Sphingosine-1-phosphate signaling in physiology and diseases

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Short Title: S1P signaling

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Abstract

Sphingosine-1-phosphate (S1P), which acts as both the extracellular and intracellular messenger, exerts pleiotropic biological activities including regulation of embryonic development, formation of the vasculature, vascular barrier integrity, vascular tonus and lymphocyte trafficking. Many of these S1P actions are mediated by five members of the G protein-coupled S1P receptors ($S1P_1$~$S1P_5$) with overlapping but distinct coupling to heterotrimeric G proteins. $S1P_1$ couples exclusively to G_i whereas $S1P_2$ and $S1P_3$ couple to multiple G proteins. $S1P_2$ and $S1P_3$ prefer $G_{12/13}$ and Gq, respectively, among others. The biological activities of S1P are based largely on the cellular actions of S1P on migration, adhesion and proliferation. Notably, S1P often exhibits bimodal effects in these cellular actions in a receptor subtype-specific manner. For example, $S1P_1$ mediates cell migration toward S1P, i.e. chemotaxis, via G_i/Rac pathway whereas $S1P_2$ mediates inhibition of migration toward a chemoattractant, i.e. chemorepulsion, via $G_{12/13}$/Rho pathway which induces Rac inhibition. In addition, $S1P_1$ mediates stimulation of cell proliferation through the G_i-mediated signaling pathways including phosphatidylinositol 3-kinase (PI3K)/Akt and ERK whereas $S1P_2$ mediates inhibition of cell proliferation through mechanisms involving $G_{12/13}$/Rho/Rho kinase/PTEN-dependent Akt inhibition. These differential effects of S1P receptor subtypes on migration and proliferation lead to

bimodal regulation of various biological responses. An observed biological response is likely determined by an integrated outcome of the counteracting signals input by S1P receptor subtypes expressed in the cells. More recent studies identified the new intracellular targets of S1P; S1P acts as the intracellular messenger to bind to the inflammatory signaling molecule TRAF2 downstream of TNF receptor and to histone deacetylases HDAC1 and HDAC2, resulting in activation of NF-κB and inhibition of histone deacteylation, respectively. Development of S1P receptor agonists and antagonists with improved receptor subtype-selectivity and their optimal drug delivery system augments useful actions and attenuates deleterious effects of S1P, thus providing novel therapeutic tactics. Inhibitors or modulators of S1P-synthesizing and -metabolizing enzymes also could be potential therapeutic tools.

Key words: sphingosine-1-phosphate, lysophospholipid, G protein-coupled receptor

Introduction

Sphingosine-1-phosphate (S1P), lysophosphatidic acid (LPA), lysophosphatidylserine and lysophosphatidylinositol constitute lysophospholipid mediators and are attracting increasing interest in cell signaling for the past decade. Among these lysophospholipid mediators, S1P and LPA are much better characterized than the others. It is now recognized that the S1P signaling system comprises S1P synthesizing/degrading enzymes [1,2], membrane S1P transporters [3,4], S1P carrier proteins in the plasma [5], and five members of the G protein-coupled S1P-specific receptor subtypes, $S1P_1$~$S1P_5$ [6-8]. S1P plays crucial roles in embryonic development and post-natal homeostasis in the cardiovascular, immune and nervous systems [9-13]. The S1P signaling system is also implicated as the target of therapeutic intervention in a variety of human diseases; multiple sclerosis, a debilitating autoimmune disease, is now treated with the S1P receptor agonist prodrug FTY720 [14], whose phosphorylation product downregulates $S1P_1$ in lymphocytes to inhibit their recirculation, thus resulting in lymphopenia and attenuated immune reaction [12]. In addition, animal studies suggest that targeting the S1P signaling system is a promising strategy for inhibiting vascular hyperpermeability and modulating angiogenesis [13, 15, 16]. Here, we will overview the signaling mechanisms underlying S1P regulation of biological functions

and the roles of S1P in diseases.

S1P synthesis and metabolism

S1P is generated within cells through phosphorylation of sphingosine by sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2) (Figure 1) [1], which share a conserved catalytic domain, but are distinct in other aspects including their structures of non-catalytic domains and expression patterns. S1P is either dephosphorylated by S1P phosphatases (SPP1 & SPP2) [17] and lipid phosphate phosphatases (LPP1~LPP3) [18] to convert into sphingosine, or degraded by S1P lyase (SPL) to ethanolamine phosphate and hexadecenal [2], the latter reaction serving as the exit from sphingolipid metabolic pathway (Fig. 1). SPPs and SPL, both of which are highly specific for S1P, reside predominantly in the endoplasmic reticulum, while LPPs dephosphorylate a range of substrates including S1P.

SphK1-knockout (KO) mice are phenotypically normal except for a 60 % reduction in plasma and serum S1P concentrations compared to wild-type mice [19]. However, tissue S1P levels in SphK1-KO mice are similar to wild-type mice. SphK2-KO mice are also phenotypically normal and exhibit a 25% reduction in plasma S1P concentrations [20]. Thus, SphK1 plays a major role in maintaining plasma and serum S1P tone, and

SphK2 compensates for SphK1 in maintaining tissue S1P in the absence of SphK1. SPL-KO mice display markedly high levels of S1P in tissues and serum with accumulation of ceramide and long chain bases, resulting in multi-organ damages with pro-inflammatory responses and altered lymphocyte and neutrophil distribution [21, 22]. LPP3 maintains S1P level at a low level in the thymus, thus playing a key role in T lymphocyte exit to the blood from the thymus [23].

SphKs and S1P-metabolizing enzymes play important roles not only in the production and degradation of S1P but also in controlling cellular levels of sphingolipids including sphingosine and its metabolic precursor ceramide. In contrast to S1P, these sphingolipid species exert growth inhibitory and proapoptotic effects when their cellular levels rise, through multiple mechanisms including activation of protein phosphatases and inhibition of Akt [1, 2, 24]. Since S1P exhibits anti-apoptotic or survival effects on a variety of cell types, the balance of S1P and ceramide levels is implicated in the determination of cell fate, i.e. survival or death under certain conditions.

Blood S1P and S1P transporters

In the mammalian body, there is a steep S1P concentration gradient across the

capillary wall [25]: the plasma S1P concentration is around 500 nM, which is considered to be markedly higher than that in the tissue interstitial fluid. The majority of plasma S1P derives from red blood cells [26], which express SphK1 but lack S1P degrading enzymes and thus serve as a supplier of S1P in blood, while the remaining of plasma S1P is released from other cells, particularly endothelial cells [27]. Indeed, anemia causes a reduction in the plasma S1P level. Release of S1P from erythrocytes strictly requires acceptor plasma proteins, mostly HDL and albumin [5]. The major part of plasma S1P is bound to HDL (~60%), albumin (~30%) and other plasma proteins, with only a few percentages of total S1P circulating in a free form. Plasma S1P is crucial in maintaining vascular integrity, which is achieved by endothelial $S1P_1$-mediated stabilization of adherence junctions [16, 28]. At least a part of beneficial effects of HDL, including activation of eNOS, atheroprotection and myocardial protection from ischemia/reperfusion injury, are suggested to be mediated by HDL-bound S1P [29].

S1P is released out of erythrocytes via a transmembrane S1P transporter. Although ABC family transporters have been implicated in S1P export from erythrocytes, the exact molecular entity of an S1P transporter remains inconclusive [3]. In a zebrafish mutant *miles apart*, loss of function mutation of $S1P_2$ results in an anomaly termed

cardia bifida (two primordial heart tissues remaining separated) [30]. In a different zebrafish mutant *ko157*, which also shows cardia bifida, the major facilitator superfamily type transporter, Spns2, was mutated and cardiac defects in *ko157* mutant was rescued by S1P injection [4]. Zebrafish Spns2 and its mammalian counterpart were found to function as a transporter for S1P and FTY720 phosphate.

Expression of S1P receptors

$S1P_1$, $S1P_2$ and $S1P_3$ are broadly expressed in most of organs and mediate diverse actions of S1P (Table 1) [6-8, 11]. Detailed expression patterns of S1P receptors in tissues were defined by analyzing mice in which β-galactosidase (LacZ) reporter gene was knocked into the receptor gene loci. $S1P_1$, which was originally cloned from vascular endothelial cells, is detected in the endothelium in the lung, heart and liver of $S1P_1^{+/LacZ}$ mice [9]. In the lung, $S1P_1$ is also expressed in vascular smooth muscle. Unexpectedly, LacZ activity is undetectable in the endothelium of the kidney, spleen and testis. The non-vascular cells, including neuronal cells including Purkinje cells and neurons in the molecular layer, astrocytes, cardiomyocytes, cells in the marginal zone in spleen, and epithelial cells in the renal collecting duct express $S1P_1$.

In normal tissues of $S1P_2^{+/LacZ}$ mice, LacZ activity is detected in various sizes of

blood vessels in a variety of organs, which include lung, brain, skeletal muscle, kidney, and liver [31]. Vascular cells are the major cell types that express $S1P_2$ in many organs. Histological analysis using combined immunohistochemistry and X-gal staining showed that the endothelium in microvessels and both the endothelium and smooth muscle of larger vessels express $S1P_2$. In addition, a limited population of bone marrow cells and a small number of non-vascular cells in the brain express $S1P_2$.

In contrast to $S1P_1$, $S1P_2$ and $S1P_3$, the expression of the other two S1P receptors $S1P_4$ and $S1P_5$ is restricted: $S1P_4$ and $S1P_5$ are primarily expressed in lymphoid tissues and the lung, and the brain (especially oligodendrocytes), leukocytes and spleen, respectively [8, 11].

Distinct signaling mechanisms of $S1P_1$, $S1P_2$ and $S1P_3$

The signaling mechanisms of $S1P_1$~ $S1P_3$ are better characterized compared with $S1P_4$ and $S1P_5$. $S1P_1$, $S1P_2$ and $S1P_3$ activate overlapping yet distinctive intracellular signaling pathways, as analyzed by expressing cloned receptors in Chinese hamster ovary (CHO) cells and other cells (Fig. 2) [6-8, 11, 32, 33]. $S1P_1$ couples exclusively to heterotrimeric G_i to activate Ras/ERK, PI 3-kinase/Akt, and Rho family small GTPase Rac. $S1P_1$ also moderately activates phospholipase C (PLC) and consequently induces

Ca^{2+} mobilization [6, 7]. In contrast to $S1P_1$, $S1P_2$ and $S1P_3$ couple to multiple G proteins, i.e. G_q, G_i and $G_{12/13}$ [11, 32, 33]. $S1P_2$ stimulates small GTPase Rho via $G_{12/13}$, PLC mainly via G_q, ERK via G_i, and JNK and p38 mitogen-activated protein kinase (MAPK) via pertussis toxin (PTX)-insensitive G protein [32]. $S1P_2$ mediates ERK activation obviously less potently compared with $S1P_1$ and $S1P_3$ [7, 33], suggesting inefficient G_i-coupling of $S1P_2$. Regardless of the G_i-coupling of $S1P_2$, $S1P_2$ increases cyclic AMP. This was found to be mediated via G_{13} [34]. Like $S1P_2$, $S1P_3$ also couples to G_q-mediated PLC stimulation, $G_{12/13}$-mediated Rho stimulation, and G_i-mediated ERK and Rac stimulation [11, 33]. $S1P_3$ decreases or increases cyclic AMP level, depending on experimental conditions. Although $S1P_2$ and $S1P_3$ similarly can couple to G_q, G_i and $G_{12/13}$ when overexpressed, obvious difference in the two receptor subtypes exists in primary cells: mouse embryonic fibroblasts (MEFs) from $S1P_2$-null mice exhibit impaired Rho activation while PLC activation is not compromised compared with wild-type MEFs [11]. On the other hand, MEFs from $S1P_3$-null mice show impaired PLC activation with Rho activation and adenylate cyclase inhibition unaffected. Although $S1P_3$ deletion does not impair Rho activation in MEFs, $S1P_2$- and $S1P_3$-double null MEFs completely lack Rho activation response, suggesting that there is partial functional redundancy between $S1P_2$ and $S1P_3$. $S1P_4$ was reported to couple

to G_i and $G_{12/13}$, which mediates ERK activation, PLC stimulation and Rho activation [11]. $S1P_5$ couples to G_i and $G_{12/13}$, resulting in adenylate cyclase inhibition and Ca^{2+} mobilization.

Since $S1P_1$, $S1P_2$ and $S1P_3$ are widely expressed, an integrated outcome of S1P signaling in a given cell type largely depends upon relative expression levels of the S1P receptor subtypes. In addition, ever growing numbers of examples of cross-talks between S1P receptor signaling and growth factor or cytokine receptor signaling have been reported. For example, under certain conditions $S1P_3$ activation leads to activation of TGFβ signaling pathway and fibrosis. Update information regarding detailed cross-talk mechanisms is available in recently published excellent reviews [8, 35].

Regulation of cell migration by S1P receptor signaling

Cell migration is a fundamental biological process essential for morphogenesis, angiogenesis, immune surveillance, inflammation, tumor cell invasion and metastasis [36]. It is regulated through receptor-mediated processes in response to a variety of ligands, which are either soluble, bound to extracellular matrix or expressed on cell surface.

One of outstanding biological activities of S1P is the ability to regulate cell

migration either negatively or positively, which was first recognized to be apparently cell type-dependent [37]. S1P potently inhibits cell migration in a variety of tumor cells including B16 melanoma, breast cancer, and glioblastoma cells, as well as vascular smooth muscle cells. By contrast, S1P induces chemotaxis in vascular endothelial cells (ECs) [28], MEFs [11], and T and B lymphocytes [10, 12].

CHO cells are an excellent model for studying mechanism of cell migration [38]. They vigorously exhibit stimulation or inhibition of cell migration, depending on stimuli. In a Boyden chamber assay in which cells are placed in the upper well, either $S1P_1$ or $S1P_3$ mediate migration of CHO cells toward S1P in the lower well, i.e. chemotaxis, with typical bell-shaped dose-response curves [38]. In contrast, $S1P_2$ mediates inhibition of cell migration directed toward a chemoattractant. This $S1P_2$ effect is dependent on a concentration gradient of S1P: $S1P_2$ mediates inhibition of cell migration toward a chemoattractant in the lower well, when S1P is placed only in the lower well. If S1P is placed only in the upper well or in both the upper and lower wells, chemotaxis is not suppressed. Therefore, $S1P_2$ mediates chemorepulsion. Prostaglandin E_2 (PGE_2) and isoproterenol, which elevate the intracellular cyclic AMP level via G_s, also inhibit chemotaxis in CHO cells. However, the inhibitory effects of PGE_2 and isoproterenol are distinct from the $S1P_2$-mediated effect in that PGE_2 and isoproterenol

effectively inhibit chemotaxis, whether these ligands are placed in the upper well or in both the upper and lower wells [39]. Thus, cell migration inhibition induced by PGE_2 or isoproterenol is not dependent on their concentration gradients and therefore differs from chemorepulsion. Rho family GTPase Rac promotes actin polymerization to induce lamellipodia formation and plays a pivotal role in cell migration. The chemoattractant receptors $S1P_1$ and $S1P_3$ mediate Rac activation via G_i, whereas chemorepellant receptor $S1P_2$ does not [38]. Importantly, $S1P_2$ but not $S1P_1$ or $S1P_3$ inhibits Rac activation induced by a chemoattractant. $S1P_2$-mediated inhibition of Rac activation and cell migration in response to a chemoattractant is abolished by the expression of dominant negative Rho mutant N^{19}Rho and inhibition of $S1P_2$-$G_{12/13}$ coupling, indicating that Rho mediates Rac inhibition in $S1P_2$-expressing cells. Detailed analysis suggests the involvement of stimulation of Rac GTPase-activating protein (GAP) in Rac inhibition in a manner independent of a Rho kinase [38]. Rac activation by a chemoattractant, whether it is a ligand for a GPCR or a receptor tyrosine kinase, is at least in part dependent on PI 3-kinase. The product of PI 3-kinase produces PI-3,4,5-P_3, which mediates recruitment and activation of signaling molecules including a Rac-guanine nucleotide exchange factor such as Tiam-1. PI-3,4,5-P_3 is de-phosphorylated by the 3'-specific phosphoinositide phosphatase "Phosphatase and

Tensin Homolog Deleted from Chromosome 10" (PTEN). PTEN was found to be stimulated by $S1P_2$ [40]. However, $S1P_2$-mediated inhibition of Rac and migration does not seem to involve inhibition of PI 3-kinase or stimulation of PTEN [41]. $S1P_2$ mediates elevation of cyclic AMP, which could mediate inhibition of cell migration. However, this is also unlikely because different from the cases of PGE_2 and isoproterenol as stated above, $S1P_2$ activation induces chemorepulsion. Various cells, e.g. endothelial cells and smooth muscle cells, express multiple S1P receptor subtypes. A net effect of S1P on cell migration is likely determined by integration of the counteracting signals input by the chemoattractant receptors $S1P_1$ and $S1P_3$ and the chemorepellent receptor $S1P_2$.

Regulation of vascular formation by S1P receptor signaling

Angiogenesis is a complex process comprising EC proliferation and migration, cell-cell adhesion, and mural cell recruitment [36]. The first discovery of an in vivo angiogenic activity of S1P came from the observation that S1P stimulated angiogenesis in the Matrigel implants in mice. S1P induced directed migration of endothelial cells via G_i and proliferation [28]. S1P also facilitates adherens junction assembly in an $S1P_1$-G_i-Rac- and $S1P_3$-$G_{12/13}$-Rho-dependent manner, leading to stimulation of

capillary-like tube formation. $S1P_1$-null mouse embryo is defective in recruiting pericytes and SMCs to vessels, i.e. vascular maturation or stabilization [9] (see below for more detail). Conditional EC-specific deletion of $S1P_1$ results in the similar vascular maturation defect to global $S1P_1$ deletion, indicating that vessel coverage by mural cells is directed by $S1P_1$ in ECs. In contrast to $S1P_1$-null mice, either $S1P_2$- or $S1P_3$-single null mice are alive without a vascular formation defect. However, compared with mice null for $S1P_1$ alone, embryos null for both $S1P_1$ and $S1P_2$, null for both $S1P_1$ and $S1P_3$, and null for all of $S1P_1$, $S1P_2$ and $S1P_3$ exhibit more severe vascular phenotypes including a vascular maturation defect and hemorrhage with earlier intrauterine death [42]. $S1P_1$ is the most important receptor for vascular development while $S1P_2$ and $S1P_3$ possess partially redundant and cooperative functions in S1P regulation of vascular formation.

S1P signaling is involved in pathological angiogenesis including tumor neovascularization. In a tumor cell implantation model in mice, $S1P_1$ is upregulated in vessels at sites of tumor implantation [15]. $S1P_1$ silencing by repeated local injections of $S1P_1$-specific siRNA suppresses tumor angiogenesis and vascular maturation. Administration of monoclonal anti-S1P neutralizing antibody inhibits tumor growth [43]. The effectiveness of anti-S1P antibody is substantial and more than that obtained

with monoclonal anti-VEGF antibody. This anti-tumor effect is likely due to inhibition of both angiogenesis and tumor cell motility, survival and proliferation [44]. Interestingly, anti-S1P antibody suppresses VEGF- and FGF-induced angiogenesis in Matrigel plugs in mice, suggesting that endogenous S1P plays a permissive role in angiogenesis or functions downstream of VEGF and FGF.

In contrast to $S1P_1$, $S1P_2$, which is also expressed in ECs, inhibits growth factor-induced Rac activation, cell migration and capillary-like tube formation via a $G_{12/13}$/Rho-dependent mechanism [38]. The $S1P_2$-selective antagonist JTE-013 enhances S1P-induced angiogenesis in Matrigel plugs in mice [36]. In murine retinal angiogenesis model, $S1P_2$ inhibits post-natal physiological angiogenesis in avascular areas of the retina [45]. Thus, different from $S1P_1$, $S1P_2$ is a negative regulator of angiogenesis. $S1P_2$ deletion enhances angiogenesis in implanted tumors with accelerated tumor growth [31]. In tumors, $S1P_2$ is expressed in ECs and mural cells in tumor vessels. In $S1P_2$-null mice, the coverage of tumor neovessels with pericytes and SMCs is enhanced compared with wild-type mice. VEGF- and FGF2-induced microvascular formation and mural cell coverage in matrigel plugs are also enhanced in $S1P_2$-null mice, suggesting that angiogenesis induced by these growth factors is egatively affected by $S1P_2$.

The ECs isolated from $S1P_2$-null mice display altered phenotypes compared with wild-type ECs: $S1P_2$-null ECs show increased cell proliferation, migration and the formation of tube-like tube structures in response to growth factors compared with wild-type ECs [31]. In $S1P_2$-null MLECs, two major changes in the intracellular signals are noted. Both the basal and S1P-stimulated activities of Rac are greater in $S1P_2$-null ECs compared with wild-type ECs. Secondly, in wild-type ECs S1P inhibits VEGF-induced activation of Akt but not ERK whereas S1P fails to inhibit Akt activation in $S1P_2$-null ECs. Thus, $S1P_2$ seems to mediate S1P-induced Akt inhibition in wild-type ECs. The Akt inhibition is probably mediated through PTEN stimulation, which reduces amount of PI-3,4,5-P_3 [40]. Thus, $S1P_2$ inhibition of angiogenesis involves the $G_{12/13}$-Rho-Rac/PTEN signaling pathway in ECs.

In addition to ECs, $S1P_2$ is also expressed in $CD11b^+$ positive bone marrow-derived cells (BMDCs) in the tumor stroma [31]. Myeloid cells including $CD11b^+$ cells participate in tumor angiogenesis through multiple mechanisms [36]. Infiltrating myeloid cells in tumors release pro-angiogenic factors including VEGFs, FGF-2, PDGFs and matrix metalloproteases (MMPs), the enzymes that contribute to angiogenesis through degradation of the extracellular matrix proteins and resultant release of VEGFs and TGFβ that has been deposited in the matrix. A subpopulation of

BMDCs is capable of transdifferentiating into vascular ECs and become incorporated into the new blood vessels in tumors. In $S1P_2$-null mice, $CD11b^+$ cells infiltrating into tumors are increased compared with wild-type mice [31]. Bone marrow chimera experiments document that $S1P_2$ in BMDCs exerts an inhibitory effect on tumor angiogenesis.

Thus, $S1P_2$ exerts inhibitory effects on tumor angiogenesis through both the EC-autonomous and myeloid cell-dependent actions. These $S1P_2$ actions open the possibility of a novel anti-angiogenic therapy to target $S1P_2$. It is an interesting possibility that S1P receptor subtype-selective pharmacological targeting strategies, i.e. $S1P_1$ inhibition in combination with $S1P_2$ activation, could lead to more effective inhibition of tumor angiogenesis. In addition to an expected anti-angiogenic action of $S1P_2$-selective agonist, $S1P_2$ stimulation in tumor cells could directly inhibit tumor progression in vivo, leading to inhibition of invasion and metastasis, as previously demonstrated [44, 46].

Regulation of vascular homeostasis by S1P receptor signaling

S1P regulates vascular tone by acting on both the endothelium and smooth muscle through multiple S1P receptors. In ECs, $S1P_1$ is most abundant with $S1P_2$ and $S1P_3$

being expressed at much lower levels, whereas in smooth muscle the expression of $S1P_2$ and $S1P_3$ are abundant with $S1P_1$ expression being very low [13]. S1P-induced relaxation is mediated through its action on the endothelium whereas S1P directly contracts smooth muscle. In ECs, S1P stimulates a calmodulin-dependent enzyme, eNOS, which produces nitric oxide (NO). NO diffuses into the underlying smooth muscle to induce relaxation through generating cyclic GMP. This S1P action is mediated via $S1P_1$ and $S1P_3$, which activate Akt through PI 3-kinase to phosphorylate eNOS [47]. $S1P_1$ and $S1P_3$ also activate PLC to mobilize Ca^{2+}, which fully activates eNOS in concert with Akt-mediated phosphorylation. Although G_q-coupled $S1P_3$ more robustly activates PLC compared with G_i-coupled $S1P_1$, the contribution of $S1P_1$ seems to dominate in eNOS stimulation because $S1P_1$ expression is higher in ECs compared with $S1P_3$. In smooth muscle, S1P activates Rho and Rho kinase via $S1P_2$/$S1P_3$ and $G_{12/13}$ [41]. Rho kinase phosphorylates the myosin targeting subunit, MYPT1, of myosin phosphatase and the myosin phosphatase inhibitor protein, CPI-17, to inhibit myosin phosphatase. The myosin light chain kinase activation by PLC-Ca^{2+}, together with myosin phosphatase inhibition by Rho-Rho kinase, efficiently increases myosin light chain phosphorylation and, thereby, vascular contraction. $S1P_2$ is also suggested to contribute to vascular tone through a mechanism involving the action on the

endothelium although the precise mechanism remains to be defined [48].

S1P contributes to vascular barrier integrity. Initially, S1P was found to enhance barrier function of an EC monolayer and to protect barrier disruption induced by the edemagenic agent thrombin [49]. This effect is mediated by $S1P_1$ and, to the lesser extent, $S1P_3$ through G_i-PI 3-kinase-Rac. In contrast to $S1P_1$, $S1P_2$, when overexpressed in vitro in ECs, disrupts barrier integrity via Rho-Rho kinase-PTEN pathway [50].

Endothelial barrier dysfunction, which increases vascular permeability, occurs in inflammation, tumor neovessels and atherosclerotic lesions. Challenge with lipopolysaccharide (LPS) or thrombin induces an increase in pulmonary microvascular permeability. $S1P_1^{+/-}$ mice exhibited reductions in barrier protection by administering a moderate dose of S1P or the S1P1-selective agonist SEW-2871, after LPS challenge [51]. In contrast, $S1P_2^{-/-}$ mice were protected from LPS-induced barrier disruption compared with wild-type mice. Barier disruption is also enhanced in SphK1-null mice. Adenoviral transduction of SphK1 into the lung protects mice from barrier disruption whereas that of SphK2 rather augments it, indicating the distinct roles of SphK1 and SphK2 [52]. The intravenous or intratracheal administration of S1P is protective against LPS-induced barrier disruption [51]. However, a higher dose of S1P or repeated administration of $S1P_1$ agonists (FTY720 and AUY954) rather exacerbates barrier

disruption by stimulating internalization and degradation of $S1P_1$ protein in a lung injury model [53], highlighting the importance of $S1P_1$ agonist concentration.

Plasma S1P concentration is another critical determinant for maintaining barrier integrity. In inducibly SphK1-deleted mice with $SphK1^{fl/-}$:$SphK2^{-/-}$/Mx1-Cre Tg+ (S1Pless mice), which show approximately 30 nM plasma S1P compared with 2.5 μM in control mice, vascular leak on anaphylaxis and administration of platelet-activating factor or histamine is augmented with impaired survival [16]. Transfusion of erythrocytes, which restores plasma S1P levels, or acute administration of an $S1P_1$ agonist reverse vascular leak and prevent death. In contrast, SphK2-null mice have a rapid recovery from anaphylaxis [54]. $S1P_2$- but not $S1P_3$- null mice also show poor recovery from anaphylaxis. S1P infusion fails to promote recovery of $S1P_2$-null mice from anaphylaxis.

Physiological levels of endothelial $S1P_1$ and SphK1-produced S1P serve a constitutive maintaining role for vascular barrier function. Exogenous supraphysiological $S1P_1$ agonists impair this mechanism by downregulating $S1P_1$. Furthermore, $S1P_2$ participates in the vascular protection from anaphylaxis although the precise mechanism of the $S1P_2$ action remains to be fully defined.

Modulation of leukocyte functions and inflammation by S1P signaling

The role of S1P signaling as significant modulator of leukocyte functions and inflammation has emerged. SphK1-derived S1P regulates pro-inflammatory signaling pathways, including activation of nuclear factor-κB [55]. $S1P_1$ regulates endothelial barrier integrity as stated above [49-52, 56]; cytokine and adhesion molecule expression, lymphocyte maturation, differentiation and trafficking, and mast cell migration. $S1P_2$ also regulates B lymphocyte survival and confinement in lymph node follicles [57]. $S1P_3$ modulate dendritic cell trafficking and activation. In addition, $S1P_5$ regulates NK cell trafficking [12].

$S1P_1$-G_i signaling pathway regulates trafficking of lymphocytes and other immune cells by directing migration of immune cells toward a compartment with a relatively higher S1P concentration. Therefore, the existence of a S1P concentration gradient between compartments, e.g. lymphoid tissue parenchyma and blood plasma/lymphatic fluid, which is created and maintained by the SphK-catalyzed S1P production by erythrocytes and vascular/lymphatic endothelial cells and SPL- and LPP3-catalyzed S1P degradation in lymphoid tissue parenchyma, is critical. $S1P_1$ expression on the cell surface of lymphocytes and other immune cells is maintained in a low S1P environment in the thymus and lymph nodes, through its inhibited internalization/degradation or

upregulation as a result of lymphocyte maturation and interaction with other immune cells within lymphoid tissues [12]. $S1P_1$ also participates in the regulation of lymphocyte recirculation through tightening the cell-cell junction of sinus-lining ECs [56]. $S1P_2$-$G_{12/13}$ pathway ensures the localization of $S1P_2$-expressing B cells in a follicular center in lymph nodes [57]: S1P concentration is higher at the follicle perimeter than the follicular center due to S1P production by stromal cells abundant at the perimeter and rapid S1P degradation by follicular B cells in the center. In the presence of this S1P concentration gradient, migration of $S1P_2$-expressing B cells from the center to the perimeter of a follicle is impeded by the chemorepellent activity of $S1P_2$ through Rho-induced Rac inhibition. The low S1P environment at the follicular center also favors survival and proliferation of $S1P_2$-expressing B cells because the mitogenic and survival signaling molecule Akt, which is negatively regulated by $S1P_2$-$G_{12/13}$-Rho-PTEN, is spared from suppression.

SphK1 are involved in inflammation through both the extracellular messenger and intracellular messenger actions of S1P [12]. In a septic model due to bacterial peritonitis, thrombin, which is produced by coagulation reaction, binds to and activates the GPCR proptease-activated receptor-1 (PAR1) on dendritic cells involved in innate immunity [58]. The activation of PAR1 in turn stimulates SphK1, S1P export to the cell exterior,

and $S1P_3$ activation, which induces amplification of inflammation by stimulating the production of IL-1 and tissue factor from dendritic cells and disrupting EC barrier function. SphK1 is also implicated in the actions of tumor necrosis factor (TNF) and other cytokines, in which intracellular S1P produced by SphK1 binds to TRAF2 and thereby activates NE-κB [55]. Disruption of SphK1 gene alleviates inflammatory diseases including colitis and arthritis, providing further support for the involvement of SphK1 in inflammatory responses [12]. In addition to the intracellular action of SphK1-generated S1P, a recent study [59] showed that S1P produced by SphK2 in the nucleus bound to the histone deacetylases HDAC1 and HDAC2 and inhibited their enzymatic activity, which suggested that HDACs are direct intracellular targets of S1P. Furthermore, S1P generated by SphK2 in mitochondria plays the important role in cytochrome-c oxydase assembly and respiration [60].

Conclusion

There is now broad consensus that S1P signaling plays a crucial role in the physiology and pathophysiology of the cardiovascular, immune and other systems. Observations obtained with gene-engineered mice and pharmacological tools to target

receptors and enzymes rapidly promote our understsanding S1P functions. Investigation in more depth into involvements of S1P signaling in various diseases, in combination with development of drugs with improved specificity and efficacy and their optimal drug delivery system, will provide new treatment strategies.

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Figure legends

Figure 1. Sphingolipid metabolism in various subcellular compartments

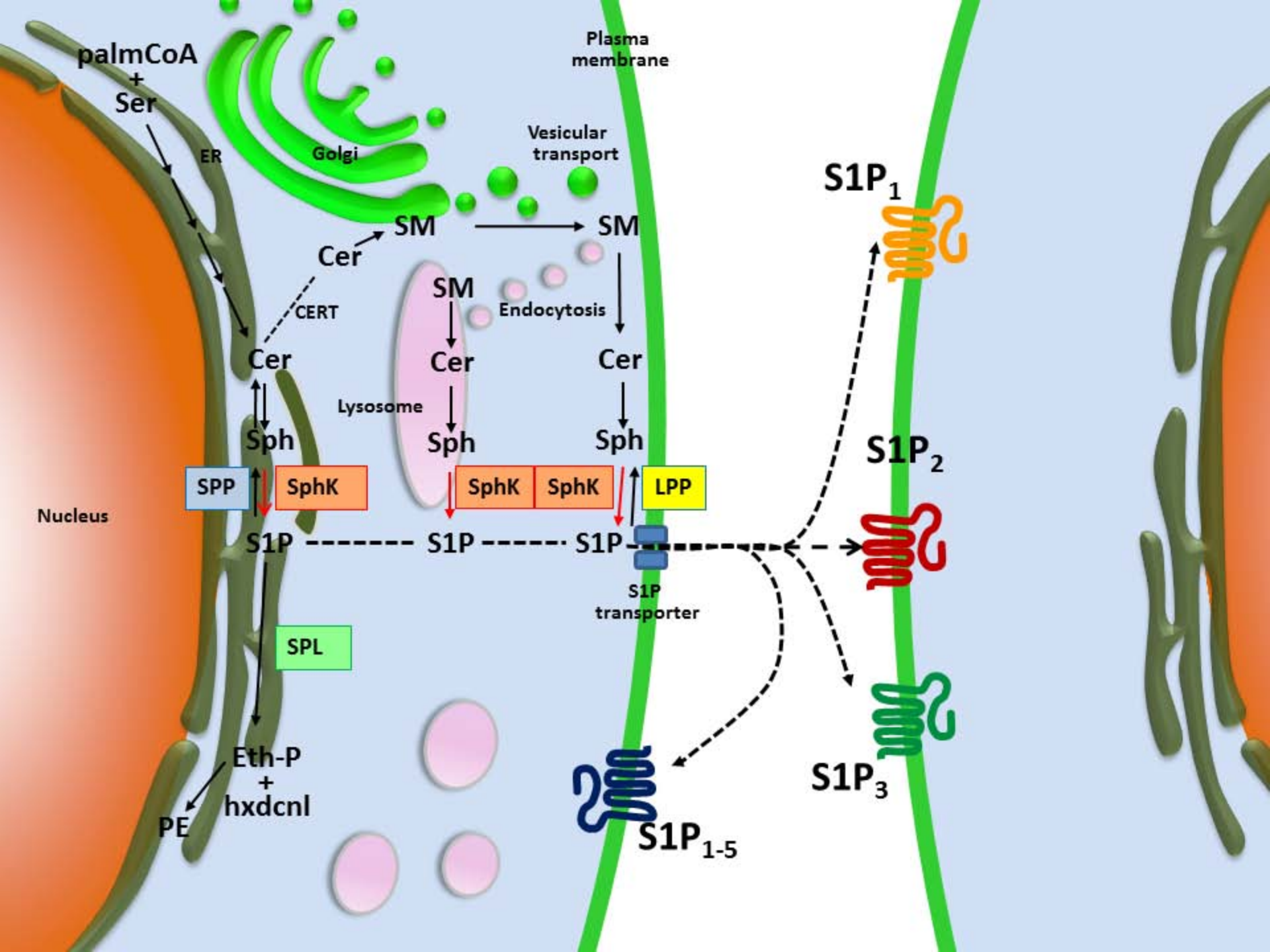
Ceramide (Cer) is produced either by de novo synthesis from palmitoyl CoA (palmCoA) and serine with sequential enzymatic reactions in endoplasmic reticulum (ER) or through degradation of sphingomyelin (SM) by the action of sphingomyelinases in the plasma membrane and intracellular membranes including lysosomes. Cer is deacylated by ceramidase to yield sphingosine (Sph), which is then phosphorylated by SphK1/2 to generate S1P. S1P is exported through a plasma membrane S1P transporter, leading to activation of the G protein-coupled S1P receptor subtypes ($S1P_1$~$S1P_5$). S1P could be either dephosphorylated by S1P phosphatase1/2 (SPP) and lipid phosphate phosphatase1-3 (LPP) back to Sph or degraded to ethanolamine-phosphate (Eth-P) and hexadecenal (hxdcnl) by S1P lyase (SPL) to leave sphingolipid metabolic pathway. SphK1 is present in both cytosolic and membrane-bound fractions, both being enzymatically active. SPPs and SPL are located in ER. At least, a subtype of LPPs exists on the plasma membrane. Intracellular transfer of Cer from ER to Golgi is facilitated by transfer proteins such as CERT, and both Cer and SM traffic between membrane compartments via vesicular transport.

Figure 2. S1P receptor subtype-specific heterotrimeric G protein coupling and intracellular signaling mechanisms

$S1P_1$ couples exclusively to G_i to activate Ras-ERK and PI 3-kinase-Akt/Rac pathways, leading to stimulation of chemotaxis and cell proliferation. $S1P_2$ couples to multiple G proteins, especially to $G_{12/13}$ to induce robust Rho activation, leading to inhibition of Rac and cell migration, and also inhibition of cell proliferation via inhibition of Akt. $S1P_2$ also couples to stimulation of adenylate cyclase via G_{13}. $S1P_3$ activates G_q-PLC-Ca^{2+} pathway, and G_i-Ras-ERK and G_i-PI 3-kinase-Akt/Rac pathways. $S1P_3$-$G_{12/13}$-Rho pathway becomes evident only when G_i is inhibited by pertussis toxin.

Table 1. Characteristics of S1P receptors

Receptor	G protein coupling	Expression	Agonist	Antagonist	Phenotypes of knockout mice
$S1P_1$	Gi	Widespread	FTY720-P SEW2871 AUY954 AAL-R CYM5442 KRP-203 RP-002	W146 VPC-23019 VPC-44116	Embryonic lethal Impaired vascular maturation Abnormal brain development Abnormal lymphocyte trafficking
$S1P_2$	$G_{12/13}$ G_q G_i	Widespread	None	JTE-013	Seizure Hearing loss Vestibular ataxia Decreased blood pressure High incidence of B cell lymphoma Small litter size
$S1P_3$	G_q $G_{12/13}$ G_i	Widespread	FTY720-P AAL-R KRP-203	VPC-23019 VPC-44116	Attenuated inflammation in sepsis Decreased nitric oxide production in vascular endothelium Disappearance of pressor response and bradycardia to S1P
$S1P_4$	G_i $G_{12/13}$	Lymphoid tissues, lung	FTY720-P AAL-R KRP-203	None	Unknown
$S1P_5$	G_i $G_{12/13}$	Brain, spleen	FTY720-P AAL-R KRP-203	None	Abnormal trafficking of NK cells


palmCoA
+
Ser
ER
Golgi
Plasma
membrane
Vesicular
transport
SM
Cer
CERT
Cer
SM
Endocytosis
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Cer
Lysosome
Sph
Sph
Sph
SPP
SphK
SphK
SphK
LPP
Nucleus
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S1P
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S1P3
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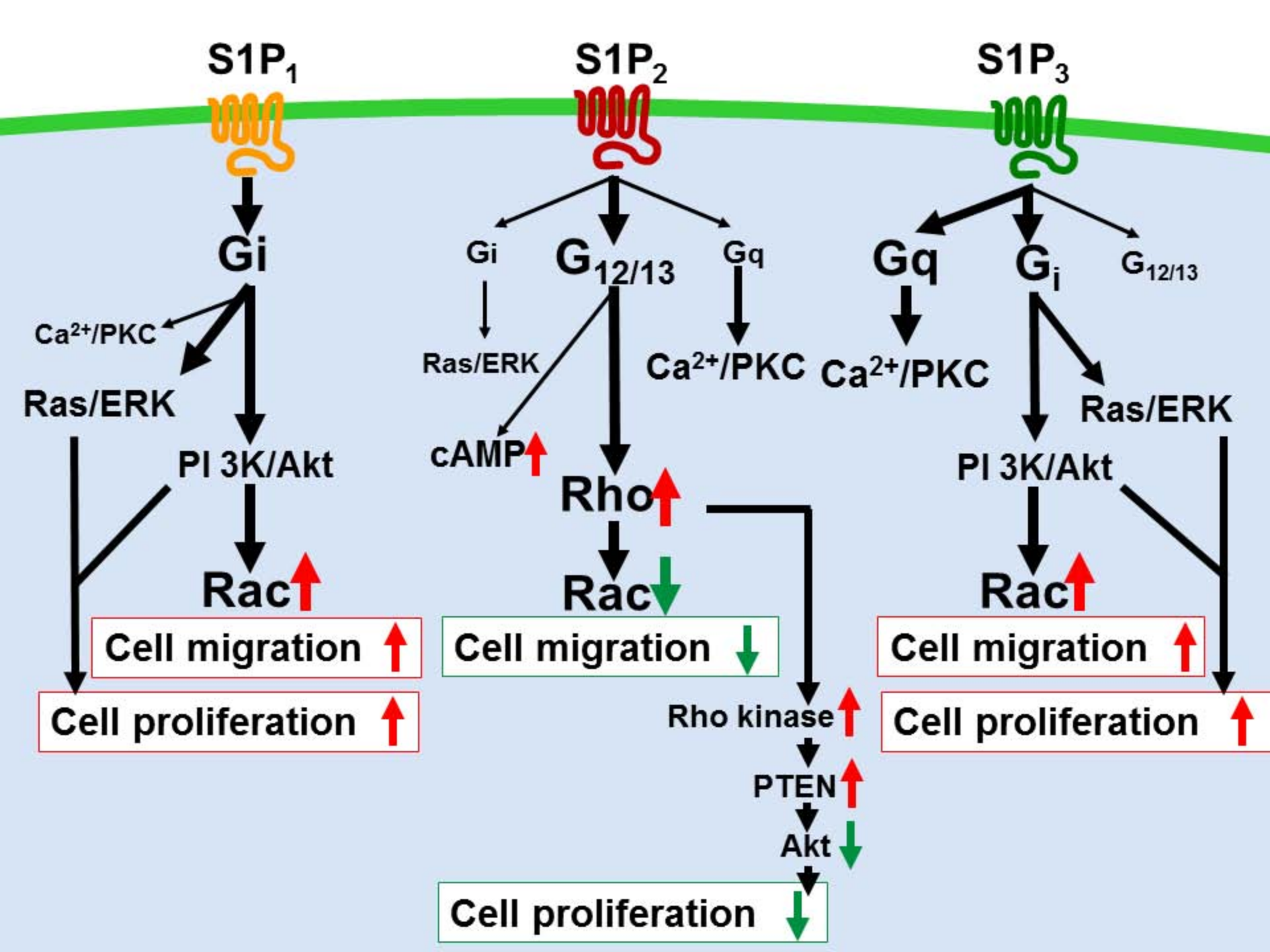
S1P1
S1P2
S1P3
Gi
Ca2+/PKC
Ras/ERK
PI 3K/Akt
Rac
Cell migration
Cell proliferation
Gi
G12/13
Gq
Ras/ERK
Ca2+/PKC
cAMP
Rho
Rac
Cell migration
Rho kinase
PTEN
Akt
Cell proliferation
Gq
Gi
G12/13
Ca2+/PKC
Ras/ERK
PI 3K/Akt
Rac
Cell migration
Cell proliferation