# Lysophospholipid Receptors as Potential Drug Targets in Tissue Transplantation and Autoimmune Diseases

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**Abstract:** New therapies directed at ameliorating or altering autoimmune diseases represent an area of significant medical need. Included amongst autoimmune diseases are problems related to transplantation rejection, as well as a number of neurological diseases such as Multiple Sclerosis (MS). A new group of molecular targets that may lead to novel therapies are lysophospholipid (LP) receptors. A large range of biological activities has been attributed to the actions of these simple phospholipids that include well-studied members lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). Documented cellular effects of these lipid molecules encompass growth-factor-like influences on cells, including but not limited to survival, migration, adhesion differentiation, as well as pathophysiological actions associated with cancer. In turn, these cellular effects have roles in developing and adult organ systems such as the nervous system, cardiovascular system, reproductive system and, of relevance here, the immune system. The mechanisms for these actions can be attributed to a growing family of cognate, 7-transmembrane G protein-coupled receptors (GPCRs), with documented validation through studies utilizing pharmacology, molecular genetics and an enlarging repertoire of chemical tools having agonist or antagonist properties. The growing literature on immunological effects of LP receptors, particularly those mediating the effects of S1P, has suggested possible therapeutic roles for this class of receptors. In particular, entry into humans of a non-selective S1P receptor agonist, FTY720, for kidney transplantation and possibly other indications (e.g., Multiple Sclerosis), has raised prospects for efficacious treatment of human diseases based on LP receptor targets. Here we provide a brief introduction to receptor-mediated lysophospholipid signaling and discuss its basic and potential therapeutic roles in autoimmune-related diseases.

**Key Words:** Lysophosphatidic acid, lysophosphatidylcholine, sphingosine 1-phosphate, sphingosylphosphorylcholine, G protein-coupled receptor, LPA, S1P, lymphocyte, FTY720.

## INTRODUCTION

Lysophospholipids are small, simple phospholipids that have long been known as components associated with cell membrane biosynthesis and metabolism [1-4]. They are present at relatively low levels, by comparison to the major phospholipids that include phosphoglycerol backbone lipids like phosphatidylcholine or phosphatidylethanlolamine, as well as those with sphingoid backbones such as sphingomyelin. Lysophospholipids share these 3-carbon backbones, but are distinguished by containing just one acyl chain that shows varied saturation and length along with a single phosphate group. Well known lysophospholipid species include lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC or lysolecithin), sphingosine 1-phosphate (S1P), and sphingosylphosphorylcholine (SPC). The biochemistry of these molecules has been discussed extensively elsewhere [5-17], and will not be covered in depth in this review.

In addition to structural roles, sporadic reports during the 1970s indicated that LPA also showed properties that were consistent with extracellular effects, including vasopressive responses in animals [18]. A variety of other properties were identified in subsequent decades, however the detergent-like lipid structure of lysophospholipids combined with an absence of identified receptors led to hypotheses involving non-receptor mechanisms that included membrane perturbation, calcium sequestration and second messenger roles (discussed in [19, 20]). These mechanisms may have relevance to specific cases as reported for the plant *Arabidopsis thaliana* [21]. However, it is now clear that the dominant mechanism that explains extracellular lysophospholipid functions in vertebrates can be attributed to the actions of specific, cell-surface receptors.

In the mid-1990s, the first lysophospholipid receptor was identified through studies of the embryonic brain [22]. This receptor, now called $LPA_1$, was cloned by degenerate PCR strategies, and its gene was initially called "ventricular zone gene-1" or vzg-1 because of its expression within the neuroproliferative region of the embryonic cerebral cortex called the ventricular zone. Other orphan receptor names for $LPA_1$ exist, most notably the "edg" nomenclature, however the consensus names for both LPA and S1P receptors now reflect their high-affinity ligands [23] and will be utilized throughout this review.

There are currently 4 LPA receptors and 5 S1P receptors. Many details on these G protein-coupled receptors (GPCRs) have been covered in recent reviews [23, 24], and will thus

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be considered here in summary form. The rich and growing range of biological effects of lysophospholipid signaling includes organismal development, and effects on a multitude of organs systems including nervous, cardiovascular, respiratory, reproductive and immune, as well as effects in disease models including cancer, neuropathic pain and multiple sclerosis. Members of both LPA and S1P receptor groups are expressed in immune compartments such as the spleen and thymus [25], however the S1P receptors encompass members that have greatest functional validation for roles in autoimmunity, and thus will be the primary focus of biological discussions to follow.

In addition to established lysophospholipid receptors for LPA and S1P, a number of other orphan GPCRs have been reported as interacting with lysophospholipids, particularly lysophosphatidyl choline (LPC) and sphingosylphorylcholine (SPC), and some of these GPCRs have clear relevance to the immune system: however, recent data suggest that they may in fact have been misidentified (reviewed in [23, 24, 26]), and thus caution should used in interpreting the role of these receptors with respect to lysophospholipids.

## Lysophospholipid Metabolism

A surprisingly large and intricate biology has emerged since early studies identified gross, pressor effects in animals. Both LPA and S1P can be found in most mammalian tissues, and this is not surprising in view of both structural as well as signaling roles for these lipids. Indeed, a challenge that continues to confront the field is differentiating lysophospholipid pools with primarily structural roles, from those that with primarily signaling roles. This difficulty is compounded by the minute quantities of these lipids compared to major membrane phospholipids. The preferred use of mass spectrometry to detect lysophospholipids in small populations of cells remains a non-trivial undertaking. Much of what is known about the major lysophospholipids comes from studying tissues or fluids that contain large amounts of lysophospholipids. For example, human serum (as distinguished from plasma) can contain micromolar concentrations of LPA that is produced by activated platelets. By contrast, S1P exists at lower human serum concentrations of ~0.5-0.8 micromolar and can be stored for release within platelets [27, 28] (reviewed in [9], 10]). Normal biological levels are certainly lower, although firm values have large uncertainties because of the aforementioned signaling vs. structural pools.

The enzymatic production of lysophospholipids, particularly that associated with signaling functions, is only just beginning to be elucidated. Biosynthesis of LPA [7, 20]) can occur *via* multiple enzymes, reflecting initial substrate availability. Documented enzymes include autotaxin, cytosolic phospholipase $A_2$ ($cPLA_2$), lysophospholipase D (lysoPLD), monoacylglycerol kinase, phospholipase $A_1$, and secretory phospholipase $A_2$ ($sPLA_2$) [8, 29]. The degradation of LPA can also occur through multiple enzymes that include endophilin, LPA acyl transferase, lipid phosphate phosphatases and lysophospholipases [8]. By contrast, while still a bioactive lysophospholipid, S1P has a generally distinct set of biosynthetic and degradative enzymes associated with its metabolism. The biosynthesis of S1P occurs by the action of sphingosine kinases on sphingosine, while it is degraded by S1P phosphatases or lyases [9, 11]. It is worth noting that even in comparatively simple organisms like *Drosophila*, many genes (at least 31) are associated with lysophospholipid metabolism [30]. Along with differences in spatial and/or temporal expression patterns, this indicates that intricate biosynthetic and degradative networks function to control levels of lysophospholipids in normal function and likely diseased states as well.

It is further important to note that there are many other actual and potential lysophospholipid species/subspecies as defined by constituent chemical groups, acyl chain position and degrees of saturation. For example, sphingosylphosphoryl choline (SPC) and lysophosphatidyl choline (LPC) are lysophospholipids that contain phosphorylcholine, and biological responses have been observed following extracellular exposure to these lipids [12, 31].

The vast range of concentrations, biosynthetic and degradative pathways along with technical hurdles to detecting minute quantities of lysophospholipids has represented a daunting challenge to mechanistic understanding of lysophospholipid functions. However, with the demonstration that lysophospholipid effects could in fact be explained by receptor mechanisms, it became possible to focus analyses on receptors, particularly through the use of molecular genetics, molecular pharmacology, associated signaling pathways and the production of chemical tools. Within the last decade, identification of multiple lysophospholipid receptors has provided a foundation for mechanistic studies of biological actions, including effects on the immune system.

## G Protein Coupled Receptors for Lysophosphatidic Acid (LPA) and Sphingosine 1-Phosphate (S1P)

The first receptor for any lysophospholipid was one that mediated effects of LPA. This receptor, known as $LPA_1$ [22], is a G protein coupled receptor that interacts with heterotrimeric G proteins, classified by their alpha subunit forms $G_i$, $G_{12/13}$ and $G_q$. These G proteins in turn activate a broad range of downstream, intracellular pathways that include the small GTPases, Rho, Rac and Ras; adenylyl cyclase (AC) inhibition, $Ca^{2+}$ mobilization through phospholipase C (PLC), mitogen-activated protein kinase (MAPK) activation, release of arachidonic acid, activation of survival pathways through Akt/PkB, and undoubtedly other activities as well [32]. Similarly, S1P receptors also interact with heterotrimeric G proteins, and can produce an equal variety of effector responses. Precisely which pathway(s) will be activated or dominant as well as which specific cellular effects will result are dependent on a range of variables that include receptor subtype usage, expression of multiple receptor types/subtypes in a single cell, actual cell type, developmental age, level of receptor and/or signaling proteins expressed, ligand availability including concentration and half-life, to name but a few variables.

For lysophosphatidic acid, there are currently four identified receptors for which clear validation exists. It is probable that there are others that exist as well. All 4 are of the GPCR class. As discussed towards the end of this section at least one intracellular, non-GPCR receptor has been reported, however validation as a specific bona fide receptor awaits further analyses.

The four lysophosphatidic acid receptors are designated $LPA_1$, $LPA_2$, $LPA_3$ and $LPA_4$ reflecting their order of discovery in the published literature. $LPA_1$ is a ~41 kd, 7-transmembrane domain protein that shows wide expression, including spleen and thymus. It is expressed phylogenetically from humans through at least amphibians [22] (reviewed in [20]). Like $LPA_2$ and $LPA_3$, it is encoded by two exons and is located on chromosome 9q32 in human and 4B3 in mouse [32, 33]. It's effects are mediated through at least three G proteins, $G_{i/o}$, $G_q$ and $G_{12/13}$ [20, 34-36]) that can increase cell proliferation, activate serum-response elements (SRE), MAPK, PLC, inhibit AC, mobilize $Ca^{2+}$ mobilization, and activate Rho, Rac and Akt [37, 38] (reviewed in [20, 36]**.**

It's expression is widespread and in adult mice and humans, where it is present within immune organs, although less is known about specific lymphoid expression, particularly under biologically relevant conditions [36]. Even within a single tissue, receptor expression can be non-homogenous [22, 39, 40]. The nervous system is a particular locus of expression, and functional analyses using genetic approaches have identified roles in diverse, multiple processes, including embryonic development of the cerebral cortex [41], and initiation of neuropathic pain [42] (reviewed in [20, 36]). Genetic deletion produces 50% perinatal lethality, along with a spectrum of other phenotypic alterations.

$LPA_2$ and $LPA_3$ were identified based on sequence homology to $LPA_1$ (reviewed in [20]). $LPA_2$ is located on chromsome 19p12 in humans and 8B3.3 in mouse. A ~39 kd protein, $LPA_2$ can activate at least $G_{i/o}$, $G_q$ and $G_{12/13}$, [20, 36]), and like $LPA_1$, it also shows expression in the mouse and human immune system, including thymus, spleen, [36] and some lymphocyte populations [43]. Based on studies of mouse embryonic fibroblasts from normal and null-mutants, $LPA_2$ produces similar signaling properties as $LPA_1$, although its expression patterns likely indicate distinct, tissue-specific roles compared to other LPA receptors. Genetic deletion of $LPA_2$ produces viable animals albeit with detectable signaling deficits [44].

$LPA_3$ is located on human 1p22.3-p31.1 and mouse 3H2. It is an ~40 kd protein expressed in the thymus in mice, although its human immunological expression is less clear [36]. It has slightly different signaling properties that are manifested through its apparent inability to couple to $G_{12/13}$ while still coupling with $G_{i/o}$ and $G_q$ [35]. It also shows some differential ligand preference whereby it is shows a preference for unsaturated acyl chains [45, 46]. $LPA_3$-null mutations have not yet been reported.[1]

$LPA_4$ is a non-homologous GPCR that was recently reported, and is located on the X chromosome of human and mouse (Xq13-q21.1 and XD, respectively). A ~42 kd protein, it shows slight expression in the thymus and broader expression during development in other tissues [47]. Its signaling properties are still being determined, however it appears to show increased $Ca^{2+}$ mobilization and, distinct from the other 3 LPA receptors, shows LPA-dependent increased cAMP levels that suggest coupling to $G_s$. Mutants for $LPA_4$ have not yet been reported.

Like the 4 LPA receptors, S1P receptors are named for their high affinity ligand and numbered based on their published appearance in the literature. Unlike most LPA receptors, they are encoded by a single exon. $S1P_1$ was identified as an orphan receptor expressed by endothelial cells following exposure to phorbol esters. Its shared homology to $LPA_1$ led to its identification as a receptor for S1P, and it is coupled primarily to the heterotrimeric G protein $G_{i/o}$ that can inhibit adenylate cyclase, and activate PLC and MAPK (reviewed in [14-16, 20]). It is located on human chromosome 1p21 and mouse chromosome 3G1. A ~43 kd protein, $S1P_1$ signaling also appears to involve PDGF-receptor interactions for its cellular responses, in at least some cell types. [48, 49]. $S1P_1$ is expressed widely, including spleen and thymus [50, 51]. Constitutive nulls for $S1P_1$ show mid-gestational embryonic lethality [52], and this death is attributable to defective vascular maturation whereby vascular endothelial cells (VECs) based on conditional deletion studies [53].

$S1P_2$ is located on human chromosome 19p13.2 and mouse chromosome 9A3. It was isolated multiple times independently as an orphan GPCR and later shown to be a receptor for S1P (reviewed in [20]). A ~39 kd protein, it shows broader coupling to G proteins than $S1P_1$, interacting with $G_{i/o}$, $G_q$, $G_{12/13}$, and perhaps $G_s$, mediating anticipated responses (reviewed in [14-16, 20]). $S1P_2$ also shows widespread expression, including spleen and thymus [50, 51]. It is evolutionarily expressed from humans through zebra fish. Genetic deletion results in a generally normal phenotype that can be associated with seizures, possibly reflecting differences in genetic background [54, 55]. There is a major disruption of S1P-dependent Rho activation but not $Ca^{2+}$ mobilization in $s1p_2^{(-/-)}$ MEFs [55].

$S1P_3$ was also first identified as an orphan GPCR gene [56], and subsequently identified as an S1P receptor based on its sequence homology. It is located on human chromosome 9q22.1-q22.2 and mouse chromosome 13B1, encoding a ~42 kd protein. It also appears to be a low-affinity receptor for SPC (reviewed in [20]). Although evolutionarily more closely related to $S1P_1$ than $S1P_2$, it has signaling properties more akin to the latter except for Rac activation [57] (reviewed in [16]), and couples with $G_{i/o}$, $G_q$, $G_{12/13}$, and possibly $G_s$ (reviewed in [14-16, 20]). Its expression is again widespread and present in spleen and thymus [50, 51]. Null mutants do not produce major abnormalities [51], however MEF studies demonstrate that S1P-dependent PLC activation and $Ca^{2+}$ mobilization are lost in $S1P_3$-null cells [51]. It is notable that both $S1P_3$ and $S1P_2$ are often co-expressed [51], and analyses of double-null mice [55] revealed an obvious phenotype of reduced litter sizes compared to single-nulls or wildtype, with ~1 pup per litter surviving after 3 weeks [55]. In addition, MEFs null for both receptors no longer are able to activate Rho through S1P. It likely interacts with PDGF receptors [58], can influence heart rate [59] and myocardial perfusion [60] and vasorelaxation [61], and can regulate MAdCAM1 endothelial cells in the organization of the splenic marginal sinus [62].

The fourth S1P receptor, $S1P_4$ was found as an orphan GPCR in mouse and human dendritic cells [63], and later shown to be a high-affinity S1P receptor and low-affinity SPC receptor [64, 65]. It is located on human chromosome 19q22.1-q22.2 and mouse 13B1, encoding a ~42 kd protein. $S1P_4$ shows a somewhat divergent amino acid sequence

similarity compared to its 4 other cohorts, which initially suggested its preferred interaction with another, preferred ligand besides S1P [20, 66]. This does appear to be the case, as the molecule phytosphingosine 1-phosphate (4D-hydroxy-sphinganine 1-phosphate) shows a 50-fold higher affinity for $S1P_4$ [67]. This ligand distinction has bearing on interpretting signalling and biological data on $S1P_4$ through the exclusive use of S1P. $S1P_4$ acts through $G_{i/o}$, $G_{12/13}$ and perhaps $G_s$. It activates adenylate cyclase, $Ca^{2+}$ mobilization, cytoskeletal rearrangements, cell motility, MAPK, PLC, and Rho [51, 64, 65, 68]. It's expression is predominantly within immune compartments including lymph nodes, spleen, and thymus [51, 63]

The fifth receptor is $S1P_5$, isolated as an orphan receptor from rat pheochromocytoma 12 (PC12) cells [69], and later identified based on sequence homology as a high-affinity S1P receptor [70-72] (reviewed in [20]). It is located on human chromosome 19p13.2 and mouse 9A3, encoding a ~42 kd protein. $S1P_5$ couples with $G_{i/o}$ and $G_{12/13}$, producing adenylate cyclase inhibition and $Ca^{2+}$ mobilization as with other S1P receptors. Notably, it is distinct from the other S1P receptors by inhibiting MAPK activation and cell proliferation [51, 70, 71, 73]. It is expressed in multiple tissues, including spleen and peripheral lymphocytes [51, 69, 70], and is also expressed in oligodendrocytes, the myelinating cells of the brain [70, 74].

## Non-GPCR Lysophospholipid Receptors

The published literature, particularly with respect to S1P, has numerous reports attributing lysophospholipid function to non-surface receptor or, indeed, non-receptor mechanisms. As of this writing, all of the major, biological phenomena in vertebrates associated with extracellular lysophospholipid exposure can be explained by the action of one or more GPCRs from the 9 identified receptors previously noted. This does not eliminate the possibility for other important mechanisms, and indeed, the existence of biosynthetic and degradative enzymes demonstrates that other molecules can interact specifically with, or to produce lysophospholipids. Notably, however, such interactions are not classically viewed as receptor-mediated phenomena. Similarly, protein-bound complexes, such as lysophospholipid binding to serum albumin, are also inconsistent with classical views of receptor-ligand interactions. For LPA, it has been reported that a nuclear hormone receptor known as peroxisome proliferator-activated receptor γ (PPARγ), serves as an intracellular receptor for LPA [75]. This identification, however, lacks specificity and biological relevance based upon available data. Other distinct lipid ligands bind PPARγ such as anionic fatty acids, components of oxidized LDLs and eicosanoids [76]. As critical, *in vivo* studies of PPARγ expression and function by knock-out and related approaches showed that PPARγ has restricted expression (e.g. in adipose tissues) [77, 78]. This expression pattern alone is not consistent with the broad range of GPCR-dependent LPA responses shown in many other tissues. Indeed, $LPA_1$ and $LPA_2$ deletion produces phenotypes that are not rescued by the continued expression of PPARγ [41, 44, 79]. Even within adipose tissue, recent data argue against a physiological role for PPARγ in mediating major effects of LPA on adipocytes (laboratories of Saulnier-Blache and Chun, JBC, in press).

S1P has been proposed as a second messenger which, along with SPC, could produce non-receptor-mediated effects, particularly on mobilization of internal $Ca^{2+}$ [80-85]. What remains uncertain is the role of both identified and as yet unidentified GPCR S1P receptors in explaining some of these prior observations, and future studies on both LPA and S1P will provide clarity for mechanistic understandings of possible non-GPCR function in vertebrate lysophospholipid action.

## Other Putative Lysophospholipid GPCRs

A substantial literature exists on orphan receptors that were identified as other lysophospholipid GPCRs. Current data indicate a need for caution in accepting the published identities. These include *G2A* [86] that was identified as a high-affinity LPC receptor and low-affinity SPC receptor [87-90]; *OGR1* (*Ovarian cancer G protein-coupled Receptor 1*) gene [91] reported to encode a high-affinity ($K_d$=33 nM) receptor for SPC [92]; *GPR4* (*G Protein-coupled Receptor 4*) gene [93, 94] reported to encode a high-affinity receptor for SPC and low-affinity receptor for LPC ($K_d$=159 nM) [95]; *GPR12* (*G Protein-coupled Receptor 12*) gene [96]. reported to be a moderate-affinity S1P receptor, unresponsive to SPC [97], or as a high-affinity SPC receptor [98]; and GPR63 reported to act as a low-affinity receptor for S1P, dihydro-S1P and dioleoylphosphatidic acid [99].

## Agonists and Antagonists LP Receptors

The development of specific agonists and antagonists for GPCRs of a given class has been instrumental understanding receptor signaling and biological consequences of a given receptor. For lysophospholipids, the field remains in its infancy, with a few partially validated compounds of variable selectivity and potency currently in existence. While non-specific compounds like suramin have been reported as specific inhibitors, its action is in fact poorly selective for lysophospholipid receptors. As expected, lysophospholipid receptors show stereoselectivity [100], despite reports to the contrary. Existent compounds for LPA receptors include an $LPA_1$ antagonist, 3-(4-[4-([1-(2-chlorophenyl)ethoxy]carbonylamino)-3-methyl-5-isoxazolyl] benzylsulfanyl) propanoicacid (Ki16425) [101]; another ethanolamide derivative $LPA_1$ antagonist [102, 103]; decyl and dodecyl fatty alcohol phosphate $LPA_2$ agonists (FAP-10 and FAP-12) [104]; a phosphothionate LPA analog that has $LPA_3$ agonist properties [105]; another $LPA_3$ agonist which was produced as a monofluorinated analog of LPA [100]; a diacylglycerol pyrophosphate $LPA_3$ antagonist, DGPP 8:0 [103, 106]; a pyrazolopyridine $S1P_2$ antagonist, JTE-013, [107]; non-specific S1P receptor agonists that are 3-(N-alkyl)aminopropylphosphonic acids [108] and an $S1P_1$-selective agonist, SEW2871 [59]. Of particular note is a myriocin analog, FTY720, that represents a drug-like compound that is currently in Phase III trials for immunosuppression of kidney transplantation [101, 109, 110] and Phase II for the treatment of multiple sclerosis (Novartis).

## AUTOIMMUNITY AND LYSOPHOSPHOLIPIDS

### Angiogenesis and Cardiovascular Development

Angiogenesis is the formation of new capillary networks from preexisting vasculature by sprouting and/or splitting of capillaries, involving coordinated proliferation, migration, adhesion, differentiation, and assembly of both VECs and their surrounding VSMCs. This process is also implicated in physiological processes including wound healing and myocardial angiogenesis after ischemic injury, and is precisely controlled by (both angiogenic and anti-angiogenic) protein growth factors such as vascular endothelial growth factor (VEGF), bFGF and PDGF. It is also influenced by the lysophospholipids, LPA and S1P. Dysregulation of angiogenesis can lead to pathological conditions such as atherosclerosis, hypertension, solid tumor growth, rheumatoid arthritis, and diabetic retinopathy (reviewed in [5, 111]).

Several lines of evidence suggest that S1P receptor-mediated signaling plays a major regulatory role in angiogenesis. First, S1P induces both proliferation and migration of VECs [27, 107, 112-115] while inducing proliferation but inhibiting migration of VSMCs [107, 113, 116, 117]. VEC migration is inhibited by antisense oligonucleotides against $s1p_1$ or $s1p_3$ [112] or by $s1p_2$ overexpression [113]. Using an $S1P_2$-specific antagonist (JTE-013), S1P-induced migration of VECs was enhanced and inhibition of VSMCs migration was reversed [107]. Overexpression of $s1p_1$ in VSMCs enhances both mitogenic and migration responses to S1P [113, 116]. Furthermore, S1P protected VECs from serum-deprived apoptosis by nitric oxide production through both $S1P_1$ and $S1P_3$ receptors [118]. These results implicate S1P receptor signaling in VEC/VSMC proliferation and migration. Second, S1P stimulates the formation and maintenance of VECs assembly/integrity by activating both $S1P_1$ and $S1P_3$. S1P-induced VEC adherens junction assembly and cell barrier integrity are blocked by antisense oligonucleotides against $s1p_1$ or $s1p_3$ [119, 120]. Expression of a dominant negative $S1P_1$ mutant inhibits S1P-induced VEC assembly and migration [114]. Although $s1p_2$ is not expressed or is expressed at only low levels in VECs [107, 112, 113, 119], overexpression of $s1p_2$ augments cell barrier integrity of VECs [120]. Third, $slp_1$–$s1p_3$ transcripts are found in embryonic brain blood vessels [121].

Different S1P responses can be explained by altered S1P receptor expression patterns in VECs and VSMCs, and with differential regulation of the two small GTPases, Rho and Rac. In general, VEC expresses both $s1p_1$ and $s1p_3$ but not $s1p_2$ [27, 112, 113, 115, 117, 119] whereas VSMCs express all three receptors with high $s1p_2$ expression levels [107, 113, 116, 117]. Functional analyses revealed that these receptors differentially regulate Rho and Rac; $S1P_1$ mediates the activation of Rac (and often Rho [112]), $S1P_2$ mediates Rho activation and Rac inhibition, and $S1P_3$ mediates the activation of both Rho and Rac. Rac activation (*via* $S1P_1$) is required for migration of both VEC and VSMC [114] whereas both Rac-mediated cortical actin assembly (*via* $S1P_1$) and Rho-mediated stress-fiber formation (*via* $S1P_3$) are essential for VEC adherens junction assembly [114, 119, 120]. $S1P_1$ plays a primary role in angiogenesis by its potent activation of Rac [114, 116], potentially through the intimate interplay with PDGF. Negative regulation of Rac by $S1P_2$ in VSMCs underlies the S1P inhibitory response in migration [107, 113].

Although LPA regulates VSMC functions [122, 123], the roles of LPA signaling in angiogenesis appear to be in pathological conditions such as wound healing (see next section) and atherosclerosis (reviewed in [124, 125]) rather than in a normal or basal conditions. However, frontal cephalic hemorrhages are observed in a significant percentage of $lpa_1^{(-/-)}$ or $lpa_1^{(-/-)}lpa_2^{(-/-)}$ embryos [44, 79], suggesting potential roles for LPA receptor signaling in some aspect of normal angiogenesis/vascular maturation.

The cardiovascular system is another major locus for LP receptor expression; at least 5 LP receptor genes ($lpa_1$, $lpa_3$, $s1p_1$–$s1p_3$), are expressed in mammalian heart [36, 51, 91, 95]. Vasoregulatory actions of LPA were described as early as 1978 in which intravenous LPA application produced hypertension in rats/guinea pigs but hypotension in cats/rabbits [18]. Later, S1P was also shown to regulate the cardiovascular system; intravenous administration of S1P decreased heart rates, ventricular contraction, and blood pressure in rats [126]. The effects of LPA and S1P are predominantly receptor-mediated. Direct evidence for S1P receptor signaling in angiogenesis and cardiovascular development comes from the phenotype of two genetic-null studies in mice and zebrafish [52, 127] (reviewed in [5, 6, 25]). The $s1p_1$-null embryos die *in utero* because of defective vascular maturation in which VSMCs/pericytes do not migrate to surround the vessels (see section 2-5). In zebrafish, the homolog of mammalian $s1p_2$ (the *mil* gene) was mutated [127], resulting in a cardiac phenotype. Normally cardiac muscle progenitor cells migrate from bilateral positions toward the dorsal midline and fuse to form a single heart tube. However, Mil mutant progenitors do not migrate to the midline as normally occurs, leading to lethality for lack of proper blood circulation. Transplanted mutant progenitors migrate normally in wild-type embryos whereas transplanted wild-type progenitors do not migrate in the Mil mutant, suggesting defects in the guidance of progenitor cell migration by surrounding paraxial cells [127]. By contrast, deletion of $s1p_2$ in mice does not produce discernible cardiovascular defects, and $s1p_2^{(-/-)}$ mice are alive and grossly normal (see section 2-6) [54, 55]. Deletion of both $s1p_2$ and $s1p_3$ in mice leads to marked perinatal lethality, despite absence of gross anatomical and histological defects in surviving double-null mice (see section 2-7) [55]. Deletion of $lpa_1$, $lpa_2$ or both in mice does not reveal obvious cardiac defects [44, 79].

### Barrier Integrity

A critical function of lysosphospholipids, that may have been a selective pressure in the evolution of the lysophospholipid- vascular axis, is the maintenance or regulation of endothelial integrity [128-133] S1P, acting through $S1P_1$-dependent activation of rac [129] tightens adherence complexes between epithelial cells, and protects pulmonary endothelial integrity in the face of thrombin or LPS-induced damage in animal models of ARDS [132-135]. In contrast, the pleiotropicity of the lysophsopholipid responses amongst differentiated endothelia perhaps upon the basis of receptor subtype distribution and coupling, suggest that there are circumstances under which endothelial permeability may be enhanced.

Other lysophospholipids such as lysophosphatidic acid, as well as S1P secreted from activated platelets, and can induce an increase in tight junction permeability in brain endothelial cells [136-138], altering the competence of blood-brain barrier. FTY720 is capable of enhancing endothelial barrier function and inducing the tightening of junctions containing VE-cadherin [139]. The striking, very early clinical response of mice with Experimental Allergic Encephalomyelitis to FTY720 [140] suggests that vascular integrity improvements may also be contributing to the mechanism of this drug by enhancing blood-brain barrier functions, although other acute mechanisms are also conceivable.

## Immunity

Consistent with their roles as pleiotropic lipid mediators, LPA and S1P have been shown to regulate immunological responses by modulating activities/functions of immune cells such as T-/B-lymphocytes and macrophages (reviewed in [141-144]). These immune cells and/or other cells involved with their normal function express several LP receptors, and their activities are regulated differentially by the expressed LP receptor subtypes. Furthermore, expression patterns of LP receptors can be altered by cell activation (reviewed in [141-143]). Through LP receptors, T-cell migration and immune responses can be influenced with high receptor sensitivity [145]. LPA and S1P may also protect T-cells from apoptosis through $LPA_1$ in combination with $LPA_2$, and $S1P_2$ in combination with $S1P_3$, respectively [146]. LPA induces migration of, and inhibits interleukin-2 (IL-2) production from unstimulated T-cells that predominantly express *lpa*$_2$. Mitogen activation of T-cells leads to down-regulation of *lpa*$_2$ as well as up-regulation of *lpa*$_1$ expression, and therefore, in activated T-cells, LPA inhibits cell migration but activates IL-2 production/cell proliferation through $LPA_1$ [147, 148]. S1P stimulates migration of T-cells that predominantly express *s1p*$_1$ and *s1p*$_4$. T cell receptor-mediated activation of T-cells suppresses expression of both *s1p*$_1$ and *s1p*$_4$, and eliminates their migration responses to S1P [149].

Immunomodulatory actions of S1P on lymphocytes represent a particularly active area of investigation (reviewed in [144, 150]). The phosphorylated metabolite of FTY720, a novel immunomodulator that causes lymphopenia has been shown to act through S1P receptors [109, 110]. FTY720 is being evaluated in human transplant studies where it induces lymphocyte sequestration from circulation to the secondary lymphoid organs (lymphocyte homing). Assayed models of autoimmunity and transplantation indicate that sequestered lymphocytes may be prevented from reacting/migrating to inflammatory chemokines at graft sites [144, 151-153]. Unlike current immunosuppressive drugs such as cyclosporine, FTY720 neither inhibits T-cell activation/proliferation nor impairs general immunological responses [153, 154]. Because FTY720 does not sequester antigen-experienced effector or memory T cells from the blood [154-157], but only entraps those in lymph nodes, the pre-existing blood effector and memory populations present within the circulation of patients with autoimmune diseases will fail to respond to this mechanism. FTY720 is thus best suited to maintenance therapy, and an induction-maintenance regimen will be needed to attenuate the flare of disease at presentation.

FTY720 is phosphorylated by sphingosine kinase *in vivo* and *in vitro*, and the phosphorylated form of FTY720 acts as a S1P receptor agonist [109, 110]. FTY720-P can bind to each of four S1P receptors ($S1P_1$, $S1P_3$–$S1P_5$) and activates them with varied potency and efficacy compared to S1P. Since S1P concentrations are exquisitely regulated both by synthesis and degradation, S1P homeostasis may contribute to the normal status of lymphocyte homing. S1P receptor specific agonists as well as enzymes involved in S1P synthesis thus represent attractive immunoregulatory targets.

## Chemical Tractability

The development of a two-site binding model for initially the $S1P_1$ receptor [158, 159], and its extension to the LPA receptor [160, 161] suggested that chemical approaches to the receptors may not be limited to lipidic structures. These data support a model whereby lysophospholipids interact *via* a series of undefined hydrophobic interactions with the ligand acyl chain deep within a transmembrane binding pocket, and then head groups making selective interactions with the formation of salt-bridges between the ligand headgroup(s) and charged amino acid side chains at the extracellular face of the receptor. Both sites contribute to ligand affinity, and presumably receptor activation

The first support for the tractability of the lysophospholipid receptors came from the Japanese company Kirin, who explored GPCR privileged scaffolds as $LPA_1$ receptor antagonists [162, 163]. Bioisoteric replacement of both hydrophobic interactions, as well as of the putative headgroup, were performed. This approach was also applied to $S1P_2$ receptor antagonists exemplified by JTE-013 [164, 165]. Koide and colleagues, interrogated chemical libraries by 3-D modeling and found compounds with $S1P_3$ antagonist activity [166].

The discovery of the target of FTY720-phosphate spurred the field to a considerable extent. The FTY720 prodrug had a 300nM binding $IC_{50}$ for $S1P_1$ [109], and was reported to be a modest agonist of $S1P_4$ [110]. The insertion of the aromatic center in optimizing the original natural product lead, myriocin, [167, 168], provided additional hydrophobic–aromatic interactions. A significant structure-activity relationship could be developed around FTY720 and its analogs [169-173]. Synthesis of 3-(N-benzyl)aminopropylphosphonic acid analogs of FTY720 separated $S1P_1$ activity from $S1P_3$, with enhancement of acute cardiovascular tolerability. An alternate approach was the identification of hydrophobic-aromatic scaffolds that were solely $S1P_1$-selective by high throughput screening using a compound-induced calcium flux assay by FLIPR, which is an $S1P_1$-selective immunosuppressive. Phospholipid-based antagonist approaches around FTY720 have also made significant recent progress. Overall, these approaches suggest a wide variety of structural solutions compatible with this relatively simple set of interactions in this receptor family. The constraints of ligand-based approaches and their associated lipidic physical properties clearly no longer exist, making this field ripe for therapeutic exploitation.

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## [1]NOTE ADDED IN PROOFS

Deletion of $LPA_3$ was recently reported: Nature 435: 104-108.

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