al. 2010; Peng et al. 2004; McVerry et al. 2004; Zhang et al. 2013; Cui et al. 2013). It is also thought that $S1P_2$ and $S1P_3$ are responsible for the increased permeability that is observed after administration of higher doses of S1P or SEW2871 (Sammani et al. 2010).

4.2 Anaphylaxis

Anaphylaxis is a serious allergic response with a rapid onset. The release of inflammatory mediators like histamine subsequently impairs the function of multiple organ systems and increased vascular permeability and fluid extravasation is a known complication (De Bisschop and Bellou 2012). There is a strong association between the plasma concentration of S1P and the concentration of circulating histamine after the anaphylactic response (Olivera et al. 2007). Indeed, *Sphk1*-deficient mice had lower S1P concentrations in blood compared to wild-type mice, while increased S1P concentrations in blood of *Sphk2*-deficient mice resulted in a faster recovery from an anaphylactic shock due to enhanced histamine clearance in blood (Olivera et al. 2007). Furthermore, pS1Pless mice displayed increased vascular leak and impaired survival after anaphylaxis, administration of platelet-activating factor (PAF) or histamine, and exposure to related inflammatory challenges. Increased leak was associated with increased interendothelial cell gaps in venules and was reversed by transfusion with

wild-type erythrocytes (which restored plasma S1P levels) and by acute treatment with an agonist for the $S1P_1$ (Camerer et al. 2009). Thus the plasma S1P-endothelial $S1P_1$ axis plays a central role in maintaining vascular barrier integrity. In contrast to $S1P_1$, it has been shown that pharmacologic blockade of $S1P_2$ reduced histamine-induced vascular leakage, and genetic deletion of $S1P_2$ attenuated vascular leakage and hypothermia in a mouse model of anaphylaxis after antigen challenge (Oskeritzian et al. 2010; Lee et al. 2009). Our studies also showed that $S1P_2$ activation in endothelial cells increases vascular permeability. The balance of $S1P_1$ and $S1P_2$ receptors in the endothelium may determine the regulation of vascular permeability by S1P (Sanchez et al. 2007). More recently, Zhang et al. also reported that inhibition of $S1P_2$ signaling dramatically decreased PLS-induced vascular permeability (Zhang et al. 2013; Cui et al. 2013). However, a recent study showed that $S1P_2$ protects mice from vascular barrier disruption elicited by either antigen challenge or PAF injection and that this protective effect of $S1P_2$ is mediated through suppression of anaphylaxis-associated endothelial nitric oxide synthase (eNOS) stimulation (Cui et al. 2013). Thus the in vivo role of $S1P_2$ in inflammatory conditions such as sepsis and anaphylaxis is not yet fully understood.

4.3 Ischemia-Reperfusion Injury

Ischemia-reperfusion (I/R) injury is encountered in a variety of settings from disease states such as transplantation, stroke, hemorrhage, cardiopulmonary bypass, and aneurysm repair. Microvascular dysfunction mediates many of the local and systemic consequences of I/R injury, while disruption of the endothelial barrier and increased hydraulic conductivity lead to compression of the vessel by interstitial edema (Seal and Gewertz 2005). In a rat model of orthotopic left lung transplantation, Okazaki et al. showed that S1P treatment of lung recipients just prior to graft reperfusion improves lung function. In comparison to vehicle-treated grafts, S1P-mediated preservation of lung graft function was associated with markedly less tissue injury as evidenced by significantly reduced vascular permeability, inflammatory cell infiltration, and EC apoptosis (Okazaki et al. 2007). Furthermore, in another report of lung I/R injury via pulmonary artery ligation and subsequent reperfusion, animals pretreated with S1P exhibited reduced BAL, inflammatory cells, BAL neutrophils, and BAL albumin content compared to controls (Moreno-Vinasco et al. 2008). Hepatic I/R injury that frequently complicates acute kidney injury (AKI) showed elevated inflammatory cytokines and increased vascular permeability during the perioperative period, while pretreatment with S1P resulted in an attenuation of systemic inflammation and endothelial injury, suggesting modulation of the S1P signaling might have some therapeutic potential in hepatic IR injury-induced kidney injury (Belvitch and Dudek 2012; Lee et al. 2011). Moreover, recent studies showed there is a highly significant inverse relationship between the level of S1P in the high-density lipoprotein (HDL) containing fraction of serum and the occurrence of ischemic heart disease

(Argraves et al. 2011; Egom 2013). Oyama et al. showed that either local exogenous S1P administration or endogenous S1P overproduction promotes post-ischemic angiogenesis and blood flow recovery mouse ischemic hindlimbs (Oyama et al. 2008). Furthermore, our group reported that the S1P in the extracellular milieu, generated by the overexpression of Sphk1, induced angiogenesis and vascular maturation (Ancellin et al. 2002). All these studies suggest the potential usefulness of S1P as an angiogenic therapeutic agent in I/R injury.

4.4 FTY720 in the Pathology of Vascular Barrier Dysfunction

FTY720 is a sphingosine analog and is phosphorylated by sphingosine kinase-2–FTY720-phosphate, which is an agonist of 4 out of 5 S1P receptors. FTY720 has been approved by the United States Food and Drug Administration in 2010 for the treatment of multiple sclerosis (Pitman et al. 2012). The clinical availability of FTY720 makes it attractive as a potential mediator for patients with vascular barrier dysfunction. Indeed, a single intraperitoneal injection of FTY720 significantly attenuated murine pulmonary injury after LPS administration (Peng et al. 2004; McVerry et al. 2004). Similarly, low concentrations of FTY720 (0.1 mg/kg) reduced lung permeability in mechanically ventilated mice (Christoffersen et al. 2011; Muller et al. 2011). However, there are limitations to the therapeutic utility of FTY720 in vascular barrier dysfunction. Prolonged exposure to FTY720 resulted in the downregulation of $S1P_1$ on the EC surface and decreased responses to S1P (Krump-Konvalinkova et al. 2008). An administration of high concentrations FTY720 (0.5–5.0 mg/kg) to mice induced a dose-dependent $S1P_1$ degradation and an increase in vascular permeability (Shea et al. 2010). This in vivo barrier-disruptive effect of high-dose FTY720 is in contrast to its barrier protective effect observed in vitro (Sanchez et al. 2003; Berdyshev et al. 2009).

5 Endothelium-Protective Function of Apolipoprotein M/HDL-Bound S1P

The concentration of S1P in the plasma ranges between 200 and 1,000 nM, and most of the S1P in plasma is protein-bound. The majority of plasma S1P ($\sim$60 %) is bound to HDL, whereas $\sim$30 % is bound to albumin and a minor fraction to very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) (Murata et al. 2000b; Murata et al. 2000a; Karuna et al. 2011). It was unknown how S1P binds to HDL, until apolipoprotein M (ApoM) was revealed as a carrier of S1P on HDL particle (Christoffersen et al. 2011) and the linkage of ApoM/HDL- bound S1P and endothelial protection was explored. ApoM is a 25 kDa protein predominantly

associated with HDL via a retained hydrophobic signal peptide (Christoffersen et al. 2008). The plasma ApoM concentration is approximately 0.9 μmol/l, and more than 95 % is bound to HDL (the remaining ApoM is bound to LDL and VLDL) (Christoffersen et al. 2006). *Apom*$^{-/-}$ mice have approximately 46 % reduced plasma S1P levels compared with wild-type mice, remarkably, S1P is absent in HDL from *Apom*$^{-/-}$ mice. Moreover, S1P is increased approximately by 71 and 267 % in mice models with either 2-fold or 10-fold increase of plasma ApoM, respectively. Also, the S1P content of HDL is confined to the ApoM-containing particles in human plasma (Christoffersen et al. 2011). Thus ApoM was defined as a carrier of S1P in HDL.

Growing evidence indicates that HDL-associated S1P is responsible for the beneficial effects on vascular integrity. HDL-associated S1P was reported to promote endothelial barrier via Gi-coupled S1P receptors and the Akt signaling pathway (Argraves et al. 2008). In addition, S1P was identified as one of the principal bioactive lysophospholipids in HDL which is responsible for about 60 % of the vasodilatory effect of HDL in isolated aortae ex vivo (Nofer et al. 2004). Moreover, the regulation of endothelial barrier by protein-bound S1P is carrier-dependent. Wilkerson et al. indicate that the duration of the barrier promotion elicited by HDL-S1P lasted longer than albumin-S1P, which may due to the specific effects of HDL-S1P on $S1P_1$ trafficking that prolong S1P-$S1P_1$ signaling involving persistent activation of Akt and eNOS (Wilkerson et al. 2012). ApoM was recently reported to mediate the S1P-dependent vascular protective effects of HDL by delivering S1P to the $S1P_1$ receptor (Christoffersen et al. 2011). S1P carried by ApoM in the HDL fraction has an important role in preserving vascular integrity, which proved that ApoM-containing HDL (with S1P) is better in activating and inducing EC migration, and formation of endothelial adherens junctions than ApoM-free HDL (without S1P). Interestingly, *Apom*$^{-/-}$ mice also display vascular leakage in the lungs, accompanying decreased plasma S1P (Christoffersen et al. 2011). Recently, the $S1P_1$ receptor was crystallized (Hanson et al. 2012). The structural information forms a new basis for understanding the interaction between ApoM-containing HDL and release of S1P from the calyx into the binding pocket of the $S1P_1$.

6 Conclusions and Future Perspectives

These studies have highlighted the generality of the regulation of endothelial cell barrier function by S1P. Future challenges include further defining molecular mechanisms which are of fundamental importance in vascular biology and therapeutic application of this knowledge base to many pathological functions in which vascular barrier function is dysregulated, including sepsis, anaphylaxis, acute lung injury, inflammation, stroke, Dengue hemorrhagic fever, etc.

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Blood, Sphingosine-1-Phosphate and Lymphocyte Migration Dynamics in the Spleen

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Abstract The spleen, the largest secondary lymphoid organ, has long been known to play important roles in immunity against blood-borne invaders. Yet how cells migrate within the spleen to ensure fast and effective responses is only now coming to light. Chemokines and oxysterols guide lymphocytes from sites of release at terminal arterioles into the lymphocyte-rich white pulp. Sphingosine-1-phosphate (S1P) and S1P-receptor-1 (S1PR1) promote lymphocyte egress from white to red pulp and back to circulation. Intravital two-photon microscopy has shown that marginal zone (MZ) B cells that are enriched between white and red pulps undergo continual oscillatory migration between the MZ and follicles, ferrying antigens. Cycles of G-protein-coupled receptor kinase-2 (GRK2) mediated S1PR1 desensitization and resensitization underlie this remarkable behavior. The findings discussed in this review have implications for understanding how splenic antibody and T-cell responses are mounted, how the immunosuppressant drug FTY720 (fingolimod) affects the spleen, and how cell shuttling behaviors contribute to immunity.

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Contents

1 Introduction

The spleen has a unique role in filtering blood for detection of antigens and splenectomy is associated with an increased propensity for overwhelming infection by systemic pathogens (Mebius and Kraal 2005). Splenic anatomy is intricately organized to foster responses against blood-borne antigens. The organ is divided into two major regions: the lymphocyte-rich white pulp that forms large sheathes around arterioles, and the surrounding macrophage and red blood cell (RBC)-rich red pulp. Unlike most tissues, the splenic blood circulation is not a closed vascular circuit. Instead, blood is released from terminal arterioles into sinuses at the margin of white and red pulps, and from here it flows through fenestrations in the red pulp-facing surface (Schmidt et al. 1985; Veerman and van Ewijk 1975). Although some terminal arterioles open directly into the red pulp, it is estimated that 90 % of the splenic inflow passes through the sinuses bordering the white pulp (Schmidt et al. 1993). Situated between the marginal sinus and the red pulp is the marginal zone (MZ), a region that contains specialized populations of B cells and macrophages (Kraal and Mebius 2006). After passing out of marginal sinus fenestrations, blood passes over the MZ cells before reaching the red pulp and returning to circulation through slits in red pulp venous sinuses (Mebius and Kraal 2005).

Splenic white pulp anatomy bears similarity to lymph nodes, with a central T zone rich in T cells and dendritic cells (DCs) and flanking B cell rich follicles (Cyster 2005). Much of the follicle surface is covered by the meshwork of marginal sinuses but there is also a region where the T zone extends between the follicles to directly interface with the red pulp. This is termed the MZ bridging channel (Mitchell 1973). Underlying the T zone and follicles is a network of chemokine producing stromal cells that promote immune cell compartmentalization. Fibroblastic reticular cells (FRCs) in the T zone produce CCL21 and CCL19 that attract T cells and DCs via CCR7. Follicular stromal cells, including the specialized antigen presenting follicular dendritic cells (FDCs) that occupy the center of the follicular stromal network, produce CXCL13 and attract B cells and follicular helper T cells via CXCR5 (Cyster 2005). B-cell migration to the outer regions of the follicle is also

promoted by an oxysterol, 7α, 25-dihydroxycholesterol, that is made by lymphoid stromal cells in these regions and attracts B cells by acting on the G-protein-coupled receptor, EBI2 (Hannedouche et al. 2011; Liu et al. 2011; Yi et al. 2012).

After entering a lymphoid tissue from the blood, lymphocytes spend 4–24 hr within the tissue before returning to circulation to travel to another lymphoid organ and continue surveying for antigens (Cyster 2005; Tomura et al. 2008). If an antigen is encountered, responding lymphocytes are retained within the tissue for days (and possibly longer) to become activated and undergo differentiation into effector cells. Real-time imaging of cell migration in lymph nodes using two-photon microscopy has shown that T and B lymphocytes migrate continually within their respective zones, surveying for antigens (Cahalan and Parker 2008).

Lymphocyte exit from lymphoid organs is promoted by the lysophospholipid, sphingosine-1-phosphate (S1P) acting via the Gi-coupled receptor, S1P-receptor-1 (S1PR1). S1P is made by the action of two sphingosine kinases, Sphk-1 and -2, present within most cell types. As well as being inside cells, S1P is exported by some cell types and it is abundant in plasma ($\sim$1 μm) and lymph ($\sim$0.1 μm) where it is carried by high density lipoprotein-associated apolipoprotein M and by albumen (Christoffersen et al. 2011; Murata et al. 2000). RBCs are the major producers of plasma S1P (Pappu et al. 2007). Blood and lymphatic endothelial cells are also critical S1P producers (Pham et al. 2010; Venkataraman et al. 2008), with release from these cells being dependent on the membrane transporter, spinster-2 (Fukuhara et al. 2012; Mendoza et al. 2012). S1P can be dephosphorylated by any of five transmembrane phosphatases (Cyster and Schwab 2012) and recent studies have shown that lipid phosphate phosphatase-3 (LPP3) plays an important role in S1P degradation within the thymus (Breart et al. 2011). Within the cell, S1P is terminally degraded by S1P lyase (Schwab et al. 2005). The high activity of the multiple S1P degradation pathways is established by the less than 15 minute half-life of S1P within blood (Venkataraman et al. 2008). Notably, RBCs lack S1P degrading enzymes (Ito et al. 2007; Pappu et al. 2007). Given that all of the S1P degrading enzymes are cell membrane associated, and their absence from RBCs, it can be anticipated that the S1P half-life within interstitial fluids will be even shorter than within blood. Consistent with this view, interstitial S1P concentrations within lymph nodes are estimated to be in the low or sub-nanomolar range (Cyster and Schwab 2012). Thus, in lymph nodes a "gradient" or difference in S1P concentration exists between the parenchyma and the lymphatic sinuses, and egressing cells are triggered to enter lymphatic sinuses by S1PR1 exposure to the higher local S1P concentration (Grigorova et al. 2010; Grigorova et al. 2009; Sinha et al. 2009). Recent findings for the spleen suggest a similar series of steps may be at work to promote lymphocyte emigration out of the white pulp and into the MZ and red pulp, as will be discussed in later sections of this review.

2 Lymphocyte Entry to the Splenic White Pulp

After their release from terminal arterioles, many B and T cells migrate into the splenic white pulp in a Gai-coupled receptor dependent manner (Cyster and Goodnow 1995). For T cells, CCR7 is critical in guiding their movement to the T zone in response to CCL21 (Forster et al. 1999). After being released in the marginal sinus, T cells travel into the T zone by following the CCL21-laden FRC network (Bajenoff et al. 2008). Although the second CCR7 ligand, CCL19, is also expressed by splenic stromal cells (Ngo et al. 1998), mice lacking this chemokine were not found to have any overt defect in T cell trafficking (unpubl. obs.). However, the much more basic nature and greater heparin-binding activity of CCL21 compared to CCL19 (de Paz et al. 2007; Patel et al. 2001) make it likely that the two chemokines will be distributed differently through the T zone and adjacent regions and it remains probable that a nonredundant role for CCL19 in splenic lymphocyte trafficking will be revealed.

For B cells, white pulp entry is promoted by CXCR5 and to a lesser degree by CCR7 and EBI2 (Förster et al. 1994; Gatto et al. 2011; Pereira et al. 2009b). By serial analysis of frozen sections prepared in the first hour after cell transfer, B cells were observed to move directly from sites of release in the marginal sinus region into the underlying follicle in a manner that was Gai-dependent and also involved aL- and a4 containing integrins (Lo et al. 2003). Some B cells may also move into the white pulp via MZ bridging channels, moving along the B-T interface for a short period before entering follicles (Bajenoff et al. 2008; Nieuwenhuis and Ford 1976). The fraction of B cells that enter the white pulp via these two paths is not yet agreed upon. It seems possible that the amount of entry via CCR7 ligand high regions (bridging channels and the B-T interface) might be increased by certain isolation and in vitro labeling conditions, given the propensity for B-cell activation to cause prompt upregulation of CCR7 and EBI2 (Gatto et al. 2011; Kelly et al. 2011). The different utilization of these entry pathways might also alter the integrin dependence of B cell entry and explain why B cells lacking the integrin interacting cytoskeletal protein, talin, that have greatly reduced integrin function, entered the white pulp with normal efficiency (Manevich-son et al. 2010).

3 Overcoming S1P-Mediated Attraction During Lymphoid Organ Entry

While S1PR1 is detectable on the surface of most lymphocytes within spleen and lymph nodes, it is undetectable on the surface of cells circulating in blood (Lo et al. 2005). This down-modulation is ligand mediated as S1PR1 remains high on the surface of blood lymphocytes in conditional Sphk-deficient mice that lack measurable blood S1P (Pappu et al. 2007). A number of in vitro studies established

that S1PR1 is remarkably sensitive to down-modulation by ligand (Liu et al. 1999; Watterson et al. 2002) and in the case of lymphocytes, internalization can be completed after 20 min of exposure to as little as 1 nM S1P (Schwab et al. 2005). Ligand-activated GPCR internalization often involves phosphorylation of serine and threonine residues in the C-terminus as a first step and studies in cell lines showed that multiple residues in S1PR1 were phosphorylated following ligand exposure (Liu et al. 1999; Oo et al. 2007; Watterson et al. 2002). Using a gene targeting approach, mice lacking a five serine motif (all converted to alanines in an allele termed $S1pr1^{S5A}$) in the S1PR1 C-terminus showed reduced sensitivity to induction of S1PR1 down-modulation by ligands, including the S1PR1 modulating immunosuppressant drug FTY720 (Thangada et al. 2010).

G-protein-coupled receptor kinases (GRKs) are the predominant enzymes involved in GPCR phosphorylation (Reiter and Lefkowitz 2006) and in vitro experiments showed GRK2 could act on S1PR1, though additional kinases were also active on the receptor (Oo et al. 2007; Watterson et al. 2002). Mice lacking GRK2 in lymphocytes revealed the critical role of this kinase in ligand-induced S1PR1 down-modulation in vivo (Arnon et al. 2011). Thus, in contrast to control cells, GRK2 deficient T and B cells retained high levels of surface S1PR1 while circulating in blood. One reason lymphocytes may need to down-regulate S1PR1 after entering the bloodstream is to be able to overcome the attractive pull of S1P and move back into lymphoid tissue. This hypothesis gained support from the finding that GRK2-deficient T and B cells had a reduced ability to enter lymph nodes. This defect reflected ongoing responsiveness to S1P because it was overcome when cells were transferred to mice lacking circulatory S1P (Arnon et al. 2011). This does not exclude roles for GRK2 in regulating other GPCRs within lymphocytes, and there is evidence for this (Penela et al. 2009), but it highlights the nonredundant role of GRK2 in S1PR1 regulation. Interestingly, the lymph node entry step that was most affected by GRK2-deficiency differed between T and B cells. For T cells, there was a significant defect in the ability to undergo rolling-to-sticking transitions in high endothelial venules (Arnon et al. 2011). In this case, sustained Gi signaling over the lymphocyte surface in response to S1PR1 engagement might be reducing the ability of endothelium-associated chemokines to promote focal activation of integrin-mediated adhesion at the site of contact with the endothelium (Alon and Feigelson 2009). GRK2-deficient blood lymphocytes showed reduced phospho-ezrin/radixin/moesin (ERM) protein levels in wildtype but not S1P-deficient hosts (Arnon et al. 2011). ERM proteins link the plasma membrane with the cortical F-actin cytoskeleton (Fehon et al. 2010). By promoting their dephosphorylation, Gi signaling disrupts the linking function and this has been suggested to cause resorption of lymphocyte microvilli (Brown et al. 2003). The reduced pERM levels in GRK2-deficient lymphocytes confirmed that Gi signaling was chronically elevated. Further studies will be needed to resolve whether the defect in rolling-to-sticking transitions reflects the need for focal Gi signaling to dominate over global Gi signaling, or whether it is a consequence of, for example, altered microvilli density.

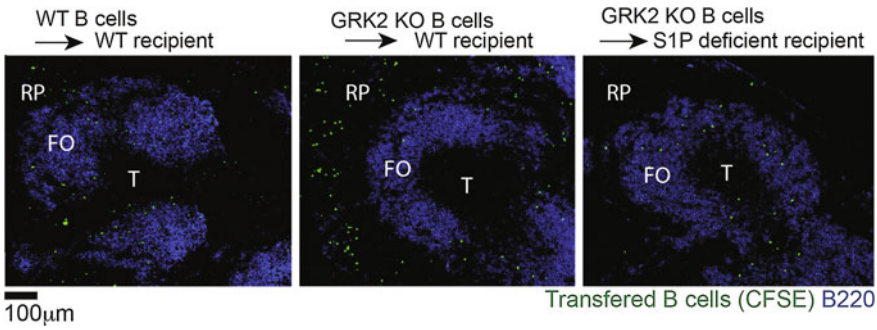

Fig. 1 *GRK2-mediated S1PR1 desensitization is required for B cell entry into splenic follicles.* WT or GRK2 KO follicular B cells (*green*) were transferred into WT or S1P-deficient recipients, as indicated. 48 hours later, the positioning of the cells in the spleen was analyzed. In contrast to WT B cells, GRK2 KO B cells were unable to enter the follicles (FO, anti-B220, *blue*) and were mostly found in the red pulp (RP). This entry defect depended on constitutive S1PR1-S1P signaling since in S1P deficient hosts transferred GRK2 KO B cells entered the FO normally

In GRK2-deficient B cells, there was less evidence for a defect in rolling-to-sticking transitions though these were not directly measured (Arnon et al. 2011). Instead, there was reduced movement of adherent cells from the vessel lumen into the lymph node. This might reflect a defect in B cell polarization toward attractive chemokine cues due to the global signaling over the cell surface by S1PR1. Future two-photon microscopy studies of B cell entry via high endothelial venules (Park et al. 2012) may help better define the nature of this entry defect.

GRK2-deficiency also diminished the ability of lymphocytes to enter the splenic white pulp. When frozen sections of spleen were examined a day after cell transfer, a greater proportion of GRK2-deficient than wildtype cells were located in the red pulp relative to white pulp (Fig. 1). This block in entry was again ligand dependent as white pulp entry was restored when the cells were transferred to Sphk-deficient hosts (Fig. 1). This finding is in agreement with the earlier observation that S1PR1 overexpression in B cells greatly reduced their movement into the white pulp (Lo et al. 2005) and is consistent with the cells being attracted too strongly by the blood-rich S1P-high red pulp to be able to respond to the chemoattractants that normally guide them into the white pulp.

4 Marginal Zone B Cell Shuttling

Splenic MZ B cells play important roles in antibody responses against encapsulated bacteria (Kraal and Mebius 2006). They also function in delivering opsonized antigens from blood into follicles for long term retention and display on FDCs. MZ B cells were so-named because of their location at the margin of the white and red pulp in the spleen (MacLennan et al. 1982). In rodents they are non-

recirculating. In humans, the MZ is a larger and more complex microenvironment and it is enriched for $CD27^{hi}$ somatically mutated B cells (Weill et al. 2009); while some $CD27^{hi}$ B cells do recirculate, it is not yet clear whether all the B cells present in the human MZ are recirculating or whether some are restricted to the spleen as they are in rodents.

The exposed location of MZ B cells situates them well for mounting rapid responses against blood-borne invaders. However, it also places upon them the special requirement of being retained in an environment that is continuously exposed to fluid flow. Since cell attachment in blood vessel lumens is universally dependent on integrins, it was perhaps not surprising that the retention of MZ B cells in the MZ required both αL- and α4-containing integrins; blocking these integrins caused release of MZ B cells into blood (Lu and Cyster 2002). The key integrin ligands involved in their retention, ICAM-1 and VCAM-1, are expressed in a lymphotoxin-dependent manner by MZ stromal cells, though the nature of these stromal cells remains poorly characterized (Lu and Cyster 2002).

Insight into the signals promoting MZ B cell positioning in the splenic MZ came from the finding that FTY720 treatment led to rapid displacement of MZ B cells from this zone (Cinamon et al. 2004; Vora et al. 2005). However, in this case, rather than causing their release into blood, the cells were found entrapped within follicles (Cinamon et al. 2004). Conditional deletion of S1PR1 in B cells led to a similar loss of cells from the MZ and positioning within follicles (Allende et al. 2010; Cinamon et al. 2008). Strikingly, when the cells lacked CXCR5 or the hosts lacked CXCL13, MZ B cells remained in the MZ even when S1PR1 function was disrupted (Cinamon et al. 2004). This observation suggested that the function of S1PR1 in MZ B cells was solely to overcome the recruiting activity of CXCL13. In an effort to improve quantitation of cell repositioning between MZ and follicles, a procedure was developed to in vivo label blood exposed cells with fluorophore-conjugated antibody; B cells within follicles are not exposed to the open blood circulation and are protected from short-term antibody exposure (Cinamon et al. 2008; Pereira et al. 2009a). This procedure led to the unexpected finding that in wildtype mice, only about 55 % of MZ B cells were actually situated in the MZ—the other ∼45 % were inside follicles (Cinamon et al. 2008). Moreover, the proportion of cells distributing in the MZ versus follicle was remarkably sensitive to the amount of S1PR1 and CXCR5 expressed by the cells and heterozygosity for either receptor led to a shift in favor of the follicle or MZ, respectively. This approach also revealed a detectable role for S1PR3 in promoting MZ B cell positioning in the MZ (Cinamon et al. 2008). The weak influence of S1PR3 on MZ B cell positioning in vivo contrasts with the strong promigratory activity of this receptor in transwell migration assays. We suspect that the greater activity of S1PR3 than S1PR1 in the in vitro assays reflects the resistance of S1PR3 to S1P-mediated desensitization; the lack of S1P compartmentalization in the transwell system likely exposes cells to nonphysiological S1P gradients. Recent work has established a role for cannabinoid receptor-2 (CB2) in positioning MZ B cells in the MZ and restricting their loss into blood (Basu et al. 2011; Muppidi et al. 2011). CB2 deficiency was not associated with a shift in the distribution of MZ B cells

between MZ and follicles, however, suggesting that it functions in a manner distinct from the S1P receptors (Muppidi et al. 2011).

The finding that MZ B cells were not all situated in the MZ was at first puzzling. Were these stable populations in each compartment, or was some exchange taking place? One observation in favor of the latter possibility was that MZ B cells had been repeatedly implicated in the rapid delivery of opsonized antigens from the MZ to follicle, yet frozen section analysis of this process had never managed to show that such noncognate antigens caused any change in the abundance of MZ B cells in the MZ (Ferguson et al. 2004; Gray et al. 1984; Guinamard et al. 2000; van Rooijen 1973). Could it be that MZ B cells were continually exchanging between compartments at a rate that would achieve prompt delivery of opsonized antigens to the follicle without any change from their steady state distribution? Initial support for this hypothesis came from findings with in vivo antibody pulse-chase labeling experiments (Cinamon et al. 2008). The inability of CXCR5-deficient MZ B cells to support opsonized antigen delivery to FDCs was also consistent with this model (Cinamon et al. 2008). However, to rigorously establish that MZ B cells undergo oscillatory movement between MZ and follicle, it was necessary to image this behavior in the intact spleen.

5 Real-Time Imaging of Cell Migration Dynamics in the Spleen

Efforts to study the dynamics of RBC passage through the spleen using intravital microscopy began as early as the 1930s (Knisely 1936). In the 1980s, bright field microscopy of rodent spleens revealed the kinetics of RBC passage from the red pulp through interendothelial slits into venous sinuses (MacDonald et al. 1987; McCuskey and McCuskey 1985). Interestingly, there were marked variations in the rate at which RBCs passed through the interendotheial slits, with cells in some occasions being caught in the sinus wall for a period of many minutes. RBC movement occurred in a series of brief discontinuous bursts separated by periods of very low flow and it was concluded that there were significant changes in the caliber of the interendotheial slits with time. White blood cells were not a focus of these studies but were occasionally visualized, held in the slits for several minutes and it was suggested that the slits were dilated by the passing white cells with the effect that there was subsequently a transient increase in flux of RBCs (MacDonald et al. 1987).

Intravital laser scanning confocal microscopy procedures were used to examine the early appearance of labeled lymphocytes in the spleen, though their subsequent migration path was not tracked and the exact anatomical regions being imaged were not well defined (Grayson et al. 2003). This approach was also used to follow the early events occurring following systemic *Listeria* infection. Subcapsular red pulp dendritic (CD11c-YFP +) cells became rapidly infected and recruited myelomonocytic (LysM-GFP +) cells swarmed around the DCs. Using labeled

dextrans, it was found that the myeloid cell accumulation caused a reduction in blood flow around the infection foci. After a period of days, antigen specific CD8 T cells interacted with the DCs (Waite et al. 2011). In another *Listeria* study, explanted spleen sections were imaged using two-photon microscopy, revealing in this case the movement of infected DCs from the MZ to the T zone and the subsequent interaction with cognate CD8 T cells in this region (Aoshi et al. 2008). The different anatomical locations of the responses tracked in these two studies highlight how multiple regions can be involved in the splenic response against even a single pathogen.

Real-time imaging of the red pulp has also yielded important insights about migration dynamics of spleen monocytes (Swirski et al. 2009). Subcapsular CX3CR1-GFP^{+} monocytes are mobilized in response to ischemic myocardial injury and a combination of confocal and two-photon laser scanning microscopy procedures revealed that the cells increase their motility prior to accessing venous sinuses (Swirski et al. 2009). A similar increase in motility and release of cells was promoted by treatment with angiotensin II, a ligand for the Gq/11-coupled AT1 receptor. Angiotensin-converting enzyme inhibitors prevented the increase in motility and release of red pulp monocytes following myocardial infarction (Leuschner et al. 2010). This work highlights the presence of subcompartments within the red pulp and it will be important in future work to understand how the monocytes organize in subcapsular clusters and to determine how AT1 signaling promotes their motility.

In contrast to the extensive two-photon laser scanning microscopy of lymphocyte migration within lymph nodes (Cahalan and Parker 2008), application of this procedure to the lymphoid regions of the spleen has been limited due to the challenges associated with penetrating through the thick splenic capsule, a sufficient distance to detect the white pulp. Although splenic slice approaches overcome this difficulty, they are not suitable for studies of cells migration in the MZ and red pulp because of the blood flow in these regions and associated exposure to shear forces, S1P, and likely other circulating factors that influence cell behavior. However, recent work showed that by extensively scanning the length of the spleen some white pulp cords could be identified that passed into the 200–300 μm critical depth window that can be accessed by current two-photon microscopes, permitting image analysis of cell migration in the MZ and adjacent follicles (Arnon et al. 2013).

Two-photon intravital microscopy of mice containing GFP-labeled MZ B cells in relation to labeled “landmark” MZ macrophages revealed that these spleen-restricted B cells continually shuttle between MZ and follicle with an exchange rate of about 20 % per hour (Arnon et al. 2013) (Fig. 2). MZ B cells migrated with similar speeds and cell shapes on both sides of the marginal sinus. Crucially, however, migration in the MZ was dependent on integrin function whereas migration in the follicle was not (Arnon et al. 2013). The limited contribution of integrins to motility within the follicles was consistent with findings for lymphocytes and DCs within lymph nodes (Boscacci et al. 2010; Lammermann et al. 2008; Woolf et al. 2007). When integrins were blocked, MZ B cells in the MZ

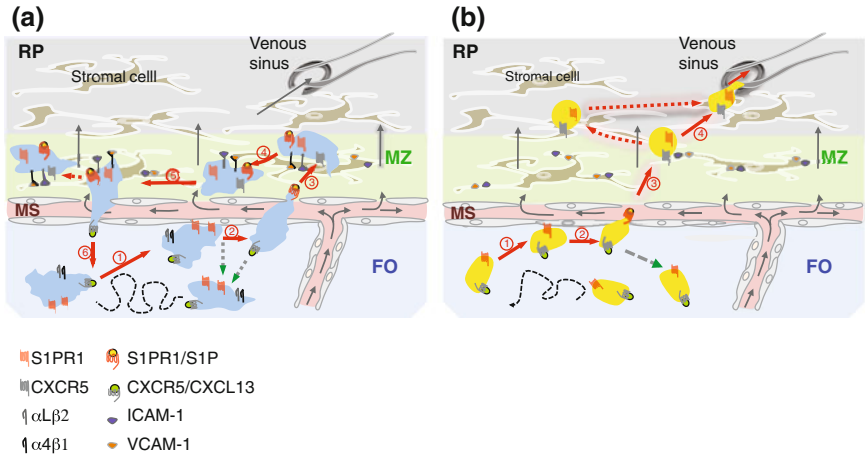

Fig. 2 *Multi-step models of marginal zone (MZ) and follicular (FO) B cell movement between FO and MZ.* Major compartments, cell types, and molecules are labeled and are described in detail in the text. Direction of blood flow is shown in *gray* shaded arrows. **a** Model of MZ B cell shuttling. *Step 1* Migrating MZ B cell (*blue*) has a large, probing morphology and comes in contact with the marginal sinus (MS). Cell movement toward the sinus could be random, or promoted by S1PR1, S1PR3, CB2, or other unknown receptors. *Step 2* MZ B cell pauses and probes the MZ sinus. Once contact has been made, the cell either moves away from the sinus (*dashed green line*) back to the FO or pauses at the MZ-FO boundary for several minutes. *Step 3* $S1PR1^{hi}$ MZ B cell commits to crossing the MS in an S1PR1-S1P dependent manner. Occasionally, a probing cell fails to cross and returns to the FO (*dashed green line*). *Step 4* MZ B cell maintains probing morphology and migrates in the MZ. Integrin-mediated adhesion confines its movement to the MZ. Exposure to S1P leads to partial downregulation of S1PR1 in a GRK2-dependent manner. *Step 5* MZ B cell crawls along MZ-FO boundary. *Step 6* $S1PR1^{lo}$ MZ B cell commits to enter FO in a CXCR5-dependent manner. At the crossing step, the cell makes a sharp turn and becomes stretched. In some cases, the MZ B cell fails to enter the FO and remains in the MZ (*dashed red line*). In the FO, the cell resumes its migration. Low concentrations of S1P in the FO lead to S1PR1 resensitization, allowing the cell to begin a new cycle. **b** Model of FO B cell egress via the MS. *Step 1* Migrating FO B cell (*yellow*) has a small, amoeboid morphology and approaches the sinus. *Step 2* FO B cell makes contact with and probes the sinus. *Step 3* FO B cell crosses the sinus in an S1PR1-dependent manner. After crossing, the FO B cell de-adheres, becomes rounded, and is "flushed" toward the RP, carried by blood flow. Lower expression of S1PR1 on FO B cells compared with MZ B cells reduces the chance of a probing cell committing to crossing and in some cases the cell is retained in the FO (*dashed green line*). *Step 4* FO B cell exit from the spleen. In the RP, the cell moves with the flow, crosses an interendothelial slit in a venous sinus and returns to circulation. In some cases the flow causes the cell to move in alternate directions (*dashed red line*), taking it around obstacles in the red pulp before reaching an exit venule. The red pulp exit vessels in the mouse are morphologically distinct from the venous sinuses in humans and it has been suggested they should be termed "red pulp venules" (Schmidt et al. 1985). However, for consistency with other current literature we have kept the more commonly used term "venous sinuses"

often became rounded and moved in the direction of the red pulp, likely having de-adhered from the integrin-ligand expressing stroma and becoming caught in blood flow (Arnon et al. 2013). The high motility in the MZ, and the similarity of

this movement to that in the follicle, established that the cells could sustain similar migration behaviors in integrin-dependent and integrin-independent fashions. Precedent for a single cell type migrating with similar characteristics in an integrin-dependent and -independent manner was provided by findings in an in vitro system with dendritic cells (DCs) (Schumann et al. 2010).

Many MZ B cells exhibited trailing membrane processes of remarkable length (Arnon et al. 2013). B cells have been shown to exhibit nanotube-like processes under conditions of strong adhesion in vitro (Cambier and Lehmann 1989; Santos-Argumedo et al. 1997) and GC B cells occasionally showed long processes during migration within the GC (Allen et al. 2007; Hauser et al. 2007). α4- and αL-containing integrin function was not required for the MZ B cell processes, pointing to the possible involvement of other adhesion molecules, such as the MZ B cell-expressed cadherin (Ohnishi et al. 2005). A recent study identified similar long trailing processes in HIV-1 infected human T cells migrating within murine lymph nodes and in this case it appeared that gp120 binding to CD4 on other cells promoted their formation (Murooka et al. 2012). Whether trailing extensions facilitate interactions with other cells such as NKT cells in the MZ (Barral et al. 2012) or with follicular B cells scanning for surface displayed antigens in the follicle (Suzuki et al. 2009) remains to be assessed. Although it has been possible to track transport of fluorescently labeled antigens by noncognate B cells in lymph nodes (Phan et al. 2007), the sensitivity of fluorescent molecule detection through the splenic capsule has so far not been sufficient to track antigen movement dynamics in the spleen.

6 What is the Basis for the MZ B Cell Migratory Oscillator?

The propensity of S1PR1 to undergo desensitization by ligand led to the hypothesis that MZ B cell shuttling might be mediated by a cycle of S1PR1 de and resensitization (Cinamon et al. 2008). Direct support for this hypothesis came from the finding that GRK2-deficient MZ B cells were localized in the MZ at the expense of the follicle (Arnon et al. 2011). GRK2-deficient MZ B cells were all S1PR1-high whereas cells in wildtype mice had a broader range of S1PR1 expression. The crucial role of S1PR1 desensitization in MZ B cell shuttling was confirmed by the finding that in mice carrying a targeted replacement of a TSS motif near the C-terminus of S1PR1 with AAA, the migratory oscillator was inactivated and MZ B cells were prevented from accessing the follicle (Arnon et al. 2011).

Despite the similar phenotype of GRK2-deficient and $S1PR1^{TSS}$ MZ B cells, it has not yet been established whether GRK2 targets the TSS motif of S1PR1. The stronger phenotype of GRK2-deficiency in protecting S1PR1 from down-modulation on cells circulating in blood suggested it targets other or additional residues (Arnon

et al. 2011), as has been indicated by in vitro studies (Oo et al. 2011; Watterson et al. 2002). The S1PR1 S5 motif may be an important site of GRK2 phosphorylation given that this motif is needed in vivo for correct S1PR1 desensitization (Thangada et al. 2010). However, this motif alone is unlikely to be the single target of GRK2 since blood-exposed lymphocytes in S1PR1 S5A mice completely down-regulated S1PR1 expression. Studies of other GPCRs such as CXCR4 have shown how phosphorylation by one kinase at one motif can sensitize the receptor for subsequent phosphorylation by another kinase at further motif(s) (Busillo et al. 2010). We suspect that similar cooperative processes are at play during the downregulation of S1PR1 and it will be important in future work to identify the other kinases involved. Beyond phosphorylation, ubiquitination of S1PR1 is important in directing its fate within the cell (Gonzalez-Cabrera et al. 2007; Oo et al. 2011; Oo et al. 2007). Arrestins have also been implicated in mediating S1PR1 internalization (Oo et al. 2007). More work is needed to fully dissect the mechanisms guiding internalization and recycling versus degradation of S1PR1 as signals that modulate this process will modulate multiple aspects of lymphocyte trafficking, as exemplified by the egress inhibitory function of CD69 (Shiow et al. 2006; Tomura et al. 2010). CD69 interacts via its transmembrane and membrane proximal domains with S1PR1 to both directly inhibit S1PR1 function and to promote its internalization (Bankovich et al. 2010; Shiow et al. 2006). Yet, how CD69 promotes S1PR1 internalization remains incompletely defined.

We propose the following model (Fig. 2) to account for MZ B cell shuttling: During active migration within the MZ, MZ B cells are exposed to blood-borne antigens and to high amounts of interstitial S1P (perhaps 10's of nM); while capturing opsonized antigens via complement receptors they undergo gradual S1P-mediated GRK2-dependent desensitization of S1PR1. Alternatively, the S1P concentration in the MZ may be heterogeneous and MZ B cells are only occasionally exposed to amounts sufficient to cause S1PR1 desensitization. When their migration path brings them in contact with cells at or near the marginal sinus they encounter CXCL13 and are attracted into the follicle. Within the follicle they encounter FDCs and can off-load any opsonized antigens they carry. The follicle is an S1P low environment (low or sub nM) and during their migration in this compartment S1PR1 resensitizes so that when their migration path brings them into contact with the marginal sinus they have the chance to probe the sinus and encounter high S1P and this frequently is followed by their commitment to cross the sinus back into the MZ. In the MZ they resist blood flow by integrin-mediated adhesion and continue their migratory behavior, and the cycle begins again.

7 Lymphocyte Egress from the Spleen

The specialized recirculation property of lymphoctyes through lymph nodes and Peyer's patches, so elegantly defined by Gowans and coworkers in the 1960s (Gowans and Knight 1964), with entry occurring mostly from the blood and exit

occurring via lymphatics, has greatly facilitated the study of egress from these organs (Cyster 2005). By selectively blocking cell entry, the rate of egress from the tissue can be measured (Lo et al. 2005). In contrast, lymphocyte entry to and exit from the spleen both occur via the blood making the study of cell egress from this organ less straightforward. An early effort to measure this process involved the tour-de-force procedure of perfusing the isolated spleen in an organ chamber system and examining the rate of flux of tritiated cells added to the perfusate (Ford 1969). This and other early studies led to transit time estimates that were similar to those of lymph nodes (4–24 h) (Nieuwenhuis and Ford 1976). Thus, despite its larger size, the amount of tissue volume explored by a given cell during each passage might be quite similar.

The discovery that FTY720 blocked egress from lymph nodes and Peyer's patches and S1PR1 and S1P were required for lymphocyte egress from these organs raised the obvious question of whether this same ligand-receptor system was involved in cell egress from the spleen. An initial interpretation of FTY720 treatment studies was that since spleen lymphocyte number decreased after treatment, egress from this organ does not involve an FTY720 sensitive pathway (Mandala et al. 2002; Morris et al. 2005; Rosen et al. 2007). However, this interpretation did not take into consideration that the spleen is made up of both white and red pulp and while lymphocytes are concentrated in the white pulp, there are also many cells situated in the red pulp at any moment in time, including cells just entering in the blood and cells about to depart via venous sinuses. Indeed, when tissue sections from mice treated with FTY720 for 1–3 days were examined, there was a marked depletion of cells from the red pulp without an obvious change in the white pulp (unpubl. obs.). Moreover, although splenic lymphocyte numbers decayed over a period of days after FTY720 treatment, the rate of decay was much slower than predicted from the estimates of splenic recirculation kinetics (Nieuwenhuis and Ford 1976). The gradual decay may in part reflect toxicity of chronic drug treatment or a need for lymphocytes to recirculate to maintain their viability (Link et al. 2007; Luo et al. 1999; Morris et al. 2005). Direct support for the conclusion that S1PR1 was needed for cell emigration from the spleen came from the finding that transferred S1PR1 deficient lymphocytes were found within the white pulp 1–2 days after transfer in similar frequencies as control cells despite their near absence at this time from blood circulation (Matloubian et al. 2004). In animals where blood S1P is intact but lymphatic S1P is depleted, lymphocyte numbers are low in the spleen compared to lymph nodes and Peyer's patches, likely because they are able to exit the spleen in response to circulatory S1P but are not able to return from other secondary lymphoid organs that depend on egress into lymph (Mendoza et al. 2012; Pham et al. 2010).

Further evidence that S1PR1 could play a role in cell egress from the spleen came from studying requirements for plasma cell trafficking. Following their induction in the spleen, early plasma cells (plasmablasts) were strongly dependent on intrinsic S1PR1 expression to appear in blood circulation (Kabashima et al. 2006). S1PR1 deficient plasma cells could still reach the splenic red pulp in a manner that involved recruitment by CXCR4 and movement to CXCL12-high

zones (Hargreaves et al. 2001) but they were defective in egressing into blood (Kabashima et al. 2006).

Recent two-photon microscopic imaging has allowed direct visualization of an S1PR1-dependent step in B cell egress from the white pulp. Follicular B cells were observed passing out of the follicle via a similar path to MZ B cells and the movement from follicle to MZ was strongly S1PR1 dependent (Arnon et al. 2013). Whether the block in white pulp egress associated with S1PR1-deficiency is as strong as the block in lymphocyte movement from the lymph node parenchyma into lymphatic sinuses (Grigorova et al. 2010; Grigorova et al. 2009; Sinha et al. 2009) is not yet clear and requires further studies. A key role of S1PR1 in promoting lymph node egress of T cells is to overcome the recruiting activity of CCR7 and additional retention signals (Pham et al. 2008). The nature of the retention signals acting on follicular B cells in the spleen are not yet defined but it seems reasonable to propose that CXCR5 and CXCL13 play a role.

In contrast to MZ B cells, egressing follicular B cells fail to adhere in the MZ and are "flushed" into the red pulp (Fig. 2). In this compartment, many of the cells are rounded and show bursts of linear displacement, consistent with episodic movement by fluid flow and capture by obstacles rather than with an actively motile behavior (Arnon et al. 2013). In this regard, their red pulp movement was similar to the previously described movement of RBCs (MacDonald et al. 1987). Cells in the red pulp sometimes vanished from the middle of the imaging volume, a behavior never observed in the white pulp, consistent with having entered a venous sinus and being carried out of the spleen in venous circulation. Further work is needed to develop methods that allow fluorescent visualization of red pulp venule endothelium and passage of egressing cells into these vessels.

The route used by T cells to exit the spleen is not known but given the evidence cited above that it will involve S1PR1, the egress decision step would again be anticipated to be at the interface between the T zone and the blood (and S1P)-rich red pulp, the MZ bridging channels. Consistent with this possibility, when the distribution of newly generated effector CD8 T cells was followed over time in frozen sections, the cells appeared to move sequentially from the T zone to the bridging channels and then to the red pulp (Khanna et al. 2007). Newly generated plasmablasts also transit out of the white pulp via this path (Jacob et al. 1991). Whether some follicular B cells use this route to exit follicles is not known though it has been suggested even in early work (Mitchell 1973). In future studies, as the imaging depth that can be achieved by available microscope systems improves, it will be important to obtain real time intravital imaging data of cell behavior in MZ bridging channels.

MZ B cells travel from follicle to MZ at a faster rate than follicular B cells (Arnon et al. 2013). What might be the basis for this differing rate of flux? At least one explanation may be the $\sim$2-fold higher expression of S1PR1 by MZ compared to follicular B cells (Cinamon et al. 2004). This may increase the propensity of MZ B cells to commit to exiting from the follicle when they encounter S1P at the follicle boundary or while probing the marginal sinus (Fig. 2). In lymph nodes, T cells encounter and probe the cortical sinuses that support egress in an S1PR1-

independent fashion but their commitment to enter the sinus is S1PR1-dependent. Only about a third of probing events are followed by a commitment to migrate into the sinus and egress the lymph node (Grigorova et al. 2010; Grigorova et al. 2009). Although it has not yet been possible to resolve the thin marginal sinus lining cells within the spleen by intravital two-photon microscopy, we anticipate that a similar decision making process takes place during B cell movement from follicle to MZ (Fig. 2). Lower S1PR1 expression compared to MZ B cells likely allows signals such as from the CXCR5 chemokine receptor to "win out" more frequently, promoting retention, and increase dwell time within the follicle.

8 Summary

The large size of the spleen has long made it a favorite source of lymphocytes for research studies, yet its size and complex blood-rich anatomy have also made it a challenging organ in which to study immune response dynamics. Work discussed above has been overcoming this hurdle. In particular, in relation to the topic of this review series, intravital two-photon microscopy has revealed an S1PR1-dependent pathway of follicular B cell egress from the splenic white pulp and shown that MZ B cells continually shuttle between MZ and follicles in an S1PR1-dependent manner. The S1PR1-modulating drug, FTY720, disrupts these processes. FTY720-mediated suppression of MZ B cell shuttling is expected to reduce the efficiency with which opsonized antigens are delivered from blood to follicular dendritic cells, possibly impacting on the induction or maintenance of antibody responses. Whether defects in opsonized antigen delivery occur in the spleens of patients treated with this immunosuppressant (Chun and Brinkmann 2011) remains to be seen.

In future work, it is anticipated that advances in deep tissue imaging will allow a more extensive analysis of B and T cell movement within and egress from the splenic white pulp. Such procedures should also help to resolve the specialized cell-cell interaction dynamics involved in mounting and sustaining responses against systemic pathogens and autoantigens. More precise measurements of interstitial S1P concentrations and further definition of the cells involved in secreting and degrading this lipid will also be needed if we are to generate a complete picture of lymphocyte egress dynamics. Ongoing work is also certain to add detail to the picture of cell positioning within the red pulp. For example, it remains to be understood how plasma cells can travel into the red pulp in a CXCR4-dependent manner yet still require S1PR1 to exit the spleen. Perhaps the CXCL12-high areas in the red pulp occupied by plasma cells are maintained as S1P-low regions and movement out of these compartments depends on sinus-associated S1P overcoming CXCL12-mediated retention. The ability of red pulp myeloid cell clusters to reduce blood flow (Waite et al. 2011) suggests one means by which S1P concentrations across the red pulp may vary. The controlled release of monocytes from subcapsular red pulp clusters following myocardial infarction

provides an example for how cell egress from the red pulp can be regulated (Swirski et al. 2009).

The finding that GRK2-mediated GPCR desensitization can underlie a process of cell movement back and forth between adjacent niches raises the question of whether this type of desensitization-based oscillatory migration will be used by other cells as a way to repeatedly explore adjacent niches. Desensitization of CXCR4 is important for cell emigration from the bone marrow and is disrupted in patients with the Warts, Hypogammaglobulinemia, Infections and Myelokathexis (WHIM) syndrome (Diaz 2005). It will be important to determine whether genetic polymorphisms affecting GPCR desensitization cause disease-associated defects in other processes dependent on the precise movement and positioning of immune cells.

S1P has long been recognized for its role in vascular biology and recent studies have provided mechanistic insight into how S1P and S1PR1 regulate vascular angiogenesis (Gaengel et al. 2012; Mendelson et al. 2013; Shoham et al. 2012). Given the complex and specialized vasculature of the spleen, it will be illuminating to explore the roles of this same ligand-receptor system in organizing and regulating the properties of splenic vessels. The recent evidence that S1P signaling in lung endothelium may quell cytokine release during viral infection (Teijaro et al. 2011) raises interesting questions about whether it has similar functions in other organs with high blood content, like the spleen.

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Cytokine Storm Plays a Direct Role in the Morbidity and Mortality from Influenza Virus Infection and is Chemically Treatable with a Single Sphingosine-1-Phosphate Agonist Molecule

Michael B. A. Oldstone and Hugh Rosen

Abstract Cytokine storm defines a dysregulation of and an excessively exaggerated immune response most often accompanying selected viral infections and several autoimmune diseases. Newly emerging and re-emerging infections of the respiratory tract, especially influenza, SARS, and hantavirus post considerable medical problems. Their morbidities and mortalities are often a direct result of cytokine storm. This chapter visits primarily influenza virus infection and resultant cytokine storm. It provides the compelling evidence that illuminates cytokine storm in influenza pathogenesis and the clear findings that cytokine storm is chemically tractable by therapy directed toward sphingosine-1-phosphate receptor (S1PR) modulation, specifically S1P1R agonist therapy. The mechanism(s) of how S1P1R signaling works and the pathways involved are subjects of this review.

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1 Introduction

Newly emerging and re-emerging infections of the respiratory tract pose considerable medical and public health concerns as well as economic hardships to humans and countries. The last century witnessed at least five pandemics: 1918/1919, H (viral hemagglutinin) 1 N (viral neuraminidase) 1 Spanish influenza; 1957, H2N2 Asia influenza; 1968, H3N2 Hong Kong influenza; 1977, H1N1 Russian influenza; and 1997, H5N1 bird influenza (reviewed Wright et al. 2007). In twenty-first century alone, two viral pandemics have already occurred—the first was in 2002 when the new viral pandemic, severe acute respiratory syndrome (SARS), appeared (reviewed Oldstone 2010), followed by the first influenza virus pandemic in 2009, H1N1 swine influenza (Dawood et al. 2012). Moreover, Hantaviruses have infected humans in the past and recently in an outbreak at Yellowstone National Park. These viral infections loom as important zoonotic human diseases with the threat of human to human transmission and excessively high mortality rates. For example, 1918/1919 H1N1 influenza infections caused the greatest loss of life from any infectious disease or medical condition known, visiting roughly 5 % of the world's population and killing 2 % or 40–50 million persons (Ahmed et al. 2007; Johnson and Mueller 2002). The most recent influenza pandemic, 2009 H1N1 swine influenza, rapidly infected millions worldwide with estimates exceeding 290,000 deaths of which more than 201,000 resulted from respiratory failure and over 83,000 from cardiovascular complications (Dawood et al. 2012). All of the above diseases in humans (Arankalle et al. 2010; Cheng et al. 2010; Lee et al. 2011) and experimental animals (Baskin et al. 2009; Kobasa et al. 2007; Marcelin et al. 2011; Zhang et al. 2012) are accompanied by early exacerbation and dysregulation of innate immune responses, a combination of events called "cytokine storm." Severe disease and death following infection correlated strongly with the cytokine storm.

Susceptibility or resistance to any viral infection is determined by the balance between the virulence of the infecting agent and the resistance of the host including the aggressiveness of the latter's immune response against the virus infection. When the immune response is limited due to either host genetics, acquired defects like lymphoid diseases, immaturity of the immune system in fetuses, newborns, or young children, or

loss of immune vigor in the aged, the advantage is firmly in the virus's court. However, usually when the infection occurs in individuals with a developed and competent immune system, the advantage is the host's, unless the infecting virus overwhelms the individual's immune system or the immune response becomes hyperactive resulting in an excessive innate and adoptive immune reaction, the "cytokine storm" phenomenon. Cytokine storm leads to immune-mediated injury (immunopathology).

When available, vaccination is useful in protecting groups of previously uninfected (naive) individuals from acute viral respiratory diseases. By this means, the spread of infection is diminished. Additionally antiviral drugs, which were developed as effective therapies to diminish or in some instances prevent ongoing infections, are reasonably effective, nevertheless come with two marked limitations. First, antiviral drugs exert selective pressure on viral progeny, promoting their mutation and selection thereby creating a new generation that is more fit and resistant to the drug (Nguyen et al. 2012; Orozovic et al. 2011). Second, the injury associated with these acute viral respiratory diseases, including influenza, results from a combination of the virus's intrinsic virulence in lysing cells it infects and the intensity of the immune response which can damage tissues and promote a cytokine storm. Antiviral drugs are effective against the virus but not against cytokine storm or immune-mediated injury.

Recently, while studying human H1N1 2009 influenza virus infection in mice (Walsh et al. 2011; Teijaro et al. 2011) and ferrets (Teijaro et al. 2013), we uncovered the first direct and definitive experimental evidence that cytokine storm, per se, was a major factor in the causation of morbidity and mortality from influenza virus and some other acute, severe respiratory infections rather than just the accompanying phenomena. Further, we documented that cytokine storm was chemically treatable using an immunomodulatory small molecule, sphingosine-1-phosphate agonist, which dramatically inhibited the production of cytokines/chemokines and the innate cellular response, thereby blunting both the innate as well as the adoptive antiviral T cell response (Marsolais et al. 2009; Walsh et al. 2011; Teijaro et al. 2011). These events successfully limited immunopathologic injury. Nevertheless, a sufficient host T cell response remained and coupled with the antiviral antibody response curtailed the acute infection while providing recall immunologic memory to any renewed insult by the virus. This review focuses primarily on our experimental work that provided these conclusions.

2 Influenza Virus Infection

2.1 Epidemiologic and Experimental Evidence for Cytokine Storm

An overly aggressive innate immune response, the early recruitment of inflammatory leukocytes to the lung and dysregulated immune gene expression were key contributors to morbidity from the 1918/1919 influenza virus onslaught, as

suggested by experimental infection of macaques with the 1918 H1N1 virus strain (Kobasa et al. 2007; Cilloniz et al. 2009). Clinical studies of humans infected by H5N1 bird influenza virus revealed a significant association between excessive early cytokine responses and immune cell recruitment as strongly predictive of poor medical outcomes (de Jong et al. 2006). Recently, similar results for influenza virus infections were reported for experimental animal models (Baskin et al. 2009; Marcelin et al. 2011, Zhang et al. 2012) and for humans (Arankalle et al. 2010; Cheng et al. 2010; Lee et al. 2011). Among reports of H1N1 2009 pandemic influenza infections in humans, that of Arankalle et al. (2010) is illuminating. Analyzing viral events and cytokine storms in critically ill-hospitalized patients, the investigators showed that those who died had no difference in influenza viral load from those who recovered. However, the patients who recovered and left the hospital had significantly lower cytokine storm profiles than the population who succumbed from the infection. My colleague, Hugh Rosen, and I reasoned that calming the host's aggressive and exaggerated cytokine storm response might provide the opportunity to shift the balance from severe morbidity and mortality to survival. Our laboratories started jointly about 7 years ago to test this hypothesis (Marsolais et al. 2008). We selected the molecule sphingosine 1-phosphate (S1P) and sought to determine if harmful immunologic processes accompanying H1N1 2009 influenza infection could be modulated by S1P receptors in the lung. We selected S1P agonists because of their documented history of modulating lymphoid trafficking by inducing sequestration of lymphocytes in secondary lymphoid regions. By that means, S1P agonists limit the migration of effector lymphocytes to areas where such cells mediate immunologic injury (Rosen et al. 2007, 2009, 2013; see Chaps. 1 (Rosen) and 6 (Cyster) in this volume)). S1P is a signaling lipid present at a concentration of 1–3 nM in plasma and approximately 100 nM in lymph. Physiologically, S1P levels are under tight homeostatic control, and S1P signals through specific S1P receptors of which there are five (S1P receptors 1–5). These five specific S1P receptors are coupled to different G proteins for the purpose of regulating a variety of downstream pathways specific for many cells, tissues, and organs (Rosen et al. 2007, 2009, 2013).

2.2 *Tracking and Kinetics of Influenza Virus-Specific CD8 and CD4 T Cells in the Lung and their Modulation by S1P Agonist*

Infiltration of lymphoid cells into pulmonary tissues accompanies influenza virus infection. To identify and quantitate CD8 and CD4 cells that specifically recognize influenza and separate these virus-specific effector T cells from the majority of CD8 and CD4 bystander T cells nonspecifically drawn into the lung by chemotoxic attractants released during virally induced damage of infected pulmonary epithelial cells, we took advantage of the wealth of reagents we and others created for

lymphocytic choriomeningitis virus (LCMV). Our colleague Yoshi Kawaoka and his co-workers used reverse genetics (Marsolais et al. 2009) to place the MHC Db-restricted immunodominant LCMV CD8 T cell epitope glycoprotein (Gp) aa31-41 and the MHC IA^b restricted immunodominant CD4 T cell epitope Gp aa65-77 into the neuroaminidase stalk of WSN influenza virus. This technology generated a recombinant WSN Flu/LCMV virus that replicated in vivo displaying the same pulmonary geography as wild-type (wt) WSN virus. The experimental plan utilized GFP- or RFP-labeled, cloned LCMV recognition lymphocytes obtained from T cell receptor mice in which >98 % of CD8 fluoroprobe-labeled T lymphocytes recognized LCMV Gp aa31-41, and >97 % of CD4 fluoroprobe-labeled T lymphocytes recognized Gp aa65-77. Such GFP/RFP-labeled, virus-specific lymphocytes were adoptively transferred into naïve H-2b C57Bl/6 mice where they resided in secondary lymphoid tissues as resting lymphocytes. Two days later the recombinant WSN Flu/LCMV was administered intratracheally. Virus replication in pulmonary epithelial cells (Fig. 1a) was followed by the infiltration of virus-specific CD8 T cells (red) and virus-specific CD4 T cells (green) (Fig. 1b) at day 6 (Marsolais et al. 2009). Kinetic study of infiltrating virus-specific CD8 T cells showed their arrival by day 4, peak amounts at days 6–8, and significant numerical decrease at day 10 postinfection (Fig. 1c). There are five S1P receptors, i.e., S1P1, S1P2, S1P3, S1P4, and S1P5. Administration of S1P permissive agonist AAL-R, which signals on S1P1, S1P3, S1P4, and S1P5 receptors but not the S1P2 receptor, significantly reduced the numbers of virus-specific CD8 T cells entering the lung (Fig. 1d). The result was significant protection from pulmonary tissue injury (Fig. 1e) and related mortality (Fig. 1f) when compared to the effects of vehicle alone or use of a control isomer, AAL-S, that is not able to be phosphorylated and cannot signal S1P receptors. Blunting of innate cytokine and chemokine responses following AAL-R treatment was evident and remarkable at day 2 postinfluenza infection (Fig. 1g). All these observations were initially made with murine H1N1 WSN virus and later confirmed by use of the non-murine adopted human pathogenic H1N1 influenza viruses A/Wisconsin/WSLH34934/09 and A/California/04/09 (Walsh et al. 2011). In studies with all these influenza viruses, although cytokine/chemokine expression was significantly blunted by S1P agonist AAL-R, AAL-R-treated mice terminated the virus infection, displayed robust virus-specific CTL responses 7 days after influenza infection, as measured by ^{51}chromium release assay, and also mounted vigorous specific memory T cell responses upon rechallenge with virus 40 days after the infection. Further, the kinetics, titers of neutralizing anti-influenza antibodies in sera, or immunoglobulin subtypes of either AAL-R or AAL-S or vehicle-treated mice were equivalent. Together, these results document the validity of our premise. That is, the permissive S1P agonist AAL-R, which signals via S1P1, S1P3, S1P4, and S1P5 receptors, when given locally into the respiratory tract, down-modulated numbers of virus-specific T cells, decreased innate cytokine/chemokine expression in the lung parenchyma, and reduced the supply of innate inflammatory cells—NK, PMN, and macrophages (Marsolais et al. 2009; Walsh et al. 2011)—sufficiently to abort cytokine storm. The successful outcome was protection of the host from influenza virus infection while still providing an antiviral response that curtailed and

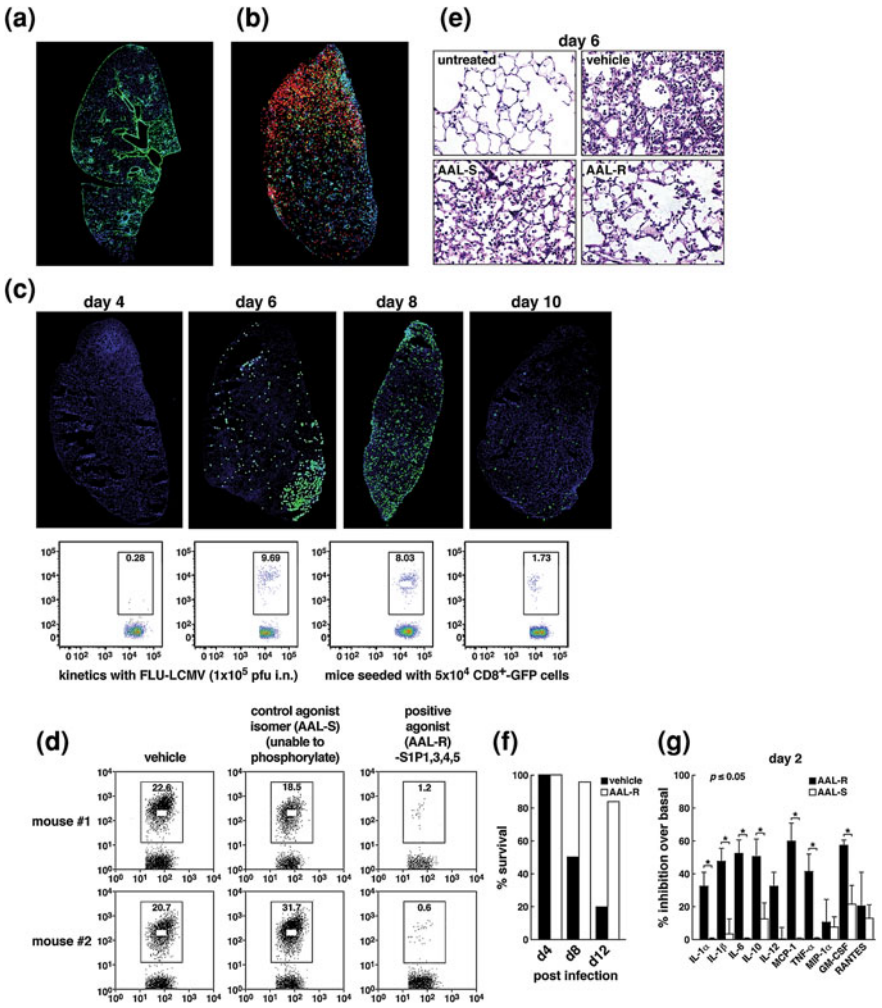

Fig. 1 *Panel a*: Distribution of viral antigen (*green*, *left*); *Panel b*: Virus-specific *CD4* T cells (*green*, *right*) and *CD8* T cells (*red*, *left*) in the lung 7 days following influenza/*LCMV* infection; *Panel c*: Kinetics of virus-specific *CD8* T cell infiltration into the lung analyzed by immunohistochemistry (*upper panels*) or *FACS* (*lower panels*); *Panel d*: The *S1P* permissive agonist *AAL-R* significantly blunts infiltration of virus-specific *CD8* T cells into the lung following influenza/*LCMV* recombinant virus infection; *Panel e*: Significant reduction of pulmonary tissue injury and preservation of alveolar air space in influenza-infected mice treated with *AAL-R*; *Panel f*: Significant protection from mortality accompanying influenza virus infection with *AAL-R*; and *Panel g*: *AAL-R* significantly dampens cytokine and chemokine content at day 2 following influenza virus infection. Figure reprinted from Marsolais et al. (2009), with permission from PNAS

eventually impeded the influenza infection. Our data indicated that 23 of 28 mice (82 %) receiving AAL-R were protected ($P = <0.001$; only five of 28 died from the infection) when compared to a dose of virus that killed approximately 80 % of

vehicle- or AAL-S-treated mice (22 of 28 mice died) (Walsh et al. 2011). Interestingly, when an optimal dose of the currently used antiviral drug Tamiflu was administered by itself, protection was significantly less effective 50 % (14 lived of 28 mice treated) compared to survival after AAL-R therapy alone (80 %). These results document a prominent role for cytokine storm as the cause of death from influenza infection. Most important is the benefit of S1P agonist therapy for the victims of multiple influenza virus strains and especially those that are resistant to anti-neuraminidase therapy. Although greater benefit was obtained in blocking cytokine storms with the S1P agonist than with Tamiflu (82 % vs. 50 %) protection, administering both the antiviral drug and the S1P agonist as combined therapy was optimal, yielding a 96 % protection rate from influenza virus challenge (27 of 28 mice survived the infection) (Walsh et al. 2011).

2.3 Pulmonary Injury and Disease Associated with Influenza and Resultant Cytokine Storm are Treatable with a Single S1P1 Receptor Agonist Molecule

All five S1P receptors couple to different G proteins require many downstream signaling pathways (Fig. 2a) (Rosen et al. 2007, 2009, 2013, Chap. 1 in this volume). The biological functions of these various S1P receptors are dependent on the cell/tissue location of the receptors, their expression, and their activation. Knowing that a broadly permissive S1P agonist AAL-R, which signals via S1P1, S1P3, S1P4, and S1P5 but not S1P2 receptors, significantly downregulated the cytokine storm and protected mice from the effects of a pathogenic human H1N1 influenza infection (Fig. 1, Panels c–g) (Walsh et al. 2011), we repeated the experiments shown in Fig. 1, Panels e–g, using two S1P1-specific agonists, CYM-5542 (Walsh et al. 2011), or RP-002 (Teijaro et al. 2011). The results are displayed in Fig. 2 and indicate that either of the two specific S1P1 receptor agonists whose signal is entirely restricted to S1P1 receptors were as effective as the broadly permissive AAL-R that signals on S1P1, S1P3, S1P4, and S1P5. The S1P1-specific agonists CYM-5442 were administered intratracheally (2 mg/kg) and RP-002 intratracheally (3 mg/kg) or orally (6 mg/kg) (Teijaro et al. 2011). Both S1P1 receptor agonist molecules provided protection against a lethal intranasal challenge with human H1N1 A/Wisconsin/WSLH34934/09 or A/California/04/09 (Fig. 2b) and blunted cytokine storm (Fig. 2c and d). The S1P1 receptor agonists significantly inhibited secretions of cytokines and chemokines associated with influenza virus infection, namely IFN-α, CCL-2, IL-6, TNF-α, CCL-3, CCL-5, CxCl-2, IL-1α, and IFN-γ. Observations from several experiments indicated that amounts of these cytokines/chemokines were inhibited to a degree similar to that from AAL-R treatment. The S1P1 selective agonists also significantly blunted the accumulation of innate infiltrating inflammatory cells (Fig. 2, Panel d). Notable were the reductions of macrophages/monocytes (marked by $CD11b^{+}$, $LyG6^{-}$,

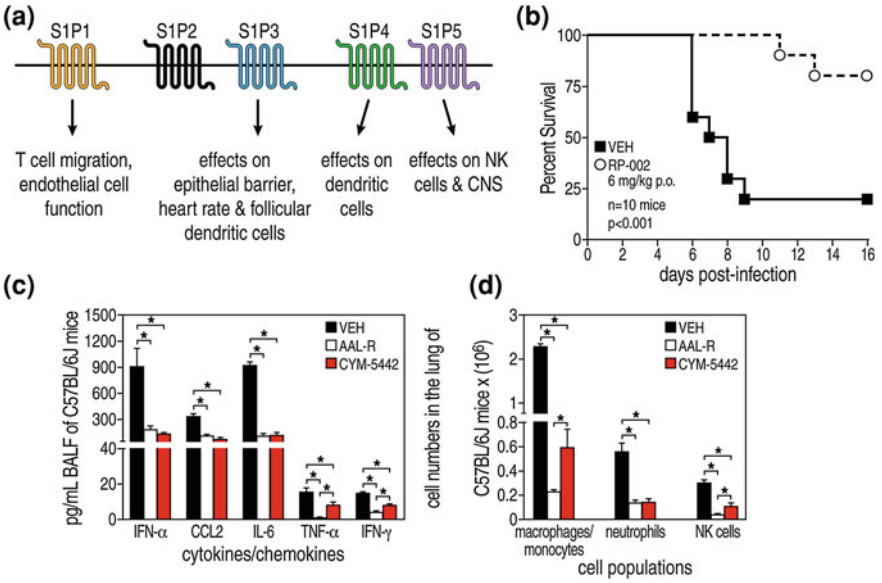

Fig. 2 *S1P1* specific agonists *CYM-5442* and *RP-002* are therapeutically equivalent to the permissive *AAL-R* agonist that utilizes *S1P1*, *S1P3*, *S1P4*, and *S1P5* receptor signaling to blunt cytokine storm and also protect mice from a lethal challenge by human pandemic H1N1 2009 A/Wisconsin/WSLH34939/09 (shown) or A/California 04/09 (not shown) viruses. *Panel a*: Cartoons of the five *S1P* receptors and their biologic effects. AAL-R signals on receptors *S1P1*, *S1P3*, *S1P4,* and *S1P5* but not *S1P2*. *Panel b*: *S1P1* receptor-specific agonist RP-002 given orally protects mice challenged with H1N1 human 2009 influenza virus A/Wisconsin. Treatment with *S1P1* receptor-specific agonist *CYM-5442* after mice are challenged with 2009 influenza A/Wisconsin inhibits their cytokine/chemokine response (cytokine storm) equivalently to treatment with the permissive *AAL-R* (*Panel c*) and also impedes the recruitment of innate immune cells into their lungs (*Panel d*). *BALF* Bronchial lavage fluid, * = $p < 0.01$. Figure reprinted from Teijaro et al. (2011), with permission from Elsevier

F480^{+}), neutrophils (CD11b^{+}, LyG6+$^{-}$, F480^{-}), and natural killer cells (NK1.1^{+}, CD3^{-}). Correspondingly, the quantity of activation marker CD69 was significantly reduced following S1P1 agonist therapy. Further, pulmonary tissues also reflected S1P1's beneficial outcome, since histologic study of mice given this remedy manifested mostly open alveolar air spaces, diminished to negligible inflammatory cell infiltrates and neither edema nor hemorrhage in the lung tissue. Importantly, S1P1 agonist treatment did not enhance viral titers. Influenza infection was effectively terminated, and both anti-influenza neutralizing antibodies and anti-influenza virus CD8 T cells were generated. Although numbers of T cells were reduced compared to infected mice not receiving S1P1 agonist therapy, they were sufficient to terminate the infection. Further, immune memory was established following this S1P1-specific therapy.

Thus, severe pulmonary injury and disease associated with influenza infection and resultant cytokine storm were treatable with a preparation composed of only S1P1 receptor agonist molecules, thereby avoiding signaling through S1P2, S1P3, S1P4, and S1P5 receptors. Pharmaceutically this may be of importance if/when individually S1P2, S1P3, S1P4, or S1P5 signaling might lead to unwanted harmful biologic effects.

2.4 S1P1 Receptors are Located on Pulmonary Endothelial Cells, Which Serve as the Gatekeepers for Cytokine Storm

Having identified S1P1rec signaling as the primary pathway for the initiation of cytokine storm, we sought to identify the cell or cells in the lung that expressed the S1P1 receptor. Since epithelial cells are the primary cells infected by influenza viruses, we suspected that S1P1 receptors might be located on those cells. To determine which pulmonary cell types bear the S1P1 receptor, we took advantage of eGFP-S1P1 receptor knock-in mice made by Stuart Cahalan in the Rosen laboratory (Cahalan et al. 2011, see Cahalan Chap. 4, this volume). In this strain of mice, the native S1P1 receptor was homologously replaced with a functional fused eGFP-tagged S1P1 receptor (Cahalan et al. 2011). Utilizing this mouse model, we could directly detect eGFP-S1P1 receptor protein expression on pulmonary cells by using antibodies to specific pulmonary cell markers and flow cytometry (Fig. 3, Panel a). Additional substantiation came from biochemical analysis of these purified pulmonary cells (Fig. 3, Panel b). S1P1-eGFP receptor expression was plentiful on lung lymphatic ($CD45^-$, $CD31^+$, $GP38^+$) and vascular ($CD45^-$, $CD31^+$, $GP38^-$) endothelial cells but, surprisingly, was absent on pulmonary epithelial cells ($CD45^-$, $CD31^-$, $EpCAM^+$) (Fig. 3, Panel a) (Teijaro et al. 2011). These results were confirmed by doing Western blots on >98.5 % pure populations of pulmonary endothelial and epithelial cells (Fig. 3, Panel b). As expected and previously reported, CD4 T cells ($CD4^+$, $CD3^+$), CD8 T cells ($CD8^+$, $CD3^+$), and B cells ($B200^+$, $CD19^+$) also expressed the S1P1-eGFP receptor (Fig. 3, Panel a). In contrast, pulmonary leukocytes, including macrophages/monocytes ($CD11c^+$, $CD11b^-$, $F480^+$), dendritic cells ($CD11c^+$, $IA\text{-}IE^+$, $CD205^+$, $F480^-$), neutrophils, NK cells ($NK1.1^+$, $CD3^-$) (Fig. 3, Panel a), and immature lymphoid cells (LIN^-, $SCA\text{-}1^+$) failed to express significant levels of eGFP-S1P1 receptor protein. S1P1-eGFP receptor expression was similar whether cells were harvested from mice that were uninfected or infected with influenza virus. Other experiments in infected mice indicated that S1P1-eGFP receptor expression was not altered during influenza virus infection. Importantly, S1P1 agonist treatment of infected eGFP-S1P1 receptor knock-in mice did not lessen expression of the S1P1-eGFP receptors indicating that administration of specific S1P1 agonist does not degrade the endothelial S1P1 receptor. These results signify that the functional agonism of S1P1, not its antagonist effect of receptor degradation, was the mechanism by which S1P1 receptor

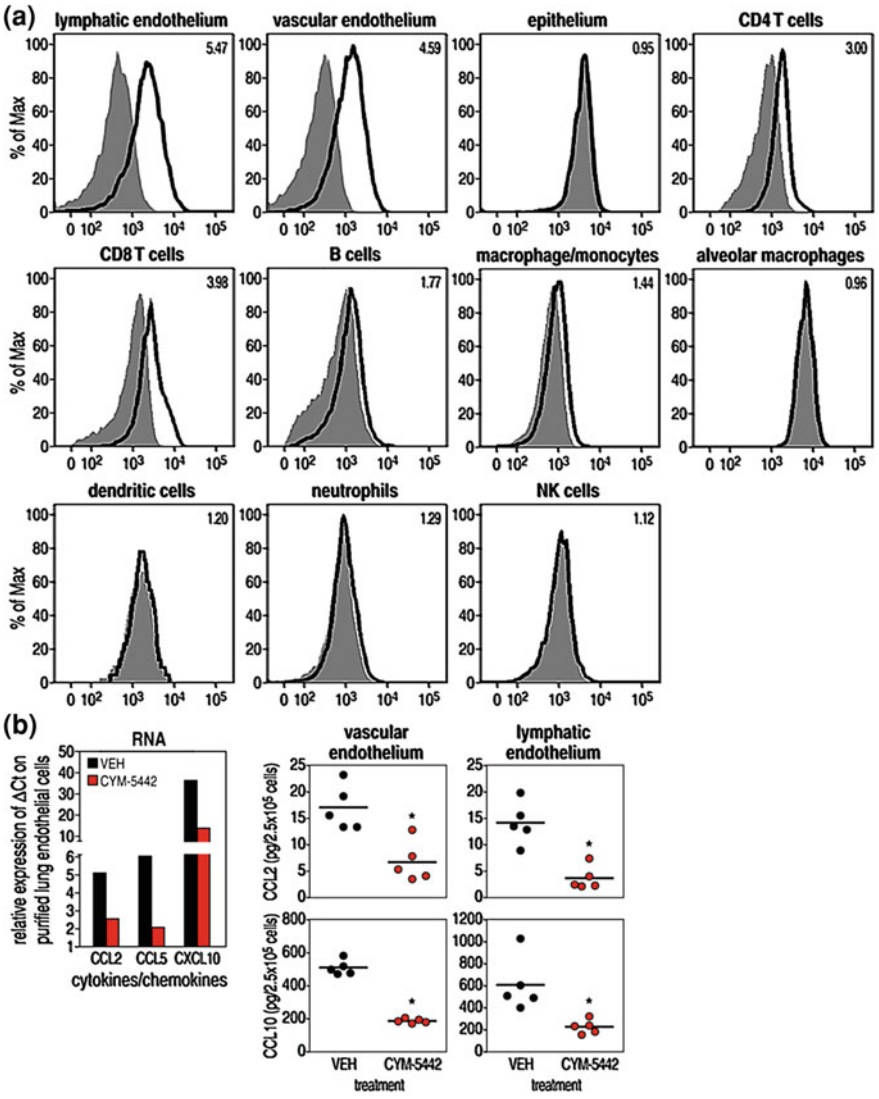

Fig. 3 S1P1 is expressed on endothelial cells and lymphocytes isolated from eGFP-S1P1 receptor knock-in mice. *Panel a*: Cell populations purified using antibodies to specific cell surface markers and FACS. Purity of all cell populations exceeded 98.5 %. See Teijaro et al. (2011) for details about reagents and experiments. As seen in *Panel a*, only endothelial cells (lymphoid and vascular) and lymphocytes ($CD4^+$ T cells, $CD8^+$ T cells) expressed the GFP-S1P1 receptor marker. Pulmonary epithelial cells, the primary target for influenza virus, do not express the S1P1 receptor. *Panel b*:S1P1 agonism inhibits chemokine expression in endothelial cells following influenza virus infection. * = $p < 0.01$. See Teijaro et al. (2011) for details. Figure reprinted from Teijaro et al. (2011), with permission from Elsevier

blocking molecules CYM-5442 and RP-002 suppressed cytokine storms. In other studies, pulmonary endothelial cells were processed to a greater than 98.5 % purity during the first 48 h following influenza virus infection and treated with S1P1 agonist. Assessment of both RNA and protein levels showed that the S1P1 agonist CYM-5442 effectively decreased amounts of cytokines and chemokines made by vascular as well as lymphatic pulmonary endothelial cells (Fig. 3b).

T and B lymphocytes as well as pulmonary endothelial cells were the only cells within the lung that expressed measurable amounts of S1P1-eGFP protein (Fig. 3 Panel a). We therefore determined whether lymphocytes expressing S1P1 receptors were involved in S1P1 agonist inhibition of cytokine storm or were merely bystander cells accompanying the innate immune response to influenza virus infection. Since Rag2-/- mice are deficient in lymphocytes, we reasoned that if such mice, when infected with influenza virus, generated a cytokine storm that could be blocked by S1P1 agonist, then lymphocytes were ruled out as initiators of cytokine storms. Our experiments documented that cytokine storm occurred in Rag2-/- mice infected with influenza virus. Importantly, treatment of infected Rag2-/- mice with the S1P1 agonist CYM-5442 significantly reduced cytokines and chemokines in the bronchial lavage fluids as well as minimalizing the infiltration of innate cells (macrophages/monocytes and NK cells). Recently, John Teijaro (2013), utilizing cell sorting and a biochemical approach, found S1P1 receptor on plasmacytoid dendritic cells (pDC) whose expression was undetectable in the S1P1-γ GFP transgenic mouse model.

2.5 Type I Interferon Signaling is Essential for the Cytokine/Chemokine Response of Cytokine Storm but is not Involved in Recruitment of Innate Inflammatory Cells into the Lung

As observed in Fig. 4a and b and detailed in Teijaro et al. 2011, amounts of type I interferon and almost exclusively the interferon-α species were elevated early after acute influenza virus infection. The release and action of type I interferon was crucial for the production of pro-inflammatory cytokines/chemokines, since blockage of the type I interferon response by using monoclonal antibody to interferon-α-β receptor (IFNAR1) significantly reduced the quantity of pulmonary cytokines/chemokines associated with acute influenza infection (Fig. 4b). Further, treatment with S1P1 receptor agonist inhibited the production of interferon-α in the pulmonary bronchial lavage fluid early after initiating influenza virus infection. Proof that this blunting of interferon-α production was a mechanism by which S1P1 receptor agonist inhibited cytokine storm derived from use of IFNAR1 receptor knock-out mice infected with H1N1 virus and treated with S1P1 receptor agonist CYM-5442. Such studies showed a significant reduction of cytokines/chemokines (IFN-α, CCL-2, IL-6 (shown Fig. 4c), IFN-γ, CCL-5, CxCl-0, not shown) in the bronchial lavage fluid when compared to results from similar

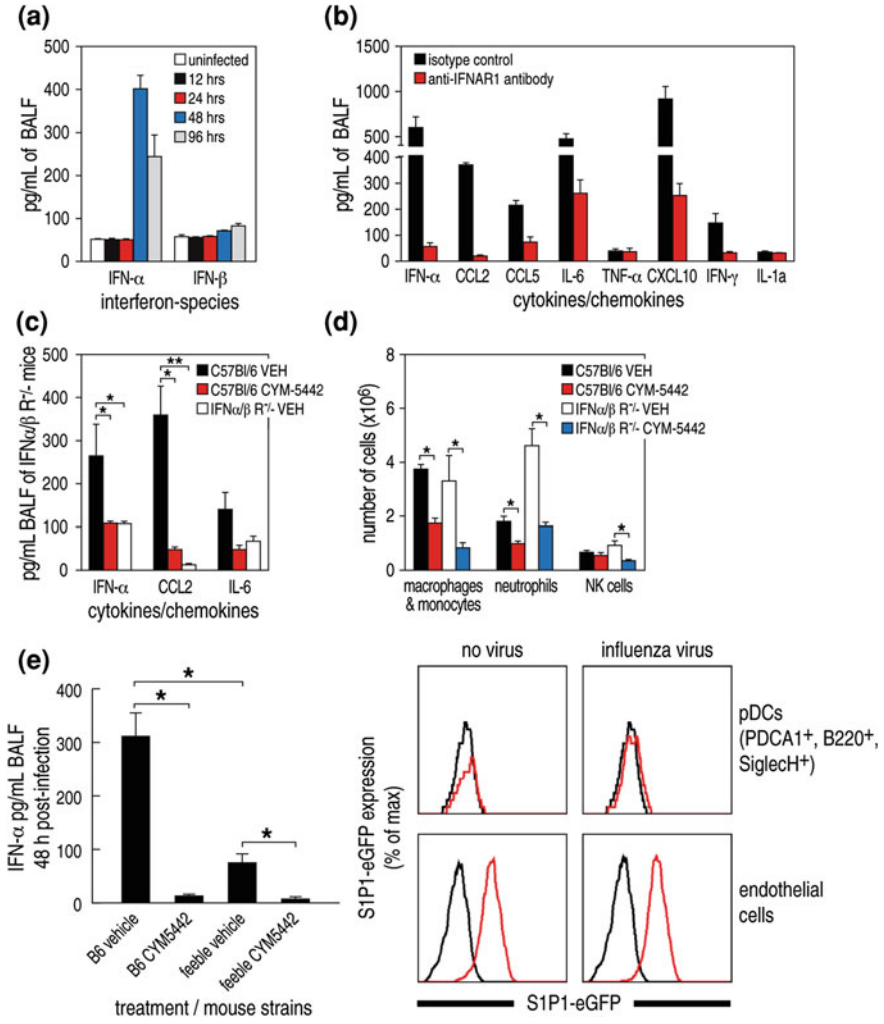

Fig. 4 Interferon-α is the predominant type 1 interferon produced early following virus infection (*Panel a*) and is associated with the dysregulation of cytokines and chemokines that causes a cytokine storm. Antibody to interferon type 1-α-β receptor significantly blocks release of cytokines and chemokines (*Panel b*). *Panel c*: *S1P1* agonist suppression of cytokines is dependent on interferon 1. *Panel d*: Innate inflammatory cell recruitment is independent of interferon-α-β receptor signaling. *Panel e*, *left*: The majority (75–85 %) of interferon-α released following influenza viral infection is from plasmacytoid dendritic cells (pDCs, use of feeble mice—see text). *Panel e*, *right*: Data from *S1P1-eGFP* knock-in mice indicating that *S1P1* receptors are not present on surfaces of pulmonary *pDCs* but are found, as expected, on surfaces of pulmonary endothelial cells. However, utilizing more sensitive techniques *S1P1* receptors are found on *pDCs* (see text). Figure reprinted from Teijaro et al. (2011), with permission from Elsevier

experimentation in mice with an intact interferon-α-β receptor signaling ability (Fig. 4c). Of interest, blockage of interferon-α-β receptor signaling did not retard pulmonary infiltration by the inflammatory cells—macrophages, monocytes, neutrophils, or NK cells—following S1P1 receptor agonist therapy (Fig. 4d). Thus, the infiltration of innate inflammatory cells was blunted only in interferon-α-β sufficient mice but not in interferon-α-β receptor knock-out mice. This outcome indicates that regulation of such cell recruitment into the lung was primarily mediated by endothelial cells and was independent of type I interferon signaling (Teijaro et al. 2011). Cytokine/chemokine production in the lung was also mediated by pulmonary endothelial cells, and S1P1 receptor agonism of such cells inhibited interferon-α production leading to the significantly diminished inflammatory cytokine/chemokine responses we observed.

Influenza virus infection induces a robust interferon type I response, despite the early induction of the viral NS1 protein that suppresses the cellular induction of and response to interferon I (Fernandez-Sesma 2007). The predominant type I interferon produced early following influenza virus infection is alpha, not beta (Fig. 4a). However, the cellular sources of interferon type I-α produced and amounts made by various cell populations have not been clear. The two major pulmonary cell populations known to make type I interferon in vivo following respiratory viral infections are pDCs and alveolar macrophages (Kumagai et al. 2007). To judge the contribution of pDCs to interferon-α production in the lung, we utilized a novel mouse model recently developed at Scripps by Bruce Beutler and termed "feeble." Feeble mice have a specific genetic defect that prevents their pDCs from producing type I interferon and pro-inflammatory cytokines upon activation of TLR7 and TLR9 ligands by influenza virus stimulation (Blasius et al. 2010). Importantly, there is no disruption of the numbers or vitality of pDCs, and the feeble mouse defect is specifically restricted to pDCs. As displayed in Fig. 4e, when wild type or feeble mice received H1N1 human 2009 swine influenza with or without S1P1 agonist CYM5442 treatment, 75–85 % of total interferon-α was produced by pDCs. Further, interferon-α release was significantly inhibited by the S1P1 agonist CYM-5442. These results were confirmed using a pDC depletion antibody (anti-PDCS-1 clone 120.68), which again resulted in significant depletion of pDCs in the lung and corresponding reductions in interferon-α, CCL2, CCL5, and IL-6. Thus, pDCs are the essential and major producers of interferon-α (75–85 %) and involved in amplification of cytokine/chemokine volumes following influenza infection. Other depletion studies indicate that most of the remaining interferon-α production (∼15–25 %) was by alveolar macrophages.

Since S1P1 agonist therapy diminished interferon-α production, and the majority of interferon-α produced was by pDCs, it was important to learn whether or not pDCs expressed S1P1 receptors on their surfaces. We know that alveolar macrophages, the other main albeit minority producers of interferon-α do not express S1P1 receptors (Fig. 2a). Plasmacytoid dendritic cells were recently found to express S1P1 receptors (Teijaro 2013). However, using the S1P1 eGFP receptor knock-in mice and pDCs of more than 98.5 % purity failed to show that these cells expressed S1P1 receptors (Fig. 4e). Thus, the S1P1-specific receptor is found

primarily on pulmonary endothelial cells with lesser amounts on pulmonary pDCs. S1P1 receptor agonist acts directly on pulmonary endothelial and pDCs and likely indirectly on alveolar macrophages. We have, as yet, been unable to detect S1P1 receptor on alveolar macrophages.

2.6 Working Model for the Initial Production of Cytokine Storm and its Chemical Tractability by Single S1P1 Molecules

A working model based on the accumulated data for the earliest events of influenza infection is provided in Fig. 5. Although there are several possible scenarios, I selected the simplest one in which S1P1 agonist signals a factor(s) that blocks [negative signal(s)] the release of interferon-α from pulmonary pDCs and the migration of innate inflammatory cells from blood vessels into the lung. This model is based on presence of S1P1 receptors on pulmonary endothelial and pDCs but their absence on lung epithelial cells and the findings that alveolar macrophages which produce type I interferon do not display S1P1 receptors on their surfaces. However, pulmonary endothelial cells and pulmonary pDCs do express S1P1 receptors on their surfaces. Cytokines/chemokines elicit the initial cytokine storm reflective of factors (viral or nonviral) produced by the influenza virus-infected lung epithelial cells per se. These factors likely activate pulmonary pDCs and alveolar macrophages to release interferon-α. As chemotoxic factors are then liberated into the site of action, infiltrating cells of the innate immune system (macrophages/monocytes, NK cells, leukocytes) are drawn into the inflammatory milieu where they release additional cytokines/chemokines.

After that initial response, a second stage occurs by day 4–8 (see 2.2) via a mechanism described in our publication (Marsolais et al. 2009). Here influenza virus-specific T cells are activated and expand numerically in the mediastinal lymph nodes and pulmonary tissues. These T cells of the adoptive immune system produce additional inflammatory molecules and lyse influenza virus-infected epithelial cells thereby augmenting cytokine storm and immune-mediated injury. This second phase of tissue injury is primarily influenza virus–specific T cell-mediated and signals through S1P1 but also likely progresses via S1P3 and 4 receptors, as by our preliminary results. However, additional data are required to ensure these observations. What is clear is that treatment with S1P permissive-AAL-R agonists signaling via S1P1,3,4,5 receptors affect adoptive immune T cell-mediated immunopathology by downregulating MHC and co-stimulatory molecules of DCs located in the mediastinal lymph nodes and the lung parenchyma thereby blunting the arming, expansion, and migration of virus-specific CD4 and CD8 T cells into the lung (Marsolais et al. 2009).

The cytokine pathways blunted by S1P1 agonist signaling are displayed in Fig. 5 by the symbol ⊢.

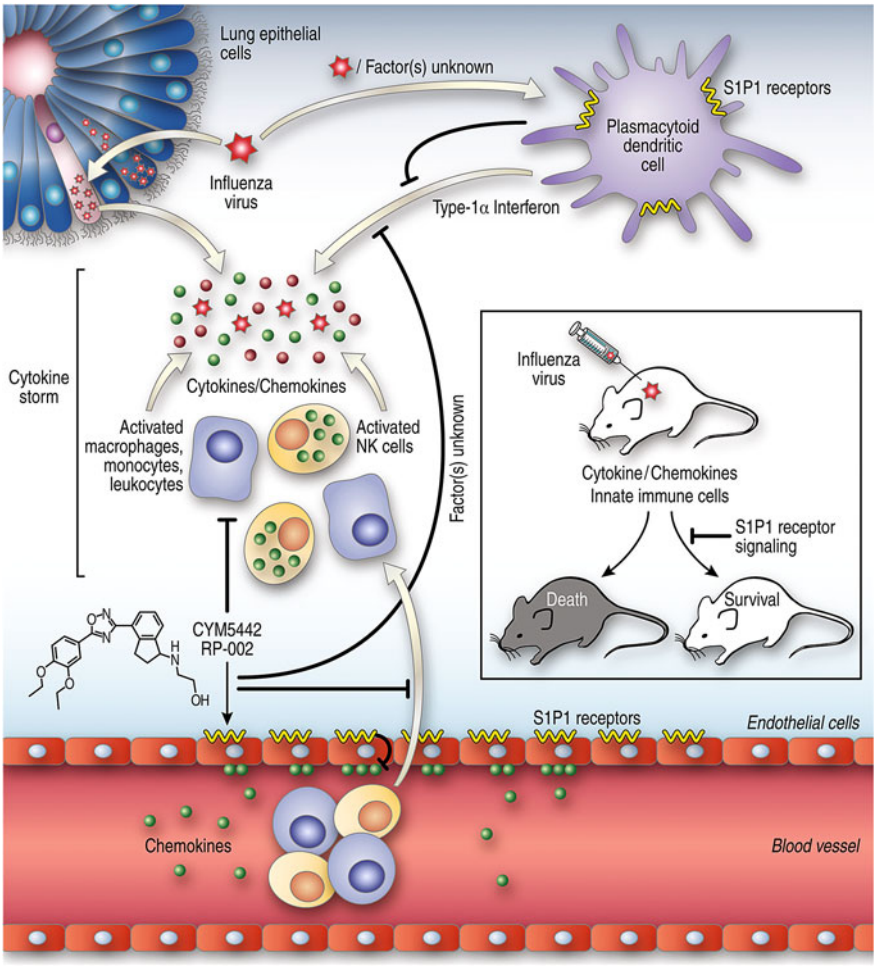

Fig. 5 Schematic of data presented in the text: Proposed pathways and cell–cell crosstalk in the lung following influenza virus infection and *S1P1* receptor agonist signaling. Initial events: Viruses infect lung epithelial cells that release one or more (currently unknown) factors that signal plasmacytoid cells (primary cell-type involved) and alveolar macrophages to release type 1 interferon-α, which dysregulates cytokines/chemokines to elicit cytokine storm. Released chemokines attract innate immune inflammatory cells that become activated and release additional chemokines/cytokines to amplify cytokine storm. Therapeutic control of cytokine storm: Pulmonary endothelial cells and *pDCs* contain *S1P1* receptors on their surfaces, but *S1P1* receptors are absent from lung epithelial cells and alveolar macrophages. The *S1P1* receptor agonist signals pulmonary endothelial cells and *pDCs* likely to release factors that negatively regulate the cytokine storm in terms of both its cytokine/chemokine release and infiltration of innate inflammatory cells

3 Conclusions and Future Studies

3.1 Conclusions

Cytokine storm plays an essential role in the pathogenesis and clinical outcome of influenza virus infection. Blockade of cytokine storm provides greater protection than does antiviral therapy, like that with a neuraminidase inhibitor, and does so without compromising the control and clearance of viruses. Moreover, optimal therapy is achieved by combining S1P agonists with anti-neuraminidase treatment. For the foregoing observations, human pathogenic H1N1 09 influenza virus and mouse adapted H1N1 influenza virus were used.

Sphingosine-1-phosphate (S1P) receptor agonists blunt cytokine storm. Importantly, cytokine storm is chemically disarmed by administering one of the five S1P receptors: S1P1.

The molecular mechanism of this event involves S1P1 receptor signaling on pulmonary endothelial cells and pulmonary pDCs but not virally infected epithelial cells or alveolar macrophages. Pulmonary endothelial cells are the major gateway combined with pulmonary pDCs to precipitate a cytokine storm. S1P1 agonism suppresses the recruitment of both cytokines, innate and adoptive immune cells. Blunting of innate immune cell function and virus-specific T cell activity lessens morbidity and prevents mortality associated with experimental models of influenza virus infection in mice and ferrets. In both species, there is a sufficient antiviral response remaining to terminate the virus infection and provide immune memory upon rechallenge.

Immune cell infiltration and cytokine production are distinct events, but both are orchestrated by endothelial and pDC cells. Pro-inflammatory cytokine responses depend on type I interferon signaling; IFN-α is the predominant interferon made. The predominant pulmonary cell making type I interferon is the pDC (over 75–85 %); alveolar macrophages make most of the rest.

3.2 Future Studies

1. Investigate interferon I as to the cellular source and signaling pathway(s) in the influenza system.
2. Dissect crosstalk and signaling between pulmonary endothelial cells, infected epithelial cells, and interferon-producing pDCs. Identify the molecules involved. See if these molecules provide potential therapeutic targets.
3. Determine generalities for other acute respiratory infections, e.g., Hantavirus, respiratory syncytial virus, SARS, pneumococcal pneumonia, in which cytokine storm plays a prominent role.
4. Study an animal model (subhuman primates) more reflective of influenza in humans. Results from our studies in ferrets (Teijaro et al. 2013) mirror the

protection supplied by S1P1 agonist therapy in defending against influenza virus infection in the murine model.
5. Define the S1P pathway and design a genetic screen to identify humans who are the most susceptible to cytokine storm.
6. Develop specific S1P receptor agonists and antagonists for human therapeutics, focusing initially on S1P1 molecules.

Acknowledgments The experimental work described in this chapter was initiated with and carried out as a collaboration between my laboratory and that of Hugh Rosen. Graduate student (Stuart Cahalan) and former postdoctoral fellows (David Marsolais, Kevin Walsh, and John Teijaro) were instrumental in the findings presented. John Teijaro continues his independent work in this area as a faculty member at Scripps. Yoshi Kawaoka and colleagues in his laboratories have been valued collaborators. This work was funded by NIH grants AI009484 (MBAO), AI074564 (MBAO, HR), MH084512 (HR), and was also supported by the NIH/NIAID under Award Number U54 AI057160 to the Midwest Regional Center of Excellence (MRCE) for Biodefense and Emerging Infectious Diseases Research (MBAO).

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Sphingosine-1 Phosphate and Central Nervous System

Roland Martin and Mireia Sospedra

Abstract The development of fingolimod, an unselective functional antagonist of the interactions between sphingosine 1 phosphate (S1P) and sphingosine 1 phosphate receptors (S1PRs), as the first oral therapy for multiple sclerosis (MS) has been a milestone. The parallel intensive research on the role of S1P, sphingosine kinases, and the five known S1PRs, their tissue distribution and expression in physiological and pathological conditions have led to a wide range of interesting findings. The initial focus of this research in the context of developing fingolimod as a treatment of MS has been on its immunological effects. The wide distribution and important roles of sphingosine, its metabolites, and their receptors in the central nervous system (CNS) in general, in myelin, and in all cell types of this organ have spurred interest to examine S1P and its five receptors in the brain as well. The present review will concentrate on the latter area and give a brief overview of what is known about S1P/S1PR interactions in the CNS in physiological and pathological conditions.

Contents

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1 Introduction

Sphingolipids, particularly sphingomyelin, are principal components of oligodendrocytes and myelin and of the CNS in general. Sphingomyelin is catalyzed to ceramide by sphingomyelinase, and ceramide metabolized further to sphingosine by ceramidase, which then can be phosphorylated by sphingosine kinases (SphK) to yield sphingosine-1-phosphate (S1P). The role of these metabolites was long enigmatic until the discovery of fingolimod, a first-in-class agonist of sphingosin-1 phosphate receptors (S1PRs). The parallel growing understanding of the functions of breakdown products of sphingomyelin, ceramide, and S1P, as signaling molecules upon binding to S1PRs, has shown that these molecules are involved in a wide range of functions of essentially all cells in our body via intracellular signaling in different pathways that are mediated by Rho-/Ras, phospholipase C, phosphatidylinositol-3 kinase, and Akt. Fingolimod binds to four of the five S1PRs, and among its pharmacological effects, immunomodulation and those on multiple CNS cells stand out. Based on the novel immunomodulatory mechanisms fingolimod was first explored as a treatment to prevent the rejection of allotransplants and then for multiple sclerosis (MS). In 2010, fingolimod was approved as the first oral treatment for MS. Already during later stage clinical development and in parallel animal model studies, it has been shown that fingolimod, besides its prominent effects on immune cell homing, has a number of mechanisms of action on CNS cells and the blood-brain barrier (BBB) (Brinkmann et al. 2004; Groves et al. 2013; Coelho et al. 2007; van Doorn et al. 2012), which are of interest not only in MS, but also in other neurodegenerative diseases including stroke (Kimura et al. 2008; Wei et al. 2011), brain/spinal cord injury (Lee et al. 2009), and glioma (Estrada-Bernal et al. 2012). S1PRs are expressed on endothelial cells at the BBB (van Doorn et al. 2012), on astrocytes (Sorensen et al. 2003; Wu et al. 2008), on neuronal cell populations and their progenitors (Kimura et al. 2007), on oligodendrocytes and their precursors (Jaillard et al. 2005), and on microglia (Kimura et al. 2007). These observations have spurred intensive further research. Currently, a series of small molecules and antibodies with more specific inhibitory profiles regarding S1PR subtype inhibition are being developed. The current state of knowledge about the role of S1P and S1PRs in the context of the CNS will be reviewed here. Since numerous excellent reviews have been written along the research in this field, a few are mentioned here for the interested reader (Groves et al. 2013; Brinkmann 2009; Chun and Hartung 2010).

2 Sphingosine-1 Phosphate Receptor Expression and Functions in the CNS

2.1 Neuronal Cell Populations

Following the discovery of the structural analog of S1P, fingolimod (FTY720), and its potent effects in MS, the role of S1P/S1PR interactions in CNS function and the expression of S1PRs on CNS cells and tissue received increasing interest. The involvement of S1P signaling in normal neural function became clear from in vivo knockout studies, from effects of fingolimod and other pharmacological inhibitors in various in vivo models, and also from in vitro studies with cultured cells (Kono et al. 2007; MacLennan et al. 2001; Ishii et al. 2002; Akahoshi et al. 2011; Edsall et al. 1997; Toman et al. 2004; Choi et al. 2011; Rau et al. 2011; Rossi et al. 2012; Callihan and Hooks 2012). Indirect evidence for a role of S1P/S1PRs in CNS function was derived from demonstration of sphingosine kinase 1 or −2 (SK1, SK2) expression in various cell types (Bryan et al. 2008) and their role in neural development (Mizugishi et al. 2005). Regarding the expression of S1PRs on CNS cells, since monoclonal antibodies (mabs) were not available until recently and are still not available for all subtypes, the/tissue-/cellular expression was examined mainly by nucleic acid-based methods (PCR, in situ hybridization), but recently also by immunohistochemistry (IHC) and western blotting using a S1PR-specific mab for staining autopsy specimens of brain tissue and parallel analysis by PCR (Brana et al. 2013; Nishimura et al. 2010). The five S1PRs are found in the developing and mature brain (Dubin et al. 2010), and constitutive knockout (ko) of S1PR1 causes a behavioral phenotype reminiscent of schizophrenia (Contos et al. 2002). S1PR1 is expressed on neuronal precursor cells and likely also mature neurons and has been found to affect neurogenesis, cell migration, and functions such a brain-derived neurotrophic factor (BDNF)-induced process extension (Chun and Hartung 2010; Deogracias et al. 2012). Fingolimod upregulates BDNF and hence probably contributes to tissue protection in EAE [31]. The fact that S1PR1 is expressed preferentially and several fold higher in the gray compared to white matter hinted at prominent neuronal expression, but detailed examination of its cellular localization revealed expression in astrocytic foot processes (Nishimura et al. 2010). Further evidence for a role of S1P/S1PR signaling includes inhibition of amyloid production by cultured neurons upon exposure to FTY720-phosphate (FTY720-P) and a more selective S1PR1 agonist KRP203-P (Takasugi et al. 2013), the development of neural tube defects upon maternal ingestion of the mycotoxin fumonisin (Callihan et al. 2012), the S1P-mediated increase in glutamate release and expression of SK1 by hippocampal neurons (Kajimoto et al. 2007), the induction of neuronal precursor cell migration to areas of spinal cord injury via increased S1P and interaction with S1PR1 (Kimura et al. 2007), and by reduced neuronal cell death upon FTY720 treatment in stroke models (Hasegawa et al. 2010). Existing data thus documents a wide range of effects of S1P/S1PR signaling on neuronal precursor cells and mature neurons in different areas of the

brain. Due to technical limitations, particularly the lack of mabs against all S1PRs, the expression of the different S1PRs on different cell types is, however, not yet clear. Developing a better understanding for the cellular distribution on neuronal cells during development of the healthy brain and during pathological conditions will be important.

2.2 Astrocytes

As already briefly mentioned above, S1PRs are widely expressed on astrocytes (Nishimura et al. 2010), a cell population that tightly interacts with neurons and endothelial cells of the BBB and is considered essential for many homeostatic processes within the brain but also tightly involved in neuroinflammation (Brinkmann 2007). Both in situ hybridization and conditional ko studies in animal models as well as data from intracerebral injection of FTY720 indicate that astrocytes are the major CNS cell type that is responsible for the beneficial effects of FTY720 in experimental autoimmune encephalomyelitis (EAE), the main animal model of MS (Choi et al. 2011; Miron et al. 2008; Wu et al. 2013). In vitro studies with human fetal astrocytes documented that FTY720 inhibits subsequent S1PR-mediated pERK1/2 signaling for a protracted period of time and thus desensitizes neuroinflammatory effects on astrocytes and their proliferation (Sorensen et al. 2003; Wu et al. 2013). Cultured murine cortical astrocytes express a wide range of lipid-activated receptors including the protease-activated receptors (PAR1-4), lysophosphatidic acid receptors (LPA1-3), and S1PR1, 3, 4, and 5, and each of these activates multiple downstream signaling pathways that participate in astrocyte proliferation and gliosis, Sorensen et al. 2003. Regarding S1PR subtypes on astrocytes, Rao et al. (2003) have demonstrated expression of S1P1, S1PR2, S1PR3, and S1PR5 with relatively higher expression of S1PR3 and S1PR1. In vitro or in vivo administration of S1P promotes the expression of glial fibrillary acidic protein (GFAP) and astrogliosis, but also astrocyte proliferation and migration (Chun and Hartung 2010). Particularly, S1PR3 appears to be involved in astrocyte proliferation and neurodegeneration during the terminal stages of Sandhoff's disease (Wu et al. 2008). During treatment with FTY720, its metabolite FTY720P induces astrocyte migration through preferential binding to S1P1, while S1P binds to both S1P1 and S1P3, indicating that the profile of FTY720 could play a role for its therapeutic effects in MS (Mullershausen et al. 2007). Since glial proliferation is a general characteristic in many experimental models of neuroinflammation and neurodegeneration and also human neurodegenerative/inflammatory diseases, it is likely that S1P/S1PR-mediated functions play a major role during these pathological conditions.

2.3 Oligodendrocytes, Myelination, and Remyelination

Lipids and sphingomyelin, the precursor of S1P, are abundant in the CNS white matter and myelin. Further, myelin and oligodendrocytes (OLG) are the main targets of the immune system during MS, and demyelination and partial remyelination are characteristic of the disease (Noseworthy et al. 2000). In one pathological subtype of MS, i.e., in pattern III, preferential loss of myelin-associated glycoprotein (MAG) and metabolic alterations have been noted (Lucchinetti et al. 2000), and due to these histopathological findings and the efficacy of fingolimod, the role of S1P/S1PR interactions have been studied extensively in recent years in vitro in oligodendrocyte cultures from adult human brain (Miron et al. 2008, 2010) and from various developmental stages in animals (Jaillard et al. 2005), in oligodendrocyte precursor cell (OPC) models (Kim et al. 2011), in embryonic stem cell-derived OPCs (Bieberich 2011), in organotypic cerebellar slices (Miron et al. 2010), and in models of toxic (cuprizone, lysophosphatidyl-choline-induced, lysolecithin) demyelination (Miron et al. 2008, 2010; Kim et al. 2011; Jackson et al. 2011). Various S1PR subtypes are expressed by human and rodent OLGs, i.e., S1P5, S1P3, and S1P1 at decreasing levels (Terai et al. 2003; Yu et al. 2004; Miron et al. 2012). Upon interaction with S1P1 $G_{i/o}$-associated signaling is mediated via Rac1 and Ras GTPase activation, which affect membrane dynamics and survival (Jung et al. 2007; Spiegel and Milstien 2003), while S1P/S1P3 and -S1P5 interactions lead to $G_{12/13}$-mediated RhoA GTPase activation (Jaillard et al. 2005; Toman et al. 2004). When OLGs from adult human brain were exposed to various concentrations of fingolimod in vitro, cyclical changes with sequential increase of membrane elaboration, retraction and recurring extension were observed at low doses (0.1–1 nmol/L), while the opposite sequence occurred at higher concentrations (10 nmol/L–1 μmol/L) (Miron et al. 2010). Further, membrane retraction could be reversed with a S1P3/S1P5 antagonist, suramine (Miron et al. 2008). In parallel, fingolimod prevented negative effects of serum and glucose withdrawal, which were again blocked by suramine (Miron et al. 2008). S1P5 (formerly Edg8) is expressed throughout oligodendrocyte differentiation, and in vitro experiments with 04-positive pre-oligodendrocytes as well as in S1P5 ko mice revealed that S1P/S1P5 interacions play a role in OLG process retraction and cell survival (Jaillard et al. 2005). Activation of S1P5 in rat neonatal cortex-derived OPCs in vitro inhibits the migration of these cells (Novgorodov et al. 2007). The systematic assessment of the effect of S1P and fingolimod on OLG differentiation from ES cell-derived neural precursor cells (NPCs) demonstrated the expression of S1P1 and protection from ceramide-induced apoptosis and preferential differentiation into the oligodendrocyte lineage upon S1P or fingolimod exposure (Bieberich 2011). When the effects of fingolimod were examined in demyelination models, i.e., in lysolecithin-induced demyelination in organotypic cerebellar slice cultures (Miron et al. 2010), in cuprizone-induced demyelination in vivo, and in mixed neural/glial aggregate cultures (Jackson et al. 2011), remyelination and increased process extension by OPCs and mature OLGs (Miron et al. 2008), reduced damage to OLGs, myelin, and axons (Kim et al. 2011), and increased de novo

synthesis of myelin proteins (Jackson et al. 2011) were observed. Together, these data indicate not only the importance of S1P/S1PR signaling in oligodendrocytes and myelin, but also that pharmacological modulation of S1P represents an interesting way to modulate OPC and OLG biology during de- and remyelination.

2.4 Microglia

Microglia are the CNS-resident equivalent of monocytoid cells in the peripheral immune system (Benarroch 2013). Any type of damage and alteration of CNS cells such as for example, protein aggregate deposition, neuronal cell death, or damage of processes are sensed by microglia and lead to their activation (Benarroch 2013). Microglia serve a wide range of functions including phagocytosis, cytokine/inflammatory mediator release, antigen presentation, Fc receptor-mediated cell killing, and migration, and hence are the main mediators of neuroinflammatory processes, although astrocytes and innate and adaptive immune cells (T and B cells) that have entered the brain also participate (Goldmann and Prinz 2013). In MS, microglia activation is widespread, and it is currently believed that this cell population is activated already during the earlier relapsing-remitting phase of MS and plays a central role in secondary (SPMS) and primary chronic progressive disease (PPMS), i.e., during the phase (SPMS) or MS type that are characterized by increasing neurological deficit in the absence of relapses (Lassmann 2013). Despite the importance of microglia for MS and also for neurodegenerative disease, relatively little attention has been devoted to this cell type in the context of S1P/S1PR interactions and its pharmacological modulation by fingolimod. In the abovementioned model of lysophosphatidyl-choline-induced toxic demyelination, microglial ferritin, the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), nitric oxide metabolites, and apoptosis mediators (caspase 3 and −7) were all reduced by fingolimod and probably represent an important factor for increasing myelination (Jackson et al. 2011). In the lysolecithin-induced demyelination in cerebellar slice cultures, fingolimod treatment interestingly increased microglia numbers and also the expression of GFAP by astrocytes, however, the number of phagocytosing microglia remained unchanged (Miron et al. 2010). If analogies can be drawn from peripheral innate immune cells such as dendritic cells (DCs), which express all five of the S1PRs (Brinkmann 2009), it can be expected that S1P/S1PR signaling and modulation by S1P agonists exerts a wide range of effects on microglia (Brinkmann 2009).

2.5 Endothelial Cells and Blood–Brain Barrier

Maintaining brain homeostasis critically depends on the blood–brain (BBB) and blood-CSF barriers, which restrict access of components of the blood to the CNS

(Engelhardt 2011), The BBB is composed of a complex cellular network including cerebrovascular endothelial cells with tight junctions, astrocytic foot processes and pericytes, which jointly restrict the paracellular and transcellular passage of molecules (Engelhardt 2011). The tight junctions together with membrane efflux pumps assure that this specialized barrier separates the CNS from potentially noxious substances within the blood and also blocks entry of immune cells (Cannon et al. 2012). In MS T and B lymphocytes as well as innate immune cells (macrophages and DCs) cross the BBB, and this transit involves a series of steps including interactions of α4-integrins, specifically very late antigen-4 (VLA-4), on activated immune cells with vascular cell adhesion molecule 1 (VCAM-1) on activated endothelial cells (Vajkoczy et al. 2001). This step is a central aspect during the formation of new inflammatory lesions in MS, and inhibition of binding of VLA-4 on activated T cells to VCAM-1 by the humanized monoclonal antibody natalizumab very efficiently blocks inflammatory disease activity and exacerbations of MS (Yednock et al. 1992; Polman et al. 2006). Other MS treatments such as interferon-β (IFN-β) have also been found to inhibit certain steps of BBB opening such as matrix metalloprotease activation (Waubant et al. 1999), although they are overall less efficient than natalizumab.

Understanding the effects of a novel drug on the BBB and the questions if it accesses the CNS compartment are therefore of high interest, and an important characteristic of fingolimod as a treatment of MS is the fact that it easily crosses the BBB due to its lipophilic nature (Meno-Tetang et al. 2006). In addition to its physicochemical characteristics, fingolimod inhibits P-glycoprotein, an ATP-driven efflux pump, which inhibits drug delivery through the BBB (Cannon et al. 2012). Studies of S1P5, which is relatively specifically expressed in the brain, in human brain tissue (autopsy material) and in vitro experiments using pharmacological modulation with fingolimod as S1P5 agonist as well as lentiviral knockdown of S1P5 in cultures of human brain endothelial cells demonstrated its involvement in BBB function (van Doorn et al. 2012). Fingolimod improved several aspects of BBB integrity and reduced the migration of inflammatory cells across endothelial cells (van Doorn et al. 2012a, b). Pharmacological modulation of S1PRs also affect other mechanisms involved in BBB function. Protein S, a vitamin K-dependent anticoagulant plasma protein, which is involved in maintaining BBB integrity during hypoxia-induced BBB damage, mediates its effects via the protein tyrosine kinase receptor Tyro3 and S1P1, and specific inhibition of S1P1 with the antagonist W146 blocked the protein S-mediated protection of ischemia-induced BBB opening (Zhu et al. 2010). During EAE, fingolimod enters the CNS compartment in a dose-dependent manner and preferentially accumulates in the white matter {Foster, 2007 #626}. Taken together, S1P and its interactions with specific S1PRs (e.g., S1P5 on endothelial cells) affect several aspects of BBB function, and functional S1PR antagonism appears to improve BBB integrity in the context of inflammatory processes.

3 Treating Multiple Sclerosis with the S1P Agonist Fingolimod

MS is considered a prototypic T cell-mediated autoimmune disease with a complex genetic background involving more than 100 quantitative trait loci conferring genetic risk (Beecham et al. 2013), but environmental factors also contribute to MS etiology (Ascherio and Munger 2008). MS pathogenesis involves the activation of autoimmune $CD4^+$ T cells by molecular mimicry or other as yet incompletely understood mechanisms (Sospedra and Martin 2005), their entry into the CNS and initiation of inflammatory CNS lesions that result in damage of myelin, axons, and neurons, astrocyte activation and gliosis as well as complex metabolic alterations (Lassmann 2013). The inflammatory processes involve innate immune cells (DCs, macrophages, microglia), perturbations of astrocyte function, $CD8^+$ T cells, and to various extents also antibody deposition and complement activation with the result of different patterns of CNS pathology (Lucchinetti et al. 2000). Permanent CNS tissue damage occurs already during the earliest stages of MS, but chronic inflammation, gradually increasing loss of axons/neurons, and incomplete remyelination characterize the later stages of MS. MS affects young adults between the ages of 20 and 40 years and women more than twice as often than men (Noseworthy et al. 2000). It is highly specific for the CNS, and the peripheral nervous system is almost never affected to a significant extent. MS can involve every functional system of the CNS including visual, motor, sensory, cerebellar, autonomic, and neurocognitive function, and consequently clinical signs and symptoms are very heterogeneous. Most patients initially present with periodic neurological deficits and a relapsing-remitting course (RRMS), which after 10–20 years often evolves into secondary progressive MS (SPMS) (Noseworthy et al. 2000). Relapses gradually disappear during SPMS, and neurological deficits and disability steadily increase during this stage. A minority of patients show primary progressive MS (PPMS). These patients worsen progressively from onset without relapses.

While many aspects of MS etiology and pathogenesis are still incompletely understood, the development of treatments for this disease has been remarkably successful during the last 20 years (Haghikia et al. 2013). Based on many lines of evidence that MS is an autoimmune disease affecting the CNS and that inflammation is the main cause of CNS tissue damage, several immunomodulatory or—suppressive treatments have been developed and approved for clinical use in RRMS. The injectable drugs IFN-β and a peptidic mixture, glatiramer-acetate (GA), which are both moderately effective, but very well tolerated have long been the mainstay of the treatment of RRMS until a humanized monoclonal antibody against VLA-4, natalizumab, was introduced in 2006 (Polman et al. 2006). The latter compound is considerably more effective than IFN-β and GA and usually well tolerated, but more than 400 patients developed a serious and often fatal complication, an opportunistic infection of the brain with the polyoma virus JC called progressive multifocal leukoencephalopathy (PML). The introduction of the oral S1P agonist fingolimod, which will be discussed in more detail below, was an

important milestone in MS treatment since it was the first orally available compound with superior efficacy when compared to the injectable first-line drugs (IFN-β and GA) and an overall good safety profile (see below). Very recently, two additional oral drugs, teriflunomide (O'Connor et al. 2011) and dimethylfumarate (DMF) (Gold et al. 2012), and a humanized monoclonal antibody, anti-CD52 (alemtuzumab) (Coles et al. 2012) have been approved for the treatment of RRMS. Based on data from the large phase III trials all appear superior to IFN-β and GA, albeit only marginally in the case of teriflunomide. Among the oral drugs, DMF appears most active based on the clinical trial data, but direct comparisons with fingolimod or teriflunomide are not available. Alemtuzumab is more active than the oral compounds and probably comparable to natalizumab. Different from natalizumab, for which PML represents the most important liability regarding safety, alemtuzumab leads so secondary autoimmune diseases in a substantial fraction of patients and to an increased rate of infections probably due to the long-lasting lymphopenia. The long-term safety of the newer agents, i.e., teriflunomide, DMF, and alemtuzumab, remains to be determined. For a more detailed overview about MS treatments the reader is referred to special reviews of the topic.

As indicated above, the introduction of fingolimod as the first oral agent to treat MS has been a significant advance. Its development process has been summarized in excellent reviews elsewhere (Brinkmann 2009; Chun and Hartung 2010), and therefore we will focus here on the most important aspects in the context of S1P and the CNS. Drug development in MS usually involves proof-of-concept in the well established and widely used EAE model. Extrapolation from the EAE model to MS has been difficult, and in many cases promising findings in EAE did not hold up during clinical trials in MS from reasons such as differences between the immune systems of rodents and humans and others. There are, however, notable examples such as VLA-4 blockade by natalizumab and functional S1P antagonism by fingolimod, for which EAE data was fully confirmed in MS (Brinkmann 2009; Yednock et al. 1992). Fingolimod has been tested extensively both as prophylactic, i.e., prior to disease development, and therapeutic, i.e., given after disease onset, intervention in various chronic and relapsing-remitting EAE models (Fujino et al. 2003; Webb et al. 2004; Brinkmann 2009; Kataoka et al. 2005). Fingolimod treatment efficiently blocks disease activity in the EAE model, and a number of mechanisms, most importantly the trapping of $CD4^+$ T helper 1 (Th1, secreting IFN-γ) and Th17 (secreting interleukin-17; IL-17) cells in lymph nodes, but also the stabilization of the BBB, and the abovementioned effects on astrocytes, oligodendrocytes and remyelination, and possibly also neurons, contribute to its efficacy as well [for review see (Brinkmann 2009)].

Following the highly promising data in the EAE model, transplant, and other animal models [for review see (Brinkmann 2009)], fingolimod was developed for the use as oral immunomodulatory agent for RRMS in a large phase IIb and—III program including multiple studies (Cohen et al. 2010; Kappos et al. 2010). Due to the highly positive results, fingolimod (Gilenya®, Novartis) was approved for RRMS in September 2010. In brief, the clinical trials showed the following: in a phase III randomized, placebo-controlled trial of fingolimod given as either 0.5 or

1.25 mg/d, the annualized relapse rate (ARR; primary outcome) was 0.18 with 0.5 mg fingolimod, 0.16 with 1.25 mg, and 0.4 with placebo (highly significant reduction) (Cohen et al. 2010; Kappos et al. 2010). Furthermore, a significant reduction with respect to cumulative probability of disability progression was observed with both doses, and other secondary outcomes (MRI) were also significantly improved (Kappos et al. 2010). In a second, similarly large phase III study intramuscularly injected IFN-β1a was compared with 0.5 and 1.25 mg/d fingolimod (Cohen et al. 2010), and the primary outcome (ARR) showed a significant reduction from 0.33 (placebo) to 0.16 (0.5 mg) and 0.20 (1.25 mg) fingolimod (Cohen et al. 2010). Secondary (MRI) outcomes supported these data, but there was no significant reduction of disability progression in the two verum groups (Cohen et al. 2010). Two fatal adverse events (disseminated varizella zoster virus (VZV) infection and herpes simplex (HSV1) encephalitis) were observed in the 1.25 mg dose, which was a main reason for later continuation of studies and filing for approval of the 0.5 mg dose, which is now in clinical use since September 2010 (for further details on adverse event profile, see below).

3.1 Immunomodulatory Effects

The immunomodulatory effects have been reviewed extensively elsewhere (Brinkmann et al. 2010) and therefore will only be briefly summarized here. The preclinical studies in EAE and later the clinical experience in MS with natalizumab, the anti-VLA-4 monoclonal antibody have highlighted the importance of keeping autoreactive T cells and other immune cells out of the brain. Therefore, other approaches including anti-LFA-1 monoclonal antibodies, small molecule VLA-4 inhibitors, and others were approached to achieve a similar outcome, and among these the oral S1P agonist fingolimod showed a promising profile due to oral availability and efficient modulation of lymphocyte migration/homing, which was expected to lead to a similar outcome, i.e., keeping autoreactive immune cells from gaining access to the CNS compartment. In brief, fingolimod interferes with a well-known sequence of events that occur during de novo activation of immune cells following antigen exposure in a peripheral organ, e.g., the skin, uptake and processing of antigen by organ-residing DCs and transport into regional lymph nodes (LN), where antigen is efficiently presented to T cells and results in T cell activation and proliferation (Matloubian et al. 2004). The containment of the latter step in LN leads to "trapping" of T lymphoctes and involves S1P/S1PR signaling and downregulation of S1P1 mRNA, which was shown by several approaches including mice with targeted deletion of S1P1 from hematopoietic cells resulting in S1P1-deficient thymocytes and T cells, in mice with genetic deletion of sphingosine kinase, which eliminated S1P, and by a series of other experimental strategies (Matloubian et al. 2004; Pappu et al. 2007; Brinkmann 2009). As a result, T cells cannot enter the LN cortical sinuses and consequently fail to enter the medullary sinuses and exit the LN via the subcapsular space and efferent lymph

(Grigorova et al. 2009). Administration of fingolimod mimicked the situation in ko mice with targeted deletion of S1P1 in hematopoietic cells (Matloubian et al. 2004) and upon binding to S1P1 led to receptor internalization in LN T cells and subsequent ubiquitinylation and degradation in the proteasome (Oo et al. 2007, 2011). Interestingly, despite the efficient trapping of T cells in LN, viral immune responses (Brinkmann 2009; Pinschewer et al. 2000), CNS immune surveillance (Bartholomäus et al. 2008), and the development of thymocytes did not appear to be compromised although the egress of thymocytes and the homing to peripheral lymphoid structures were delayed (Metzler et al. 2008). Treatment with fingolimod primarily traps $CCR7^+$ $CD45RA^+$ naïve and $CCR7^+$ $CD45RA^-$ central memory T cells (T_{CM}) in LN, while $CCR7^-$ $CD45RA^+$ and $CCR7^-$ $CD45RA^-$ effector memory T cells (T_{EM}) remain relatively unaffected (Mehling et al. 2008). The recruitment into and regular passage through LN of naïve and central memory T cells via CCR7 play an important role in the relative subtype specificity of fingolimod for lymphocyte homing to LN. Functional testing of fingolimod-exposed T cells demonstrated reduced production of IL-2 and proliferation, but unperturbed release of the proinflammatory cytokine IFN-γ (Brinkmann et al. 2001; Mehling et al. 2008). Overall, the effects of fingolimod on S1P/S1P1 interactions preferentially affect $CD4^+$ naïve and T_{CM} T cells, but left the $CD4^+$ T_{EM} population and $CD8^+$ cytolytic T cells functionally unperturbed (Brinkmann 2009).

Regarding the effects on T cell subsets, particular attention has been given to Th17 cells, which are defined by the production of IL-17 and IL-22 and the expression of the signature transcription factor RORγt (Sallusto et al. 2012). Th17 cells play a prominent role in the EAE model (Peters et al. 2011), but their role is less clear in MS (Lovett-Racke et al. 2011). Th17 cells can cross the BBB and kill neurons and contribute to CNS inflammation by recruiting other immune cells (Kebir et al. 2007). S1PR inhibition by fingolimod efficiently traps Th17 cells in LN and reduces their numbers to less than 5 % in the peripheral blood of MS patients (Hohlfeld et al. 2011) and also in CNS and PNS tissue in experimental models. Other immune cells also express S1PRs (Mehling et al. 2008) and are affected by treatment with fingolimod. B cells are retained in bone marrow and LNs, show reduced germinal center reaction, and upon vaccination with KLH and a pneumococcal vaccine a delayed production of specific IgG was observed (Sinha et al. 2009; Boulton et al. 2012). In fingolimod-treated MS patients, comparable vaccination efficacy was observed during influenza vaccination, and influenza-specific T cell numbers and IgM titers increased in both fingolimod- and placebo-treated patients (Mehling et al. 2008). DCs express all five S1PRs, but it is currently not clear to what extent the redistrubition between antigen-draining LN, where DC numbers drop, and peripheral tissues are mainly due to alterations in T cell homing and numbers or to effects on DCs (Brinkmann 2009). In macrophages, which express S1P1 and S1P2, fingolimod reduces the production of proinflammatory cytokines (Durafourt et al. 2011; Michaud et al. 2010). Monocytes are also affected and show a decrease in the peripheral blood, increased numbers in LN and bone marrow, and reduced expression of CD40 and TNF-α (Lewis et al. 2013). Regarding natural killer (NK) cells, another innate immune cell population that plays important roles during

anti-viral defense and also in immunoregulation, e.g., during pregnancy, global NK cell numbers are not altered during fingolimod treatment in MS, but a subset of NK cells expressing $CD56^{bright}$ $CD62L^{+}$ $CCR7^{+}$ are reduced, probably by trapping in LN as it is observed for CCR7-expressing T cells (Johnson et al. 2011). NK cell effector functions (cytokine release, cytolysis) are not affected in fingolimod-treated MS patients, but the migratory properties of these cells are reduced (Johnson et al. 2011).

In summary, S1P modulation in vitro and in vivo has a broad range of effects on immune cells, and the data from experimental models as well as from fingolimod-treated MS patients indicate that the trapping of CCR7-expressing naïve and central memory T cells in LN and their drop in peripheral blood play a major role in reducing inflammatory disease activity and relapse rates in MS. Whether the inhibition of certain subpopulations of immune cells, e.g., the above mentioned CD56bright NK cells, which have been shown to be beneficial in the context of anti-CD25 blockade, another treatment approach in MS, and/or the effects on T and B cells and monocytes are involved in rare infectious adverse events (see below) remains to be determined.

3.2 Possible Neuroprotective Effects

Eight different immunomodulatory treatments are now available for the treatment of RRMS, but none for the chronic progressive diseases (SPMS, PPMS). Alterations in CNS tissue, which are often referred to as neurodegeneration, including axonal transections, neuronal loss, glial proliferation, de- and partial remyelination, and metabolic changes in neurons occur already during the earliest stages of MS and are the main causes of long-term disability and chronic progression. Hence, treatments that are neuro- or myelin-protective and prevent astrogliosis are urgently needed. Different from the reduction of new inflammatory lesions, which can be easily measured by decrease in contrast-enhancing or new T2-weighted MRI lesions in the brain (Stone et al. 1995), it is more difficult to document the influence of a treatment on the neurodegenerative aspects. Several measures have been proposed including the reduction of brain atrophy and brain volume loss, which are considerably higher in MS than in healthy individuals (0.5–1 % annual loss in MS patients vs. 0.1–0.2 % in controls), the reduction of lesions with signs of permanent tissue damage (so-called T1 holes), the improved recovery with respect to T1 hypointensity of new MRI lesions (Barkhof et al. 2009), and the reduction of retinal nerve fiber layer thickness loss, which is measured by optical coherence tomography (OCT) (Young et al. 2013). Each of these is considered useful to document neuroprotection, but measuring small changes accurately over time is technically demanding, and therefore it has remained difficult to document neuroprotective effects for a given treatment.

When considering the cellular/molecular aspects of neuroprotection, it can be defined as the lack of newly occurring damage or improvement of function and

structural integrity of already damaged neurons and axons. Neuroprotection may involve many different direct, i.e., functions of neurons/axons themselves, or indirect mechanisms, i.e., functions of cells such as astroglia that metabolize excitatory neurotransmitters and/or provide trophic support such as astrocytes and oligodendrocytes. The reduction of autoimmune inflammatory mechanisms in the brain also results indirectly in neuroprotection, but will not be considered in the following brief summary of potential neuroprotective effects of S1P/S1PR interactions and treatment with functional S1PR antagonists such as fingolimod. As summarized above, several lines of evidence from in vitro experiments with various CNS cells types including neurons and their precursors, astrocytes, oligodendrocytes and OPCs, microglia, and cerebrovascular endothelial cells, but also in vivo animal models point at potentially beneficial effects in MS, EAE, and other CNS diseases (see below). Further, S1P1 and S1P3 are expressed at higher levels in MS brain tissue (van Doorn et al. 2010) indicating primary or secondary contributions of S1P/S1PR signaling during the pathologic processes in MS. Support for mechanisms of fingolimod that may result in neuroprotection stem from the phase III clinical trials, in which an attenuation of brain volume loss by MRI was observed after 2 years (Cohen et al. 2010). Another measure, i.e., persistent T1 hypointensities, so-called T1 holes, which indicate focal permanent CNS tissue damage and probably primarily the destruction of axons (Kappos et al. 2010), has also been explored. The accrual of T1 hypointense lesions and their volume was attenuated after 2 years in the FREEDOMS study, i.e., fingolimod versus placebo (Kappos et al. 2010), but no significant change was observed in the TRANSFORMS study, i.e., fingolimod versus IFN-β1a, after one year of treatment (Cohen et al. 2010). Together, these data indicate that modulation of S1P/S1PR interactions by fingolimod not only exerts indirect neuroprotective effects via the reduction of CNS inflammation, but may also protect axons/neurons and also myelin directly.

3.3 Adverse Event Profile

Balancing the risks versus the benefits has become more and more important with the introduction of more effective therapies in MS. While the previous first-line therapies, IFN-β and GA, are only moderately effective, 20 years of clinical experience have shown that they are very safe, and serious adverse events (AEs) are extremely rare. The newer treatments of MS such as natalizumab, alemtuzumab, but also fingolimod show increased clinical efficacy, but this comes at the price of more frequent and more serious side effects, among the latter particularly notable the occurrence of PML in natalizumab-treated patients (Kleinschmidt-DeMasters et al. 2012). Considering the importance of S1P/S1PR signaling in almost every cell and tissue and the potent immunomodulatory activity of functional S1P inhibition by fingolimod, it is not too surprising that a treatment with such pleiotropic effects has also resulted in a number of AEs. During clinical testing, total lymphocyte numbers drop to about 50 % of the normal threshold and in most individuals return

to normal numbers within 45 days after treatment cessation after long-term therapy, however, some patients remain lymphopenic for prolonged periods of time from reasons that are not understood (Mehling et al. 2011). Lymphocytopenia was observed in 1 % (0.5 mg/d) and 4 % (1.25 mg/d) of fingolimod-treated patients in the TRANSFORMS study (Cohen et al. 2010) and in 3.5 % (0.5 mg/d) and 5.4 % (1.25 mg/d) in the FREEDOMS study (Kappos et al. 2010). Other notable AEs included bradycardia, arrhythmias and atrioventricular blocks, macular edema, epilepsia, a hint toward increased rates of malignancies or premalignancies, and abnormal liver enzymes. Probably as a consequence of the immunomodulatory effects, an increase in herpes virus infections, particularly those with varizella zoster virus (VZC) and herpes simplex virus type 1 (HSV1) were noted, while no general increase of infections was found. Two deaths, one from generalized VZV infection and one with HSV1 encephalitis, occurred in the TRANSFORMS study (Cohen et al. 2010), and increased reactivation of herpes viral infections have also been observed during postmarketing surveillance of fingolimod-treated MS patients. That the immune control of herpes viruses is reduced by fingolimod is supported by a recent study, which documented a reduction of VZV- and EBV-specific T cell reactivity and more frequent reactivation of latent VZV and EBV infection under fingolimod treatment (Ricklin et al. 2013). Furthermore, two cases of fatal hemophagocytic lymphohistiocytosis, which is characterized by a hyper-inflammatory state due to uncontrolled T cell, macrophage, and histiocyte activation, accompanied by excessive cytokine production (Rosado and Kim 2013) have recently been reported as another immune system-related AE (Novartis, Adverse Even reporting). These occurred after 9 and 15 months fingolimod therapy with the approved dose of 0.5 mg/d, and both cases suffered from concomitant viral infections (Novartis, Adverse Even reporting). A case of hemorrhagic encephalitis with subsequent epilepsy (Leypoldt et al. 2009) and the occurrence of HSV1 encephalitis [(Cohen et al. 2010), own unpublished case] under fingolimod indicate that the CNS-related mechanisms of action may in certain, predisposed patients contribute to CNS side effects. A rigorous pharmacovigilance program has therefore been started, and several safety measures are taken. Periodic dermatological and ophthalmological examinations and cardiac monitoring are required during fingolimod treatment, and patients are instructed with respect to the possibility of herpes viral reactivations, however, it is currently not clear how patients at risk to develop these side effects can be identified prospectively. In the case of the reactivation of infections with herpes viruses probably a number of the immune effects of fingolimod, i.e., perturbed migration and LN homing of T cells, B cells, and DCs, changes in the composition of NK cells (Johnson et al. 2011) and other as yet unknown factors contribute. Further research in this direction to develop biomarkers and other means to define risk profiles are clearly needed. The fact that very specific infectious complications occur with specific drugs, e.g., PML under natalizumab and increased reactivation of herpes virus infections under fingolimod, probably also teach us important lessons, which components of the immune system play important roles during physiological immune control of these specific agents and how to avoid these complications in the future.

4 Potential Role of S1P and S1P Receptors in Models of Neurological Diseases and CNS Tumors

Fingolimod is, at the moment, the only approved S1P/S1PR-modulating agent, and MS is the only indication. There is, however, also promising data from using fingolimod or examining the role of S1P/S1PR interactions in models of other CNS diseases and from studies in the context of glioma. B cells that are deficient in S1PR expression fail to disseminate prion proteins (Mok et al. 2012), in animal model of heart failure interrupting TNF-α/S1P signaling inhibited vasoconstriction and improved cerebral blood flow (Yang et al. 2012), elevated S1P levels are involved in ethanol-induced neuroapoptotic effects in the developing brain (Chakraborty et al. 2012), and fingolimod reduces CNS inflammation and promotes functional recovery in a spinal cord injury model (Lee et al. 2009). Further, fingolimod phosphate blocks neurotoxicity induced by amyloid-β aggregation via release of brain-derived neurotrophic factor (BDNF) (Doi et al. 2013). Effects that might be beneficial in the context of stroke are that fingolimod treatment results in improvement of long-term outcome in stroke models primarily via its anti-inflammatory rather than direct effects on neurons (Wei et al. 2011), the contribution of S1P/S1PR signaling in hypoxic preconditioning via pathways involving hypoxia-inducible factor (HIF), sphingosine kinase- and CCL2 signaling-related signaling (Wacker et al. 2012). Neuromodulatory effects such as antinociception, hypothermia, catalepsy, and reduced locomotion are also at least in part mediated by S1PR-mediated (Sim-Selley et al. 2009).

Several lines of evidence show that S1P/S1PR interactions play a role in brain tumors and that fingolimod may be promising in glioblastoma. Fingolimod induces apoptotic death of glioblastoma stem cells (Estrada-Bernal et al. 2012), and S1P regulates the invasive growth of glioblastoma cells via urokinase plasminogen activating mechanisms (Young et al. 2009; Bryan et al. 2008).

Together, these preliminary data indicate that fingolimod or newer, more specific S1P/S1PR modulating agents may be useful in other CNS diseases such as stroke, Alzheimer‘s and other neurodegenerative diseases, traumatic brain/spinal cord injury, and in glioblastoma.

5 Conclusions

Research on the role of S1P/S1PR interactions in many cells and tissues including the CNS and, in parallel, the development and approval of the functional S1P antagonist fingolimod as the first oral immunomodulatory treatment of MS have been very rewarding. These studies have not only led to many fundamental findings regarding basic biological mechanisms in health and disease, but also opened a new area of therapeutics, which may be effective not only in MS, but also a number of other conditions. Further research is ongoing with respect to

modulating S1P/SP1R interactions more specifically than with fingolimod, but also with respect to mechanisms that are involved in overall rare, but sometimes serious side effects.

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RORs in Autoimmune Disease

Mi Ra Chang, Hugh Rosen and Patrick R. Griffin

Abstract The retinoic acid receptor-related orphan receptor (ROR) subfamily of nuclear receptors are transcription factors involved in the maintenance of circadian rhythm and are essential for proper immune function. The T cell-specific isoform, RORγt, is required for T helper 17 cells (T_H17) development and it has been implicated in the pathogenesis of autoimmune diseases including multiple sclerosis and rheumatoid arthritis. Thus, pharmacological repression of RORγt may provide a strategy for therapeutic intervention in autoimmune disorders. This chapter provides a summary of the current status for target validation and development of new chemical entities targeting RORγt.

Contents

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1 Nuclear Receptors

Nuclear receptors (NRs) are a highly conserved superfamily of ligand-dependent transcription factors that control a diverse set of biological activities by translating dietary and endocrine signals into changes in expression of gene networks. NRs have been implicated in a range of diseases and disorders including diabetes and obesity, cancer, inflammation, and atherosclerosis. The superfamily contains 48 members in the human genome and these receptors bind a range of ligands from retinoids, fatty acids, sterols, and vitamins. NRs are characterized by a multi-domain architecture comprised of an N-terminal ligand-independent Activating Function 1 (AF1) domain, DNA-binding domain (DBD), hinge, and ligand-binding domain (LBD) containing the ligand-dependent AF2 (Evans 1988). The canonical domain structure of the NR superfamily is shown in Fig. 8.1. The AF1 and hinge regions of NRs are the most divergent in sequence and length across the superfamily, are considered intrinsically disordered (Krasowski et al. 2008), and their function and significance have been reviewed (Moore et al. 2006; Warnmark et al. 2003; Tremblay et al. 1999; Clinckemalie et al. 2012; Zwart et al. 2010). The DBD is the most highly conserved sequence among NRs and contains two zinc finger motifs to bind distinct DNA response elements. NR response elements are commonly arranged as either direct or inverted repeats of a consensus half-site (RGGTCA; R = purine). NRs can bind DNA as monomers, homodimers, or heterodimers with the retinoid X receptor alpha (RXRα).

The activity and function of NRs can be modulated upon binding small lipophilic ligands. This feature makes the superfamily attractive as therapeutic drug targets. For a majority of the family members examples of controlling their activity by exogenous synthetic small molecules have been published. Interestingly, nuclear receptors are the molecular target of approximately 10–15 % of drugs currently approved by the FDA, highlighting their tractability for therapeutic intervention (Overington et al. 2006). The ligand binding domain has been the focus of drug discovery efforts as it is structurally conserved across the superfamily, containing an internal hydrophobic cavity to which small molecule ligands bind (Moore et al. 2006). The ligand-dependent AF2 structural element that is contained within the LBD is the surface of the receptor directly involved in interactions with coregulatory proteins that have either intrinsic chromatin remodeling activity or that tether in enzymes such as histone acetyltransferases (HATs) or histone deacetylases (HDACs). Coactivator proteins contain a highly conserved hydrophobic LXXLL motif known as a "NR box." This motif is involved in direct interactions with the AF2 surface of NRs when they are in an active conformation (e.g., when receptor is liganded to agonist) (Heery et al. 1997). Coactivators like steroid receptor coactivator 1 (SRC-1) facilitate acetylation of histones. This aids in relaxing chromatin to allow recruitment of the basal transcription complex to the initiation site of target genes of a particular NR (Spencer et al. 1997). In contrast, corepressors like the nuclear receptor co-repressor (NCoR) and silencing mediators of retinoid and thyroid (SMRT) contain a slightly different hydrophobic motif referred to as

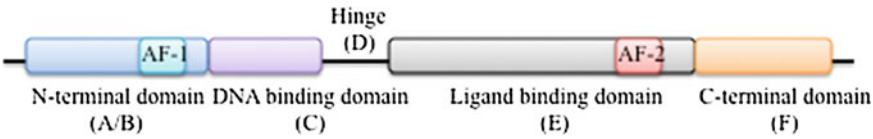

Fig. 8.1 Structural organization of nuclear receptors: *A/B* domain contains *AF-1* (activation function) whose action is independent of the presence of ligand; *C* domain contains zinc fingers that bind to specific sequences of DNA (HRE: hormone response elements); *D* region to be a flexible domain that connects the DBD with the LBD; *E* domain contains ligand binding cavity and *AF-2* whose action is dependent on the presence of bound ligand; *F* domain is highly variable in sequence between various nuclear receptors

"CoRNR boxes" that interact with high affinity at the AF2 when the receptor is in the inactive conformation (e.g., when receptor is liganded to antagonist or inverse agonist) (Hu and Lazar 1999). SMRT and NCoR tether HDAC3 to promoters keeping chromatin compact leading to repression of basal transcriptional activity (Privalsky 2004).

2 The NR1F Subfamily of NRs

The first member of the NR1F subfamily of nuclear receptors was identified in the early 1990s based on sequence similarities to the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), hence the name "retinoic acid receptor-related orphan receptor alpha" or RORα (Giguere et al. 1994; Becker-Andre et al. 1993). Two additional members of this subfamily were subsequently identified, RORβ and RORγ, (Carlberg et al. 1994; Hirose et al. 1994). The three RORs display modest sequence homology and are conserved across species, with each ROR gene encoding multiple isoforms as a result of alternative promoter usage and splicing. The RORs display distinct patterns of tissue expression with RORα being widely expressed and is abundant in liver, skeletal muscle, skin, lungs, adipose tissue, kidney, thymus, and brain (Hamilton et al. 1996; Steinmayr et al. 1998). The expression of RORβ is extremely restricted and is limited to the central nervous system (Andre et al. 1998a, b). Two forms of RORγ are found in both humans and mice (RORγ1 and RORγ2) with RORγ2 commonly referred to as RORγt as it was originally identified in the thymus (Jetten et al. 2001). RORγt has been the focus of considerable attention due to its role in T helper 17 cells (T_H17) development and the pathology autoimmune disease. RORγ, specifically RORγ2 or RORγt, is highly expressed in immune tissues, including the thymus, but there is significant expression of RORγ in the liver, skeletal muscle, adipose tissue, and kidney and this receptor is also involved in metabolic pathways and adipogenesis (Jetten 2009). All RORs recognize and bind to specific sequences of DNA termed ROR response elements or ROREs and these ROREs typically consist of an AGGTCA "half site" with a 5′ AT-rich extension. Unlike most NRs that bind response

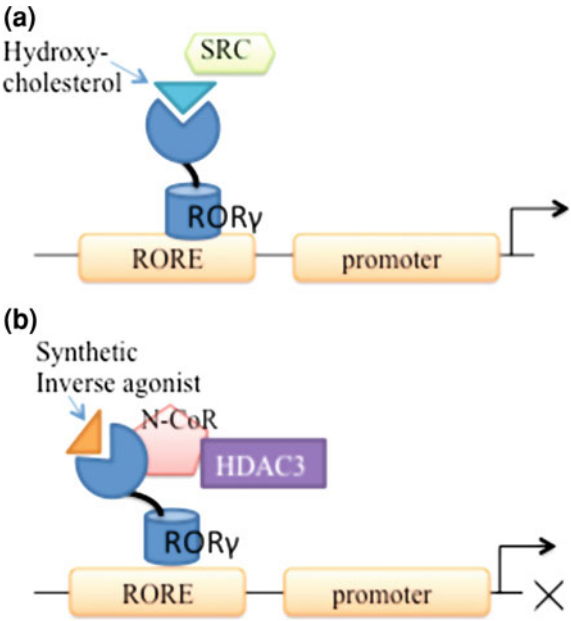

Fig. 8.2 Mechanism of repression of RORs by synthetic ligands. (**a**) ROR agonists drive recruitment of transcriptional coactivators such as SRC2 (**b**) Inverse agonists of ROR displace coactivator and drive recruitment of transcriptional repressors such as NCoR/SMRT

elements as homodimers or heterodimers, the RORs bind to DNA as monomers. As shown in Fig. 8.2a, when RORs are bound to ROREs within the promoter of a target gene, they recruit coactivators independent of ligand status resulting in constitutive transactivation of target gene expression (Jetten 2009; Wang et al. 2010). ROR binding to inverse agonists would repress target gene expression by driving binding to the corepressor NCoR and tethering HDAC3 as shown in Fig. 8.2b. It is interesting to note that another subfamily of orphan nuclear receptors, the Rev-erbs, bind to the same response elements as the RORs as constitutive repressors (constitutive interaction with NCoR) and they functionally antagonize the action of the RORs (Burris 2008; Raghuram et al. 2007).

3 RORγ and T_H17 Cells

Acquired immune responses orchestrated toward protection against various classes of pathogens are facilitated by differentiation of naïve CD4 T cells into cytokine-secreting effector T_H cells. Effector T_H cells historically are classified into T_H1 and T_H2 subsets. T_H1 cells produce interferon γ (IFNγ) and regulate antigen presentation and cellular immunity whereas T_H2 cells secrete IL-4, IL-5, and IL-13, which together regulate humoral and anti-parasite immunity. Recently, T_H17 cells have been identified as an inflammatory T_H subset. Several transcription factors including RORγ are required for the differentiation of T_H17 cells from naïve CD4 T cells (Yang et al. 2008). The innate immune response is an antigen-nonspecific

defense mechanism that a host uses immediately after exposure to microbe. Unlike adaptive immunity, innate immune cells present pattern recognition receptors (PRRs) that recognize molecules broadly shared by pathogens. Phagocytic cells including neutrophils, monocytes, and macrophages, basophils, mast cells, eosinophils and natural killer (NK) cells are part of the first line defense immune cells against pathogens. Interestingly, the expression of RORs is induced in these cells upon infection (Barish et al. 2005).

RORγ is essential for survival of intrathymic CD4 + CD8 + DP cells and for differentiation of T_H17 cells in periphery (Ivanov et al. 2006; Sun et al. 2000; Yu et al. 2004). While both T_H17 cells, and macrophages as well, play important roles in host defense against bacterial and fungal infections, they have been linked to several autoimmune diseases, including psoriasis, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease (Korn et al. 2009; Tesmer et al. 2008). Therefore, pharmacological repression of RORγ might be attractive starting point for the development of a novel therapeutic for the treatment of inflammatory diseases.

4 Ligand Modulation of the RORs

Given the specific tissue distribution of each ROR isoform, and their role in pathophysiological conditions, the utility of synthetic ligands that modulate the activity of these receptors is apparent. As expected, development of small molecule synthetic ligands, including agonists, antagonists, and inverse agonists as dual RORα/RORγ or as isoform selective modulators, is occurring at a rapid pace. These efforts are briefly summarized below. For a more detailed discussion on the state-of-the-art modulators please see a recent review by Kamenecka et al. (2013).

Recently, a well-characterized agonist of LXRα and LXRβ, T0901317, was shown to be a dual RORα/γ inverse agonist (Kumar et al. 2010, a). T0901317 repressed RORα/γ-dependent transactivation of an ROR promoter-reporter gene in HEK293 cells and in HepG2 cells reduced recruitment of the steroid receptor coactivator-2 (SRC2) by RORα at an endogenous ROR target gene (*G6Pase*). Thus, T0901317 represented a novel chemical starting point for the development of selective dual RORα/γ and isoform-specific modulators. More importantly, this finding suggested for the first time that small molecules could be used to target the RORs for potential therapeutic treatments in immune disorders.

A range of compounds with improved selectivity and improved potency emerged from the T0901317 scaffold. SR1001 was the first to be reported as a T0901317 analog devoid of LXR activity (Solt et al. 2011; Griffin et al. 2011). Removal of the sulfonamide alkyl group led to complete loss of LXR activity. In a competitive radioligand binding assay, SR1001 dose-dependently displaced [^{3}H]25-hydroxycholesterol (25-OHC) binding to RORα and RORγ with K_i's of 172 and 111 nM, respectively, and the compound inhibited the development of murine T_H17 cells, as demonstrated by inhibition of interleukin-17A (IL-17a) gene expression and protein production. More importantly, SR1001 was shown to

effectively delay the onset and clinical severity of autoimmune disease (EAE) in a MOG-induced mouse model of multiple sclerosis. This data demonstrates the feasibility of targeting the orphan receptors RORα and RORγt to inhibit specifically T_H17 cell differentiation and function, and indicate that this novel class of compound has potential utility in the treatment of autoimmune diseases.

Using a modular chemistry approach, modifications to the SR1001 scaffold were made to improve potency on RORγ, diminish RORα activity, and maintain selectivity over LXR. Two compounds that emerged from these efforts have been described in the literature (SR2211 and SR1555) (Kumar et al. 2012; Solt et al. 2012). SR2211 and SR1555 were screened in a radioligand binding assay in a scintillation proximity assay (SPA) format. The calculated Ki values for SR2211 and SR1555 were 105 nM and 1 μM on RORγ, respectively. Neither small molecule could displace the radioligand from RORα demonstrating its specificity for RORγ. Both compounds can repress RORγ target genes in cells and minimal activation of LXRα can be detected at the highest concentrations tested. These data demonstrate that SR2211 and SR1555 are selective for RORγ with SR2211 being significantly more potent. Both SR2211 and SR1555 were capable of repressing the expression of Il17a in stimulated EL-4 cells. Interestingly, SR1555 was also shown to induce regulatory T cell populations when cultured splenocytes were treated with T regulatory cell polarizing conditions (TGFβ and IL-2). This unique feature of SR1555 (this effect was not observed with SR2211) may offer additional benefits above and beyond RORγt mediated repression in the treatment of autoimmune disorders. Figure 8.3 summaries the evolution of these interesting ROR modulators from the LXR agonist T0901317.

5 RORs in Multiple Sclerosis

Multiple sclerosis (MS) is a neuroinflammatory disease in which the insulating covers of nerve cells in the brain ad spinal cord are damage by one's own immune system results in loss of muscle control, vision, balance, and sensation. Thus, the condition is called an autoimmune disease. In MS, the immune system attacks the brain and spinal cord. The blood–brain barrier (BBB) disruption is an early and central event in MS pathogenesis. Proinflammatory cytokines such as IL-17 and IL-22 are key factor in immunopathogenesis of MS. Auto-reactive T_H17 cells can migrate through the BBB by the production of proinflammatory cytokines, which disrupt tight junction proteins in the central nervous system (CNS) endothelial cells. T_H17-mediated inflammation is characterized by neutrophil recruitment into the CNS and neurons damage. EAE (experimental autoimmune encephalomyelitis) animal model has been used for the observation of the role of T_H17 cells in MS pathogenesis. As mentioned above, the dual RORα/RORγ inverse agonist SR1001 demonstrated the ability to delay the onset and clinical severity in the EAE model (Solt et al. 2011).

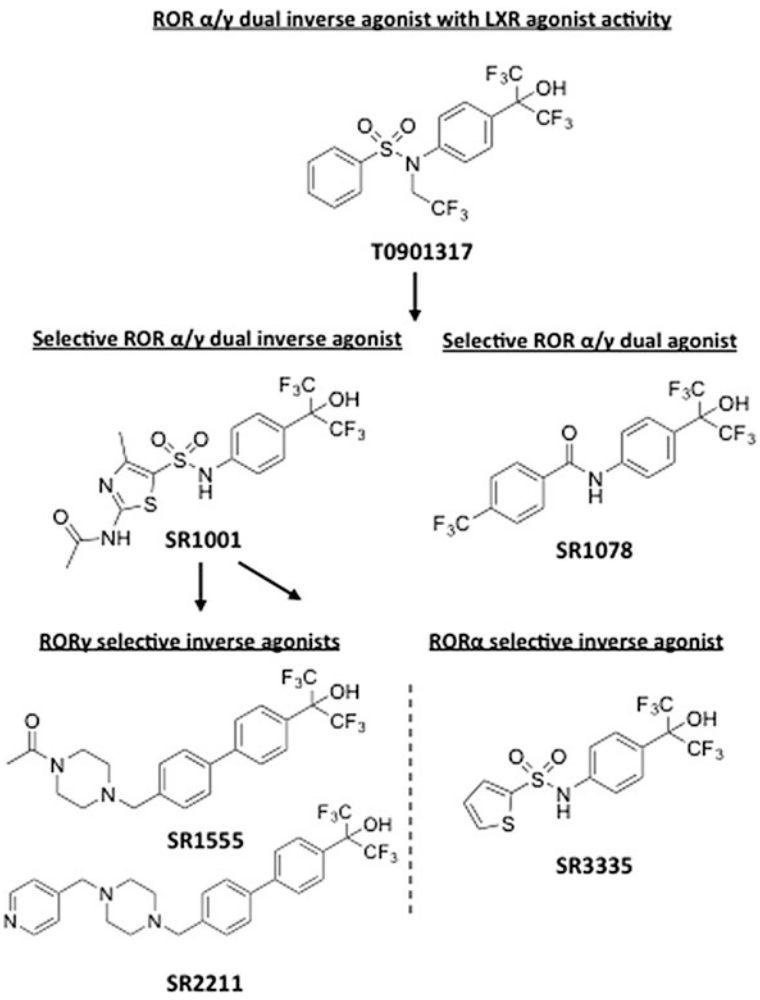

Fig. 8.3 The evolution of ROR modulators from the LXR agonist T0901317

6 RORs in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an inflammatory disease that is characterized by extensive synovial hyperplasia, cartilage damage, bone erosion, and functional joint disability (Smolen et al. 1995). The inflammation in RA results from infiltration of inflammatory cells and the production of proinflammatory cytokines, prostaglandins, and nitric oxide (Park et al. 2010). The cytokine TNFα has been shown to play a major role in the pathophysiology of RA and increased exposure to TNFα leads to degradation of cartilage and bone (Dayer et al. 1985; Bertolini et al. 1986). The efficacy of anti-TNFα therapy in the treatment of RA is well

documented and exemplified by clinical use of infliximab (Remicade), etanercept (Enbrel), and adalimumab (Humira). However, chronic administration of these anti-TNFα agents is directly associated with an increased risk of urinary tract and respiratory infections, and pneumonia. In addition to targeting TNFα, repression of other inflammatory cytokines such as IL1-β (Joosten et al. 1999) IL-6 (Kishimoto 2005; Alonzi et al. 1998), LTαlβ2 (Takemura et al. 2001), and IL-17A (van den Berg and Miossec 2009) have shown efficacy in various animal models of arthritis. Targeted sequestration of IL-17A, commonly referred to as IL-17, using antibodies has gained significant momentum recently. The receptor for IL-17 (IL-17RA) was found to be overexpressed in peripheral whole blood of RA patients and the receptor was detected locally in synovium of the same patients (Gaffen 2008; Toy et al. 2006). IL-17 is an inflammatory cytokine produced by T_H17 cells and it has been shown that IL-17 is present at sites of inflammatory arthritis and it synergizes the inflammatory response induced by other cytokines such as TNFα (Miossec 2007; Fossiez et al. 1996; Kolls and Linden 2004). T_H17 cells differ from T_H1 and T_H2 lineages in that they develop under the influence of TGFβ, IL6, and IL1. Further, these cells have IL23 as a maturation factor and exclusively express the T cell-specific isoform of RORγ, RORγt (Ivanov et al. 2006). T_H17 cell differentiation and function in humans is associated with susceptibility to inflammatory bowel disease, rheumatoid arthritis, and psoriasis (Duerr et al. 2006; Nair et al. 2009; Stahl et al. 2010). Recently, the therapeutic potential of anti-IL-17 therapy was evaluated in a phase I study as adjunct therapy to patients taking oral disease-modifying anti-rheumatic drugs (DMARDs). As compared to placebo, patients given LY2439821, a potent anti-IL-17 antibody, had reduced joint inflammation and erosion (Genovese et al. 2010).

In addition to T_H17 cells, other cell types play major roles in inflammation. Macrophages are specialized differentiated mononuclear phagocytic cells that perform key roles in antimicrobial defense, autoimmunity, and inflammatory disease (Fujiwara and Kobayashi 2005). It has been shown that macrophages can produce a wide range of inflammatory cytokines including TNFα and IL-17. Several studies have shown a role for RORs in regulating macrophage activation (Song et al. 2008; Gu et al. 2008). Of relevance to the pathogenesis of RA are the effects of IL-17 in driving osteoclastogenesis, leading to bone resorption (Kolls and Linden 2004; Kotake et al. 1999). Prior reports have shown that neutralization of IL-17 in mice decreases the severity of antigen-induced arthritis (Koenders et al. 2005). Further, the severity of collagen-induced arthritis was decreased in IL-17-deficient mice and mice administered IL-17 neutralizing antibodies (Lubberts et al. 2005). Despite the complex etiology of RA, IL-17 has been shown to be associated with the severity of RA (Hot and Miossec 2011; van de Veerdonk et al. 2011).

As discussed above, SR2211 was effective at suppressing IL-17 and IL-23R gene expression in EL4 cells (Lubberts et al. 2005). Based on this SR2211 was evaluated in the CIA mouse model. As shown in Chang et al. (2014) administration of SR2211 was efficient at pharmacological repression of RORγ activity affording a therapeutic effect in CIA mice. In the published studies, repression of T_H17 cell differentiation by SR2211 also resulted in induction of IFNγ production

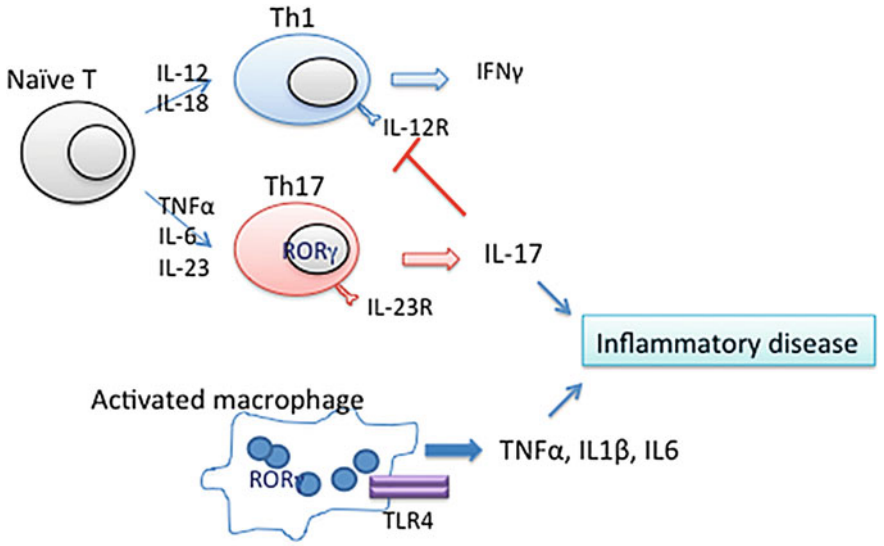

Fig. 8.4 A proposed model for targeting RORγ for autoimmune disease therapy

in murine draining lymph nodes an observation that is consistent with the relationship of T_H17 cells to T_H1 cells. It was also demonstrated that treatment of cells in culture or tissues ex vivo with SR2211 inhibits T_H17 cell differentiation, IL-17 and IL-23R expression, reduces inflammatory cytokines expression in activated macrophages, and systemic activation of T_H1 cells as shown by the induction of IFNγ. A proposed mechanism by which pharmacological repression of RORγ impacts the inflammatory process is shown in Fig. 8.4.

7 Summary and Perspective

While most NRs are considered druggable, selective modulation of target genes involved in disease has been difficult to achieve. For example, pharmacological activation (agonism) or repression (antagonism or inverse agonism) of a specific NR impacts directly the expression of target genes of interest but often alters many target genes not involved in disease leading to pleiotropic effects. There is a wealth of structural data on the LBD of the RORs that can aid the design and development of selective and potent binders, but this information does not provide insight into functional selectivity. Detailed analysis of the proteome and transcriptome upon pharmacological modulation of the RORs should provide detailed information on pathways critical to controlling genes of interest. Finally, while genetic and pharmacological repression of RORg has been shown powerful in reducing inflammation in rodents, there is still no clinical evidence that will translate to humans.

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