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Regulation of mammalian physiology, development, and disease by the sphingosine 1-phosphate and lysophosphatidic acid receptors

Victoria A. Blaho and **Timothy Hla***
Center for Vascular Biology, Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, NY 10065

1. Introduction

The moniker lysophospholipid incorporates two broad families of membrane-derived lipids: the glycerophospholipids and the sphingolipids[1]. Primary representatives of these two arms are lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), the majority of whose activities are mediated via multiple G-protein-coupled receptors with a high degree of specificity for either LPA or S1P [2,3]. Unlike S1P, which is a single molecular species (2*S*-amino-1-(dihydrogen phosphate)-4E-octadecene-1,3*R*-diol), LPA (1-*O*-acyl-2-hydroxy-*sn*-glycero-3-phosphate) is actually a diverse group of molecules consisting of either a saturated (e.g. 16:0; 18:0) or unsaturated (e.g. 16:1; 18:1; 18:2; 20:4) fatty acid chain esterified at the *sn*-1 or *sn*-2 position of a glycerol backbone [4,5]. This can also be modified to alkyl or alkenyl at the *sn*-1 position [6]. Other bioactive members of the LP family include the LPA analogues cyclic phosphatidic acid (cPA), sphigosylphosphotidylcholine (SPC), lysophosphatidylcholine (LPC), lysophosphatidylserine (LPS), and lysophosphatidylinositol (LPI) [1,5,7]. Numerous emerging reports indicate roles in mammalian biology for other, less well-characterized LP members, as well [8]. In the past decade, our understanding of LP biology has expanded exponentially, fueled by the identification of LP receptors, generation and analysis of LPA and S1P-receptor knockout mice, and small molecule compounds functioning as receptor-specific agonists or antagonists [3,9,10].

Specific receptors for LPA and S1P were cloned and characterized beginning only about 15 years ago. The description of the first LPAR was reported in 1996, and the orphan receptor EDG-1, which was cloned as an immediate-early gene from endothelial cells, was identified as the first S1P receptor in 1998 [11-13]. Since then, four more S1P receptors and at least five more LPA receptors have been identified [3]. The two families have been best characterized in specific organ systems; namely, LPARs in the nervous system and cancer, and S1PR in vascular biology and immunology. That is not to say that their contributions in other fields are minor, only that they are incompletely understood.

2. Synthesis and degradation of LPs

The synthesis of LPA and S1P occurs both intracellularly and extracellularly [14-1617]. S1P and LPA can be found in high nanomolar to low micromolar concentrations in the blood and lymph and low concentrations in normal tissue [18-20]. This LP gradient is utilized by some

*Corresponding author: Timothy Hla, Center for Vascular Biology, Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, 1300 York Avenue, Box 69, New York, NY 10065; ph: 212-746-9953; fx: 212-746-2830; tih2002@med.cornell.edu.

cell types, especially hematopoietic/immune cells, as a migratory cue and will described later.

A significant fraction of the plasma and lymph concentrations of both S1P and LPA have been attributed to production by erythrocytes and platelets, respectively [21-25]. Vascular and lymphatic endothelial cells also contribute to the production of plasma and lymph S1P, respectively, and neural cells and adipocytes significant quantities of LPA [26-29]. Both LPA and S1P can also be found in ovarian cancer ascites at 5-to 10-fold the typical plasma concentration, although the specific cell types responsible for this production are unknown [30,31]. Synthesis of LPs by neutrophils, mast cells, mononuclear cells, intestinal, lung, and uterine epithelium, testis, ovaries, and kidneys has also been reported [32-39]. It is likely the production by these, and other as yet unidentified cell types, is responsible for managing the recruitment of specific cells to sites in the microenvironment within certain tissue structures, or to stimulate growth in isolated areas, such as the LPA-mediated neurogenesis of post-mitotic cortical neurons [40]. Tissue-specific knockouts of the synthetic enzymes and gene silencing studies should help to expand this list or to confirm its exclusivity.

2.1 Synthetic enzymes- autotaxin, and sphingosine kinases

Lysophopholipase D, also referred to as autotaxin (ATX), is a secreted enzyme Phospholipase A1 and A2 are responsible for the de novo synthesis of the precursor LPC [41]. Deletion of the ATX gene is embryonic lethal in the mouse at e 8.5-9.5 [18,42]. The production of S1P from sphingosine is mediated by two kinases, Sphk1 and 2 (Figure 2) [43,44]. Mice carrying deletions of only Sphk1 or 2 do not have gross abnormalities, but global deletion of both isoforms results in embryonic lethality at e10.5, similar to ATX knockouts, implying that in single knockouts, compensatory mechanisms exist [45]. Additionally, no S1P was detected in *Sphk1/Sphk2* double-knockout embryos, suggesting that there are no alternative pathways for S1P synthesis, at least at the embryonic development stage. Studies have utilized “triple allele” knockouts of Sphk1 and 2, where both copies of one kinase and one copy of the other kinase have been deleted, allowing for some analyses to be performed in a partial loss-of-function scenario, as well as studies using knockouts of either isoform [46-50].

2.2 Degradative mechanisms

Degradation of either LPA or S1P can occur in either a reversible or irreversible fashion. Dephosphorylation is mediated by a number of lipid phosphate phosphohydrolases (LPPs), all of which are integral membrane proteins, but one of which, LPP1, localizes with its active site facing the extracellular space [51,52]. This indicates the possibility that it functions to dephosphorylate extracellular LPA or S1P close to membrane receptors, and thereby regulate signaling [52]. For S1P, this results in conversion to sphingosine, which can then be converted back to S1P. LPA, however, is irreversibly dephosphorylated to monoacylglycerol. What could be termed “reversible degradation” of LPA occurs via lysophosphatidic acid acyltransferase beta (AGPAT2), which converts LPA to PA, which can then reenter the lipid cycle to be converted to the LPA precursor LPC[53]. Irreversible degradation of S1P can occur intracellularly by the lysophospholipase S1P lyase (SPL), an enzyme found in every mammalian tissue examined thus far, with the exception of platelets and erythrocytes [54-56]. In many immune cells, stimulation with endotoxin can modulate the expression of multiple lysophospholipid metabolizing enzymes, including SPP, Sphk1, Sphk2, SPL [57].

3. Receptor-ligand affinities and extracellular transport

Although collectively known as lysophospholipids, the S1PR and LPAR ligand recognition is highly specific, with dissociation constants (K_d) in the nanomolar range [58,59]. Activation can occur across family lines; for example, LPA can activate $S1P_1$, but with a K_d one to two logs greater than that of S1P and thus unlikely to be physiologically relevant[60,61]. This low level promiscuity likely reflects the shared ancestry of these divergent receptors [62-64].

If synthesized intracellularly, how is S1P transported to the extracellular environment to exert autocrine and paracrine effects? In vitro studies using rat and human mast cell lines postulated that the ATP-binding cassette transporter protein ABCC1 was responsible for the transport of S1P from the site of synthesis in the cytoplasm or endoplasmic reticulum, through the membrane, and into the extracellular space where it could exert its influence via the GPCRs [65]. Further in vitro studies also pointed to ABCA7 as a transporter in platelets and erythrocytes [66,67]. However, although studies using pharmacological antagonists of either ABCA1 (glyburide) or ABCC1 (MK571) demonstrated significant suppression of endogenous S1P release, in vivo studies utilizing ABCA1-, ABCA7-, or ABCC1-null mice failed to demonstrate decreases in either plasma or lymph S1P concentrations [68].

It is important to note that such conclusions are made largely from the use of chemical inhibitors which are not specific at the doses employed [69,70]. Importantly, the Spinster 2 (Spns2) protein was identified in zebrafish as an S1P transporter necessary for S1P-mediated migration of myocardial precursors [71]. Recently, it was shown that human Spns2 transfected into CHO cells could transport S1P and other phosphosphingolipids (e.g. DH-S1P, phyto-S1P), as well as FTY720-P, making it the first transporter shown to have such activity [72]. In the same study, Hisano, et al. demonstrated the inability of ABCA1, ABCB1, ABCC1, or ABCG2 to effectively transport S1P or FTY-P from cells over-expressing each transporter. Several possibilities exist for these conflicting data. In vivo, compensatory mechanisms, by these or other, as of yet unknown, transporters might exist that are not otherwise evident in an in vitro, single-cell system. Similarly, results for different transporters could be cell-type specific [73,74]. Additionally, data obtained using MK-571 as an inhibitor of ABCC1 are unreliable, as its ability to antagonize cysteinyl leukotriene receptors and the downstream signaling pathways (e.g., calcium flux, cellular proliferation) is evident at concentrations one to two logs below the typical concentration used to block ABCC1 transport activity, a fact particularly relevant in mast cell studies [75,76]. Thus far, Spns2 is the only bona fide S1P transporter, as demonstrated by both in vitro and in vivo studies [77].

4. Chaperones and carriers

Much effort has focused on determining how these lipids can arrive at the appropriate cell at the appropriate time to initiate specific signaling sequences. Lipoproteins such as high-density lipoprotein (HDL) or low-density lipoprotein (LDL) are known to be critically important for modulating vascular homeostasis [78]. Whereas oxidized LDL stimulates platelet and endothelium activation and can have cytotoxic effects, HDL exerts cytoprotective and anti-atherogenic effects [78-80]. Several studies using aortae from pigs, rabbits, or endothelial cell culture demonstrated that the critical component mediating this cytotoxic effect was LPC in oxLDL (the precursor of LPA) activating a GPCR[81-83]. Conversely, the vasoprotective qualities of HDL were not due solely to it's ability to induce cholesterol efflux from cells, but also the presence of high concentrations of S1P found bound in HDL [84-86]. In fact, HDL-bound S1P accounts for approximately 65% of plasma S1P, but it was unknown how S1P was bound to this carrier, since HDL is composed of several apolipoproteins, as well as phospho- and other lipids [87].

4.1 S1P and ApoM

A recent study has shown that the apolipoprotein (apo) M is the HDL constituent responsible for the transport of S1P [88]. HDL from mice lacking apoM contained no S1P, whereas over-expressing apoM resulted in higher concentrations of bound S1P that was not a result of increased HDL-cholesterol or other HDL components, as these were not significantly affected. In vitro internalization studies using cells transfected with GFP-tagged $S1P_1$ illustrated the ability of apoM-bound S1P to activate downstream signaling cascades, whereas functional assays of endothelial cell migration and adherens junction formation determined that S1P in complex with either apoM alone or HDL was necessary for full cellular responses. Finally, an in vivo assay of vascular barrier function confirmed this, finding that mice lacking apoM demonstrated increased vascular leakage as compared to wild-type controls. This result mirrors those in plasma S1P-deficient mice and supports the idea that apoM-, not albumin-bound S1P is the necessary functional pairing for vascular barrier maintenance in vivo [21]. As to how apoM mediates delivery of S1P to $S1P_1$, and possibly to other receptors is not known, the most likely scenario involves membrane docking of the HDL particle, either directly or via binding of scavenger receptor class B type 1 (SR-B1)[89]. Additionally, how and where S1P is loaded onto apoM is unknown, since many cells posses the capacity for S1P synthesis. Future studies are needed to explore the transfer mechanism and it's specificity with regard to different S1P receptors.

4.2 LPA and autotaxin

Autotaxin's property as a transporter is ancillary to its enzymatic activity in the biosynthesis of LPA. In vitro binding assays showed ATX binding to lymphocytes or activated platelets in an integrin-dependent fashion, and in both instances, ATX was found in association with these cells in vivo, in high endothelial venules or thrombi, respectively [14,90]. Both of these cell types respond to LPA and express multiple LPAR, making it likely that ATX is able to bind numerous cell types via integrin associations [91]. Nishimasu, et al. state that the crystal structure of ATX suggests the intimate association of the ATX hydrophobic channel with the plasma membrane, subsequently allowing for the directed capture of LPA by it's receptors on the cell surface [92]. It is not known if the ATX-LPA-LPA receptor association allows for cell- or receptor subtype-specific delivery of agonist, and if so, what its involvement in homeostasis or disease pathogenesis may be, and how might it be manipulated.

5. Lymphatic and cardiovascular systems

Effects on the vasculature often “read-out” as effects in other systems. Changes in vessels, large and small, can have far-reaching effects throughout an organism. Thus, to a great extent, the S1PR and LPAR can collectively modulate a multitude of responses just by altering vasculature. In these systems, the most characterized is the role of $S1P_1$ in barrier function and vessel maturation.

5.1 S1P receptors

$S1P_1$, originally named Edg1, was cloned from phorbol 12-myristate 12-acetate (PMA)-stimulated HUVEC in the search for immediate early genes regulating endothelial cell (EC) differentiation, thus the name “endothelial differentiation gene” [12]. The importance of endothelial cell-expressed $S1P_1$ is now well-appreciated; *S1p1*$^{-/-}$ embryos die in utero at e12.5- E14.5 as a result of edema and hemorrhage, despite development of a vascular network [93]. Close examination of *S1p1*$^{-/-}$ embryos revealed defective coverage of vessels by pericytes and vascular smooth muscle cell (VSMC) in several large vessels, including the dorsal aorta and the cerebral artery [93,94]. Further studies with EC- or VSMC-specific deletion of S1P1 in vivo revealed that EC-intrinsic S1P1 is required for vessel coverage by

VSMC [95]. It is now known that S1P1 regulates both vessel maturation by regulating recruitment of mural cells needed for blood vessel stabilization, and the integrity of vessel barrier function by regulating adherens junctions (AJ).

5.1.1 S1PR in vascular barrier function and contractility—The signaling mechanisms involved in both cell migration and cell-cell junction formation are complex, involving the coordinated signaling of many synergistic and sometimes opposing pathways. Both junctional assembly and migratory responses are positively regulated by $S1P_1$, and to some extent, $S1P_3$, whereas destabilization and migration inhibition are mediated by $S1P_2$ and possibly $S1P_3$ signaling [96-98]. Engagement of $S1P_1$ activates solely $G_{\alpha i}$, but the downstream signaling outcome can be cell-specific [99-102]. Thus, $S1P_1$ ligation can lead to increased intracellular Ca^{2+} mobilization and PI3K activation leading to Akt and/or ERK 1/2 phosphorylation, or Rac activation, subsequently inducing actin rearrangement, lamellapodia formation, and redistribution of junctional proteins, such as Zonula Occludens-1 (ZO-1), VE-cadherin, α- and β-catenin, and p-120 catenin to lipid rafts in the plasma membrane or the cell surface, culminating in the formation of endothelial AJ or TJ, or the migration of VSMC or EC. This can lead to decreased vascular permeability or increased vessel stabilization/angiogenesis [96,103-106].

The contribution of $S1P_3$ to vascular barrier function has been more difficult to dissect, as it couples not only to G_i, but also to G_q and $G_{12/13}$[99]. Mice deficient in $S1P_3$ do not display any gross phenotypic abnormalities [107,108], and when *S1p1*$^{-/-}$*S1p3*$^{-/-}$ embryos were generated, the bleeding phenotype was only slightly more severe than the *S1p1*$^{-/-}$single mutant, likely indicating a supportive role for $S1P_3$ in the maintenance of vascular barrier homeostasis and vascular development. Early in vitro studies demonstrated that $S1P_3$-mediated RhoA activation was specifically required for the formation of stress fibers, in concert with $S1P_1$-induced cortical actin assembly, and mouse embryonic fibroblasts (MEF) from *S1p3*$^{-/-}$ mice had defective activation of PLC and intracellular $Ca2^{+}$ mobilization [96,107,108]. Recent reports in other systems support this earlier work, showing that RhoA-mediated mechanical force and activated Rac are necessary for the formation and stability of AJ [109,110,111].

In vivo studies utilizing a model of acute stress, LPS administration, demonstrated that $S1P_3$ /RhoA/ROCK activation dramatically increased vascular permeability [112]. This was mediated by suppression of activated protein C (apC), a clotting regulatory factor that protects from endotoxin-induced lethality by countering the resultant thrombin-mediated vascular leak. Mice deficient in apC were more susceptible to LPS-induced vascular leak than wild-type mice, a phenotype that could be countered by the delivery of an $S1P_1$ agonist or mimicked by $S1P_3$ deficiency [113]. The proposed mechanism for the barrier disruptive action is the stimulation of $S1P_3$ activating RhoA, and its subsequent association with ROCK1, which is known to destabilize endothelial barrier function, and has also been demonstrated in vitro with LPS stimulation or TNFα administration to increase ROCK1 in lung EC [114,115].

Although results of these studies are seemingly at odds with work suggesting the cooperative effort of $S1P_1$ and $S1P_3$, it is not difficult to imagine a model taking into account the necessity of a dynamic barrier with the ability to withstand constant fluctuations in mechanical forces of homeostatic stress with relatively constant concentration of S1P present in the circulation. Under these conditions, $S1P_1$ and $S1P_3$ can function coordinately to allow the maintenance of barrier integrity while ensuring the passage of macromolecules into the interstitial compartment and the sampling of the blood by tissue-resident immune cells in the extravascular compartment. Under inflammatory conditions, possibly with increased S1P concentrations and altered concentrations of other inflammatory mediators in

plasma, this balance could be manipulated. For example, splice variants of CD44, the hyaluronic acid receptor, differentially associated with and transactivated $S1P_1$ or $S1P_3$, depending upon the inflammatory environment [116]. Consistent with this premise, whereas many studies show barrier-protective effects of S1P, high S1P concentrations can be barrier-disruptive in concert with LPS administration in vivo, an affect that can be attenuated by $S1P_3$ knockdown or $S1P_2$ knockout [117]. Additionally, prolonged agonism of $S1P_1$ has recently been shown to induce pulmonary vascular leakage and exacerbate lung injury in a bleomycin-induced injury model [118].

$S1P_2$ appears to oppose many of the actions in the vasculature resulting from $S1P_1$ signaling, due to the ability to couple with $G_{12/13}$, initiating activation of the RHO/ROCK/PTEN signaling cascade [99,119,120]. Although its biological functions are not as well-characterized, *S1p2*$^{-/-}$ mice have no obvious defects; however, they become deaf by one month of age due to defective vascularization of the stria vascularis, the tissue responsible for maintaining the endocochlear potential essential for audition [121,122]. Evidence of the cooperative functions of $S1P_2$ with $S1P_1$ or $S1P_3$ is found through studies using double- or triple-null mice. Whereas the $S1P_2$ deficiency alone has little effect, *S1p1*$^{-/-}$*S1p2*$^{-/-}$ embryos demonstrated earlier hemorrhage at E10.5 and dramatically increased perinatal lethality as compared to the *S1p1*$^{-/-}$ single gene deletion [94,108]. *S1p2*$^{-/-}$*S1p3*$^{-/-}$ double deletion also resulted in increased perinatal lethality compared to either single null, with few pups surviving to weaning age, presumably due to defective RhoA activation, as demonstrated using MEFs in vitro[108].

Although *S1p2*$^{-/-}$ deficiency does not have the developmental effects of *S1p1*$^{-/-}$ deletion, the counter-regulation of $S1P_1$ versus $S1P_2$ has been well-illustrated using models of endothelial or vascular smooth muscle cell migration, vascular permeability, or vasoconstriction. In vitro, stimulation of EC $S1P_2$/Rho/ROCK/PTEN disrupted AJ while stimulating stress fiber formation and increasing paracellular permeability [120]. In vivo, H_2O_2-induced pulmonary vascular permeability was inhibited in mice treated with the $S1P_2$ antagonist, JTE-013. Similarly, JTE-013 administration protected against S1P- and histamine-induced venular leakage in the cremaster muscle, in accordance with the enhancement of barrier function resulting from stimulation of $S1P_1$ with FTY720, or the $S1P_1$-specific agonist SEW2871[123]. Similarly, whereas $S1P_1$ agonism or $S1P_2$ deficiency protected against LPS-induced pulmonary vascular leak, $S1P_1$ heterozygosity resulted in significant vascular barrier disruption, with or without exogenously administered LPS [117] However, another study challenged these conclusions, stating that platelet activating factor (PAF)-induced vascular permeability, which could be prevented by exposure to S1P, could only be reversed by $S1P_1$ antagonism, and not antagonism of $S1P_2$ or $S1P_3$ [124].

The discrepancies between these studies may suggest specific actions of different vascular stress stimuli. PAF receptor signaling occurs primarily via G_i, which could affect $S1P_1$ signaling heterologously, whereas the histamine receptors may signal using several heterotrimeric G proteins [125,126]. H_2O_2 stimulation induces not only PAF signaling, but VEGF and eNOS production and PLD activation, which could have diverse effects on S1P receptor signaling [127]. Additionally, whereas $S1P_1/G_i$ activation can induce Akt and Rac-regulated eNOS phosphorylation and cell motility, $S1P_2/G_{12/13}$ or $/G_q$ can inhibit chemotaxis by activating Rho [98,128]. Thus, it would be expected that these different inducers of vascular permeability might engage signaling by different S1P receptors. In vivo, these would all be working in concert to determine the level of barrier permissivity. Additionally, vascular beds from different organs can exhibit differential responsiveness to S1P and other vasculature modifiers [129-131].

This is certainly true with regards to vascular contractility, which appears to vary depending upon the level of expression of S1P receptor subtypes. Activation of G_i/Akt induced eNOS-dependent vasorelaxation via agonism of $S1P_3$ in isolated rat aortae or $S1P_1$ in bovine lung EC [104,132]. $S1P_3$-deficent mice demonstrated significantly blunted vasodilatory responses after intraarterial administration of HDL or isolated sphingolipids [132]. Conversely, coronary flow was decreased, and cerebral, mesenteric, or renal arteries were shown to contract ex vivo and in vivo in response to S1P signaling via $S1P_1$ or $S1P_3$, with $S1P_3$ possibly antagonizing eNOS induction by $S1P_1$ in some systems [131,133-136]. Basilar arteries from *S1p3*$^{-/-}$ mice were refractory to S1P-induced contractile responses, whereas arteries from *S1p2*$^{-/-}$ mice responded with increased contractile sensitivity in response to S1P [131]. Interestingly, this same study demonstrated that cerebral arteries, but not arteries isolated from the periphery, contracted in a dose-dependent manner in response to phospho-FTY720[131]. Another study demonstrated that lungs from wild-type mice treated ex vivo with JTE-013 and lungs from $S1P_2$-deficicent mice demonstrated decreased S1P-induced pulmonary vasoconstriction, while treatment with the $S1P_3$ antagonist VPC23019 had no effect [137]. However, the specificity of both JTE-013 and VPC23019 has been called into question [131,138]. These studies further highlight the differential responsiveness of vascular beds from various organ systems, as well as the complexities encountered when employing pharmacological tools.

5.1.2 S1PR regulation of angiogenesis—Cardiovascular and lymphatic angiogenesis are also regulated by S1P receptors, mediated primarily through regulation of cell motility, migration, and proliferative responses. Lymphangiogenesis may be positively regulated by $S1P_1$/PLC-induced Ca^{2+} increases, as $S1P_1$ RNA interference or $S1P_1$ antagonism prevented $S1P_1$-mediated lymphatic EC migration and proliferation in vitro [139]. Many studies have documented the ability of $S1P_1$ signaling to induce the migration of vascular EC and SMC, both of which are required for the development of new vessels and their stabilization [95,140-142]. For example, in vitro antagonism of $S1P_1$ inhibits vessel tube formation, whereas in vivo antagonism prevents angiogenesis in several model systems [142-145]. Embryos with EC-specific $S1P_1$ deletion display hemorrhage phenocopying that of the global *S1p1*$^{-/-}$ embryos [94,95]. $S1P_2$ may also play a role in the recruitment of cardiomyocyte progenitors: in zebrafish, the mutation of the $S1P_2$ orthologue *miles apart* (*mil*) results in defective primitive heart tube formation evident as cardia bifida [146].

5.2 LPA receptors

The role of LPARs in the vasculature is not as well-defined, despite the fact that studies of ATX-deficient mice clearly demonstrated severely impaired embryonic vasculogenesis, and LPA induced decreased vascular barrier integrity via Rho/RhoA kinase signaling in HUVEC [18,42,147]. Signaling via chicken orthologues of LPA_{1-3} in the chorio-allantoic membrane (CAM) assay demonstrated that LPA was angiogenic, most likely through LPA_3 [148]. Although agonism of HUVEC LPA_1 and LPA_3 induced adhesion molecule and pro-inflammatory cytokine up-regulation, LPA_1, LPA_2, or LPA_3 deficiency do not result in the dramatic lethality evident in *S1p*$_1^{-/-}$ mice, thus originally making it difficult to determine the relevance of these receptors in the vascular system [149-152]. No defects in blood or lymph vessel formation were grossly apparent in any of these mutant mice, although a small percentage of *Lpa*$_1^{-/-}$ pups demonstrated frontal hematomas, a phenotype that was compounded in double deletants *Lpa1*$^{-/-}$*Lpa2*$^{-/-}$ [150,151]. Interestingly, zebrafish with LPA_1 knocked down did not have abnormal blood vessel development, but rather defective embryonic lymphatic vasculogenesis [153]. More recently, it was reported that almost 20% of *Lpa*$_4^{-/-}$ embryos at E14.5 demonstrated increased perinatal lethality, with embryos displaying edema and hemorrhage at numerous anatomical sites [154]. These phenotypes were the result of dilated blood vessels, defective lymphatic vessel development, and impaired

mural cell recruitment. Interestingly, a previous study had shown that LPA induced VSMC migration and proliferation via receptor(s) that couple to G_q, although LPA_4 had not yet been discovered and demonstrated to couple to G_q [155,156]. This contrasts with findings from another group using *Lpa4*$^{-/-}$ MEFs, which demonstrated increased Akt/Rac signaling-mediated migratory potential [157]; however, these cells were derived from mice with *Lpa4*$^{-/-}$ on a mixed genetic background, which did not display the same perinatal lethality of *Lpa4*$^{-/-}$ on a pure genetic background. How the genetic background affects vascular effects of LPA_4, or indeed, any effects of the LP receptors, illustrates the need for cautious investigation.

Despite the lack of dramatic vascular defects in *Lpa1-3*$^{-/-}$ mice, studies have shown the importance of these receptors for maintenance of vascular homeostasis and modulation of inflammatory tone. Most recently, antagonism of LPA_3 alone, or both LPA_1 and LPA_3, demonstrated that these receptors could positively regulate angiogenesis by down-regulating MVEC expression of CD36, the thrombospondin receptor and a potent anti-angiogenic signal [158]. In other models of vascular dysfunction, such as neointima formation, expression of LPA_1 and LPA_2 by SMC modulates their migration to sites of vascular injury and neointimal lesions, whereas LPA_3 in rat ASMC appears to mediate LPA-induced dedifferentiation [159,160]. Additionally, bleomycin-induced pulmonary vascular leakage was dramatically reduced in *Lpa1*$^{-/-}$ mice [161].

LPA_5 and LPA_6 have only recently been described and determined to be bona fide LPA receptors, and thus far they do not appear to play any significant role in the regulation of endothelial cells [162-164]. However, LPA_5 was recently shown to regulate platelet activation, which can have dramatic effects on the cardiovascular system overall[165].

6. Immune system

Many of the S1P and LPA receptors have been shown to play important roles in immune cell biology, especially with regard to the modulation of cell recruitment [30,166-169]. In fact, some of the receptors are expressed with near exclusivity on cells or in organs of the immune system. Perhaps the most comprehensively explored role of an LP receptor in regulating immunity is the ability of $S1P_1$ to regulate T cell migration into and out of the lymph node [167]. However, LPAR and S1PR appear to play key roles in modulating the activity and migration of both innate and adaptive immune cells. Thus, in this review we will address the innate immune system first, as the first stage of the immune response.

6.1 Innate immunity

6.1.1 Neutrophils—Neutrophils are considered the first responders in most inflammatory responses. Because these granulocytes possess the ability to produce potent lipids, chemotactic factors, catabolic enzymes, and reactive oxygen and nitrogen species, their recruitment and activation are tightly controlled [170]. Multiple studies have found LPAR and S1PR mRNA expression by human neutrophils, but the ability of LP to modulate neutrophils via GPCRs appears to depend upon their activation status[171-173]. Incubation with LPA or S1P in isolation is relatively ineffective for inducing changes in cell morphology, chemotaxis, or activation status [171,172]. However, the response of neutrophils exposed to inflammatory or chemotactic stimuli, such as IFNγ or IL-8, is modulated by treatment with LPA or S1P through G protein signaling [173,174]. Additionally, neutrophil expression of LPA_1 and $S1P_3$ were dramatically increased in neutrophils isolated from peripheral blood of patients with pneumonia, and were more responsive to chemotactic stimuli than those from healthy subjects [173]. These differences are intriguing in light of the recent study demonstrating that neutral sphingomyelinase, a modulator upstream of S1P synthesis, polarizes the distribution of Rac1/2 versus RhoA at the leading edge of elongated neutrophils [175]. Additional evidence of the effect of S1PR on neutrophil migration was

demonstrated in studies using *Sgpl-/-* mice [176]: S1P lyase deficiency increased neutrophil numbers in circulation by impairing homeostatic chemotaxis into tissues. As this phenotype was partially normalized by $S1P_4$ deficiency, it is possible that $S1P_4$ is responsible for regulating neutrophil migration during inflammatory responses that increase S1P concentrations.

6.1.2 Eosinophils—Eosinophils (EO) and mast cells (MC), granulocytes important for anti-parasite and anti-bacterial immunity, are thought to play important roles in the development and propagation of many inflammatory conditions where S1P or LPA are implicated, such as airway and skin inflammation [177-181]. Although treatment with FTY720 or $S1P_1$-specific antagonists can reduce airway inflammation, in some instances this can be attributed to reduced inflammatory status of the endothelium, and subsequently decreased chemokine production or inhibition of lymphocyte migration [182,183]. In fact, a recent report states that administration of S1P can aggravate airway inflammation in a mouse model of allergen challenge [184]. Thus, the many players of the inflammatory response and the models used can complicate understanding of LPAR and S1PR on EO and MC.

In IL-5 transgenic mice, the resultant eosinophilia can be reversed by treatment with S1P receptor modulators FTY720 or SEW2871 [185]. EO from these mice express $S1P_4 \ll S1P_1 < S1P_3$, and migrate toward S1P in vitro, a response that can be largely attenuated by pre-treatment of the mice with FTY720 or SEW2871. Additionally, human EO isolated from patients with allergic rhinitis also expressed $S1P_1$ and $S1P_{3\text{-}5}$, and this expression was up-regulated upon allergen challenge [186]. S1P alone induces chemotaxis of human EO, but also induces up-regulation of other chemotaxis-related proteins, such as CCR3 and RANTES [187]. Human EO also express LPA receptors, and it was reported that LPA induced migration, CD11b up-regulation, and induction of respiratory burst in vitro via LPA_1 and LPA_3 [188]. However, in studies using a collagen matrix, although LPA induced significant activation of RhoA, it was unable to induce EO migration through the 3D matrix [189].

6.1.3 Mast cells—The role of S1PR and LPAR in the regulation of mast cell biology has been extensively characterized. Both LPA and S1P have both been described as modulating mast cell activation state upon FcεRI ligation [190,191]. Human MC express $LPA_{1\text{-}5}$, and their development from monocyte precursors can be accelerated by addition of LPA to growth medium, increasing granule development and tryptase content, *Kit* expression, and proliferative capacity via LPA_1 and/or LPA_3 in a G_i-dependent manner [192,193]. However, histamine release and chemokine production by human MC in response to LPA was IL-4-dependent, and regulated by LPA_2 signaling [194]. This was also true in rat and mouse MC, where LPA induced histamine release in a ROCK dependent-mechanism [195]. Most recently, it was reported that LPA_5 was the most abundantly expressed LPA receptor on human MC, and production of the chemokine MIP-1β was almost entirely due to LPA_5 agonism [193].

S1PR appear to play a similar role in the activation of MC, including inducing the synthesis of the ligand via activation of sphingosine kinase, supporting an autocrine feedback loop [196]. In vitro studies found that FcεRI ligation resulted in Sphk1 trasnlocation and production of S1P; however, a more recent study using Sphk1 and Sphk2 knockout cells and mice suggested that Sphk2 was necessary for intracellular S1P signaling, regulating FcεRI Ca^{2+}-dependent effector responses, such as LTB_4 and IL-13 release, whereas Sphk1-derived S1P from sources other than MC was necessary for S1PR activation of histamine release and the anaphylactic response [46,197]. Exogenous S1P also induced the production of several CC chemokines, including MIP-1β, and induced cortical actin rearrangement and migration in bone marrow-derived mouse MC [197]. In both human and rodent MC, $S1P_1$ was able to induce cell migration, whereas $S1P_2$ signaling regulates other activities, such as degranulation and chemokine release [197,198]. Interestingly, whereas $S1P_2$ was necessary for

the induction of passive systemic anaphylaxis, induced by sensitization and subsequent challenge with IgE, it was subsequently found to be necessary for recovery from histamine-induced anaphylaxis, which obviates mast cell degranulation, leading to the hypothesis that $S1P_2$ is necessary for histamine clearance, possibly via modulation of renal vascular tone [47,198].

6.1.4 Macrophages—Less is known about the role of LP receptors on macrophages, but much of what we understand of the actions of S1P and LPA in these cells have been determined through the use of autoimmune and infectious disease models or the use of the agonist/functional antagonist FTY720. Mouse macrophage expression of S1PR mRNA is dependant upon their origin; bone marrow macrophages express $S1P_1$ and $S1P_2$, whereas thioglycolate-elicited macrophages express $S1P_{1\text{-}4}$, with $S1P_1$ and $S1P_2$ being expressed at equally high levels [199-201]. Expression by alveolar macrophages from rat and mouse differed, with $S1P_2$ and $S1P_4$ being the only S1PR mRNA expressed by rat, but mouse alveolar macrophages expressed $S1P_{1\text{-}4}$, with $S1P_2$ predominating [202,203]. Human peripheral blood monocytes (PBM) and PBM-derived macrophages produced varying levels of mRNA for $S1P_{1\text{-}5}$ and $LPA_{1\text{-}5}$ expression which could be altered dependent upon phenotype, i.e., differentiation to foam cells could increase or decrease expression of certain receptors, and human alveolar macrophages also exhibited a different receptor pattern [81,204,205]. Differences in receptor expression can likely be explained by cell location and isolation methods, as monocytes and macrophages are notoriously susceptible to alterations in activation status and protein expression caused by manipulation [206,207].

S1P2 negatively affects macrophage migration in vitro in response to C5a complement component or CXCL12, as *S1p2*$^{-/-}$ macrophages did not display the increased cAMP or decreased Akt phosphorylation evident in WT cells [199]. This is evident in models of atherosclerosis, where *S1P*$_2^{-/-}$*ApoE*$^{-/-}$ mice demonstrate reduced plaque formation and lipid deposition possibly due to an increased ability of macrophages to exit atherosclerotic vessels and/or regulation of the inflammatory phenotype, as *S1p2*$^{-/-}$ mice had decreased levels of serum pro-inflammatory mediators [200,208]. Furthermore, $S1P_3$ deficiency had no affect atherosclerosis plaques size, but significantly decreased macrophage recruitment to lesions and impaired chemotaxis and chemokine production in vitro; however, another study utilizing the NOD mouse model found that *S1P*$_3^{-/-}$ monocytes were not defective in their ability to adhere to aortic endothelium ex vivo, and rather, $S1P_1$ was responsible for this interaction, as demonstrated using the $S1P_1$ agonist SEW2871 [209,210].

Interestingly, apoptotic cell-derived S1P was shown to induce an anti-inflammatory “M2” phenotype in RAW264.7 macrophages via $S1P_2$, as demonstrated by siRNA knockdown or JTE-013 antagonist [211]. Primary human macrophages were also themselves protected from apoptosis, presumably via $S1P_1$, as pre-incubation with phospho-FTY720 blocked this protection [212]. In another study, however, S1P decreased LPS-induced pro-inflammatory cytokine production through $S1P_1$ agonism, thus inducing an M2 phenotype through this receptor [201]. Some of these differences might be due in part to the inflammatory stimulus, and have implications for the mechanism of continued monocyte/macrophage activation in atherosclerosis and chronic inflammatory disease, as the number of necrotic versus apoptotic cells increases with the severity of atherosclerotic disease [213]. $S1P_2$ also appears to modulate the macrophage response to infectious agents, as *S1p*$_2^{-/-}$ alveolar macrophages expressed reduced levels of Fc receptors, and were compromised in their ability to phagocytose the fungal pathogen *Cryptococcus neoformans* [203].

Although LPA has long been known to act as a macrophage survival factor, it also modulates the activity of mouse and human macrophages [202]. Through a G_i-dependent pathway, LPA up-regulated ROS production in mouse and human alveolar macrophages as

well as Ca^{2+} mobilization, IL-1β and TNFα transcription and protein expression in the mouse J774A.1 macrophage cell line and human monocyte-derived macrophages [81,202,204,214]. Cell migration was also controlled by LPA_1 and/or LPA_3, as monocytic cells appeared to migrate toward concentrations of LPA < 1 μM, whereas migration is inhibited above this concentration in vitro [215]. The potential relevance of these receptors to the modulation of monocyte and macrophage recruitment during disease states is evident in models of atherosclerosis, where it was recently shown that macrophages in human atherosclerotic plaques expressed both of these receptors [216]. Thus, LPA participates in a neatly organized amplification feedback loop: with LPAR expressed by both endothelial cells and monocytes/macrophages, LPA can activate both cell types, activating endothelial cells to produce myeloid recruiting chemokines, as well as LPA itself. These chemokines then coordinately recruit myeloid cells, which along with LPA, can also activate these cells to produce pro-inflammatory mediators, such as IL-1β and TNFα, further activating the nearby endothelium, and amplifying the response [149,216,217].

6.1.5 Dendritic cells—Despite evidence that large amounts of both S1P and LPA are produced during some immune and inflammatory responses, the role of LPA in dendritic cells has not been widely explored. Immature dendritic cells (DC) derived from human peripheral blood monocytes express mRNA for LPA_{1-5}, and this expression is modulated upon maturation, with LPA_3 becoming significantly down-regulated [218-220]. Accordingly, LPA differentially affects the activity of these different sets of DC: in immature DC, unsaturated LPA induces intracellular Ca^{2+} mobilization, actin polymerization, and chemotaxis [218,220]. When added to maturation medium, those DCs matured in the presence of LPA induced a Th_2 phenotype in T cells, and augmented their proliferative capacity in vitro [218,219]. Thus, LPA may play an important role in determining the tone of the nascent acquired immune response, but more study is needed to see how it specifically affects DC biology.

Many of the activities of S1P on DCs are reminiscent of those of LPA, with important roles in migration and phenotype. In fact, $S1P_4$ was cloned from mature human DC [221]. Human and mouse immature and mature DC express mRNA and protein for $S1P_{1-5}$ [222-224]. Similar to LPA, S1P induces intracellular Ca^{2+} mobilization, actin polymerization, and migration in immature DC, and appears to induce a shift from Th_1 to Th_2 phenotype in DC that are matured in the presence of LPS [222]. Perhaps one of the more intriguing differences is the ability of S1P to induce the migration of DCs that are matured with TNFα and PGE_2 versus those matured with LPS, and DC that originate from the peripheral blood, bone marrow, or skin [222,224-226]. For instance, the migration of LPS-matured DC appears to be dependent upon $S1P_3$ and not $S1P_1$, as demonstrated using cells from $S1P_3$ knockout mice, but TNFα/PGE_2-matured DC appeared dependent upon $S1P_1$; however, the mRNA of both receptors is up-regulated upon stimulation [223,225,226]. In vivo studies using FITC-dextran recruitment or adoptive transfer of *S1p1*$^{-/-}$ or *S1p3*$^{-/-}$ DC illustrated the differential dependence of in vitro matured DC versus mature skin DC on both $S1P_{1/3}$ or $S1P_1$ alone, respectively, in their migration to lymph nodes [226].

Tissue-resident DC, such as Langherhans cells (LC) or lamina propria DCs, are phenotypically different from other DC, and as such might be expected to be differentially affected by S1P [227]. In fact, whereas SEW2871 prevented LC migration to draining LN, implying that LC require only $S1P_1$ for migration, lamina propria DC appear to require both $S1P_1$ and $S1P_3$ to exit the gut and migrate into mesenteric LN, as $S1P_3^{-/-}$ mice have impaired DC migration from LP in response to LPS stimulation, but LC migration is unaltered [225,226]. A separate study stated that skin DC were impaired in their ability to chemotax in response to various chemokines in the presence of the $S1P_1$-specific agonist SEW2871 [224].

Interestingly, $S1P_1$-mediated LN migration is negatively regulated by CD69, which performs the same role in T cells, a regulatory mechanism specific for $S1P_1$ [228,229].

S1P mediates other aspects of DC biology besides migration, although it does not appear to be necessary for maturation [226]. LC that had been exposed to FITC-dextran and migrated to LN expressed higher levels of mRNA for the $S1P_1$ and $S1P_3$ than did LC still resident in the skin [225]. Although partially reflective of LC dependence on the S1PR for migration, it was also reported that $S1P_3$ was necessary for endocytosis, a step that would naturally occur before these cells traveled to the LN for antigen presentation to T cells: phospho-FTY720 impaired FITC-dextran endocytosis, whereas SEW2871 did not [223]. Studies using $S1P_3$-deficient mice demonstrated that $S1P_3$-regulated IL-1β production by DC is also a critical regulator of systemic inflammation induced by sepsis, illustrating that $S1P_3$ signaling likely plays a role in modulating the production of various DC-derived cytokines [113,222].

6.1.6 Natural killer cells—Natural killer (NK) cells are innate immune cells important for killing transformed cells, primary defense against infectious organisms, especially viruses, and directing the acquired immune response through the production of interferons and other cytokines and chemokines [230]. Not much is known about how these cells are regulated by LPAR and S1PR, although human and mice NK cells do express the mRNA for $LPA_{1\text{-}3}$, and $S1P_{1\text{-}5}$ [231-233]. As in many other immune cells, LPA induces intracellular Ca^{2+} mobilization, F-actin polymerization, and migration in a concentration- and G_i-dependent manner [231,234]. Curiously, although LPA increases NK cell IFNγ production, it decreases perforin production and cytotoxic activity through LPA_2 agonism and subsequent cAMP/PKA activation [231,234].

Although NK cells appear to express all of the known S1P receptors, the receptor known to have any major role in the regulation of NK activity is $S1P_5$ [235]. $S1P_5$ expression in NK is regulated by T-bet, a transcription factor critical for the decision between CD4 and CD8 cell fate [236,237]. NK cells migrate in response to S1P, and the expression of $S1P_5$ regulates bone marrow and lymph node egress of NK cells [232,233,237]. Other roles for $S1P_5$ or the other S1PR have not yet been reported.

6.2 Acquired immunity

6.2.1 Natural killer T cells—Natural killer T cells are a cellular bridge between the innate and acquired immune systems. They are thymocytes; however, they express the NK receptor and are characterized by a restricted T cell receptor (TCR) which recognizes only glycolipid antigens presented on the MHCI-like CD1d [238]. Little is known about how the expression of LP receptors might affect these cells; indeed, it is unknown whether they express any of the LPAR, although pulsing antigen-presenting cells with LPC induced increased cytokine production by NKT clones and primary polyclonal human peripheral blood NKT cells [239]. Although it has been demonstrated that NKT express $S1P_{1,\ 2,\ \&\ 4}$ mRNA, $S1P_1$ is thus far the only receptor whose role has been examined [240]. Using inducible $S1P_1$ knockout mice, it was demonstrated that $S1P_1$ is not necessary for NKT precursor development and migration to the thymus, but is required for their normal distribution throughout the tissues [240]. Although a subsequent paper reported that FTY720 treatment did not impair NKT migration but did alter cytokine responses, these responses occurred in a G_i-dependent manner, indicating that other S1PR, including $S1P_5$, could be involved [241]. It is curious that, despite the important role of $S1P_5$ in regulating NK migration, this receptor has not yet been examined in NKT cells.

6.2.2 T cells—A great deal is known about the role of S1PR in the regulation of T cell responses. The predominant S1P receptors on T cells appear to $S1P_1$ and $S1P_4$, although

mRNA for the other receptors can be found, albeit at low levels [242]. T cell surface expression of $S1P_1$ is perhaps the major determinant of T lymphocyte egress from the thymus and secondary lymphoid organs, overriding the retention signals transduced by CCR7 signaling, and varies in a cyclical manner, inversely correlating with the S1P concentration gradient [19,242-248]. Whereas single-positive (SP) $CD4^+$ or $CD8^+$ T cell $S1P_1$ expression is high in the thymus, it is low in the blood, up-regulated once again upon entry into the LN, and then decreases once again upon exit into the lymph [245]. This cyclical expression is orchestrated, in part, by CD69 expression, which is up-regulated immediately upon entry into the LN, and negatively regulates $S1P_1$ through direct interaction with the receptor, facilitating the retention of cells within the lymphoid organs [229,249,250]. Forced expression of $S1P_1$ on double positive (DP) and immature SP cells, which under normal circumstances is directly controlled by the transcription factor Kruppel-like factor (KLF)-2, is sufficient to induce premature egress from the thymus [251,252]. Specifically in differentiated Th_2 cells within the LN, extracellular matrix protein-1 (ECM1) drives $S1P_1$ re-expression after antigen education via KLF-2 induction, permitting LN egress [253]. Additionally, expression of $S1P_1$ modulates the ability of effector T cells to migrate out of inflamed tissues into afferent lymphatics, and is a factor in the decreased chemotactic capacity of T cells from old versus young human subjects [254-256].

$S1P_1$ expression is also important for T cell maneuvering within the LN and tissue-specific homing. Entry into $LYVE1^+$ cortical sinuses is also regulated by $S1P_1$ expression, as $S1P_1$-deficient cells display sinus probing behavior similar to that of WT cells, but their entry into sinuses was ≤ 20% that of WT controls [257]. $S1P_1$ signals determine the migration of conventional SP T cells to become intraepithelial residents of the intestine, and T cell rolling and sticking in peripheral LN high endothelial venules (HEV) was reduced in $S1P_1^{-/-}$ mice, but was unaffected in the Peyer's patches (PP) [258,259]. Interestingly, Halin, et al. also indicated the potential involvement of other S1PR in the modulation of PP T cell trafficking, as treatment with FTY720 enhanced PP T cell retention in both WT and $S1P_1^{-/-}$ mice.

$S1P_1$ can also modulate T cell subsets, which is especially evident in $S1P_1$ T cell-specific Tg mice [260]. Transduction of $S1P_1$ into a non-expressing T cell line lead to S1P-mediated reductions in IFNγ and IL-4 production by murine splenic $CD4^+$ cells and inhibition of proliferative responses to anti-CD3/CD28 stimulation [261]. In mice bearing both the OTII OVA-specific TCR Tg and the T cell-specific $S1P_1$ Tg, the Th_{17} response was enhanced, with increased numbers of Th_{17} cells producing greater concentrations of IL-17 as compared to single Tg OTII OVA mice [262]. Similarly, WT T cell development of the Th_{17} lineage in vitro was induced by S1P to the same extent as that induced by IL-23, and this differentiation could be recapitulated by SEW2871, or prevented by phospho-FTY720, indicating that $S1P_1$ may be an important modulator of the Th_{17} inflammatory response [263].

T regulatory (T_{reg}) cell development also appears to be dependent upon $S1P_1$ signaling. Administration of FTY720 increased the number and suppressive activity of splenic $CD4^+CD25^+Foxp3^+$ T_{regs}, which express lower levels of $S1P_1$ and $S1P_4$ mRNA, and higher levels of $S1P_3$ mRNA than do $CD4^+$ effector cells [264,265]. Recent studies have demonstrated that $S1P_1$ blocks the thymic generation of $CD4^+CD25^+Foxp3^+$ T_{regs} by activating the AKT-mTOR pathway, preventing the differentiation of $CD4^+CD25^+Foxp3^-$ precursor cells [266]. Sustained stimulation of mTOR and suppression of TGFβ-Smad3 signaling by $S1P^1$ in peripheral T cells leads to the preferential generation of Th_1 effectors over induced $CD4^+CD25^+Foxp3^+$ T_{regs} [267]. Although both conventional SP cells and T_{regs} lose expression of $S1P_1$ upon TCR complex engagement, T_{regs} appear to down-regulate $S1P_1$ more slowly [266]. This elegant regulatory system prevents immune suppression from occurring too early during the immune response, but leads to its eventual termination.

LPAR also have a role in regulating T cell responses, although it is not as well understood as that of S1PR. Human $CD4^+$ and $CD8^+$ SP express predominantly LPA_1 and LPA_2, and low levels of LPA_3 [268,269]. LPA suppressed Bax-mediated apoptosis in a human T lymphoblastoma line, inhibited activation-induced $CD4^+$ IL-2 production, and augmented IL-13 production via LPA_2 in human peripheral blood T cells [180,268,270,271]. LPA_2 also stimulated $CD4^+$ cell migration, but LPA_1 blocked migration of Jurkat T cells toward RANTES (CCL5) [272]. The LPA_5 receptor is the most highly expressed LPAR in $CD8^+$ cells of the gut epithelium; however, no further studies have been reported with regards to LPAR in this group of cells [162]. The effects of LPAR on T cell immunity are likely to be highly significant, as ATX is expressed on HEV of LN, and administration of an enzymatically inactive form of ATX impaired lymphocyte homing to secondary lymphoid organs (SLO) [90].

6.2.3 B cells—The role of S1PR and LPAR in the regulation of B cell responses has not been explored as thoroughly as in T cells. In fact, very little is known about the role of LPAR in normal B cells, as most research has been conducted using B cell cancer lines. LPA_1 is up-regulated in and protects from apoptosis chronic lymphocytic leukemia (CLL) cell lines or primary patient CLL [273]. $LPA_{1,\ 3,\ \&\ 4}$ are expressed in a human B cell line, which also expressed functional ATX, and mouse primary splenic and bone marrow B cells mobilize Ca^{2+} in response to LPA, although it is unknown which receptor this occurs through [274,275].

More information is available regarding the role of S1PR in B cells. Expression of both $S1P_1$ and $S1P_4$ increases with maturation stage, whereas $S1P_3$ is most highly expressed in immature B cells [276,277]. These changes in S1PR subtype expression may underlie the differential responsiveness of B cells at various stages of maturation. $S1P_1$ is involved in the migration of immature B cells into the bone marrow vascular compartment and subsequently into the peripheral blood [21,276]. B cell-specific knockout of *S1p1* resulted in fewer immature B cells in the circulation and spleen without a concomitant increase in the number of immature cells present in the bone marrow parenchyma, although $S1p1^{-/-}$ B cells were less efficient at egress from the bone marrow [276,277]. This lack of reciprocity was evident in $S1P_1$-deficicent mice, as well as WT mice treated with FTY720, and was explained by increased apoptosis of immature B cells that could contact the bone marrow vasculature, but not enter [276,277]. Interestingly, $S1p1^{-/-}$ immature B cells chemotax toward S1P, whereas *S1p3* deletion rendered immature B cells unresponsive to S1P in vitro, and $S1p3^{-/-}$ mice had significantly fewer circulating immature B cells [276], Additionally, $S1P_3$ expression by marginal zone (MZ) MAdCAM-1^+ endothelial cells was required for their organization around B cell follicles, whereas migration and localization of the B cells themselves necessitated $S1P_1$ expression [278,279]. Curiously, despite several studies demonstrating reduced migratory capacity of $S1p3^{-/-}$ bone marrow and MZ B cells in chemotaxis assays, expression of this receptor has not yet been shown to have an intrinsic, in vivo effect on B cells [276,278,279].

Upon differentiation of mature activated B cells to IgG plasma cells, or antibody-secreting cells (ASC), differential $S1P_1$ expression is regulated by KLF2 [280]. $S1P_1$ then determines whether a specific ASC will be retained in the SLO, or migrate into other organs, such as the BM or possibly the mucosa, an effect seen with both $S1p1^{-/-}$ mixed bone marrow chimeras and FTY720 treatment [280]. With regard to the intestinal mucosa, studies using FTY720 treatment demonstrate that S1P regulates the trafficking of peritoneal B cells and Peyers' Patch plasmablasts (immature plasma cells) into the intestinal lamina propria, thus regulating IgA production [281,282]. Although no receptor-specific agonists, antagonists or genetically-modified mice were used in the studies examining ASC migration in the gut

mucosa, the above-referenced study by Kabashima, et al. imply that $S1P_1$ will likely play a role in the retention of these cells.

7. Stem cells

Hematopoietic stem and progenitor cells (HPC) have been shown to constitutively migrate through the blood, BM, peripheral tissue, and lymph [283]. During an inflammatory response to zymosan or mobilization induced by G-CSF, S1P levels in peripheral blood increased, due in part to activation of the complement cascade, mobilizing HPC from the bone marrow [284]. Other reports demonstrated that S1P-induced homing of $CD34^+$ HPC was partially regulated by $S1P_3$-mediated modulation of the CXCR4-JAK/STAT pathway, leading to increased SDF-1 responsiveness [285,286]. Mononuclear cells from $S1P_3$-deficicent mice were unable to home to sites of ischemic injury, and FTY720 pre-treatment increased homing of transferred $CD34^+$ HPC injected via the tail vein or peritoneum to the bone marrow and sites of ischemic injury [285,286]. Administration of FTY720 could improve neovascularization by patient-derived endothelial progenitors, as well as support proliferation and homing of HPC by drawing them out of the out of the lymph and blood into extramedullary tissues [283,285,286].

S1PR are also specifically involved in the migration of osteoclast precursors. SEW2871 treatment stimulated migration of osteoclast precursors from bone marrow into the blood, and mice lacking $S1P_1$ expression by osteoclast precursors demonstrated increased osteoporosis and osteoclast attachment to bone surfaces [287]. Additionally, FTY720 administration ameliorated bone loss in a mouse model of post-menopausal osteoporosis [287]. Conversely, JTE-013 blocked $S1P_2$ opposition $S1P_1$-mediated chemotaxis of RAW264.7 cells (used as a model of osteoclast precursors) at high S1P concentrations [288]. In vivo, JTE-013 administration largely reversed bone density loss induced by RANK ligand administration, an effect that was absent in *S1p2*$^{-/-}$ mice [288]. Thus, osteoclast precursor mobilization in the marrow spaces appears to be reciprocally regulated by $S1P_1$ and $S1P_2$, as $S1P_1$ attracts osteoclast precursors to the blood and $S1P_2$ serves to repulse precursors from blood and maintain them in the bone marrow [287,288].

Early studies demonstrated that LPA_1 expression correlated with HPC ability to invade stromal cell layers in vitro [289]. Interestingly, LPAR also have a role in regulating bone morphogenesis, but do not appear to regulate migration. Rather, the $LPA_{1/3}$ antagonist Ki16425 inhibited human mesenchymal stem cell (MSC) differentiation to osteoblasts, and as these cells did not express LPA_3 mRNA, this was attributed to LPA_1, whereas shRNA against LPA_4 allowed for osteogenesis, indicating a negative role for this receptor [290]. This was evident in *Lpa*$_4^{-/-}$ mice, which had increased bone volume and density [290]. Additionally, mice lacking LPA_1 exhibited skeletal abnormalities and decreased bone content, due to decreased osteoblast differentiation from precursors [291]. Additional roles for LPAR and S1PR in SC differentiation have been proposed, based upon data from nervous system and will be discussed in that context.

8. Nervous system

Of the LP receptors, LPAR predominate in regulation of nervous system biology. LPA_1 was cloned from a neuronal cell line derived from the ventricular zone (VZ), and in the mouse embryonic neocortical VZ displayed enriched expression correlating with the stage of neuronal development, and was therefore originally named ventricular zone gene-1 (*vzg-1*) [11]. Since that time, expression of LPA receptors by most cell types of the nervous system has been reported, with pleiotropic effects on the different neuronal cell types, including their development, migration, and signaling [63,292].

8.1 Neural progenitors

Neural progenitor cells (NPC) express high levels of LPA_1, LPA_2, and LPA_4 mRNA, and therefore are capable of transducing diverse signals [63]. The embryonic telencephalon, as a whole, expresses LPA_{1-5} mRNA [293]. LPA induces neurite retraction, nuclear rounding, and positioning of VZ neuroblast nuclei to the apical surface in vivo, and stimulates NPC survival in vitro through LPA_1/Rho activation[294-297]. Interestingly, the ability of LPA_1 and LPA_2 to coordinately inhibit VZ NPC cell death in the cerebral cortex are illustrated by the enhanced cortical growth and folding which results from exogenous LPA administration to cerebral explants, and the lack of folding in cortices of *Lpa1*$^{-/-}$*Lpa2*$^{-/-}$ mice [298]. NPC fate decision between neuronal and oligodendroglial is also determined by LPA, with $LPA_1/G_{\alpha i}$ signaling leading to induction of neuronal differentiation while suppressing glial differentiation, as demonstrated using pertussis toxin or Ki16425 [299]. Many of these responses are dependent in part or in whole, upon Ca^{2+} mobilization or Ca^{2+} influx, and both LPA_1 and LPA_2 have been shown to evoke Ca^{2+} responses in NPC in vitro [293]. Stable expression of LPA_4 allowed for LPA-mediated, Ca^{2+}-dependent morphological changes, including cell rounding, neurite retraction, aggregation, and N-cadherin-mediated cell-cell adhesion via the Rho/ROCK pathway [300]. In differentiated mouse neuroblastoma cells intrinsically expressing or with stable transfection of LPA_1, neurite retraction was mediated by LPA_1 induction of PLC/Pyk2/GSK-3β or PKA signaling pathways [301,302].

S1P also plays an important role in the developing nervous system and NPC recruitment, as *S1p*$_1^{-/-}$ embryos demonstrate significantly increased apoptosis and decreased mitosis in the neuroepithelial layers of the developing telencephalon [45]. NPC expressed mRNA for $S1P_{1-5}$ and respond to S1P by induction of Ca^{2+} mobilization similar to LPA stimulation [293,303]. NPC also responded to S1P by forming aggregates and undergoing proliferation and differentiation, which could be prevented by PTX or inhibition of ROCK in vitro [303]. In vivo, shRNAi silencing of $S1P_1$ prevented the migration of transferred NPC to sites of spinal cord injury [304]. Because of their known function in the regulation of hematopoietic stem cells, future studies are likely to uncover more functions of the S1PR in the developing nervous system.

8.2 Astrocytes

Astrocytes are cells that contribute to information processing and regulation of neural activity by networking with neurons and regulating brain metabolism [305]. These cells express LPA_{1-5}, as well as $S1P_1$, $S1P_{3-5}$ [306,307]. Although some debate exists as to the ability of LP to induce proliferation in vitro, in vivo injection of either LPA or S1P leads to astrogliosis [307]. Induction of proliferation in vitro occurs through activation of G_i-coupled pathways, and both LPA and S1P can induce Ca^{2+} mobilization [307-309]. Incubation with LPA mediated DNA synthesis and astrocyte proliferation while inhibiting their ability to uptake glutamate; the former was mediated by LPA_1, as demonstrated by $LPA_1^{-/-}$ cells; however, the LPAR responsible for the regulation of glutamate uptake was not identified [306,310]. Coordinated LPA_1 and LPA_2 signaling in astrocytes also induced the production of a factor that encouraged neuronal differentiation, as medium from WT astrocytes primed with LPA induced astrocyte differentiation, whereas the medium from similarly primed, *Lpa1*$^{-/-}$*Lpa2*$^{-/-}$ astrocytes did not [311].

Information regarding the role of S1P in these cells is not abundant; however it is known that S1P signaling via Rho/ROCK inhibits primary striatal astrocyte gap junction formation in vitro [312]. Additionally, a recent study utilizing CNS cell lineage-specific deletion of $S1P_1$ reported that non-immune mediated efficacy of FTY720 in models of EAE could be mediated through astrocyte-expressed $S1P_1$ [313]. Further studies will likely be aimed at

determining the precise mechanism by which FTY720 modulates astrocyte function and autoimmune inflammation in the CNS.

8.3 Microglia

Another glial cell type, microglia, are the tissue resident macrophages derived from primitive myeloid precursor cells, and are likely important for normal brain development [314]. The level of expression of the LPAR varies between species; human microglial cell lines are able to express $LPA_{1\text{-}3}$ [315,316]. LPS stimulation of rat microglia increased production of LPA_3 mRNA, implicating a role for this receptor in inflammatory responses [317]. LPA administration to microglia in vitro can induce Ca^{2+} signaling, proliferative responses, ATP release, ERK signaling, and membrane ruffling [315,317-319].

Microglia also express $S1P_{1\text{-}3,\,5}$ mRNA, and upon stimulation with LPS or agonism with S1P, $S1P_1$ and $S1P_3$ mRNA are decreased [317]. S1P dose-dependently induced TNFα release from rat microglia, activated p38 signaling, and also altered expression of the mRNA for LPAR, demonstrating the interconnectedness of these two signaling pathways [317]. Other studies on the role of S1PR in microglia have specifically examined the effects of FTY720. Although FTY720 induced down-regulation of phospho-ERK with a concomitant increase in phospho-MLC II in DC and macrophages, microglia p-ERK and p-MLC II were unaffected [319]. FTY720 also increased the numbers of microglia in an ex vivo model of demyelination while decreasing their phagocytic capacity via $S1P_1$ and $S1P_5$, as determined by the use of SEW2871 and a Novartis $S1P_5$-specific agonist [320].

8.4 Oligodendrocytes and Schwann cells

Oligodendrocytes and Schwann cells perform the task of myelination in different branches of the nervous system, CNS and PNS, respectively [321]. The myelin sheath is composed approximately 70:30 of lipid, and LPA_1 expression correlates with oligodendrocyte maturation stage, and subsequently, myelination status [322]. Differentiated oligodendrocytes express LPA_1, LPA_3 and very low levels of LPA_2, and LPA-initiated Ca^{2+} signaling and p-ERK are induced by MAPK/PLC signaling in a PTX-insensitive manner [323,324]. LPA was also found to increase myelin basic protein (MPB) mRNA and the production of myelin sheets in vitro, although evidence for a direct effect on myelination by oligodendrocytes in vivo is lacking [324].

The first description of $S1P_5$ stated that it was highly expressed in the white matter of the brain, implying expression in oligodendrocytes [325]. Subsequently, studies utilizing cells from $S1p_5^{-/-}$ mice demonstrated that S1P-mediated induction of process retraction and inhibition via Rho/ROCK of immature oligodendrocyte migration, and promotion of mature oligodendrocyte survival via G_i/AKT signaling occurred via $S1P_5$ signaling [326,327]. Administration of FTY720 increased proliferation of mature oligodendrocytes and induced phosphorylation of AKT and ERK via $S1P_1$ signaling, whereas it promoted the maturation of oligodendrocyte precursors, which express high levels of $S1P_5$ and low levels of $S1P_1$, indicating that $S1P_5$ might play a role in the beneficial effects of FTY720 by promoting the oligodendrocyte differentiation [328].

Although it does not affect oligodendrocyte survival, LPA is a critical Schwann cell survival factor, signaling via LPA_1/G_i/PI3K/AKT [329]. LPA_1/Rho/ROCK signaling also mediates Schwann cell actin cytoskeletal changes, induces focal adhesion assembly, and N-cadherin-based cell-cell adhesion [330,331]. The role of LPA_1 in vivo was confirmed in $Lpa_1^{-/-}$ mice, which demonstrated increased apoptosis of sciatic nerve Schwann cells [150]. S1P, however, has not been shown to play a prominent role in Schwann cell biology, despite their expression of $S1P_1$ and possibly $S1P_2$ [329]. S1P also induced major morphological changes

due to actin cytoskeletal rearrangement, although this was reportedly due to activation of Rac1 [331].

8.5 Neurons

Many of the neuronal effects of LPA, such as growth cone turning, neurite retraction, and chemorepulsion maybe be mediated by receptors other than LPA_1, as cortical neurons from *Lpa*$_1^{-/-}$ mice were responsive to LPA [40,302,332]. LPA_3 is expressed on motor neurons of the spinal cord and the cortex and is up-regulated upon spinal or brain injury [333]. LPA_1 has, however, been shown to play an important role in nociception. Studies in *Lpa*$^{-/-}$ mice demonstrated that the LPA_1/Rho/ROCK pathway mediated allodynia and hyperalgesia induced by LPA, as well as demyelination and neuropathic pain initiated by substance P production, sciatic nerve injury or compression of the trigeminal ganglion [334-337]. Interestingly, LPA_1 is involved in behavioral alterations unrelated to pain, including the suckling impulse of neonates, possibly regulated by LPA_1-mediated development of the cerebral cortex or olfactory bulb, and regulation of exploratory behavior, spatial learning, and working memory [150,338].

There have been few reports claiming an important role for specific S1PR signaling in adult neurons. Dorsal root ganglion and sensory neurons express mRNA for $S1P_{1-4}$, and S1P augments action potential of specific neurons by suppressing outward potassium current [339]. In another study, in vitro agonism of $S1P_1$ with SEW2871 increased the excitability of roughly half of the rat adult sensory neurons examined, also implicating other S1PR in the determination of neuronal sensitization [340]. In *Xenopus* retinal ganglion cells (RGC), anti-$S1P_5$ blocking antibody studies illustrated that the $S1P_5$/RhoA/Lim kinase signaling cascade mediated growth cone chemorepulsion to S1P, and ex vivo S1P was an axonal guidance cue in retinal explant cultures [341]. S1P also elicited a cation current mediated by PLC activation and iCa^{2+} in chicken Amacrine cells (retinal interneurons), possibly via both $S1P_1$ and $S1P_3$, as treatment with SEW2871 induced current, whereas suramin, a $S1P_3$ antagonist, reversibly blocked current induction [342]. However, caution is required when examining data obtained using suramin, as numerous other, non-S1PR-mediated effects have been reported [343,344].

9. Reproductive system

LPAR and S1PR have important roles in the process of reproduction under normal and disease conditions, in the parents as well as the offspring. Although early reports describe fluctuations in the concentrations of LPA, LPC, and S1P in the reproductive organs reflecting reproduction receptivity and capability, there is not much known about the contribution of specific receptors [345].

9.1 Female reproductive biology and gestation

Embryo implantation in the endometrium is a critical milestone in the pregnancy timeline and can be a primary contributor to infertility [346]. Delayed embryonic development and increased embryonic death due to abnormal implantation and spacing resulted from maternal LPA_3 deletion, consequently down-regulating COX-2 [152]. Pregnant *Lpa*$_3^{-/-}$ mice administered exogenous PGE_2 and PGI_2, COX-2 products required for implantation and decidualization, demonstrated increased successful embryo implantation [152,346]. Studies in pigs indicated that LPA_3 expression may be spatially and temporally regulated, dependent upon the region of the uterus and phase of the estrus cycle [347]. Interestingly, although LPA_3 is highly expressed in oviduct, placenta, and uterus, it is absent from the ovary, where LPA_4 is highly expressed in both human and mouse tissues [152,348]. In humans, endometrial stroma and first trimester trophoblasts expressed only LPA_1 mRNA, and LPA signaling induced in vitro IL-8 production, angiogenesis, and immune cell migration [349,350]. These studies

demonstrate that both maternal and fetal LPAR signaling cooperate to ensure successful pregnancy.

Signaling via S1PR also plays an important role in pregnancy. $S1P_1$ was up-regulated in sheep endometrium and localized to areas of angiogenesis [351]. $S1P_{1, 2, \& 3}$ mRNA were up-regulated in murine decidua and in uterine stromal cells, and immunohistochemistry demonstrated that $S1P_1$ and $S1P_2$ colocalized with COX-2 at various points of the maternal-fetal interface, indicating that S1P signaling may participate with LPA_3 in regulating COX-2 expression in the uterus [352]. However, a subsequent paper reported early pregnancy loss in *Sphk1*$^{-/-}$*Sphk2*$^{+/-}$ mice due to defective decidualization, but no differences in uterine COX-2 expression nor PGE_2 were detected [353]. During late stages of pregnancy, $S1P_1$, $S1P_2$, and $S1P_3$ were also detected in human decidua, with $S1P_3$ expression altered according to gestational age [354]. Additionally, S1P metabolism increased with gestational age, with highest decidual activity at term, which might prepare the decidum for parturition [351]. This is an interesting notion, as COX products are critical for the induction of labor, and therefore S1PR may be important for inducing COX regulation of later stages of pregnancy [346].

9.2 Male reproductive biology

The male reproductive system is also regulated by LP receptors. High mRNA expression of LPA_{1-3} and low expression of LPA_4 and LPA_5 were detected in mouse testis, as well as the germ cells themselves [355]. Single knockout males demonstrated a small decrease in sperm count at 6 months of age, and double knockout further decreased sperm numbers. $LPA_{1/2/3}$ triple knockout males exhibited numerous reproductive deficiencies, including decreased mating activity, age-related testicular degeneration and decreased sperm counts related to increased germ cell apoptosis [355].

S1P is also a regulator of the male reproductive organs, although little is known about the role of specific receptors. S1P inhibits male germ cell apoptosis, and Sphk1 is expressed in the acrosomes of spermatozoa [356,357]. Mature spermatozoa also express mRNA for $S1P_{1-3}$ and $S1P_5$, and $S1P_1$ is highly expressed in seminiferous tubules and spermatids in the testis [358]. S1PR may also play a role in male sexual function, by virtue of eNOS phosphorylation. Human corpus cavernosum expresses $S1P_{1-3}$, and S1P signaling potentiates acetylcholine-induced eNOS activation, which in turn regulates the hemodynamics required for erectile function [359]. Thus, S1PR and LPAR regulate the development and maintenance of both male and female fertility and reproduction.

10. Inhibitors, agonists, and antagonists

Development of S1PR- and LPAR-modifying compounds with in vivo utility has been hindered by numerous challenges, such as the lack of ligand-bound GPCR crystal structures, carrier/chaperone affinities, off-target effects, and conformational changes [360,361]. Despite these difficulties, these receptors remain attractive targets, due to their disease state correlation, potent functional effects in numerous organs/systems, and prior amenability to modulation by pharmacological compounds. There are numerous agonists and antagonists of LPAR and S1PR, a few of which will be discussed herein according to receptor specificity/preference.

10.1 ATX inhibitors

Because of the proposed ability of autotaxin to deliver LPA directly to LPAR, some of the newest pharmacological tools, the autotaxin inhibitors, must be addressed in the discussion about LP receptor-modulating drugs. Previously, studies of ATX/LPA had been hindered by the lack of ATX inhibitors with in vivo potency [362,363]. PF-8380 was obtained from a screen of the Pfizer compound collection and subsequent structure-function studies. Maximal

inhibition of LPA synthesis in plasma occurred 15-30 minutes after administration, and a 30 mg/kg dose in rats showed efficacy equal to the same dose of naproxen in a model of adjuvant-induced arthritis [364]. Small molecule screening and derivative optimization resulted in the synthesis of HA130, a boronic acid-based inhibitor targeted to the T210 active site of ATX. This compound, administered IV at 1 mol/g resulted in approximately a 75% decrease in plasma LPA within 10 minutes; however, no in vivo functional assays were performed in this report [365].

The most recent advance in autotaxin inhibitor development was the design of stable analogues (compounds 22 and 30b) of S32826 [4-(tetradecanoylamino) benzyl] phosphonic acid, a molecule that demonstrated an IC_{50} in the nanamolar range in a cell-free system, but poor stability precluded in vivo analyses [366,367]. Both compounds 22 and 30b were highly specific for ATX, exhibited long plasma half-lives of approximately 10.5 hours, inhibited ATX-dependent invasion of HUVEC monolayers by rat hepatoma in vitro, and decreased metastatic foci in the B16-F10 melanoma model [367]. The authors suggest that continuing compound evolution originating from the same benzyl- and naphthylphosphonic acid scaffold may yield ATX inhibitors with extended half-lives, and thus greater utility in the tumor microenvironment, characteristics which would likely prove useful in other disease models.

10.2 LPAR agonists and antagonists

10.2.1 LPAR antagonists—AM095 is a LPA_1-specific antagonist from with efficacy in mouse models of fibrosis, scleroderma and bleomycin-induced pulmonary fibrosis without effects on wound healing [368,369]. This compound led to the development of AM152, a LPA_1 antagonist that has recently completed Phase I clinical trials for the treatment of systemic sclerosis (http://www.amirapharm.com/articles/AmiraAM152Phase1.html), demonstrated efficacy in numerous mouse models of fibrosis and was granted Orphan Drug Status by the FDA for the treatment of idiopathic pulmonary fibrosis [370,371]. AM152 is the only LPA_1 antagonist currently in clinical trials.

Ki16425 is a $LPA_{1/3}$ receptor antagonist that demonstrates specificity for $LPA_{1/3}$, little to no action on LPA_2, with no antagonism of S1PR [372]. It is widely used in in vitro studies of LPAR function in different disease states, including various cancers, formation of neointima and atherosclerosis development, and fibrosis [161,216,373-375].

VPC12249 is a competitive $LPA_{1/3}$ antagonist, the development of which resulted from a series of LPAR agonists and antagonists evolving from an agonist parent compound, *N*-acyl ethanolamide phosphate (NAEPA) and involves the 2-substitution of a bulky benzyl-4-oxybenzyl substituent in the *S* configuration [376]. This compound blocked Ca^{2+} mobilization and inhibited forskolin-induced cAMP accumulation in HEK293T cells [376]. It demonstrates similar effects to Ki6425, inhibiting fibroblast chemotaxis after bleomycin administration and chemotaxis of human pancreatic cancer cells [161,377].

10.2.2 LPAR agonist—OMPT (1-oleoyl-2-*O*-methyl-*rac*-glycerophosphothionate) is a LPA 18:1 analogue with the hydroxy group converted to an *O*-methoxy group, and the phosphate to a phosphothionate, resulting in potent and selective agonism of LPA_3 [378]. Administration of OMPT to mice before induction of renal ischemia/reperfusion injury resulted in exacerbated pathology and was able to block the in vivo protective effects of VPC12249 administration [379].

10.3. S1PR agonists and antagonists

10.3.1 S1PR agonists—FTY720 (Fingolimod/Gilenya; Novartis) is the first LP receptor modulator approved for clinical use. It is a novel oral drug approved by the FDA in September 2010 for the treatment of relapsing-remitting multiple sclerosis [10,380]. FTY720 is phosphorylated in vivo by Sphk2 to the active form, FTY720-P (FTY-P) [381-383]. Although FTY-P is an agonist of $S1P_1$ and $S1P_{3-5}$, binding of FTY-P to $S1P_1$ results in down-modulation and degradation of this receptor, amounting to functional antagonism [145,384].

Originally, FTY720 was targeted for use as an anti-rejection treatment in renal transplants; however, clinical trials were halted when it was determined that it provided no increase in prophylaxis of transplant rejection, but did lead to adverse side effects in some patients, including macular edema, bradycardia, reduced renal arterial function, and effects on the pulmonary vasculature [385-387]. The apparently immunosuppressive actions of the drug prompted researchers to examine efficacy in experimental autoimmune encephalomyelitis (EAE), a model of human MS, and found that it prevented pathology at concentrations several fold lower than those required for prevention of transplant allografts [382]. The precise mechanism of FTY720 in MS is still debated: the sequestration of autoreactive cells in the LN has been considered the primary mechanism of action. However, some studies support the involvement of direct effects on the nervous system itself, including discrepancies between the timing of lymphopenia and amelioration/restoration of symptoms and the accumulation of FTY720 and FTYP in brain tissue [388,389]. $S1P_1$ and $S1P_3$ protein up-regulation was found on lesional astrocytes of MS patients, and cell-specific deletion of $S1P_1$ in astrocytes ameliorated EAE pathology to a similar extent as FTY720 administration [313,390]. For in-depth discussion of the putative mechanisms of FTY720 in EAE/MS, we refer you to several excellent reviews on the subject that have recently been published: [10,391-393].

FTY720 has shown efficacy in the prevention or amelioration in other models of disease, such as airway inflammation, atherosclerosis, renal ischemia/reperfusion injury, dermal allergic responses, models of Type-1 and -2 diabetes, and autoimmune uveitis [183,185,209,265,394-396]. Future studies will likely examine the ability of FTY720 to modulate many more disease pathologies, as well as help determine the potential role of the S1PR in such systems.

AUY954 is a selective $S1P_1$ receptor modulator and an aminocarboxylate analog of FTY720, effective at low nanomolar concentrations [397]. Thus, it is a useful tool to dissect the effects of FTY720, or to attribute outcomes specifically to agonism of $S1P_1$ [113,182,246,313]. However, it also induces receptor internalization and degradation and therefore also acts as a functional antagonist [398]. Administration of AUY954 in EAE and models of airway inflammation or autoimmune neuritis recapitulates the effects seen with FTY720 [182,313,399].

SEW2871 is a reversible, moderate-affinity, $S1P_1$-specific agonist that binds to the same site as S1P [400,401]. Because of this specificity, unlike FTY720, it does not induce deleterious side effects that result from agonism of $S1P_3$, such as bradycardia [133]. It also resembles S1P in that it induces signaling and receptor internalization without degradation, also unlike FTY720[401]. Although long-term administration results in lymphopenia resembling that induced by FTY720 treatment, the potency and duration of action of SEW2871 are not as prolonged as the effects of FTY720 [402].

10.3.2 S1PR antagonist—As the only known compound with $S1P_2$ specificity, agonistic or antagonistic, JTE-013 has been a critical tool for the dissection of $S1P_2$ inhibitory effects on migration and the complex G-protein signaling pathways involved [403]. It has also

allowed for assessing the relative contribution of $S1P_2$ activation in vivo, such as the regulation of B cell homeostasis and in models of atherosclerosis [200,208,288,404]. Although JTE-013 has been regarded as a $S1P_2$-specific antagonist, the $S1P_2$ specificity of the compound has recently been questioned [138,405]. Ex vivo studies utilizing basilar arteries demonstrated that JTE-013 inhibited vascular contraction induced by not only S1P, but also KCl and a thromboxane analogue, a result obtained using arteries from both wild-type and *S1p2*$^{-/-}$ mice [131]. Additionally, Long, et al. reported that JTE-013 inhibited S1P-induced ERK-1/2 phosphorylation in MDA-MB-453 breast cancer cells, a result reiterated by $S1P_4$, but not $S1P_2$ knockdown, indicating that JTE-013 may be better classified as an $S1P_{2/4}$ dual antagonist [406].

11. Receptor-independent actions of S1P and LPA

Although the signaling events downstream of S1PR and LPAR ligand binding have been extensively examined, S1P and LPA were originally proposed to transduce non-receptor-mediated signals. Early studies indicated that S1P and precursor molecule ceramide were important second messengers in multiple mitogenesis and apoptosis pathways [407,408,409,410,411]. Likewise, LPA was a known inducer of platelet activation and vasopression, via signaling of intracellularly synthesized LPA or modulation of cellular membrane architecture, respectively [412,413]. Although later studies would demonstrate the role of GPCR signaling in these effects, others appear to be initiated, in part or whole, directly by intracellular LPs [56,101,412,414,415,416].

One such effect is the release of intracellular Ca^{2+} stores. Although activation of some LPAR and S1PR can lead to intracellular Ca^{2+} mobilization, intracellular LPA or S1P are capable of eliciting Ca^{2+} flux via store-operated or non-store-operated Ca^{2+} entry channels in diverse cell types, such as neutrophils, mast cells, macrophages, vascular smooth muscle cells, and pulmonary endothelial cells [46,171,172,417-420]. Whether such effects are direct signaling function of the LPs on channel proteins or indirectly through membrane effects is not known. Recent in vitro studies have identified intracellular S1P as a negative regulator of histone deacetylase activity, a necessary component of the NF-kB activation cascade via activation of the E3 ubiquitin ligase TRAF2, a modulator of Rhodopsin 1 endosomal trafficking, and is necessary for assembly of the electron transport chain and mitochondrial function [421-424]. PPARγ (peroxisome proliferator-activated receptor γ) agonism as a result of binding LPA was demonstrated in RAW264.7 monocytic cells and CHO cells, and in vivo using LPAR knockout mice in a model of chemically-induced arterial wall remodeling [425-427]. The relevance of such interactions to physiology and disease is not clear at present.

In some cases, the physiological contributions ascribed to non-GPCR-mediated S1P or LPA signaling are still unclear, due to the difficulties in differentiating between true second messenger signaling versus intracellular synthesis and subsequent autocrine or transcellular GPCR activation. For instance, mast cells produce and secrete S1P, which is thought to bind S1PR to modulate FcεR-mediated degranulation and inflammatory mediator synthesis, and in vivo studies using *Sphk1*$^{-/-}$ or *Sphk2*$^{-/-}$ mice yield conflicting results [46,428,429]. Similarly, in studies utilizing preadipocytes, LPA binding to LPA_1 repressed PPARγ in vitro, and mice with adipocyte-specific deletion of ATX demonstrated increased adipocity with a concomitant increase in PPARγ expression, implying that the LPA that affects PPARγ is produced extracellularly and signals via LPAR[430,431]. Additionally, there have been reports of intracellular expression of LPA_1 and $S1P_5$ [432,433]. Thus, disentangling the relative contributions of GPCR- versus non-GPCR-mediated regulation of some cellular responses to either LPA or S1P will require meticulous dissection in both in vitro and in vivo systems.

12. Conclusions and perspectives

In the current review, we have attempted to comprehensively address the current state of our understanding of all bona fide members of the S1P and LPA receptor families, with emphasis on their contribution to the regulation of specific cell types in various systems. The past decade has seen a vigorous expansion in the study of S1P and LPA receptors. An increasing repertoire of agonists and antagonists, as well as the development of knockout and knockin animal models, has lead to our appreciation of these receptors as critical players in the regulation of homeostatic and disease states of most of the major body systems. Although the initial foci of S1PR and LPAR studies were the vascular or nervous systems, respectively, oncology, reproduction, immunology, and stem cell biology are just a few of the other fields that these receptors have been shown to dramatically influence.

Despite our increasing knowledge, the complexity and heterogeneity of LP receptor expression and signaling patterns demands intense investigation to fully comprehend the regulatory networks controlled by these receptors. New pathways and networks will likely emerge with further interrogation of the less-well characterized receptors, $S1P_4$ and $S1P_5$, and $LPA_{4\text{-}6}$, expanding the known overlapping or unique functionality of each family and individual receptor. The recent FDA approval of the S1PR modulator FTY720 as a first-line treatment for MS has heightened interest in the field of lysophospholipids and their receptors, and is likely a prelude to increased research and the future introduction of other LP receptor modulators into therapeutic use.

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Biographies

Victoria Blaho obtained her undergraduate B.S. in biochemistry from the University of Missouri. She completed her graduate studies in the laboratory of Dr. Charles Brown at the University of Missouri and received a Ph.D. in Molecular Microbiology and Immunology in 2007. She is currently a post-doctoral scientist mentored by Dr. Timothy Hla at Weill Cornell Medical College. Her current studies include examining the role of the sphingosine 1-phosphate receptors in the regulation of immune cell homeostasis.

Timothy Hla is a Professor of Pathology and Laboratory Medicine and the Director of the Center for Vascular Biology at the Weill Cornell Medical College, Cornell University in New York City. He received his Ph.D. in Biochemistry at the George Washington University in Washington, D.C. and did his postdoc with Thomas Maciag at the American Red Cross Holland Laboratory, Rockville, MD. His major contributions include the molecular characterization of human cyclooxygenase-2 enzyme, the discovery of the sphingosine 1-phosphate receptor and the elucidation of vascular effects of bioactive lipids. His laboratory continues to investigate the vascular biology of bioactive lysophospholipids and the role of RNA binding proteins in gene expression and cellular behavior.

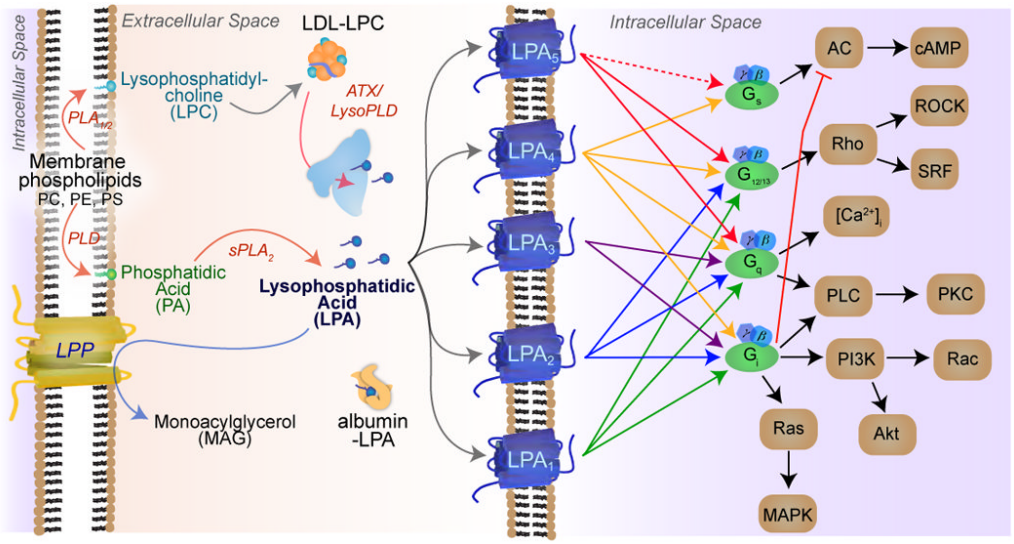

Figure 1. Metabolism of LPA, LPAR G-protein coupling and down-stream signaling pathways

PC, phosphatidylcholine; PE, phosphoethanolamine; PS, phosphatidylserine; PLD, phospholipase D; $PLA_{1/2}$, phospholipase $A_{1/2}$; PA, phosphatidic acid; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid; MAG, monoacylglycerol; LPP, lipid phosphate phosphatase; LDL, low-density lipoprotein; ATX/lysoPLD, autotaxin/ lysophospholipase D; AC, adenyl cyclase; cAMP, cyclic adenosine monophosphate; ROCK, RhoA-associated kinase; SRF, serum response factor; PLC, phospholipase C; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; eNOS, endothelial nitric oxide synthase. Dotted red line indicates suggested LPAR-G-protein coupling pathway.

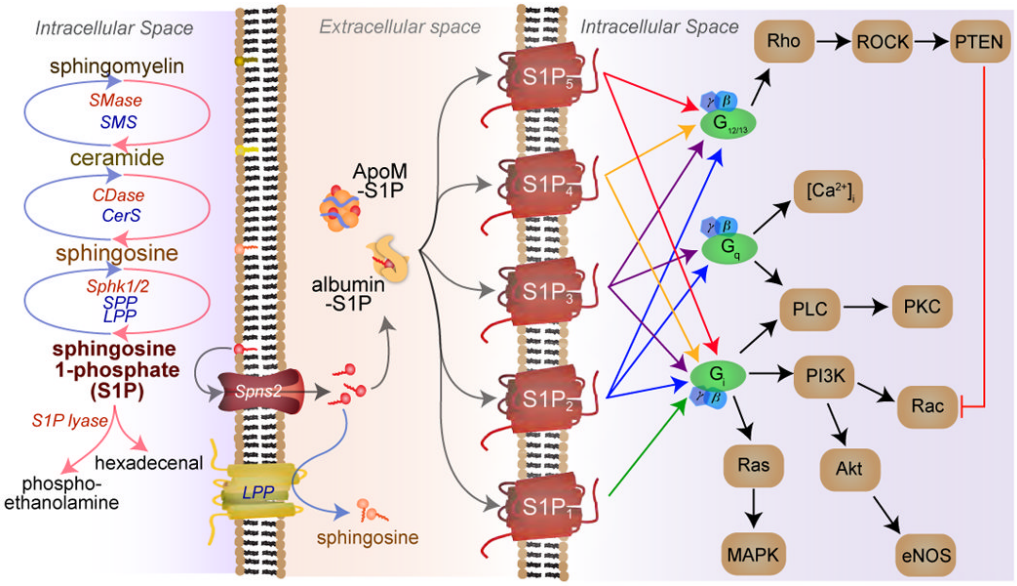

Figure 2. Metabolism of S1P, S1PR G-protein coupling, and down-stream signaling pathways

SMase, sphingomyelinase; SMS, sphingomyelin synthase; CDase, ceramidase; CerS, ceramide synthase; Sphk1/2, sphingosine kinase 1 and 2; SPP, sphingosine 1-phosphate phosphatase; LPP, lipid phosphate phosphatase; S1P, sphingosine 1-phosphate; Spns2, spinster 2 protein; ApoM, apolipoprotein M; ROCK, Rho-associated kinase; PTEN, phosphatase and tensin homolog; PLC, phospholipase C; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; eNOS, endothelial nitric oxide synthase.