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Shaping the landscape: Metabolic regulation of S1P gradients

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Abstract

Sphingosine-1-phosphate (S1P) is a lipid that functions as a metabolic intermediate and a cellular signaling molecule. These roles are integrated when compartments with differing extracellular S1P concentrations are formed that serve to regulate functions within the immune and vascular systems, as well as during pathologic conditions. Gradients of S1P concentration are achieved by the organization of cells with specialized expression of S1P metabolic pathways within tissues. S1P concentration gradients underpin the ability of S1P signaling to regulate in vivo physiology. This review will discuss the mechanisms that are necessary for the formation and maintenance of S1P gradients, with the aim of understanding how a simple lipid controls complex physiology.

Keywords

sphingosine-1-phosphate; sphingolipid; signaling; gradient; receptor; metabolism

1. Introduction

Sphingosine-1-phosphate (S1P)[1] is the terminal product of sphingolipid metabolism, a process that occurs, in some form, in all mammalian cells. Produced intracellularly, S1P can be transported to extracellular compartments where it functions as a signaling molecule by regulating important aspects of normal physiology, including lymphocyte trafficking and vascular barrier function, and of disease states, such as inflammation and cancer [1–5].

The cellular S1P signaling pathways are directed by a family of high affinity G-protein coupled S1P receptors (S1PR1-5) [6]. The S1P receptors are among the most abundant and most widely expressed of the greater than 300 nonodorant G-protein coupled receptors, and are particularly concentrated in the immune and vascular systems [7]. Diverse signaling responses are elicited by S1P through distinct combinatorial expression patterns of receptors

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[1]Abbreviations: S1P, sphingosine-1-phosphate; ER, endoplasmic reticulum; SphK, sphingosine kinase; ABC, ATP-binding cassette; LPP, lipid phosphate phosphatase

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on different cell types and the unique heterotrimeric G protein coupling capacity of each receptor subtype [8].

S1P receptor signaling is critical for regulating the in vivo physiology of the immune and vascular systems [9–14]. S1P receptor expression on lymphocytes is required for their continuous recirculation throughout the body as they search for their cognate antigens [5, 15]. S1P receptor signaling on endothelial cells has been found to be critical for maintaining a tight vascular endothelial barrier during development and homeostasis, and for maintaining or restoring barrier function when the vasculature is confronted with conditions that induce leakage, such as during anaphylaxis and inflammation [16–19]. If S1P can be made by all cells, and the S1P receptors are ubiquitously expressed, how is S1P signaling regulated in vivo? S1P concentration gradients appear to be a key determinant. It was first recognized through the work of Stoffel [20] that S1P formed in most tissues was rapidly degraded by an S1P lyase, a process that would be expected to keep tissue levels of S1P low. Later, it was shown by Yatomi et al. that serum and plasma had very high levels of S1P due to the lack of S1P degradation capacity in cells in circulation that synthesized S1P [21]. These observations formed the initial biochemical parameters of a gradient of high S1P concentrations in circulation with much lower levels within tissues. However, the functional importance of the gradient was not understood until Schwab et al. showed that an inhibitor of S1P lyase, which raised lymphoid tissue S1P levels, blocked lymphocyte egress from these tissues into blood and lymph [22]. These results cemented the concept that the S1P concentration differential between tissues and the circulation was functionally important for the regulation of the in vivo biological functions of S1P.

Both S1P receptor-dependent lymphocyte egress and vascular barrier function depend on the ability of lymphocytes and endothelial cells to sense S1P in a spatially defined manner. The S1P ligand provides a signal to lymphocytes, instructing them to migrate out of primary and secondary lymphoid organs (low S1P zones) into the circulation, both blood and lymph (high S1P zones). The concentration of S1P in blood is in the micromolar range [22]. In lymph fluid, the S1P concentration has been reported to be greater than 100 nanomolar [22]. Interstitial concentrations have not been systematically measured, but in lymphoid tissues that support S1P receptor-mediated lymphocyte egress, the functional S1P levels are believed to be in the very low or subnanomolar range [22]. For vascular barrier function, the levels of S1P in the circulation are essential, although exactly how endothelial cells sense the S1P signal is not known [23]. Beyond its specific immune and vascular effects, S1P is also a pleiotropic mediator [3]; it elicits responses in most cells of the body due to the activation of particular combinations of the five widely expressed S1P receptors. In order to carry out timely and effective responses through efficient activation of these receptors by acute increases of S1P, baseline S1P levels in tissue interstitial space must be kept low. Failure to maintain high S1P levels in circulatory fluids and low levels in tissue interstitial space results in dramatic phenotypes such as lymphopenia, neutrophilia, vascular barrier dysfunction, and other tissue malfunctions [22, 24–27].

We will summarize the latest findings that have begun to unravel the intricacy of how distinct concentration zones or gradients of S1P are created and maintained. Furthermore, we will discuss the involvement of cells that can temporarily alter S1P homeostasis in tissues or microenvironments, enabling S1P to function in pathological processes.

2. S1P Metabolism and transport

The metabolism of S1P is complex (Fig. 1). It can be produced both homeostatically and acutely. After synthesis, which occurs in different cellular compartments, it has several

possible fates: it can be degraded; its sphingoid backbone can be recycled; or it can be secreted, where its metabolism continues. Here we describe the metabolic pathways of S1P.

2.1 Synthesis of sphingosine

Sphingosine, the direct precursor substrate for S1P synthesis, is formed only from the degradation of ceramide by ceramidase [28]. Because this reaction is the only one leading to the formation of sphingosine, ceramidase activities are essential for the generation of S1P. Ceramide is produced from the de novo synthetic pathway initiated by serine palmitoyl transferase in the endoplasmic reticulum (ER), or from the degradation of complex sphingolipids, such as sphingomyelin and glycosphingolipids, in other compartments. Ceramide degradation to sphingosine is performed by any of five ceramidases: acid, neutral, and alkaline ceramidases 1, 2, and 3 [28]. These enzymes are ubiquitously expressed in tissues, except for alkaline ceramidase 1, which is found mainly in skin [28]. These ceramidases have different subcellular locations, which suggests that they may deal with different pools of ceramide (de novo or recycled). Acid ceramidase is found mainly in lysosomes [29], although its release from endothelial cells has been proposed [30]. Neutral ceramidase is a plasma membrane enzyme with its catalytic center exposed to the extracellular space [31]. In the intestine, its active site is exposed to the lumen, where it is engaged in the degradation of dietary sphingolipids [32]. Alkaline ceramidase 1 is localized to the ER [31], whereas alkaline ceramidase 3, also known as phytoceramidase, is localized both to the ER and Golgi apparatus [33]. Human alkaline ceramidase 2 is exclusively a Golgi enzyme [34]. As a consequence of the subcellular locations of the ceramidases, sphingosine formation can potentially take place through the action of alkaline ceramidase 1 and 3 in the ER (where the machinery for de novo ceramide synthesis resides), at the plasma membrane by neutral ceramidase, in lysosomes by the action of acid ceramidase, and in the Golgi by alkaline ceramidase 2 and 3. How each ceramidase contributes to S1P gradients is not known.

2.2 Synthesis of S1P

S1P is generated by the ATP-dependent phosphorylation of sphingosine by sphingosine kinase (SphK). Two SphK isoforms have been described, SphK1 and SphK2; each has distinct catalytic activities, intracellular locations, and tissue distributions [35], suggesting unique functions. In vivo, SphK1 and SphK2 are at least partially redundant [36], because the double *Sphk1/Sphk2* knockout shows lethality, in contrast to the respective single knockout mice, which are viable [36, 37]. The global deletion of *Sphk1* in mice results in the decrease of S1P levels in serum [37], suggesting a role for SphK1 in the synthesis of circulating S1P. Surprisingly, deletion of *Sphk2* increases S1P levels in blood [38, 39] due to an impaired uptake of S1P across endothelial and tissue cells, a process that involves dephosphorylation of S1P, re-phosphorylation by SphK2 and irreversible degradation of the newly formed S1P [38]. These observations may reflect a division of functions for the SphKs, with the S1P synthesized by SphK1 destined for secretion, and S1P synthesized by SphK2 destined for intracellular metabolism [38, 40, 41]. In this scenario, deletion of *Sphk2* could result in the redirection of some its substrate, sphingosine, to an SphK1-mediated secretory pathway for S1P.

In unstimulated cells, SphK1 is a cytosolic enzyme [42], although a minor fraction is constitutively exported from endothelial cells, possibly contributing to S1P gradient formation [43]. SphK2 is found mainly intracellularly in the cytoplasm, the nucleus, and in other compartments depending on the cell type, and can be also activated by epidermal growth factor and engagement of the high affinity receptor for IgE [44, 45].

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S1P levels can be elevated by an acute increase in SphK activation by several stimuli. In particular, SphK1 is translocated to the plasma membrane in response to agonists such as tumor necrosis factor alpha, phorbol esters, and lipopolysaccharide, producing S1P that is either utilized intracellularly or exported outside of the cell [40, 46–48].

Surprisingly, S1P newly made either at the ER or at the plasma membrane seems to follow the same metabolic route inside the cells, via action by S1P lyase and S1P phosphatases in the ER [49]. The implication of this finding is that S1P, after synthesis, moves rapidly between cell compartments.

2.3 Intracellular degradation of S1P

2.3.1 S1P lyase—At the final step in the metabolic pathway of sphingolipids, S1P lyase, an ER-resident enzyme, irreversibly breaks down S1P to generate phosphoethanolamine and hexadecenal (or hexadecanal, in the case dihydrosphingosine-1-phosphate). It is encoded by the *Sgpl1* gene in mice, and is highly conserved through eukaryotic evolution [50]. It is expressed throughout embryonic development [51] and widely in tissues in adult mice [25]. S1P lyase activity has not been found in platelets or red blood cells [21, 52].

S1P lyase degradation is the only "way out" of the sphingolipid metabolic pathway [49]. Indeed, when the *Sgpl1* gene was deleted, homozygous *Sgpl1*$^{-/-}$ mice accumulated large amounts of S1P in serum and in tissues [25–27]. Levels of S1P in some tissues reach several hundred-fold greater than control tissue, demonstrating the importance of S1P lyase in maintaining low tissue levels of S1P. Pharmacologic inhibition of S1P lyase in mice using 2-acetyl-4-tetrahydroxybutyl imidazole also greatly increased S1P levels in tissues [22]. In the *Sgpl1* knockout mice, not only S1P, but also sphingosine, ceramide, and sphingomyelin, were found highly elevated in liver and serum, consistent with the reutilization of the sphingosine backbone for the synthesis of other sphingolipids in the absence of the final degradative enzyme of the pathway [25].

2.3.2 S1P phosphatases—Intracellular S1P is also metabolized by the action of S1P phosphatase 1 (Sgpp1) and Sgpp2, two S1P-specific phosphohydrolases localized in the ER that are responsible for the desphosphorylation of S1P back to sphingosine [53–56]. Sgpp1 was shown to regulate the synthesis of long-chain ceramide levels by acting mainly on S1P generated by SphK2 [41, 57]. Knockdown of *Sgpp1* has also been shown to increase the amount of secreted S1P from HEK293 cells [58]. In vivo, both S1P phosphatases have different expression patterns in adult tissues: *Sgpp1* mRNA is ubiquitously expressed, with high levels found in lung, placenta, and kidney [56, 58], whereas *Sgpp2* mRNA has a more restricted pattern of expression, mainly in heart, kidney, and small intestine [56]. However, their individual roles *in vivo* are largely unknown.

2.4 Export of S1P

S1P is largely produced inside the cell. In order to maintain elevated extracellular concentrations for the formation of a gradient, S1P is secreted via specific transporters.

2.4.1 ABC transporters—In vitro studies have suggested that some members of the ATP-binding cassette (ABC) transporter family are responsible for S1P export [59]. These are transmembrane proteins using ATP to translocate diverse substrates across membranes, including sterols, drugs, peptides, and lipids. Among them, ABCC1 transporter has been found to release S1P from mast cell lines [60], ABCA1 to release S1P from astrocytes [61], and ABCA7 to release S1P from red blood cells [62] and presumably from platelets [63]. Despite this evidence, the study of single knockout ABCC1, ABCA1, and ABCA7 mice

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demonstrated normal S1P concentrations in blood and tissues and failed to reveal a role of these proteins in vivo in maintaining the S1P levels [64].

2.4.2 Spns2 transporter—Recently, a transporter for S1P was identified in zebrafish. Known as two of hearts (toh), it is essential for the migration of heart cell precursors during embryogenesis [65, 66]. The gene (*Spns2*) encodes a member of the Spinster-like family of multipass transmembrane proteins that was first identified in *Drosophila* [67]. When expressed in CHO cells, both *Spns2* and the human orthologue (*SPNS2*) enabled cellular secretion of S1P [65, 68].

Global deletion of the *Spns2* gene in mice resulted in about half the normal concentration of S1P in plasma [69]. Similar results were obtained with an endothelial cell-specific *Spns2* knockout, indicating that Spns2 on endothelial cells releases S1P into the blood, and that endothelial cells are responsible for a substantial fraction of the S1P concentration in blood [69]. This transporter was found not to be responsible for the release of S1P from hematopoietically derived cells [69].

2.5 Extracellular degradation of S1P by lipid phosphate phosphatases

It has been recognized that S1P can be rapidly dephosphorylated in the extracellular media [70] or when injected intravenously into mice [83, 84]. Dephosphorylation of S1P seems to be necessary for uptake of sphingosine and further metabolism inside cells [70, 71]. S1P *in vitro* can be dephosphorylated by unspecific phosphatases (i.e alkaline phosphatase) [72], but in the extracellular media is likely to occur by members of the lipid phosphate phosphatase (LPP) family, which are located at the plasma membrane. LPP1, LPP2, and LPP3 have six transmembrane domains and a catalytic site facing the extracellular side. These lipid phosphatases are able to act on S1P as well as on phosphatidic acid, lysophosphatidic acid, ceramide 1-phosphate, and FTY720-phosphate [73]. LPP3 has been implicated in the formation of an S1P gradient functioning in the egress of thymocytes, as will be discussed below (see section 2.6.3). Thus, extracellular dephosphorylation of S1P seems to be mediated by more promiscuous phosphatases, while only SphK specifically controls the synthesis of S1P.

2.6 Cellular regulation of extracellular S1P gradients

Sphingolipid metabolism exists in some form in all cells. However, some cell types are specialized metabolically to produce high extracellular levels of S1P. Other cell types are metabolically geared toward keeping the extracellular S1P levels low. The organization of cell types with varying S1P metabolism underlies the formation of S1P gradients.

2.6.1 Red blood cells—Most blood cells can produce S1P and, unlike other types of cells, export S1P efficiently in vitro [52, 74]. It is, however, red blood cells that contain about half of all blood S1P [52] and represent the major cell source for S1P in the vascular system [23, 52, 75]. The critical role of red blood cells in the homeostasis of plasma S1P levels was elegantly demonstrated by adoptive transfer experiments of lethally irradiated *Sphk2*-deficient mice with a conditional deletion in *Sphk1*. These mice have no circulating S1P, a deficiency that could be restored in blood (although not in lymph) only by reconstitution with red blood cells from normal donors [23]. The correlations between the levels of plasma S1P and the number of red blood cells, hemoglobin content, and hematocrit in normal individuals [76] and the differences in S1P levels between anemic and normal individuals [77] are also indicative of the important contribution of red blood cells to the regulation of blood S1P levels. What makes red blood cells ideal for this function is not only that they are the most abundant cell type in blood, but also that they are equipped with the right combination of enzymes to favor the generation and preservation of S1P. Red blood

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cells contain high SphK activity (mostly SphK1 [19, 78]) and lack the degradation enzymes S1P lyase and the S1P phosphohydrolases. Furthermore, the presence of alkaline ceramidase activity [79], the only ceramidase expressed in these cells [52, 79], could ensure an intrinsic supply of sphingosine for the generation of S1P. Intrinsic alkaline ceramidase was found to contribute to the formation of S1P by erythroid cells (differentiated in vitro from human leukemia K562 cells) [79], but surprisingly not in red blood cells from whole human blood [75]. Mice treated with an inhibitor of alkaline ceramidase demonstrated reduced plasma and erythrocyte S1P [79]; however, these *in vivo* studies do not exclude the participation of alkaline ceramidase activities in cells other than mature red blood cells. In fact, red blood cells can very avidly take up sphingosine from plasma [52] or potentially from other cells. Plasma sphingosine may be generated by secreted ceramidase, by neutral ceramidase in the outer leaflet of nonerythrocyte plasma membrane [80], or secreted by other cells proximal to the blood vessel via alkaline or acid ceramidase [79] activities. Thus, the exact source of sphingosine for the synthesis of S1P by red blood cells is still unclear.

Besides their intrinsic ability to generate and accumulate S1P without degradation, red blood cells contain an efficient system to transport S1P to and from plasma (Fig. 2). The exact mechanism for this transport and its regulation is not completely understood. Studies suggest that most of the S1P in red blood cells is present in the outer leaflet of the plasma membrane [77] and released into the media continuously, in a stimulus-independent [63] and carrier-dependent manner, since it is not observed in plasma-, serum-, or albumin-free media [63, 75, 77]. Whereas experiments using inside-out membranes from red blood cells indicated a vanadate- and glyburide-dependent transport for S1P characteristic of an ABCA1 transporter [63], other studies using intact red blood cells indicated an ABC-independent but scramblase-dependent efflux of C17-S1P into plasma [77]. An involvement of both ABCA1 and ABCC1 transporters was also described in Syrian hamster red blood cells [81]. Thus, it is possible that more than one transporter participates in the export of S1P from red blood cells, a conclusion that is supported by the observation that mice deficient in ABCA1, ABCA7, or ABCC1 show no reduction in S1P levels [82]. Unlike the active transport by ABC transporters, the scramblase would be involved in the flipping of S1P from the inner to the outer leaflet of the membrane, from where carrier proteins in plasma can extract it. The reasons for the discrepancies between these studies are unclear, but potentially both mechanisms (ATP-dependent and -independent) could be operating as has been described for platelets [63].

The carriers in plasma responsible for the extraction of S1P from the outer leaflet of red blood cells are albumin (which carries about 30–40% of S1P in plasma) and the lipoprotein HDL (which carries more than 50% of the S1P in plasma) [63, 75, 77]. In particular, crystal structure studies have demonstrated that the apolipoprotein apoM binds S1P specifically [83], and genetically deficient apoM mice or human apoM transgenic mice expressing high apoM concentrations show, respectively, substantial reduction or increase in HDL-associated S1P in the serum, indicating that apoM is a carrier of S1P in HDL particles [83, 84]. However, blood samples from Caucasian individuals have not shown a statistically significant correlation between S1P and apoM concentrations [84]. This lack of correlation could be explained by the molar excess of apoM over S1P concentrations and potentially a participation of other apoproteins either in the association with S1P, the metabolic regulation of apoM and cholesterol levels, or the distribution of S1P into different types of HDL molecules. For example, a trend for a positive correlation between apoM and S1P levels was observed in a subpopulation of blood samples with HDL-cholesterol or apoA-I levels below the median, which could reflect an effect of the limiting apoA-I in these individuals on the regulation of apoM levels [84]. Other studies from normal Japanese individuals found a strong correlation between S1P levels and HDL-cholesterol and apoA-I [85], although these correlations were weak or not found, respectively, in larger studies from Caucasian [84] or

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Japanese individuals [76]. However, in the latter study, total cholesterol, LDL-cholesterol, and apoB (the apoprotein mostly present in LDL particles), as well as apoCI and apoCII, were found to correlate positively with plasma S1P levels [76]. Furthermore, biochemical studies have implicated fraction 2.2 of fractionated human HDL lipoproteins, which contains apoCI and apoCII, in the efflux of S1P from red blood cells [77]. Thus, the specific roles for all these lipoproteins in the regulation of efflux of S1P from red blood cells or other cells and their role in the distribution of S1P into potentially different populations of HDL or other lipoproteins seem complex and remain to be further clarified.

2.6.2 Endothelial cells—Endothelial cells constitute a physical barrier between environments with very different concentrations of S1P and, because of their strategic position, they also play important active roles in the maintenance of this gradient. They contribute to both the supply of S1P (and probably sphingosine) to circulating fluids and the control of the S1P that crosses the endothelium into the abluminal side of the blood vessel. Thus, it is not surprising that endothelial cells show a robust expression of the enzymes involved in the synthesis (SphK1 and 2) and degradation (S1P lyase, Sgpp1 and 2) of S1P, indicating a high metabolic rate of S1P in these cells [86]. Indeed, whereas S1P turnover within blood cells is not very high, S1P is rapidly dephosphorylated upon contact with endothelial cells or when injected intravenously in mice [71, 86]. Endothelial cells can take up the resultant sphingosine and rephosphorylate it (presumably via SphK1) and export it back to the circulation through the Spns2 transporter [68], or route it for degradation via rephosphorylation by SphK2 and cleavage by S1P lyase [38]. This constant “filtration” of S1P across the endothelial membrane allows for multiple checkpoints in the regulation of S1P in the luminal and abluminal side of the vessels. Endothelial cells subjected in vitro to laminar shear stress downregulate the expression of S1P lyase and Sgpp1 and accumulate and release S1P [86]. Thus, it is possible that normal blood flow persistently favors the replenishment of S1P into circulation; however, additional conditions or stimuli could modify this function, favoring other fates for sphingolipid metabolites.

As discussed above (see section 2.6.1), adoptive transfer experiments from wild-type donors into irradiated S1P-less mice demonstrated a major contribution of red blood cells to the high S1P in blood; however, they also indicated the contribution of a radio-resistant source for the high S1P concentrations in the lymph [23]. The source of lymph S1P was later identified as lymphatic endothelial cells by showing that *Sphk2*$^{-/-}$ mice with *Sphk1* conditionally deleted in these cells (via expression of *Lyve 1*-driven Cre-recombinase) lack lymph S1P but have normal levels of S1P in blood [87]. Moreover, a potential contributing role for the endothelium in the maintenance of blood S1P was suggested by the observation that adoptive transfer of *Sphk1*- and *Sphk2*-deficient bone marrow (unable to generate S1P from red blood cells) into irradiated wild-type mice resulted in sufficient S1P in blood to support normal egress of lymphocytes into blood and to downregulate their S1P receptors [23]. In addition, the finding that conditional deletion of the S1P transporter Spns2 in endothelial cells reduces the levels of circulating S1P by approximately 50% demonstrates that the export of S1P from the endothelium is important in the maintenance of blood S1P [69]. Further evidence includes the restoration of the levels of circulating S1P in *Sphk1*$^{-/-}$ mice (which exhibit about half the normal levels of S1P) when adenoviral expression of SphK1 was induced predominantly in liver sinusoidal endothelial cells and hepatocytes [86]. Altogether, the data clearly implicate the endothelium in the regulation of S1P in circulatory fluids to a large extent by synthesizing S1P and exporting it through Spns2 transporters [69], and potentially by secreting small quantities of SphK into circulation [43]]. Both of these processes could be actively regulated depending on the physiological or pathological environment, but how and when this may happen is yet unknown.

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Although the function of synthesis and export may occur mostly at the luminal side of the endothelium, one can also envision that this should take place in a more regulated manner at the abluminal side of the endothelium to maintain S1P levels between the circulation and the tissue (Fig. 2). Along these lines, the recently demonstrated role of endothelial Spns2 in the egress of T and B cells suggests that export of S1P into the abluminal side of the vessel is critical for directing lymphocytes into blood [69], as the reduction in blood S1P levels in *Spns2*-deficient mice (on the luminal side), similar to the *Sphk1*-deficient mice, is not sufficient to compromise lymphocyte trafficking [23, 88]. Endothelial cells express three variants for SphK1 and two for SphK2, all of them mostly cytosolic [43]. SphK1b and c show enhanced plasma membrane localization in human umbilical vein endothelial cells [43] as compared with SphK1a, which translocates to the plasma membrane after activation [35], but it is not known whether the expression of these variants is polarized toward the luminal or abluminal side of the endothelium (which could indicate a differential regulation at either side). However, the finding of SphK1 isoforms in association with the cell membrane might be consistent with their potential role in the export of S1P, whereas the expression of SphK2 variants in association with the ER and nuclear envelope [43] is more consistent with the described role for this isoform in the degradation of S1P [38].

2.6.3 Pericytes and epithelial cells—The metabolism of S1P also contributes to the maintenance of the low level S1P environment in the tissues. S1P lyase deficiency causes very large increases in S1P in tissues as compared with circulatory fluids, indicating the importance of this enzyme in tissue S1P regulation, a task likely accomplished not only by endothelial cells but also stromal and parenchymal cells [22, 25–27]. Apart from the role of stromal cells and parenchymal cells degrading S1P, two specialized cell types in the thymus, the neural crest-derived pericytes that ensheath thymic blood vessels and cells from the epithelial wall surrounding the vessels, have been proposed to control S1P concentration zones at the abluminal side of blood vessels, guiding lymphocytes into circulation [89, 90] (Fig. 2). The evidence for this role is the substantial accumulation of mature T cells in the thymus after targeted deletion of *Sphk*s in thymic vessel pericytes [89] or conditional deletion of the phosphatase LPP3 in epithelial or endothelial cells [90]. These targeted deletions do not result in changes in S1P concentrations in blood [89] or the overall concentration of S1P in the tissues [90]; however, they cause, respectively, an increase [89] or a reduction [90] in the plasma membrane expression of thymocyte S1PR1, indicating S1P alterations in the thymic local microenvironments. Interestingly, thymocytes overexpressing the receptor S1PR1, which show enhanced migration toward S1P and enhanced thymus egress, accumulate between the basement membrane of the blood vessels and associated pericytes and the epithelial basement membrane, as if they were responding to S1P available in this location. Furthermore, the polarized localization of LPP3 toward the basolateral side [90, 91] could suggest that a perivascular gradient of S1P produced by pericytes (and endothelial cells) at the abluminal side of the vessels and degradation of S1P by epithelial cell LPP3 at the rear (farthest side of the perivascular area) may efficiently guide T cells into circulation by ensuring the maintenance of a leading edge of signal for migration (Fig. 2). Although this role for pericytes and endothelial cells has been shown in thymus, it remains to be explored whether similar mechanisms exist in the vascular beds of other tissues, when they may occur, and what cell types may be involved.

2.6.4 Platelets—Platelets have high SphK activity and no S1P lyase and thus, like red blood cells, they can accumulate S1P [21]. However, they do not seem to be an important source for circulating S1P in homeostatic conditions, because mice with depleted platelets resulting from injection of an anti-GPib antibody [86] or mice lacking platelets ($Nfe^{-/-}$ mice) [23] had normal S1P levels. The release of S1P by platelets, unlike red blood cells, is not continuous but rather stimulus-dependent [52, 63], although a continuous mechanism of

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release for S1P mimetics such as FTY720-P has also been described [92]. Even though platelets do not seem to be a source for S1P under normal conditions, their ability to store and release S1P upon activation and to respond to a damaged endothelium suggest the possibility that they may alter S1P levels under pathological conditions. For example, patients with systemic sclerosis, an autoimmune and inflammatory disease associated with activated platelets, present higher levels of circulating S1P than healthy controls; however, the impact of this increase on disease progression is not known [93]. In addition, rabbits with hypercholesterolemia, a condition that increases the risk for cardiovascular disease in humans, show increased platelet activity, and these platelets release significantly more S1P than normal platelets [94]. However, the role, if any, of platelet-derived S1P in the development or prevention of atherogenesis is still not well understood [92, 94]. Another proposed role for platelet-released S1P is endothelial angiogenesis and wound healing during clotting [92, 95]. Because thrombin induces S1P release in platelets and S1P enhances the effects of thrombin in endothelial cell tissue factor release, platelet-released S1P may contribute to the coagulation cascade [96].

2.7 Altering S1P homeostasis in tissues

Even though interstitial levels of S1P in the tissue environment are believed to be low, under certain pathophysiological conditions local increases in S1P may occur, altering responses in tissue-resident or circulating immune cells. The metabolism of S1P is complex and subject to regulation by numerous stimuli, and thus it is reasonable to assume that changes in the tissue environment can shift the tight control of S1P metabolism in the tissues. Membrane expression of S1PR1 in lymphocytes has been used as a tool to infer changes in the interstitial levels of S1P in lymphoid organs [22, 23]; however, interstitial levels of S1P in other tissues have not been directly or unequivocally measured. Nevertheless, lines of evidence suggest changes in tissue S1P during inflammation and other disease processes, including ischemia reperfusion, atherosclerosis, and cancer. Here we will briefly summarize some of the evidence suggesting these changes in two of these processes, inflammation and cancer, and discuss the potential sources of the increased S1P and the likely impact on the tissue physiology.

2.7.1 Local inflammation—Inflammation is a process that occurs as a consequence of a complex response to tissue damage due to injury or pathogens, allergens, or other substances infiltrating the tissue. This response includes vascular changes, recruitment and activation of immune cells, and various histological changes. Because the vasculature and tissue are altered under these conditions, changes in the local homeostasis of S1P may occur. Indeed, during acute inflammation induced by injection of splenocytes or adenoviral particles in the ear pinna [97] or thioglycollate in the peritoneum [98], S1P levels were substantially elevated but subsided after inflammation was resolved. Similarly, exudates from paws injected with carrageenan, which induces inflammation and vascular permeability, contained at least seven-fold more S1P than sham-injected paws [99]. S1P may also be elevated during allergic inflammation and, along these lines, S1P was found elevated in the bronchoalveolar lavage of asthma patients after an allergic challenge [100] as well as in the synovial fluid of the joints of rheumatoid arthritis patients [101].

The functional consequences for the increases in S1P at the site of inflammation may be manifold, although a clear cause-effect relationship has not been established. In addition to affecting the vasculature, extracellular matrix composition, and other functional aspects of cell tissue physiology (reviewed in [102–104]), local changes in S1P can drastically affect the immune response [1]. For example, increased S1P and/or expression of S1PRs can result in the accumulation of a number of immune cells in the local site by two mechanisms: chemoattraction of a variety of immune cells from distal tissue sites [1, 15, 102]; and

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prevention of the exit of mature T cells from peripheral tissues to the efferent lymphatics, an effect that occurs by activating their S1PR1 and increasing their adherence to the lymphatic endothelium [97]. A mechanism also involving S1PR1 regulation in hematopoietic stem cell progenitors may occur in the peripheral tissue during infection to prevent their continuous recirculation via efferent lymphatics, thus allowing their differentiation into innate effector cells [105]. This temporary retention or recruitment of immune cells can be beneficial for the host. Furthermore, S1P may also cause fundamental changes in the type of immune response elicited depending on the insult, the types of cells populating the inflammation site, and the propagating signals. For instance, experimental evidence suggests that S1P can skew responses toward allergic phenotypes by causing a shift toward T_H2- and T_H17-cell responses while disfavoring T_H1-cell responses [1]. This can, in part, be mediated indirectly through the reported effects of S1P on dendritic cell maturation [1, 106]. On the other hand, S1PR1 activation in the presence of transforming growth factor beta inhibits the differentiation of suppressive T cells (Foxp3$^+$ $T_{reg)}$ to promote the development of T_H1 cells [107], suggesting an inflammatory role for S1P toward another type of T helper lineage. It is unknown at the moment how all these effects play out in the inflamed tissue, but it is possible that the shaping of the response may vary depending on cells present at the inflammation site, the type of insult, and the cytokine environment.

In contrast to this potential role of tissue-generated S1P in promoting and skewing the immune cell phenotype, other findings suggest an anti-inflammatory role for S1P. For example, in models of allergic contact dermatitis and asthma, S1P or FTY720 applied topically to the skin [108] or by inhalation [109], respectively, inhibits the migration of dendritic or Langerhans cells to the lymph nodes, thus preventing the initiation of an allergic immune response in the skin or the airways. This is consistent with the finding that lesional skin biopsies of individuals with atopic dermatitis [110, 111] and psoriasis [112] show increased expression or activity of S1P lyase and S1P phosphatase 2, respectively. However, at the moment it is difficult to distinguish if the increase in the expression of enzymes involved in the catabolism of S1P contributes to the development of the disease by reducing the presence of an "anti-inflammatory" S1P, or if this is a compensatory mechanism to suppress disease by eliminating a "proinflammatory" S1P and producing other lipid products, such as ceramide [113, 114] or other lipids, that may improve the symptoms. Other reports also suggest anti-inflammatory effects of S1P, including a switch from proinflammatory M1 to the anti-inflammatory M2 macrophage subtype [115], a decrease in the production of chemokines within the lungs during airway inflammation [116] preventing recruitment of immune cells, and a decrease of proinflammatory cytokines by endothelial cells and neutrophils [112].

2.7.2 Cancer—A body of evidence indicates that the expression of SphK1 is increased in cancer cells of multiple origins and correlates with disease progression and poor prognosis for survival (reviewed in [117, 118]), whereas in some tumor cells S1P lyase expression is downregulated [119, 120]. A genetic link, however, for the dysregulated expression of these sphingolipid metabolic enzymes has not been demonstrated; it seems more likely that growth factors and cytokines produced by the tumor cells or present in the tumor environment can regulate the transcription and activity of these enzymes, which in turn, results in the potentiation of disease. These alterations in the sphingolipid metabolism, which in homeostatic conditions is set to keep S1P low in tissues, may lead to the production and secretion of S1P into the tissue environment. For example, the breast cancer cell line MCF-7 was found to release S1P to the media through the transporters ABCC1 and ABCG2 when the cells were activated with estradiol, but not under normal growth conditions [121]. A direct link between SphK1 activity intrinsic to the tumor cell and tumor progression was suggested by the finding that subcutaneous injection in nude mice of a lung carcinoma cell line stably expressing shRNA to knockdown SphK1 produced smaller volume tumors than

control cells and showed increased sensitivity to docetaxel treatment [122]. Another indication that S1P may be increased in the interstitial tumor environment is the finding of a high expression of intracytoplasmic S1PR1 and S1PR3 in tumor cells from patients with estrogen receptor-positive breast cancer (particularly those with worse survival prognosis), suggesting that these receptors have been internalized following activation by extracellular S1P [123]. A recent study also found an upregulation of S1PR1 in Stat3-positive tumors and showed that prevention of S1PR1 activation by antagonists or genetic deletion inhibits tumor growth, metastasis, and persistent Stat3 activation, suggesting that the ligand S1P is present in the tumor environment [124]. The role of S1P in tumor formation is consistent with the finding that a monoclonal S1P antibody attenuated human xenograft progression and angiogenesis in vivo [125]; however, because the antibody was administered systemically, it does not clarify the source of S1P.

How can S1P in the microenvironment amplify tumor formation? S1P induces new vessel formation, promotes growth, and protects against apoptosis, all of which potentiate tumor growth, as reviewed extensively elsewhere [118, 126, 127]. Another intriguing mechanism for the promoting activity of S1P recently unraveled is through activation of S1PR1, whose expression is induced by the transcription factor Stat3, which leads to interleukin 6 secretion by tumor and infiltrating immune cells perpetuating the expression of STAT3. Permanent, dysregulated Stat3 expression further facilitates transformation [124]. In addition, S1P elevations may enhance transformation by contributing to the polarization of tumor-associated macrophages toward an M2 (or anti-inflammatory) phenotype which shows a disabled ability for killing tumor cells [115, 128]. This type of macrophage promotes tumor development by producing immunosuppressive cytokines and angiogenic factors, and their presence in tumor lesions is associated with poor prognosis. The source of the S1P for this effect has been proposed to be malignant dying cells in early tumors [129, 130] in the tumor environment, although demonstration in vivo is lacking. It has been proposed that S1P secreted by dying cells activates S1P receptors in surrounding macrophages, inducing tyrosine kinase receptor A (TRKA) shuttling to the plasma membrane. The activation of TRKA by cancer cell-derived nerve growth factor, leads to interleukin 10 production, turning on the switch toward immunosuppressive macrophages [131]. However, it is also possible that S1P in developing tumors comes from growing cancer cells with increasingly dysregulated S1P metabolism.

2.7.3 Mechanism by which S1P levels in the tissues are altered—From the discussion above, it is apparent that the mechanisms and cells implicated in the proposed increases in S1P in inflamed or tumor tissue are poorly understood. During inflammation, an increased vascular permeability causes edema, and thus S1P from blood exudates can be an obvious path for increases in tissue S1P [99]. Infiltrating blood cells [74], which have the potential to produce and release important amounts of S1P, could be stimulated in the inflammatory or cancer environment to alter the tissue S1P concentration. Similarly, as explained above, stromal or cancer cells can respond to these environmental cues to turn on the production of S1P while attenuating its degradation. Furthermore, the mast cell, an innate-immune, tissue-resident cell that provides host defense against pathogens and is also found in association with tumors [132], constitutes another likely candidate for altering S1P homeostasis in the interstitial tissue, particularly in allergic inflammation [133, 134]. Mast cells are considered effector cells, particularly in IgE-associated immune responses, by releasing a plethora of modulators of the immune response [132, 135]. S1P can attract mast cells to the site [136], which in turn can release large amounts of S1P when activated by antigen via Fc epsilon RI, the high affinity receptor for IgE, and possibly other signals [133]. Unlike blood cells such as red blood cells and platelets, mast cells do not store S1P, and the levels of S1P in these cells are low. However, IgE stimulation induces a rapid and sustained activation of both SphK1 and SphK2, leading to the de novo production of S1P and its

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release into the extracellular space by a mechanism in part mediated by the transporter ABCA1 [133, 134]. In the tissues, mast cell activation and S1P release may not be restricted to antigen stimulation, since mast cells respond to a variety of stimuli that may be present in the tissue under different circumstances.

Altogether, although not directly demonstrated, the findings suggest that the normal homeostasis of S1P in the tissue interstitium can be altered by multiple contributing cells and mechanisms during conditions such as the development of inflammation and cancer. In addition, the consequences of the disruption of the normal S1P levels may be to trigger multiple responses and serve to amplify and modulate cellular responses with beneficial or detrimental consequences for the host.

2.8 Conclusions and directions

The metabolic pathway of S1P is complex and tightly regulated (Fig. 1). Depending on the cell type, the pathway may be biased toward S1P accrual and secretion, or continued intracellular metabolism leading to degradation or recycling back to ceramide. At the organism level, cells with metabolic pathways specialized in generating high S1P extracellular concentrations are found in the proximity of circulatory fluids, whereas cells with metabolism geared toward keeping the levels of S1P low are generally present within the tissue parenchyma. In concert with mechanisms of sphingolipid transport at the interface between circulatory fluids and tissues, this constitutes a highly organized metabolic system that maintains distinct S1P concentration zones (Fig. 2). A metabolically controlled gradient of lipid phosphates that guides the migration of germ cells has also been described in *Drosophila* embryos [137].

This metabolic mechanism for producing S1P concentration gradients affords a high degree of regulation that is not achievable with simple gradients based on diffusion. With a metabolic system, it is possible to create highly compartmentalized zones with extremely high S1P levels adjacent to extremely low regions. A concentration difference of 100–1000 fold [22] may exist across a distance of a few cells that separate the circulation from the interstitial fluids (Fig. 2). By distributing cells with capability to produce extracellular S1P around the blood vessels, it is possible to “shape” the gradient and to superimpose it across barrier cell layers (Fig. 2). A notable feature of the sphingolipid metabolic pathway is exquisite responsiveness to external stimuli [3, 138, 139]. This affords a mechanism for the acute generation of S1P for signaling in low S1P environments by altering the metabolic pathway toward the production and secretion of S1P.

Our understanding of the regulation of S1P gradients is still incomplete, and important questions remain. What is the source of cellular sphingosine for extracellular S1P production? Do the S1P phosphatases have a role in forming an S1P gradient? Under what pathologic conditions does the S1P gradient change, and how does this alter disease progression? Can “reshaping” the gradient be harnessed as a therapeutic strategy? Development of methods to localize S1P production and its concentration zones in vivo will help answer these important questions.

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Highlights

- S1P is both a lipid metabolite and a signaling molecule.
- An S1P concentration gradient exists between circulation (high) and tissues (low).
- S1P gradients are produced by differential expression of metabolic pathways.
- S1P receptor signaling regulates normal physiology and pathogenesis.
- S1P gradients underlie the regulation of S1P receptor signaling.

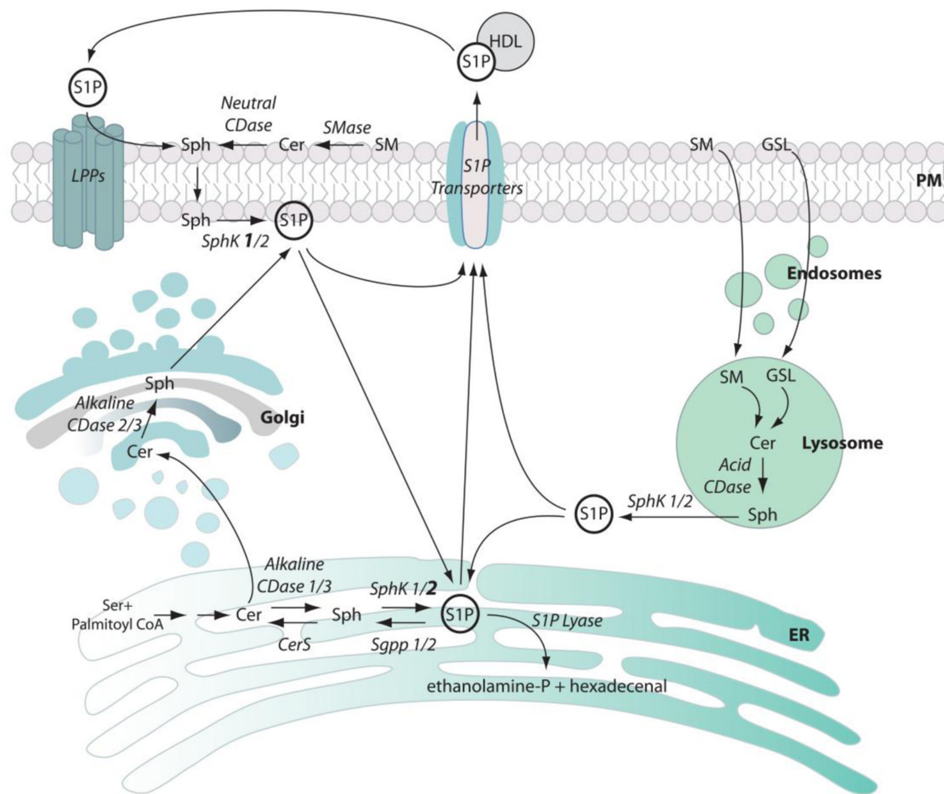

FIGURE 1. Cellular S1P metabolism

Cellular synthesis and degradation of S1P involves multiple enzymes, some expressed as various isoforms with different biochemical properties and cellular locations, indicating a highly complex metabolism. S1P synthesis by Sphk1 and 2 can occur after the degradation of ceramide, which takes place in the ER and in the Golgi after *de novo* synthesis, or in the lysosomes and at the plasma membrane, during catabolism of sphingomyelin and glycosphingolipids. Sphk1 activity (bold) predominates at the plasma membrane, while Sphk2 activity (bold) predominates for ER associated S1P metabolism. Within the cell, S1P may move rapidly between cell compartments. Intracellular S1P can be degraded by S1P lyase, dephosphorylated by S1P phosphatases to recycle the sphingoid base for ceramide synthesis, or else secreted. S1P is transported outside the cell by members of the ABC family of transporters and by Spns2. Once in the extracellular space, S1P can be dephosphorylated by a group of lipid phosphatases, LPPs, liberating sphingosine, which can be rephosphorylated back to S1P. Sph, sphingosine; Cer, ceramide; ethanolamine-P, ethanolamine phosphate; SM, sphingomyelin; GSL, glycosphingolipids; Ser, serine; LPPs, lipid phosphatases; CerS, ceramide synthase; CDase, ceramidase; SMase, sphingomyelinase; PM, plasma membrane.

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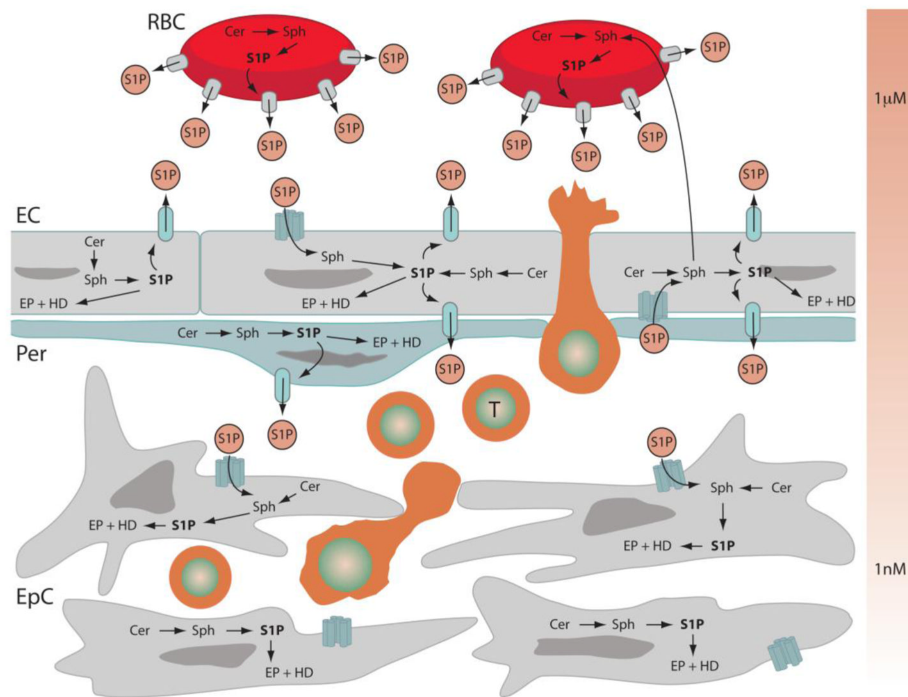

FIGURE 2. Cellular regulation of S1P gradients

S1P concentration is maintained at a high concentration in blood (~1 μM) mostly by red blood cells and endothelial cells. Whereas the active transport of S1P from red blood cells can occur via ABC-type transporters (grey ovals), endothelial cell-derived S1P is transported into the luminal side of blood vessels via Spns2 transporters (blue ovals). S1P in circulation is predominantly bound to HDL or albumin (extracellular S1P is depicted generically as orange circles indicating either carrier-bound S1P or free S1P). These carrier proteins in serum can also extract S1P directly from the red blood cell membrane in a transporter-independent manner. Endothelial cells face two distinct S1P environments (illustrated as a color gradient on the right side of the figure), one rich in S1P at the luminal side and one about two to three orders of magnitude lower at the abluminal side, and they are key cellular components contributing to this sharp concentration differential. S1P from blood enters the endothelial cells constantly after dephosphorylation by plasma membrane lipid phosphate phosphatases (six-transmembrane domain structures). The resultant sphingosine is converted to S1P mainly by SphK2 and routed for irreversible degradation by S1P lyase. Similarly, S1P lyase present in various types of parenchymal cells also degrades S1P that traverses the endothelial barrier. In addition to the general skewing toward S1P production at the luminal side and S1P degradation at the abluminal side of blood vessels, new findings indicate the presence of microgradients of S1P at the perivascular space in the thymus to facilitate the egress of mature thymocytes into circulation. These gradients are maintained by endothelial cells and pericytes, which produce S1P and export it into the abluminal side of vessels via Spns2 transporters, as well as reticular epithelial cells at the farthest end of the perivascular area, which dephosphorylate S1P via the lipid phosphate phosphatase LPP3. Thus, the concerted actions of all these cells using distinct metabolic enzymes and transporters result in a concentration gradient of S1P critical for the exit of mature T cells from thymus. Cer, ceramide; Sph, sphingosine; EP, ethanolamine phosphate; HD hexadecenal; T, thymocyte; Per, pericyte; EpC, epithelial cell; RBC, red blood cell; EC, endothelial cell.

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