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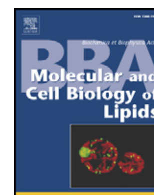

Review

Biological roles of lysophospholipid receptors revealed by genetic null mice: An update

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ABSTRACT

Two lysophospholipids (LPs), lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), are known to affect various cellular events. Their actions are mediated by binding to at least ten bona fide high-affinity G protein-coupled receptors referred to as $LPA_{1–5}$ and $S1P_{1–5}$. These LPs are expressed throughout the body and are involved in a range of biological activities including normal development, as well as functioning in most organ systems. A growing number of biological functions have been uncovered in vivo using single- or multiple-null mice for each LP receptor. This review will focus on findings from in vivo as well as in vitro studies using genetic null mice for the LP receptors, $LPA_{1,2,3}$ and $S1P_{1,2,3,5}$, and for the LP producing enzymes, autotaxin and sphingosine kinase 1/2.

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1. Introduction

Lysophospholipids (LPs) are a quantitatively minor lipid species that have been known for decades as components in the biosynthesis of cell membranes [1]. Two of the best characterized LPs are lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). LPA and S1P are both abundant in blood (0.2–5 μM) as well as tissues (0.2–100 nmol/g) [2–6], and are produced by activated platelets and other cell types, including erythrocytes [5,7,8].

In addition to their roles as metabolic intermediates, these lipids also function as extracellular signals through G protein-coupled receptors (GPCRs) and there are currently 10 identified lysophospholipid receptors, named $LPA_{1–5}$ and $S1P_{1–5}$ [9–12]. Many of the LP receptors are expressed in various cell types and activate signaling pathways to be coupled with different G-proteins [9,12,13]. Consequently, LPA and S1P trigger a variety of biological activities under physiological and pathological situations, including vascular/nervous system development, reproduction, angiogenesis, immunity/transplantation, asthma, autoimmune diseases, cancer, cardiovascular diseases [14,15], hearing loss [16–18], and pain transmission [19–27].

LPA and S1P receptors are expressed and function widely throughout the body, but the specific function of each receptor still needs to be elucidated. Using genetic null mice for individual LP receptors allows direct examination of their systemic roles in vivo and further study of LP-receptor specific signaling pathways in receptor-disrupted primary cells. Targeted gene disruption in mice has been utilized to uncover the biological functions of each lipid receptor-mediated signaling pathway in vivo and to identify the specific signaling pathways for each LP receptor in primary cells. In this review, we will focus on LP receptor functions from numerous studies that have created and studied genetic null mice. To date, single- or multiple-null mice have been reported for most of the known LPA and S1P receptors, including LPA_1-, LPA_2-, LPA_3-, $S1P_1$-, $S1P_2$-, $S1P_3$-, and $S1P_5$-null mice. In addition, as signaling mediators in vivo, the production and degradation of lysophospholipids is enzymatically controlled, and two LP producing enzymes, sphingosine kinase 1/2 (Sphk1/2) and autotaxin (ATX), play a role in tuning the level of S1P and LPA, respectively [28–34]. Therefore, we will also discuss biological functions that have been revealed using genetic null mice for those enzymes.

2. LPA_1

LPA_1 was the first receptor identified for LPA [35]. It shows broad gene expression in organ tissues such as brain, heart, lung, stomach, small intestine, spleen, thymus, testis, and skeletal muscle in adult mice [36] and has also been detected in human tissues including brain, heart, placenta, spleen, kidney, colon, small intestine, prostate, testis, ovary, pancreas, skeletal muscle, and thymus [37]. LPA_1 has been broadly studied using a heterologous expression system [36,38]. Its signaling induces cell proliferation, serum-response element (SRE) activation, MAPK activation, adenylyl cyclase (AC) inhibition, PLC/PKC

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activation, Akt activation, and Rho activation through three types of G proteins, $G_{i/o}$, G_q, and $G_{12/13}$ [39,40].

The original LPA_1-null mice were generated by deletion of exon 3 which contains the transmembrane domains I–VI [41]. About 50% of the LPA-null mice show perinatal lethality, and survivors exhibited abnormal phenotypes, such as reduced body size, craniofacial dysmorphism with shorter snouts and wider-spaced eyes, and reduced brain mass [12,41]. The majority of LPA_1-null mice pups also exhibited a suckling defect, which was evidenced by little or no milk in their stomachs. Impaired suckling behavior from defective olfaction, may explain to neonatal lethality and reduced body size [41].

Since LPA was known to induce Schwann cell survival through the G_i and phosphoinositide 3-kinase/Akt signaling pathway and overexpression of LPA_1 decreases Schwann cell apoptosis in response to serum deprivation [42], the effects of LPA_1 deficiency on Schwann cell survival was examined [41]. Young adult mouse sciatic nerve sections were examined for cellular apoptosis using in situ end labeling ($ISEL^+$), which labels fragmented DNA [43]. There was an 80% increase in the percentage of $ISEL^+$-positive cells in LPA_1-null mice compared to that of wild-type, although overall, this represented a low frequency of 18% of Schwann cell apoptosis in LPA_1-null mice compared to 10% in wild-type mice [41]. No grossly abnormal movement was observed [41], reflecting a need for more profound nerve fiber loss to reveal an overt phenotype. These data indicated in vivo effects on Schwann cells while indicating that loss of LPA1 is not sufficient to produce a more pronounced myelination defect.

LPA_1 was identified in the cortical neurogenic region of the embryonic cerebral wall, the ventricular zone, which was reflected in its original name, "ventricular zone gene-1 (vzg-1)" [35]. When neural progenitor cells (NPCs) are exposed to LPA they show cell rounding, process retraction and retraction fibers. These cytoskeletal changes bear similarity to interkinetic nuclear migration in the ventricular zone (VZ) that is associated with neurogenesis [44]. LPA_1 has been studied to understand the role of LPA in cortical development [44,45]. No obvious abnormalities in the cerebral cortex were observed in LPA_1-null mice, except for sporadic reductions in cerebral wall thickness in embryonic and neonatal LPA_1-null mice [41,46]. However, a recently identified variant of LPA_1-null mice (called "Málaga variant" or $maLPA_1$-null mice) has been reported [47]. These $maLPA_1$-null mice were spontaneously and stably generated during extended breeding of the original LPA_1-null mice [41,47]. Like their predecessors, similar phenotypic abnormalities were observed in the $maLPA_1$-null mice including reduced size and body mass, craniofacial defects such as shorter snouts and wider-spaced eyes, and reduced brain volume and mass [41,47]. Most notably, the absence of LPA_1 in the $maLPA_1$-null mice results in defects of cortical development including reduced proliferative populations, and increased cortical cell death that results in a loss of cortical layer cellularity in adult mice [47].

Since LPA is known to be a lipid metabolite released following tissue injury, LPA_1 plays an important role in the initiation of neuropathic pain [48,49]. LPA_1, unlike LPA_2 or LPA_3, is expressed in both dorsal root ganglion (DRG) and dorsal root neurons [48]. Using the antisense oligodeoxynucleotide (AS-ODN) for LPA_1 and LPA_1-null mice, it was found that LPA-induced mechanical allodynia and hyperalgesia is mediated in an LPA_1-dependent manner [48]. Neuropathic pain is mediated by the Rho-ROCK pathway. Pretreatment with Clostridium botulinum C3 exoenzyme (BoTXC3, Rho inhibitor) or Y-27632 (ROCK inhibitor) completely abolished the allodynia and hyperalgesia in nerve-injured mice [48]. LPA also induced demyelination of the dorsal root, which was prevented by BoTXC3. The dorsal root demyelination by injury was not observed in LPA_1-null mice or AS-ODN injected wild-type mice [48]. However, the precise relationship between demyelination and LPA-initiated neuropathic pain is still being determined. LPA signaling appears to induce important neuropathic pain markers such as protein kinase Cγ (PKCγ) and a voltage-gated calcium channel $\alpha_2\delta_1$ subunit ($Ca\alpha_2\delta_1$) in an LPA_1 and Rho-dependent manner [48].

Pulmonary fibrosis has been studied in LPA_1-null mice because the level of LPA remarkably increases in bronchoalveolar lavage (BAL) fluid after bleomycin-induced lung injury [50]. LPA_1 is the most highly expressed LPA receptor in lung fibroblasts among the 5 known LPA receptors (LPA_{1-5}), and LPA-induced chemotaxis of mouse embryonic fibroblast (MEF) cells are mediated by LPA_1 signaling [50,51]. Migration of fibroblasts into the fibrin wound matrix is an essential step in the wound healing process in injured tissues [52]. Tager et al. [50] showed that bleomycin-challenged LPA_1-null mice were markedly protected from pulmonary fibrosis. The mortality of wild-type mice at 21 days after administration of bleomycin was 50%, whereas LPA_1-null mice were 0%. In addition, the accumulation of fibroblasts was dramatically reduced in the injured lungs of LPA_1-null mice, and the persistent vascular leak produced by bleomycin-induced injury was notably attenuated in LPA_1-null mice [50]. Thus, LPA_1-mediated signaling was shown to have an important role between lung injury and the progression to pulmonary fibrosis. However, no significant differences in the numbers of total leukocytes, macrophages, or neutrophils were observed in the BAL of wild-type and LPA_1-null mice [50].

Astrocytes have a response to cAMP-elevating reagents that changes morphology and induces the glial fibrillary acidic protein (GFAP), indicating astrocyte differentiation. LPA has been studied in cultured astrocytes which express the five known LPA receptors (LPA_{1-5}) [53–55]. In the differentiated astrocytes induced by cAMP-elevating reagents, the levels of LPA_{2-4} were markedly reduced, whereas LPA_1 was not affected [55]. In fact, LPA-induced DNA synthesis was notably reduced in the astrocytes derived from LPA_1-null mice, indicating that LPA-induced astrocyte proliferation is mediated by LPA_1 [55].

3. LPA_2

LPA_2 was identified from sequence homology searches using LPA_1 [12,56]. LPA_2 is expressed in the embryonic brain, testis, kidney, lung, thymus, spleen, and stomach in mice [36]. In humans it is detected in the testis, pancreas, prostate, thymus, spleen, and peripheral blood leukocytes [37]. LPA_2 induces cellular signaling through three G proteins, $G_{i/o}$, G_q, and $G_{12/13}$, similar to LPA_1 [36,38].

LPA_2-null mice were generated by deletion of exon 2, containing putative transmembrane domains I to VI [57]. LPA_2-null mice were born normally, at the expected Mendelian frequency, and showed no obvious phenotypic abnormalities [57]. However, LPA-induced PLC activation and Ca^{2+} mobilization are notably reduced in MEF cells derived from LPA_2-null mice [57]. When LPA_1/LPA_2 double-null mice were generated, no additional phenotypic abnormalities were detected when compared to LPA_1 single-null mice [57]. Thus, LPA_1 and LPA_2 may have redundant functions in mediating LPA signaling, such as PLC activation, Ca^{2+}mobilization, proliferation, JNK activation, Akt activation, and stress fiber formation at least within MEFs [57]. Use of the double-null mutants in an ex vivo cerebral cortical culture system for studying embryonic neural functions of LPA signaling identified these receptors as important for LPA effects. In wild-type embryos, LPA exposure increased NPC terminal mitosis and decreased cell death resulting in induced cortical folding and thickening [46]. However, LPA_1/LPA_2 double-null mice lost these cortical growth responses [46].

Recently, cerebral cortical astrocytes have been studied towards understanding possible indirect effects of LPA on neuronal differentiation of cortical NPCs through requisite LPA_1 and LPA_2 signaling in astrocytes [58]. When neural progenitor cells were co-cultured with astrocytes that had been previously primed by LPA, the population of β-tubulin III positive neuron-like cells were increased by 41% without changing the overall cell number. Additionally, comparable effects

were observed using conditioned-medium from LPA-primed astrocytes [58]. No significant effects were observed using cerebral cortical astrocytes derived from LPA_1/LPA_2 double-null mice, but the potency of astrocytes to induce neuronal differentiation of neural progenitor cells was rescued by expression of LPA_1 or LPA_2 into LPA_1/LPA_2 double-null astrocytes [58]. This suggests that astrocytes encouraged by LPA are able to produce a soluble factor, which can induce neuronal differentiation of neural progenitor cells by LPA receptor-mediated signaling [58].

4. LPA_3

LPA_3 was cloned as a third LPA receptor by degenerate polymerase chain reaction (PCR)-based cloning and homology searches [59,60]. LPA_3 is expressed in testis, kidney, lung, small intestine, heart, thymus, brain, and in the female reproductive system such as the oviduct, placenta, and uterus in adult mice [36]. It is also found in heart, pancreas, prostate, testis, lung, ovary, and brain in humans [59,60]. Unlike LPA_1 and LPA_2, LPA_3 couples to $G_{i/o}$ and G_q protein, but not to $G_{12/13}$ [40]. LPA_3 signaling mediates PLC activation, Ca^{2+}mobilization, AC inhibition/activation, and MAPK activation [40,59,60].

LPA_3-null mice were generated by targeted deletion of the fragment containing an untranslated region and the start codon in exon 2 [61]. LPA_3-null mice were born with expected Mendelian frequency without sexual bias [61]. However, LPA_3-deficient female mice showed delayed embryo implantation, altered embryo spacing, and reduced litter size [61]. Prostaglandins E_2 and I_2 (PGE_2 and PGI_2) are known to play a crucial role in embryo uterine implantation [62]. Those PGs (PGE_2 and PGI_2) are generated by cyclooxygenase-2 (COX-2). Interestingly, COX-2 mRNA levels were notably decreased in LPA_3-null mice, with reduced levels of PGE_2 and PGI_2,caused by decreased COX-2 levels [61]. When PGE_2 and carbaprostacyclin (a stable analogue of PGI_2) were exogenously delivered in the LPA_3-null female mice, delayed implantation was rescued but defects of embryo spacing were not rescued [61]. This indicates that LPA_3-mediated signaling has a pivotal role on embryo implantation through PG signaling.

5. Autotaxin

ATX was originally characterized as a tumor cell-motility-stimulating factor [63]. Recently, ATX was shown to have a role as lysophospholipase D (lysoPLD), which converts lysophosphatidylcholine (LPC) to LPA, and potentially generates S1P from SPC [31,32,64]. Indeed, ATX can promote tumor cell motility through LPA_1 [51], and can modulate S1P-mediated motility of cells positively or negatively depending on S1P receptor subtypes [64,65]. ATX is broadly expressed, with highest levels detected in brain as well as placenta, ovary, and intestine [66–68]. In the adult brain, ATX is expressed in secretory epithelial cells, such as the choroids plexus, ciliary, iris pigment, and retinal pigment epithelial cells [66,69].

Since ATX-null mice are embryonically lethal around embryonic days 9.5–10.5 with vascular defects in yolk sac and embryo, the physiological role of ATX was studied with ATX-null embryos [33,70]. Early blood vessels formed normally, but maturation of vessels failed in ATX-null embryos [33]. In addition, ATX-null-embryos showed allantois malformation, neural tube defects, and asymmetric head folds [70]. The ATX activity and LPA levels in the plasma of heterozygotes was about half of wild-type, while S1P levels were unchanged [33,70]. These data support ATX as a critical factor in vascular development through LPA signaling [33,70], although its earlier reported functions as a nucleotide pyrophosphatase/phosphodiesterase may contribute to the null phenotype [71].

6. $S1P_1$

$S1P_1$ is highly expressed in spleen, brain, heart, lung, liver, and adipose tissues and moderately in thymus, kidney, skeletal muscle, and uterus of adult mice [72–74]. Notably, $S1P_1$ is highly expressed in the developing central nervous system (CNS) and embryonic cardiovascular and skeletal structures [74,75].

Of all currently characterized LP receptors, $S1P_1$-null mice have the most severe phenotype that includes embryonic hemorrhage, resulting in the death of all $S1P_1$-null embryos in utero between E12.5 to E14.5 [75]. Despite normal vasculogenesis and angiogenesis, $S1P_1$-null embryos displayed defects in vascular maturation. This is caused by a deficiency in the ensheathment of the nascent blood vessel by vascular smooth muscle cells (VSMCs) and pericytes [75], pointing to the essential role of $S1P_1$ in vascular development. Since deletion of $S1P_1$ results in embryonic lethality, and thus cannot be used as a constitutive null, a conditional $S1P_1$-knockout mouse has been created using the Cre-loxP system, which makes it possible to investigate $S1P_1$-mediated defects in specific cell types. In fact, endothelial cell-specific deletion of $S1P_1$ revealed that the vascular abnormality observed in conditional $S1P_1$-knockout mice was due to a maturation defect in vascular endothelial cells, rather than in VSMCs [76].

One of the well-known roles of $S1P_1$ is the regulation of cell motility. S1P enhanced migration of MEF cells that express three S1P receptors ($S1P_{1,2,3}$) via Rac activation, but these outcomes were largely diminished in MEF cells from $S1P_1$-null embryos. In addition, the critical role of $S1P_1$ in migration through crosstalk with platelet-derived growth factor (PDGF) receptor has been evaluated. PDGF-enhanced migration of MEF cells was markedly reduced by the targeted deletion of $S1P_1$ in MEF cells accompanied with the reduced Rac activation [77], implicating the S1P/$S1P_1$ axis in the downstream signaling pathway of PDGF-induced migration. This suggests that this crosstalk may be important in vascular maturation since $S1P_1$ or PDGFR single null mice show a similar lethal phenotype (vascular defect-related lethality) [75,78,79]. However, other data indicate a more complex picture, since loss of $S1P_1$ did not block the PDGFR activation-enhanced migration of VSMC and MEF cells [80].

Another interesting role of $S1P_1$ in migration has been reported recently. The S1P/$S1P_1$ axis has a pivotal role in neural stem cell migration toward an injured brain area, indicating a therapeutic potential of this receptor and related signaling [81]. However, since this role was revealed by $S1P_1$ silencing using lenti viral infection, it would be instructive to reproduce this result using conditional knockout mice to derive $S1P_1$-null neural cells.

In the immune system, studies using conditional knockout mice or adoptive transfer of $S1P_1$-deficient hematopoietic precursor cells present in the fetal liver revealed that $S1P_1$ is important in lymphocyte trafficking [82–84]. T cell-specific deletion showed that $S1P_1$ was crucial for mature T cell egress from the thymus to the blood and peripheral lymphoid organs [82]. Similarly, a study using fetal liver chimeric mice with specific deletion of $S1P_1$ from hematopoietic cells showed the same defect in T cell egress as well as in B cells [84]. Moreover, normal mice with adoptively transferred $S1P_1$-deficient T cells have a defect in exiting the secondary lymphoid organs, indicating that $S1P_1$ is intrinsically required for appropriate lymphocyte egress [84]. These two reports strongly indicate a regulatory role of $S1P_1$ in T lymphocyte egress. As in T cells, $S1P_1$ is important for B cell trafficking. $S1P_1$-null B cells that were adoptively transferred into normal mice did not exit from the secondary lymphoid organs [84]. More recently, these studies were extended to identify an important role for $S1P_1$ in cell trafficking of antibody secreting cells (ASCs). Thus, loss of $S1P_1$ in B cells did not affect the induction and localization into secondary lymphoid organs of IgG ASCs, but reduced recirculation and homing into blood and bone marrow, respectively [83].

7. $S1P_2$

Expression of $S1P_2$ is observed in a variety of organs, including heart, lung, thymus, brain, liver, kidney, spleen, adipose tissues, and all other tissues tested in adult mice [72,73]. In the CNS, the $S1P_2$ expression level

is highest in the embryonic brain, decreases postnatally, and is almost undetectable in the adult brain [72,85–88]. But even in adult mice, heart and lung have a high level of $S1P_2$ expression.

$S1P_2$-null mice that have been developed by three different groups do not have any obvious abnormalities in appearance, gross anatomy, or nervous system development. However, $S1P_2$-null mice showed a slight, but significant, decrease in the litter size, and moreover, deletion of $S1P_2$ and $S1P_3$ resulted in a marked reduction of litter size, indicating a role for these receptors in the reproductive system despite no clear mechanism. In vivo or in vitro studies using $S1P_2$-null mice have also revealed the role of $S1P_2$ in vestibular-cochlear function [16–18], seizure activity [87], vascular function [89,90], and wound healing [91]. Interestingly, a single point mutation in the $S1P_2$-related mil gene led abnormal heart development in zebrafish [92], though this defect was not observed in $S1P_2$-null mice [93].

Recently, three independent groups have reported that $S1P_2$ is indispensable for maintenance of vestibular and auditory functions in vivo [16–18]. Two groups have revealed that $S1P_2$-null mice have progressive vestibular defects as they age (more than 10 weeks old) [16,17]. The $S1P_2$-null mice exhibited persistent head tilt, defects in tail hanging posture, loss or reduction in swimming ability, reduction in rearing behavior, defects in contact righting, and loss of otoconia. All groups have shown the same results regarding hearing loss in $S1P_2$-null mice [16–18]. Analyses of auditory brainstem response (ABR) or acoustic startle response (ASR) revealed profound deafness in young $S1P_2$-null mice (3 ~ 4 weeks old). This hearing loss is closely related to structural defects in the ear that include cochlea hair cell loss and vascular disturbance. These phenotypes are degenerative rather than developmental since both vestibular and auditory systems appear normal in young animals. The precise mechanism for this degenerative defect has yet to be identified.

$S1P_2$-null mice in the C57BL/6 background display spontaneous and sporadic seizures accompanied by electrophysiological defects that are occasionally lethal [87]. The whole cell patch clamp approach revealed that hyperexcitability occurred in neocortical pyramidal neurons obtained from $S1P_2$-null mice at a physiological condition, indicating the possible role of $S1P_2$ in neuronal excitability. Further study is required to identify the specific roles of $S1P_2$-mediated signaling in this process.

Another interesting finding is the role of $S1P_2$ in vascular function. The involvement of $S1P_2$ has been implicated in embryonic vascular development based on the finding that $S1P_1$/$S1P_2$/$S1P_3$ triple-null mice have more severe vascular defects and lethality than $S1P_1$ single-null mice [94]. This concept was supported in adults, since absence of $S1P_2$ induces vascular dysfunction in adult mice [89]. The studies revealed that lacking $S1P_2$ resulted in decreased vascular tone in vivo and blunted responsiveness to vasoconstrictor agents in vivo and ex vivo despite no morphological difference compared to wild-type mice. Another group has shown that $S1P_2$-null mice have no significant abnormalities during normal vascular maturation of the retina, but $S1P_2$ plays an important role under certain pathological conditions [90]. Hypoxia-induced intravitreal neovascularization was decreased in $S1P_2$-null retinas, but there was an enhanced intraretinal revascularization in the $S1P_2$-null [90]. Normalized angiogenesis shown in $S1P_2$-null mice is closely related to the reduction of hypoxia-induced endothelial gaps, immune cell infiltration, and inflammatory responses [90]. Taken together, these studies reveal important roles for $S1P_2$ both in normal vascular development and in pathological neovascularization.

$S1P_2$-null mice have a defect in wound healing during liver injury [91]. This study demonstrated that $S1P_2$ is involved in wound healing, hepatocyte proliferation, and matrix remodeling and not in the development of the liver injury. Despite no difference in necrosis, inflammation, and hepatocyte regeneration between wild-type and null mice, $S1P_2$-null mice display a reduced accumulation of hepatic myofibroblasts (hMF) which are the cells responsible for wound healing and express $S1P_{1-3}$. The in vitro study further revealed that $S1P_2$-mediated wound healing was related to the mitogenic effect of S1P/$S1P_2$ signaling in hepatocytes. It is also noteworthy that targeted disruption of $S1P_3$ does not affect any aspect of this model, suggesting the unique role of $S1P_2$ in wound healing following liver injury.

A significant defect of S1P/$S1P_2$ signaling was found in vitro in MEF cells [73]. MEF cells from $S1P_2$-null mice display decreased Rho activation in response to S1P but no effect on PLC activation, Ca^{2+} mobilization, and adenylyl cyclase inhibition, indicating a critical role of $S1P_2$ in Rho activation. Like $S1P_1$, $S1P_2$ signaling also interfaces with PDGFR signaling, but these receptors have opposite roles. In contrast to $S1P_1$ [77,95], in vitro studies using MEF cells from $S1P_2$-null mice demonstrated that $S1P_2$ has a role as a negative regulator in PDGFR-mediated proliferation, migration, and Sphk1 induction [96]. These studies suggest that PDGFR dependent S1P production via Sphk1 can produce opposing signals, depending on which receptor, $S1P_1$ and $S1P_2$, is activated. It should be noted that in another study, PDGFR activation- and/or S1P-induced $S1P_2$ activation promoted cell proliferation via Sphk in hepatic myofibroblasts, which were blunted in $S1P_2$-null cells [91]. Therefore, the role of S1P/$S1P_2$ in cell proliferation can be cell type specific.

8. $S1P_3$

Expression of $S1P_3$ has been reported in a variety of organs including spleen, heart, lung, thymus, kidney, testis, brain, and skeletal muscles in adult mice [72,73], and in humans, in heart, placenta, kidney, liver, pancreas, skeletal muscle, lung, and brain [97].

$S1P_3$-null mice are grossly normal, but lack some of the S1P-mediated responses. The first $S1P_3$-null mice reported [73] showed that $S1P_3$ loss results in viable and fertile mice that develop normally with no obvious phenotypic abnormalities except for the small, but significant, decrease in the litter size. This confirms that $S1P_3$, unlike $S1P_1$, is not required for normal organismal development and function. Despite no apparent phenotype, cells derived from $S1P_3$-null mice have defects in S1P signaling, indicating functions for this receptor that have been revealed by perturbing the basal state of the animal in development, endothelial and epithelial barrier integrity, cardioprotection, and sepsis.

Signaling defects in vitro have been studied in MEF cells from $S1P_3$-null mice [73]. S1P-induced PLC activation, Ca^{2+} increases, and inhibition of adenylyl cyclase that are observed in wild-type MEF cells are significantly reduced in $S1P_3$-deficient MEF cells, but, in contrast to $S1P_2$, $S1P_3$ in MEF cells is not involved in Rho activation [73]. In addition to the PLC/Ca^{2+}signaling pathway, $S1P_3$ is required for Akt activation in MEF cells, which is dependent on PDGFR activation [98]. Considering the negative role of $S1P_2$ in PDGF-mediated migration [96], there may be a complex interplay between S1P- and PDGF-receptors.

$S1P_3$-mediated increases in intracellular calcium and Akt activation have been reported in cardiac endothelial cells, which is connected to NO-mediated vasodilation [14]. An ex vivo study using aortae also showed the vasodilative effect of high-density lipoprotein (HDL), which was abolished by $S1P_3$ deficiency, indicating a role for $S1P_3$ in vascular tone regulation. In addition to the control of endothelial barrier integrity, $S1P_3$ has an epithelial barrier function [99]. In $S1P_3$-null mice, S1P-induced pulmonary leakage under normal and inflammatory conditions was abolished and was mediated by blocking the disruption of the alveolar epithelial junctions. These findings indicate an important role of $S1P_3$ in the maintenance of epithelial barrier integrity.

Another interesting feature of $S1P_3$ is its role in cardio protection. The first direct evidence was reported by Theilmeier et al. [15] who expanded their initial finding of the beneficial effect of HDL/$S1P_3$ on NO-mediated vasodilation into reducing ischemia/reperfusion injury. Thus, in vivo studies using $S1P_3$-null mice revealed that exogenously

added HDL or its sphingolipid component, S1P, dramatically attenuated infarction size via $S1P_3$. Presumably, this is mediated by the release of NO based on the vasodilative effect of HDL. In addition to the extrinsic pathway, the intrinsic S1P/$S1P_3$ axis is important for cardio-protection. Another group reported that the protective effect of endogenously produced S1P in response to ischemia/reperfusion requires both $S1P_2$ and $S1P_3$ receptors since an increase in ischemic damage is not seen unless both $S1P_2$ and $S1P_3$ receptors are deleted [100]. Therefore, both extrinsic and intrinsic signaling pathways of $S1P_3$ are related to cardio-protection.

Very recently, a new aspect of intrinsic $S1P_3$ signaling was demonstrated [101]. In an lipopolysaccharide-induced septic model, activation of the protease activated receptor (PAR)1 in dendritic cells (DCs) is a main factor for inflammatory response-driven lethality. Interestingly, $S1P_3$ acts as a downstream component in PAR1-mediated septic lethality, revealed by the adoptive transfer of DCs from PAR1- or $S1P_3$-null mice or by chemical agonism of $S1P_3$. Adoptive transfer of PAR1-deficient DCs into $S1P_3$-null mice had no adverse effect on survival of these mice, but agoinst stimulation of $S1P_3$ on adoptively transferred PAR1-deficient DCs was sufficient to induce lethality. They also revealed that Sphk1 is another component in this process. These findings identified a coordinated interplay among PAR1, Sphk1, and $S1P_3$.

A series of studies using multiple S1P receptor null mice implicated their functional redundancy in vivo [93,94]. $S1P_2$/$S1P_3$ double-null mice showed a clear phenotype of reduced litter sizes compared to single null crosses owing to perinatal lethality, although double null survivors lacked any obvious phenotype [93]. This protection is not present in single receptor-null mice under these conditions. Another study using $S1P_1$/$S1P_2$/$S1P_3$ triple-null mice showed more severe defects in vascular development compared to $S1P_1$ single-null mice and earlier embryonic lethality at E10.5–11.5 [94]. However, $S1P_2$ and $S1P_3$ single-null mice show no evidence of this phenotype [73,87]. At a cellular level, $S1P_2$ deletion, not $S1P_3$ deletion, partially impaired Rho activation, but deletion of both receptors completely abolished it in MEF cells [93]. Taken together, these studies indicate the coordination of S1P signaling by these three S1P receptors.

9. $S1P_5$

Expression of $S1P_5$ is restricted to specific tissues including brain, spleen, and peripheral blood leukocytes in humans, and brain, skin, and spleen in the rat and mouse [73,102,103]. $S1P_5$-null mice developed normally and were fertile [104]. Functional studies using these null mice have been reported in oligodendrocytes [104] and natural killer (NK) cells [105] where $S1P_5$ is highly expressed.

$S1P_5$ is highly expressed in brain white matter, especially in oligodendrocytes, the main myelination cell type in the CNS [103]. However, $S1P_5$-null mice do not have any evident myelination defects, although immature oligodendrocytes that lacked $S1P_5$ by siRNA silencing have lower responses to S1P in vitro [104]. Additionally, there is no apparent behavioral deficit in these mice. Nevertheless, further studies on the function of $S1P_5$ in the brain, especially in the myelination process, may reveal other functions that might be expected in view of the abundance of $S1P_5$ in oligodendrocyte lineages [103,104]. In vitro studies using siRNA-based knock-down of $S1P_5$ in these cells has shown migration defects [106]. It is also possible that the S1P/$S1P_5$ axis may be providing a beneficial effect against demyelinating injuries, in addition to normal physiological processes.

$S1P_5$ is also highly expressed in mouse and human NK cells, indicating that this receptor-mediated signaling is important for the immune system [105]. This study demonstrated that $S1P_5$ is involved in NK cell trafficking in steady-state and inflammatory situations. $S1P_5$-null mice have a lower population of NK cells in blood, spleen, and lungs and a higher population in bone marrow and lymph nodes compared to WT mice. Additionally, inflammation induced an expansion of NK cells in inflamed livers of WT, but in $S1P_5$-null mice, this homing process was defective. Even though the exact mechanism for these phenotypes is not yet clear, it appears that $S1P_5$ has a prominent role in NK cell trafficking in vivo.

10. Sphk1/2

Sphks are highly conserved enzymes found in throughout phylogeny [107–110] and produce S1P by catalyzing the phosphorylation of sphingosine. To date, two distinct isoforms of Sphk have been identified in mammals, referred to as Sphk1 and Sphk 2 [28,30]. Northern blot and quantitative PCR analyses have revealed that Sphk1 and Sphk2 differ in developmental and tissue expression [30,111]. Sphk1 mRNA was high at E7 and then decreased, but Sphk2 mRNA expression was still high in later embryonic development [30]. In adult mouse tissues, Sphk1 was highest in lung and spleen, but not in liver, whereas Sphk2 was predominantly expressed in liver and heart [30,111]. However, the expression of both enzymes was observed in most tissues despite the different levels of expression, including brain, kidney, blood, and lymph nodes and the activity of both enzymes was detected in all mouse tissues tested.

Individual loss of either Sphk1 or Sphk2 does not have an abnormal phenotype, but, Sphk1/2 double-null mice are lethal prior to E13.5 with severe vascular and neural tube defects, resembling S1P receptor null mice [112,113]. Again this indicates the requirement of S1P in the development of the vascular and central nervous systems and reveals the functional redundancy of the two Sphk isoforms.

Further analysis of Sphk deficient mice revealed early pregnancy loss by vascular defects. $Sphk1^{-/-}Sphk2^{+/-}$ mutant females, but not mutant males nor any other mutant female combination ($Sphk1^{-/-}Sphk2^{+/+}$ and $Sphk1^{+/-}Sphk2^{-/-}$), were infertile. The infertility was caused by increased cell death in decidual cells and massive rupture of decidual blood vessels, leading to early embryonic lethality [114]. Interestingly, identification of similar reproductive defects in Drosophila Sphk mutants identified the highly conserved roles of these enzymes [107,115]. These findings suggest the importance of sphingolipid metabolism in reproduction.

Sphk1/2 have been verified to be critical for producing S1P in vivo. In mice, S1P was not detectable in embryos deficient for both Sphk1 and Sphk2, but deletion of Sphk1 caused a reduction in S1P plasma levels to 50% of those observed in WT mice [112,113], indicating that Sphk2 likely accounts for the remaining levels of S1P in Sphk1-null mice, at least within plasma. A recent report verified the cell type for S1P production by these enzymes: conditional gene deletion of both Sphk1 and Sphk2 identified erythrocytes as a major source of S1P in plasma, while lymph S1P is produced by an as yet unidentified radiation-resistant cell population [116,117].

A well-studied function of Sphk1/2 is the regulation of immune system lymphocyte trafficking, especially as revealed by FTY720 effects, an experimental prodrug that can produce lymphopenia and which has been reviewed in great detail elsewhere [20,23,118]. FTY720 is known to be phosphorylated by Sphk1/2 in vivo and exerts its effect by interacting with four of the five known S1P receptors, namely $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ [20, 119]. In vivo studies using genetic null mutant mice for Sphks have revealed that Sphk2 is the major isoform for FTY720 phosphorylation. FTY720 caused lymphopenia in Sphk1-null mice, but not in Sphk2-null mice, indicating that Sphk2, and not Sphk1, is the primary kinase responsible for phosphorylating FTY720 in vivo [112,120,121]. Besides their roles in FTY720 efficacy, these enzymes are also important physiologically for lymphocyte trafficking. Conditional deletion of Sphk1 and 2 in mice prevented lymphocyte egress from the thymus and peripheral lymphoid organs via the reduction of plasma and lymph S1P levels [116]. In addition, surface lymphocyte expression of $S1P_1$ isolated from lymphoid organs of Sphk deficient mice is high compared to WT mice,

Table 1
Summary for defects in biological function of LP receptor null mice

Gene	Phenotypes	Revealed biological functions	Refs.
LPA_1	50% of perinatal lethality	Initiation of neuropathic pain	[41,46–49, 50,55,58]
	Reduced body size	Pulmonary fibrosis	
	Craniofacial dysmorphism	Astrocyte proliferation	
	Reduced brain mass	Migration of MEF cells	
	Impaired suckling behavior	Indirect effect on neuronal differentiation	
	Increase in SC apoptosis		
	Defects in cortical development		
LPA_2	No abnormalities	Indirect effect on neuronal differentiation	[57–58]
		Signaling in MEF cells	
$LPA_{1/2}$	Same phenotypes with LPA_1-null mice	Cortical growth	[46]
LPA_3	Delayed implantation		[61]
	Altered embryo spacing		
	Reduced litter size		
$S1p_1$	Complete embryonic lethality (defect in vascular endothelial cell maturation)	Migration of MEF and neural stem cells	[75–77, 81–84]
		Trafficking of T- and B-cells[a, b]	
$S1p_2$	Reduced litter size	Vasoconstriction	[16–18,87, 89–91,96]
	Vestibular defects	Angiogenesis under hypoxia	
	Hearing loss	Wound healing during liver injury	
	Sporadic seizures (C57BL/6 mice only)	Signaling in MEF cells	
	Vascular dysfunction in adult mice	Blockade of PDGFR activation-induced proliferation and migration in MEF cells	
		Enhancement of PDGF- or S1P-induced proliferation in hepatic myofibroblast	
$S1p_3$	Reduced litter size	Signaling in MEF cells	[14–15,73, 99,101]
	Disruption of alveolar epithelial junctions	HDL-induced vasodilation	
		Disruption of alveolar epithelial barrier function	(Nature 2008)
		Cardioprotection by HDL or S1P against ischemia/ reperfusion	
		Downstream signal of PAR1-mediated sepsis[c]	
$S1p_{2/3}$	Reduced litter size	Cardioprotection against ischemia/reperfusion	[100]
$S1p_{1/2/3}$	Embryonic lethality		[93]
$S1p_5$	Defect in trafficking of NK cells		[94,105]
ATX	Embryonic lethality (defect in vascular vessel maturation)	S1P production	[33,70]
Sphk1	No abnormalities	S1P production	[112–114]
	Reproductive defects[d]		
Sphk2	No abnormalities	S1P production	[112–113, 120–121]
		FTY720-induced lymphocyte trafficking	
Sphk1/2	Embryonic lethality	S1P production in erythrocytes[a]	[101,112–113,116]
	Defect in lymphocyte trafficking[a]	Downstream signal of PAR1-mediated sepsis[c]	

[a] A finding from study using conditional knockout mice.
[b] A finding from study using adoptive cell transfer of $S1P_1$-null lymphocytes.
[c] A finding from study using adoptive cell transfer of $S1P_3$-null dendritic cells.
[d] This defect was observed only in embryos from $Sphk1^{-/-}Sphk2^{+/-}$ mutant females.

suggesting a coordinated interplay between receptor expression and available S1P levels [116]. Sphk1 is also involved in the PAR1/$S1P_3$ regulation of dendritic cells that is important for systemic inflammation and lethality in a sepsis model [101].

The role of Sphks in mast cells, another immune cell type, has been also revealed [122]. Studies using liver-derived mast cells that were deficient in Sphk1 and/or Sphk2 demonstrated a role for Sphk2, not Sphk1, in intracellular S1P production and consequent mast cell responsiveness, including Ca^{2+}influx, cytokine production, and degranulation. These findings indicate an important role of Sphk2 as an intrinsic regulator in mast cell function. However, the reduction of circulating extrinsic S1P levels in Sphk1-null mice and the resistance of these mice to anaphylaxis contrasts with conditions observed in Sphk2-null mice that show an increase in circulating extrinsic S1P levels and no resistance to anaphylaxis. These results indicate an extrinsic role of S1P regulated by Sphk1 in mast cell responsiveness. Furthermore, Sphk2-deficient mice with one functional allele for Sphk1 ($Sphk2^{-/-}Sphk1^{+/-}$) prevented Sphk2-null phenotype, indicating that a Sphk1-mediated increase in circulating S1P levels can rescue the intrinsic defects of Sphk2-null mice in mast cells. [122]. Taken together, these reports suggest different roles of Sphk1 and Sphk2 in regulating mast cell function by both intrinsic and extrinsic control of S1P levels.

11. Concluding remarks

Studies on the role of LPs are increasing, and significant effort has been made to elucidate their biological functions by taking advantage of genetic null mice. Herein we summarized the biological function of LP receptors revealed by knockouts ($LPA_{1,2,3}$ and $S1P_{1,2,3,5}$) and the related important LP producing enzymes (ATX and Sphk1/2). Biological functions identified in these studies are pervasive, including normal development of the vascular and nervous systems, as well as proper functioning of the cardiovascular, immune, reproductive, and nervous systems (summarized in Table 1). Some of these functions are expected based on in vitro findings, but there are still discrepancies between in vitro and in vivo studies, which should be clarified in the future. Further studies using multiple receptor-null mice could better elucidate this interaction. In fact, studies using LPA_1/LPA_2, $S1P_1$/$S1P_2$/$S1P_3$ or $S1P_2$/$S1P_3$ multiple-null mice have verified some of the specific biological roles of each receptor and uncovered hidden functions in the single-null mice. Likewise, it may prove useful to generate multiple-null mice by crossing single LPA and S1P receptor-null mice since the interplay or compensation between LPA and S1P receptors is likely to be present. It would also be of interest to identify the interplay between LP receptors- and other receptor-mediated signaling mechanisms. One might also consider the coordinated interplay of the LP producing or degrading enzymes with LP receptors by the regulation of intracellular, as well as extracellular LP levels. Null mice for LPA_4, LPA_5, and $S1P_4$ will surely follow in the near future. In addition, LP receptors have been regarded as viable therapeutic targets in a growing number of diseases, and the use of LP receptor-mutant mice should aid in identifying and validating involved receptors and mechanisms for future therapies.

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