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# Vascular and Immunobiology of the Circulatory Sphingosine 1-Phosphate Gradient

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## Abstract

Vertebrates are endowed with a closed circulatory system, the evolution of which required novel structural and regulatory changes. Furthermore, immune cell trafficking paradigms adapted to the barriers imposed by the closed circulatory system. How did such changes occur mechanistically? We propose that spatial compartmentalization of the lipid mediator sphingosine 1-phosphate (S1P) may be one such mechanism. In vertebrates, S1P is spatially compartmentalized in the blood and lymphatic circulation, thus comprising a sharp S1P gradient across the endothelial barrier. Circulatory S1P has critical roles in maturation and homeostasis of the vascular system as well as in immune cell trafficking. Physiological functions of S1P are tightly linked to shear stress, the key biophysical stimulus from blood flow. Thus, circulatory S1P confinement could be a primordial strategy of vertebrates in the development of a closed circulatory system. This review discusses the cellular and molecular basis of the S1P gradients and aims to interpret its physiological significance as a key feature of the closed circulatory system.

### Keywords

sphingolipid; lipid mediators; G protein-coupled receptor; vascular biology; angiogenesis; immune cell trafficking; HDL; lipid chaperones

## INTRODUCTION

In contrast to the open circulatory system of invertebrates, that of vertebrates warranted the efficient transport of nutrients and gases to various organs as well as precise control of such processes. This required drastic changes in vascular structure, control mechanisms, and the development of novel hematopoietic cell trafficking paradigms. Vertebrates developed a true endothelium, a continuous layer of endothelial cells (ECs) connected by specialized junctional complexes. ECs are ensheathed with mural cells, namely pericytes and vascular smooth muscle cells (VSMCs), which allow specialized properties of organ-specific ECs (1). The closed system also necessitated that the vessels withstand the high pressure, whereas the permeability of solutes and fluids is finely controlled to enable rapid and selective exchange

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between blood and tissues. In addition, precise organ-specific regulation of blood flow is needed. Furthermore, new immune cell trafficking systems are developed in a site- and context-specific manner.

Sphingosine 1-phosphate (S1P) is synthesized intracellularly by the sphingosine kinase (SphK) enzyme(s)-dependent phosphorylation of sphingosine. Many species of yeast, plants, invertebrates such as *Caenorhabditis elegans*, and vertebrates produce S1P as a key metabolic intermediate linking sphingolipids to glycerophospholipids (2). However, vertebrates (and perhaps chordates) started to use S1P as an extracellular bioactive lipid mediator by activating G protein-coupled receptors (GPCRs) localized in the plasma membrane of target cells (3). In mammals, S1P is spatially compartmentalized in the blood (~1 μM) and lymph (~0.1 μM) circulation. Meanwhile, it is estimated to be much lower in interstitial fluids of tissues (<1 nM), creating a sharp S1P gradient across the endothelial barrier (4, 5).

Why and how do vertebrates develop this circulatory S1P gradient? Recent studies have begun to unravel the complex mechanisms underlying the establishment and maintenance of the circulatory S1P gradient. In addition, critical functions of circulatory S1P in the maturation and homeostasis of the vascular system are now appreciated. Notably, the regulation and function of S1P are tightly linked to blood flow, a major biophysical stimulus of the vascular system (6, 7). An S1P gradient is also critical for the immune cell trafficking between peripheral tissues, lymph, and blood circulation (4, 8). Thus, circulatory S1P confinement seems to be a primordial strategy of vertebrates to adapt to the closed circulatory system. In this review, we summarize the current state of knowledge of the cellular and molecular basis for a circulatory S1P gradient. We also aim to interpret its physiological significance in vascular development and maintenance as well as immune cell trafficking. We discuss recent studies that reveal the spatial S1P distribution patterns in vascular and extravascular microenvironments.

## CIRCULATORY COMPARTMENTALIZATION OF SPHINGOSINE 1-PHOSPHATE

S1P is present in a variety of species and cell types. How do vertebrates achieve the compartmentalized enrichment of S1P in the circulatory system? Recent advances in conditional knockout technology together with identification of S1P transporters and chaperones have begun to unravel the mechanisms involved.

### Cellular Sources of Blood S1P

Red blood cells (RBCs) and ECs are the major sources of plasma S1P (Figure 1*a*). Significant progress toward understanding the cellular basis of circulatory S1P was made by the establishment of a mouse strain lacking S1P in plasma by the conditional gene deletion of two SphK enzymes, *Sphk1* and *Sphk2* (pS1Pless mice) (9). Adoptive transfer of wild-type RBCs to pS1Pless mice restored plasma S1P to the normal levels (9).In addition, a 30% reduction of plasma S1P levels was seen in irradiated wild-type mice reconstituted with RBC-specific *Sphk1*/*Sphk2* double knockout fetal liver (10). The contribution of

nonhematopoietic cells, especially of ECs, to plasma S1P was implicated by hematopoietic cell depletion experiments and cell culture experiments (11, 12). Accordingly, EC-specific *Sphk1*/*Sphk2* double knockout mice displayed a 30% reduction of plasma S1P levels (10).

Platelets may also participate in the circulatory enrichment of plasma S1P, especially under nonhomeostatic conditions (13). Platelets have high SphK activity (14) and lack S1P lyase that irreversibly degrades S1P (15, 16). In addition, S1P release was enhanced by platelet activation (15–17). Serum S1P levels are consistently higher than those in plasma (13). However, platelets are unlikely to contribute to plasma S1P levels under homeostasis because mice that lack platelets display normal plasma S1P levels (9). Acute platelet depletion by antibodies against platelet glycoprotein GPIba also failed to change plasma S1P levels (12). Consistently, *Sphk2* knockout mice lacking platelet S1P did not show plasma S1P reduction (18). Instead, local exaggerated generation of S1P by platelets may regulate platelet activation or thrombus formation (18). Importantly, local S1P production by platelets is also essential for the barrier function of high endothelial venules (HEVs), a specialized vasculature through which lymphocytes home to lymph nodes (LNs) and secondary lymphoid organs (SLOs), as discussed below. Additional cellular sources of circulatory S1P may exist and await further conclusive experiments using tissue-specific SphK enzyme knockouts.

Two SphK enzymes, SphK1 and SphK2, show unique contributions to blood S1P levels. Although these enzymes show redundant functions, they display differential tissue distribution and subcellular localization. Pharmacological or genetic targeting of SphK1 caused a >50% decrease in circulatory S1P (18–23). Surprisingly, elevated circulatory S1P levels (1.5- to 4-fold) were observed in *Sphk2* knockout mice (20, 21, 23–27). Acute treatment with SphK2 inhibitors increased blood S1P levels (1.5- to 3-fold) in wild-type mice (23, 27). However, ablation of both *Sphk1* and *Sphk2* resulted in the complete loss of plasma S1P, as mentioned above (9). SphK2 inhibitor also substantially reduced blood S1P levels in *Sphk1* knockout mice (23, 27). Therefore, SphK1 is necessary for the SphK2 inhibition-dependent increase of plasma S1P.

How can these observations be explained? It is conceivable that SphK2 blockage may lead to compensatory upregulation of *Sphk1* expression in select cell types. Indeed, some cell types or tissues from *Sphk2* knockout mice displayed substantially increased *Sphk1* expression (10, 26). However, this might not be the sole mechanism for the SphK2 blockage effects. For example, *Sphk2* deletion did not change *Sphk1* mRNA expression or activity in various tissues and cell types, including RBCs (18, 20, 24). In addition, an increase of SphK1 activity is unlikely to be the entire reason for elevated plasma S1P levels, as the transgenic overexpression of *Sphk1* failed to change plasma S1P levels (28). Furthermore, SphK2 inhibitors rapidly (<3 min) increased blood S1P levels (23, 27), which is too rapid for transcriptional induction of *Sphk1*. Alternatively, it is conceivable that SphK2 blockage increases its substrate sphingosine, which might be redirected to other cells for SphK1-dependent conversion to S1P. *Sphk1* knockout RBCs have virtually no S1P (18), so RBCs produce S1P predominantly by SphK1. Significantly, *Sphk2* knockout mice displayed increased levels of sphingosine and S1P in RBCs (24). This observation supports the idea of sphingosine redistribution to SphK1 when SphK2 is inhibited. Contrasting phenomena were

observed in platelets. *Sphk2* deficient platelets have virtually no S1P, indicating the dominant role of SphK2 in platelet S1P production (18). Conversely, *Sphk1* knockout mice showed a 50% increase of platelet S1P levels (18). Together, these observations suggest that blockage of a single isoform of SphK enzyme results in the redistribution of its substrate to the other isoform. Substrate redistribution might occur not only intracellularly but also in a transcellular manner. When wild-type mice were injected with labeled S1P–loaded RBCs, S1P redistributed from RBCs to lymphoid tissues (24). This process seems to require S1P dephosphorylation and rephosphorylation (24). On the other hand, *Sphk2* knockout failed to redistribute RBC-S1P to lymphoid tissues (24). Notably, S1P is more susceptible to degradation in lymphoid tissues than in RBCs (discussed below). Therefore, SphK2 blockage might facilitate the escape of S1P from degradation-prone tissues (e.g., lymphoid tissues) to S1P secretion-oriented cells (e.g., RBCs). This hypothesis is further supported by the observation that pharmacological or genetic targeting of SphK2 substantially lengthens the S1P half-life in circulation (27).

Taken together, these studies provide us with various insights into the SphK subtype- or cell type-specific regulation of the circulatory S1P. However, the precise molecular mechanisms are not fully understood. For example, it is hypothesized that S1P synthesized by SphK1 or SphK2 appears to be destined for secretion or intracellular metabolism, respectively (24). If so, how are these distinct fates determined? Furthermore, how does the differential subcellular localization of SphK isoenzymes affect this phenomenon? Better understanding of how plasma S1P is regulated is important to fully appreciate its biological, pathological, and pharmacological impact.

## S1P Export Into Plasma

S1P produced in the intracellular space is exported via specific transporters (Figure 1*a*). To date, only spinster homolog 2 (SPNS2) is proven as a bona fide S1P transporter (29, 30). SPNS2 is highly expressed in ECs (31, 32). *Spns2* global or EC-specific knockout mice showed substantial reduction (20–60%) in plasma S1P (31–34). S1P release from ECs is constitutive and fully dependent on SPNS2 (31, 32). Notably, laminar shear stress induced S1P release within 5 min in ECs (12). Although the underlying mechanism is still unknown, shear stress may be critical for the constitutive supply of circulatory S1P (Figure 2*a*). Nonetheless, S1P release from RBCs was unchanged in *Spns2* global knockout mice (32). Several members of the ATP-binding cassette (ABC) superfamily were reported as other candidate S1P transporters in some cell types (35–39). However, their identity as S1P transporters is tentative. First, mice that lack these ABC-type transporters showed normal plasma S1P levels (11). Moreover, heterologous overexpression of these transporters failed to promote S1P secretion (40). In RBCs, S1P seems to be present at the outer surface of plasma membrane (41), and S1P can be extracted into a soluble form with the help of S1P chaperones (discussed below). Thus, unidentified transporters/phospholipid scramblases might flip S1P to the outer leaflet to facilitate continuous enrichment of circulatory S1P.

## S1P Chaperones in Blood Circulation

Circulating S1P is bound to plasma protein chaperones, which function not only as S1P carriers but also activate its receptors in a ligand-dependent manner to evoke specific

biological responses (42–46) (Figure 1*a*). Recent studies suggest that chaperones in part determine blood S1P levels. More than half (50–60%) of plasma S1P is found in the high-density lipoprotein (HDL) fraction, approximately 30–40% in the albumin fraction, and much smaller amounts in other lipoprotein fractions (47). In the HDL particles, S1P is exclusively bound to apolipoprotein M (ApoM) (48), which is found only in 5%of plasma HDL particles (49). S1P is absent in the HDL fraction of *Apom* knockout mice, which show an approximately 45% reduction in plasma S1P levels (48). Moreover, transgenic mice overexpressing *Apom* show increased plasma S1P levels in a transgene dosage-dependent manner (48). These data suggest that S1P chaperones determine the S1P concentration in blood. How do they determine the blood S1P levels? First, plasma S1P chaperones may stabilize S1P by limiting access to degradative enzymes. Indeed, overexpression of ApoM in HepG2 cells increased S1P levels in a conditioned medium via the specific blockage of S1P degradation (50). The complete docking of S1P into ApoM, as shown in crystal structure (48, 51), likely protects S1P from degradation. Another possibility is that chaperones accelerate S1P release from cells. This hypothesis is supported by the observations that S1P was not released from platelets (52) or RBCs (41) in the absence of carrier proteins. Furthermore, a monoclonal S1P antibody also extracted S1P from cells (41). Remarkably, HDL showed faster and more efficient extraction of S1P from RBCs compared to albumin (41). This property of HDL (presumably facilitated by ApoM) may explain why S1P is more abundant in ApoM, a minor protein in plasma, than in albumin, the most abundant plasma protein. However, the precise mechanisms by which S1P is transferred to chaperones are not yet determined.

## Limiting S1P Levels

S1P enzymatic degradation in tissues is a key component in the formation of the circulatory S1P gradient. In tissues, intracellular S1P levels are limited mainly by the irreversible degradation to phosphoethanolamine and hexadecenal via S1P lyase located at the endoplasmic reticulum (16) (Figure 1*b*). Either pharmacological or genetic targeting of S1P lyase induced a 10–1000-fold elevation of S1P levels in various tissues (4, 53–55). S1P lyase is expressed in a wide range of tissues (55), whereas RBCs lack S1P lyase activity (13, 56). This distribution of S1P lyase expression can partly explain the formation of the circulatory S1P gradient. In addition, laminar flow markedly downregulates endothelial S1P lyase expression in ECs (12). Therefore, blood flow would facilitate the continuous provision of circulatory S1P by limiting endothelial S1P degradation while allowing S1P release (Figure 2*a*). There are additional intracellular S1P degrading enzymes, namely S1P phosphatase 1 and 2 (57); however, their contribution to the S1P gradient is not yet clear. By contrast, extracellular S1P is dephosphorylated by three lipid phosphate phosphatases (LPP1–3) located on the cell surface (58). Among them, LPP3-dependent S1P degradation is essential for the maintenance of local perivascular S1P levels to support immune cells' egress into circulation (59) Circulatory S1P is continuously degraded by cell-dependent degradation mechanisms (12). Thus, the balance between the continuous supply and clearance of S1P establishes the S1P gradients.

## Embryonic S1P

During embryogenesis, blood S1P compartmentalization seems to differ from that of the postnatal period. RBC-specific *Sphk1*/*Sphk2* double knockout embryos displayed almost complete reduction (>95%) of embryonic S1P (10). This study used *Sphk1* RBC-specific knockout in the background of *Sphk2* global knockout, which might mask the contribution of SphK2 in specific cell types to plasma S1P. Nevertheless, this observation suggests that RBCs are an exclusive source of embryonic S1P and that ECs begin to contribute to the blood S1P enrichment after vascular network maturation. Embryonic vascular structure is immature, with ECs undergoing dynamic processes of angiogenesis, remodeling, and pruning (60). The cellular or molecular mechanisms underlying this transition of circulating S1P sources are unknown. Embryonic and adult ECs may express different levels of factors involved in S1P production, secretion, and degradation. Furthermore, protein chaperones for embryonic S1P were not determined. It is noteworthy that albumin production becomes prominent only at late embryonic stages, whereas α-fetoprotein is another major plasma protein in embryos (61, 62). Although *Apom* gene expression is initiated at embryonic day 10 (E10) (63), its contribution to embryonic circulatory S1P is unclear. Either α-fetoprotein or ApoM might not be the sole protein chaperone in the embryos. This scenario is supported by the observation that *Afp* (the gene coding α-fetoprotein) or *Apom* knockout embryos normally develop (48, 64) unlike *Sphk1*/*Sphk2* double knockouts (10, 65) or S1P receptor(s) knockout embryos (66–69). As mentioned above, S1P chaperones are more than just S1P carriers; they are thought to stabilize, extract, and direct specific S1P signaling functions. Therefore, embryonic stage-specific S1P chaperones might facilitate the unique regulation and function of embryonic circulatory S1P.

## S1P in Lymph Circulation

The cellular source of lymph circulation is different from that of blood circulation. The main source of S1P in lymph is proposed to be lymphatic EC (LEC) (70) (Figure 1*c*). Distinct compartmentalization of lymph and blood S1P was inferred from the observation that adoptive transfer of wild-type bone marrow to S1Pless mice failed to restore lymph S1P levels (9). Indeed, LEC-specific *Sphk1/Sphk2* knockout mice showed fiftyfold lower levels of S1P in lymph circulation with normal S1P levels in blood circulation (70). Global or EC-specific *Spns2* knockout mice displayed substantial reduction in lymph S1P levels, suggesting that lymph S1P is secreted by SPNS2 from LECs (33). Accordingly, lymphocytes from EC-specific *Spns2* knockout mice showed tenfold higher cell-surface S1P1 expression in lymph (33). This observation can be explained by their exposure to lower levels of extracellular S1P, which promotes receptor endocytosis.

The composition or function of S1P carrier(s) in lymph also might be different from those in blood. Lymph has a generally similar protein composition to that of blood plasma albeit with different concentrations (71). Indeed, ApoM is found in lymph at approximately half of the plasma levels (45). However, lymph S1P levels were not changed in *Apom* knockout mice (45). Therefore, ApoM might be a minor S1P chaperone in lymph. Future studies on lymph S1P chaperones may provide valuable insights into general roles of S1P chaperones in immune and lymphatic functions.

## VASCULAR CENTRIC FUNCTIONS OF S1P RECEPTORS

At present, five receptors for S1P ($S1P_{1–5}$) were identified. The differential but overlapping intra-cellular signaling pathways and expression patterns of each receptor enable S1P to exert its diverse and redundant functions (72–75). $S1P_1$ exclusively couples with heterotrimeric Gαi/o proteins, whereas $S1P_2$ and $S1P_3$ couple with Gαi/o, Gαq, and Gα12/13, and $S1P_4$ and $S1P_5$ with Gαi/o and Gα12/13. Although $S1P_{1–3}$ receptors are widely expressed and are the predominant S1P receptors in vascular cells, $S1P_4$ and $S1P_5$ expressions are more restricted mainly to the lymphoid and central nervous systems, respectively. Thus, studies in the cardiovascular system have focused on $S1P_{1–3}$. ECs primarily express $S1P_1$ and $S1P_3$, whereas $S1P_2$ and $S1P_3$ are expressed on VSMCs. However, endothelial expression of $S1P_2$ was also confirmed in larger vessels of normal tissue, lung microvasculature, and tumor and inflamed vasculature (76). Therefore, these receptors seem to have different functions depending on the vascular bed or physiological context as discussed below. For more detailed information on S1P receptor signaling and tissue/cell distributions, the reader is referred to other reviews (72–75).

### Recycling and Degradation of $S1P_1$

Among five S1P receptors, the trafficking regulation of $S1P_1$ is well studied. After S1P stimulation, $S1P_1$ is desensitized by phosphorylation of its C-terminal tail and subsequent internalization by β-arrestin- and clathrin-dependent mechanisms (77–79). Most of the internalized $S1P_1$ is recycled back to the plasma membrane (77, 79). Only a small portion is targeted to lysosomal/proteasomal degradation (77, 79). Importantly, $S1P_1$ is highly sensitive to internalization and desensitization compared with other GPCRs. This unique characteristic of $S1P_1$ facilitates its distinctive roles in the immune cell movement in and out of the circulatory systems. Moreover, $S1P_1$ cell surface level is an indicator of S1P exposure status to cells because S1P availability and $S1P_1$ cell surface expression are correlated inversely (4, 33, 59, 70, 80).

Modulation of activated $S1P_1$ fates, recycling, or degradation by pharmacological agents is a well-known mechanism applied to clinical practice. Fingolimod (FTY720/Gilenya), a structural analog of sphingosine, is phosphorylated by SphK2 to FTY720-P (20, 81, 82). FTY720 was approved as the first-line oral therapeutic for relapsing-remitting multiple sclerosis (MS) by the US Food and Drug Administration (83, 84). Although FTY720-P acts as a high-affinity agonist on $S1P_1$ and $S1P_{3–5}$ (85, 86), it also acts as a functional antagonist for $S1P_1$. In contrast to S1P, FTY720-P induces persistent internalization (87). This is followed by polyubiquitinylation and degradation of $S1P_1$, leading to a marked decrease in $S1P_1$ expression (79, 88). This functional antagonism on $S1P_1$ leads to the trapping of autoreactive lymphocytes in the lymphoid organs, away from the central nervous system, which seems to explain the clinical efficacy of FTY720 (89).

### Innovative Reporter Systems for $S1P_1$ Activation and S1P Availability in Vivo

Two unique reporter mouse models utilizing $S1P_1$ were recently developed. Proia and colleagues used the “Tango” system developed by another team (90), which detects interactions between GPCR and β-arrestin. They adopted the Tango system to an in vivo

context using mouse genetics (91). In this $S1P_1$-green fluorescent protein (GFP) reporter mouse, a synthetic $S1P_1$ signaling pathway was genetically engineered so that a transcription factor is released from activated $S1P_1$ to turn on a nuclear-targeting GFP reporter gene. Therefore, the $S1P_1$-GFP reporter mouse displays mapping of $S1P_1$ activation status in vivo. $S1P_1$-GFP reporter mice validated the high-activation status of $S1P_1$ in previously expected cell types including ECs (91). In addition, they also revealed that endothelial $S1P_1$ activation is locus- and flow-dependent (46). Nonetheless, Schwab and colleagues (92) aimed to define S1P geography in vivo. As mentioned above, researchers took advantage of the unique $S1P_1$ trafficking properties to define local S1P availability by cell surface $S1P_1$ levels. However, this approach is based on the assumption that total cellular $S1P_1$ expression is similar among comparison groups. Moreover, the loss of cell surface $S1P_1$ does not always indicate $S1P_1$ activation because GPCRs can internalize even in the absence of their ligands. Moreover, we cannot determine S1P availability in areas where $S1P_1$ is not normally expressed. Schwab et al. (92) overcame these issues by designing a reporter mouse model in which GFP-tagged $S1P_1$ and RFP-tagged S1P–unresponsive $S1P_1$ mutants were overexpressed at equal levels. By checking the $S1P_1$ trafficking status by ratiometric imaging measurements, they estimated relative S1P concentration in vivo. Using this system, they found that S1P concentration is kept low by the lipid phosphatase LPP3 in the splenic tissue of red pulp (92). This is unexpected because the red pulp, the area filled with RBCs, was previously assumed to be rich with S1P. These elegant reporter systems are expected to provide additional insights to understand the spatiotemporal control or S1P levels in complex tissue microenvironments.

## Chaperone-Dependent Signaling of $S1P_1$

The $S1P_1$-dependent functions of S1P seem to be carrier dependent: Albumin-bound and HDL-bound S1P show situation-dependent signaling persistence or biased signaling. In cultured EC experiments, HDL-bound S1P displayed sustained $S1P_1$ signaling and barrier activity longer than albumin-bound S1P (42–44). In addition, HDL-bound S1P may enhance the pulmonary barrier integrity more efficiently in vivo (48, 93), as discussed below. More recently, our laboratory reported the chaperone-dependent anti-inflammatory roles of S1P–$S1P_1$ signaling (46). In human umbilical vein endothelial cells (HUVECs), HDL-bound S1P, but not albumin-bound S1P, suppressed cytokine–induced NF-κ activation and adhesion molecule expression (46). S1P induced rapid phosphorylation of ERK irrespective of its carrier (46). However, only HDL-bound S1P increased the association of $S1P_1$ with β-arrestin 2 (46). Accumulating evidence now supports the idea that GPCRs can activate G protein-independent but β-arrestin-dependent intracellular signaling (94). Consistently, β-arrestin 2 knockdown ablated the anti-inflammatory effects of HDL-S1P in HUVECs (46). However, albumin-bound S1P, but not HDL-bound S1P, inhibited adenylyl cyclase, possibly by a Gαi/o-dependent pathway (46). These observations imply the existence of a biased-agonism depending on the different S1P chaperones. In addition, HDL-bound S1P, but not albumin-bound S1P, suppressed lymphopoiesis in the bone marrow, although it is not necessary for normal lymphocyte trafficking in vivo (45).

How does S1P bound to various chaperones facilitate differential signaling persistence or biased agonism? One of the possible mechanisms is that coreceptors for $S1P_1$ might tether

S1P–bound HDL in proximity to $S1P_1$. Indeed, two of the HDL receptors, scavenger receptor-BI and endothelial lipase, were shown to be essential for HDL–induced and S1P–dependent cellular responses in cell culture experiments (42, 95). These tethering proteins might facilitate the more efficient delivery of S1P to $S1P_1$. Moreover, this mode of signaling may lead to the differential fate of $S1P_1$ after activation, internalization, recycling, or degradation. This hypothesis is supported by the aforementioned in vitro EC experiments. In these studies, $S1P_1$ treated with HDL-bound S1P was more protected from the internalization or degradation than $S1P_1$ treated with albumin-bound S1P (44, 46). However, there are other possible mechanisms, including HDL-dependent cholesterol uptake from cells and lipid microdomain modulation.

## Blood Flow-Dependent Regulation of $S1P_1$

Blood flow can increase transcription and protein levels of $S1P_1$ (Figure 2*b*). $S1P_1$ was shown to be induced by fluid shear in HUVECs (96). Accordingly, an increase of $S1P_1$ protein and a response to S1P were observed in shear-treated HUVECs (97, 98). Flow-dependent regulation of $S1P_1$ expression is seen in the retinal vascular plexus, where *S1pr1* expression displayed high expression in the flow-positive, mature regions of the vascular network and low expression in the sprouting vascular front region (99). These results suggest that blood flow induces *S1pr1* transcription via flow-regulated transcription factors. In lymphocytes, Kru¨ppel-like factor 2 (KLF2) is a critical transcription factor for *S1pr1* expression (100–102). Notably, KLF2 is a major flow-regulated transcription factor (103, 104), coordinating approximately half of the gene expression programs evoked by shear stress (104). However, the detailed mechanisms by which endothelial $S1P_1$ expression is regulated, including the involvement of KLF2, have not yet been addressed even though *Klf2* and *S1pr1* knockout mice showed similar vascular developmental anomalies (66, 105). As mentioned above, blood flow also downregulates S1P lyase transcripts. Therefore, future studies on the mechanistic basis for flow-mediated $S1P_1$ and S1P lyase transcriptional regulations are needed to reveal one of the remarkable intersections of S1P and mechanotransduction systems.

Shear stress also enhances or even directly activates $S1P_1$ signaling in ECs (Figure 2*b*). In the mouse aorta, $S1P_1$ expression was observed on the plasma membrane of ECs in the descending aorta, which is subject to steady laminar shear stress (99). However, $S1P_1$ is barely detected on the plasma membrane; instead, it is found intracellularly in the endocytotic vesicles in areas of turbulent shear (lesser curvature) of the aorta that is subjected to disturbed or turbulent flow (99). Concomitantly, in the observation of the aorta from $S1P_1$-GFP-reporter mice, substantial numbers of ECs had nuclear GFP in turbulent shear areas but not in the laminar shear stress area (46). Taken together, these data suggest that disturbed flow enhances $S1P_1$ activation and internalization in vivo. Shear stress might even directly activate $S1P_1$ in a ligand-independent manner.

In HUVECs, laminar shear stress induces the adherens junction assembly and alignment of EC monolayers to the direction of flow (99). Laminar flow also activates ERK, Akt, and the endothelial isoform of nitric oxide (NO) synthase (eNOS) (99). Importantly, all of these flow-mediated effects were abolished by pharmacological or shRNA-mediated targeting of

$S1P_1$ (99). Moreover, the overexpression of an S1P nonbinding $S1P_1$ mutant restored shear responsiveness, suggesting that $S1P_1$ can work as a component of shear stress sensing machinery in a ligand-independent manner (99). What are the molecular mechanisms for the ligand-independent mechanosensory role of $S1P_1$? It is conceivable that there mightbemechanosensitive coreceptors for $S1P_1$.In this scenario, proper $S1P_1$ expression might facilitate mechanosensory signaling by recruiting components of the mechanosensory complex (106). Alternatively, $S1P_1$ might sense mechanical stimuli by itself, as is proposed for other GPCRs. If so, how can different stimuli to $S1P_1$ (e.g., chaperone-bound S1P versus shear stress) be organized to address the physiological function of $S1P_1$ in vivo? The answers await future studies, especially those searching for the binding partners of $S1P_1$ or the combinatorial usage of pS1Pless and $S1P_1$-GFP reporter mice.

## SPHINGOSINE 1-PHOSPHATE IN VASCULAR DEVELOPMENT

S1P plays critical roles in embryonic vascular development. For example, genetic deletion of *S1pr1* in mice caused embryonic lethality at E12.5 to E14.5 as a result of severe hemorrhage (66). Because EC-specific knockouts of *S1pr1* phenocopied *S1pr1* global knockouts, $S1P_1$ function in the endothelial compartment seems to be critical for the regulation of embryonic vascular stability (67). On the contrary, no clear developmental defects were observed in either *S1pr2* or *S1pr3* single knockout embryos (68, 69, 107). However, simultaneous deletion of *S1pr2* and *S1pr3* resulted in approximately 50% embryonic hemorrhage and death at approximately E13.5 (68, 69). Moreover, embryos lacking all of the *S1pr1–3* showed the most severe bleeding, which occurs much earlier than the *S1pr1* single deletion (69). These results indicate the supportive and/or redundant roles of $S1P_2$ and $S1P_3$ in embryonic vascular development. Similar to the compound S1P receptor(s) knockout phenotypes, global or RBC-specific *Sphk1/Sphk2* double-knockout embryos also displayed severe defects in vascular development (10, 65). Therefore, it is likely that circulatory S1P activation of S1P receptors is a fundamental event in early vascular development.

### $S1P_1$ as a Negative Regulator of Sprouting Angiogenesis

$S1P_1$ inhibits angiogenic sprouting and thus influences the collective endothelial cell behavior during embryonic vascular development. In the murine retinal angiogenesis model, EC-specific deletion of *S1pr1* leads to a hypersprouting phenotype: The vascular plexus becomes overly dense, resulting in inadequate growth of the vasculature into the retinal tissue (99, 108). The endothelial hypersprouting phenotypes are also observed in multiple locations of *S1pr1* knockout embryos, including the hindbrain, neural tube, and limbs (108–110). It is notable that embryonic *S1pr1* deletion leads to aortic hyperbranching (108). This is the most distinctive vascular phenotype of *S1pr1* knockout embryos that was not reported in other gene mutants. However, this phenotype was recapitulated in RBC-specific *Sphk1/Sphk2* double-knockout embryos (10). Together, the embryonic lethality of these mutants seems to bemainly due to exaggerated and ectopic endothelial sprouting.

S1P contributes to vascular stabilization by repressing the sprouting behavior of ECs after the initiation of blood flow. This is distinct from the well-documented roles of Notch signaling at the angiogenic front (60, 111). Indeed, *S1pr1* deletion did not change the

expression of Notch-target genes in embryonic microvessels or retinal tissues (99, 108). The aortic hyperbranching phenotype of *S1pr1* knockout embryos was not observed in the EC-specific deletion of Notch target *Rbp-j* (108). Thus, the $S1P_1$-dependent regulation of sprouting angiogenesis appears to be an independent pathway that is not epistatic to Notch signaling. Notably, the onset of the *S1pr1*-knockout phenotype occurred after the establishment of major vascular networks. Because S1P is compartmentalized in blood circulation, perfusion of the newly constructed vessels would initiate the S1P signaling.In this process, the RBC-dominant supply of embryonic blood S1P likely initiates S1P signaling after vascular perfusion. Furthermore, blood flow itself might support this process by enhancing expression and activity of $S1P_1$.

### The Role of Endothelial $S1P_1$ in The Mural Cell Investment of Nascent Vasculature

Endothelial $S1P_1$ regulates mural cell investment, another important step in vascular stabilization. In coculture experiments with murine embryonic ECs (MEECs) and mural cells, S1P induced mural cell binding with MEECs from wild-type but not from *S1pr1* knockout embryos (112). Mechanistically, S1P induced the polarized distribution of N-cadherin on the apical EC surface, which should strengthen the EC-mural cell interactions (112). Consistently, the mislocalization of N-cadherin or mural cell coverage defects was observed in the dorsal aorta of *S1pr1* knockout embryos (66, 67, 112). However, these mural cell coverage defects may be secondary to the hypersprouting phenotype described above. In contrast, pericyte-deficient mice displayed much milder and later onset vascular abnormalities (113, 114). Understanding of the postnatal function of $S1P_1$ on mural cell investment still awaits further studies.

## SPHINGOSINE 1-PHOSPHATE IN PHYSIOLOGICAL VASCULAR INTEGRITY

S1P is a major regulator of vascular barrier function. Many in vitro studies have shown that S1P induces barrier protection by the EC cytoskeleton and assembly of cell–cell junction proteins (115, 116). Indeed, circulatory S1P is essential for the basal vascular integrity (48, 117, 118). Endothelial $S1P_1$ plays a major role in the physiological maintenance of barrier integrity (45, 88, 93, 99, 119, 120). By contrast, $S1P_2$ is involved in barrier disruption in most vascular beds during anaphylaxis (25, 121–123), and in MS (124) and stroke (125). Here, we focus on and discuss the role of circulatory S1P in the physiological maintenance of vascular barrier integrity.

### Maintenance of Endothelial Barrier Integrity by Blood S1P

Blood S1P plays a pivotal role in the maintenance of constitutive vascular integrity. Indeed, pS1Pless mice showed a basal vascular leak in the lung, which can be rescued by the restoration of plasma S1P levels (118). Basal pulmonary leakage phenotypes were observed even in mice showing partial (approximately 50%) reduction in blood S1P levels, namely *Sphk1* (117) or *Apom* knockout mice (48). However, virtually no exaggerated pulmonary leakage was observed in EC-specific *Spns2* knockout mice showing a 23% reduction of plasma S1P level (33). These data suggest that blood S1P regulates basal barrier integrity in a dose- and/or chaperone-dependent manner. This hypothesis is further supported by the observation that EC-specific *S1pr1* knockout mice showed much higher pulmonary leakage

than *Apom* knockout mice (45). It is also conceivable that S1P might regulate barrier integrity in a cellular source-dependent manner. RBCs supply circulatory S1P predominantly via SphK1. By contrast, the SPNS2-mediated S1P supply should be exclusively from ECs. Therefore, the S1P supply from RBCs might have a more significant role in endothelial barrier maintenance. Furthermore, HDL-bound S1P might have a larger contribution to the constitutive barrier integrity than albumin-bound S1P. This scenario is supported by cultured EC experiments as discussed above. Further studies are needed to elucidate the dose-, source-, and carrier-dependent role of circulatory S1P in barrier maintenance.

Among five S1P receptors, $S1P_1$ plays a central role in the endothelial barrier homeostasis. Indeed, the pharmacological blockage of $S1P_1$ induced pulmonary edema in mice (88, 119). Global or EC-specific deletion of *S1pr1* increased vascular permeability under basal conditions in the colon, retina, and lung (45, 93, 99, 120). Moreover, basal leakage phenotypes of pS1Pless and *Apom* knockout mice were rescued by selective agonists for $S1P_1$ (93, 118). Clinical use of FTY720 showed adverse events, including respiratory symptoms and macular edema (83, 126). Importantly, the FTY720–induced increase of lung vascular permeability was dramatically reduced in $S1P_1$-S5A knockin mice in which genomic $S1P_1$ is replaced with an internalization-defective mutant of $S1P_1$ that resists FTY720–induced degradation (88). In accordance with the protection from pulmonary edema, $S1P_1$ protein degradation by FTY720 was substantially ablated in the mutant lungs (88). Therefore, targeting lymphocyte $S1P_1$ while sparing endothelial $S1P_1$ may be a better therapeutic strategy for the control of various autoimmune diseases.

## The Blood-Brain Barrier

The role of S1P in the physiological barrier function in tissues with a specialized vascular barrier (e.g., brain, spinal cord, retina, testis) is not known. The blood-brain barrier (BBB) is a highly selective permeability barrier that protects the central nervous system from variations in blood composition and toxins. In cultured human brain ECs, S1P enhanced the barrier integrity via $S1P_5$ activation (127). However, $S1P_2$ signaling rather diminished BBB integrity in human brain ECs (124). Using the in situ rodent brain perfusion model, the administration of S1P increased the brain uptake of substrates of P-glycoprotein, an ATP-driven drug efflux pump (128, 129). The effect is mediated by the $S1P_1$-dependent rapid and reversible reduction of P-glycoprotein activity (128, 129). However, the in vivo role of S1P in physiological barrier function has not been studied. We have recently shown that *Apom* knockout mice have normal BBB integrity under physiological conditions (45). Therefore, unlike its role in normal vascular barrier integrity, the impact of ApoM-bound S1P on BBB maintenance appears dispensable. BBB is composed of ECs together with pericytes, astrocytes, and neurons. Hence, these components might promote BBB function through multiple S1P receptors and the potential local perivascular supply of S1P.

## Barrier Maintenance in High Endothelial Venules by Locally Released S1P

In HEVs, postcapillary venules for lymphocytes homing to LNs and SLOs, endothelial barrier integrity is regulated by S1P in a unique manner. As described above, platelets do not contribute to plasma S1P levels. However, platelet-derived local S1P has a critical role in the maintenance of HEVs' barrier integrity. Indeed, pS1Pless mice showed severe leakage and

bleeding in LNs after an immune challenge (130). This barrier dysfunction was completely rescued by the daily transfusion of platelets from wild-type mice but not from pS1Pless mice (130). Mechanistically, extravasated platelets in the perivenular space of HEVs interact with the perivenular sleeve of fibroblastic reticular cells and release S1P (130). The locally released S1P maintains vascular en-dothelial (VE)-cadherin expression and tightens the HEVs' barrier in an $S1P_1$-dependent manner (130). Different vascular beds have distinct perivascular components and requirements for barrier maintenance. Thus, the perivascular local supply of S1P might facilitate the unique barrier regulation by cooperating with more exhaustive regulation through constitutive circulatory S1P.

## VASCULAR TONE REGULATION BY SPHINGOSINE 1-PHOSPHATE

S1P potentially regulates vascular tone in both directions: vasodilation or vasoconstriction. *Sphk1* or *Sphk2* knockout mice showed normal mean arterial blood pressure (25). Thus, a partial reduction or increase in circulatory S1P levels is unlikely to affect homeostatic blood pressure. However, exogenous application of S1P induces vascular tone changes in arteries from various tissues. Therefore, it is conceivable that circulatory S1P controls local vascular tone in vivo. Indeed, *S1pr2* knockout mice displayed a regional increase of blood flow (131), as discussed below. Generally, S1P induces vasodilation through $S1P_1$ and $S1P_3$ on ECs, by activation of eNOS and the production of NO, a strong vascular relaxant (132). In contrast, S1P causes vasoconstriction through $S1P_2$ and $S1P_3$ activation on VSMCs at higher concentrations, presumably under conditions of endothelial injury/damage (132). This dichotomy likely depends on the anatomical location of vascular beds and the pathophysiological context.

### $S1P_1$ As a Vasodilatory Receptor

S1P exerts vasodilative action by acting on $S1P_1$, presumably by cooperating with flow-dependent mechanotransduction systems. Using cultured ECs and isolated blood vessels, many studies have shown that S1P induces a marked production of NO via activation of eNOS (133–136). Pharmacological or siRNA-mediated inhibition studies also have elucidated the key role of $S1P_1$ in this process (137, 138). Indeed, the $S1P_1$ antagonist substantially reduced basal eNOS activity, increased basal tone, and enhanced the vasoconstriction response to α-adrenergic stimulation in murine mesenteric arteries (138). Notably, flow-mediated vasodilation was blunted by an $S1P_1$ antagonist (138). Thus, $S1P_1$ activation is indispensable for the well-established vasodilatory role of blood flow. These data suggest cooperation between the aforementioned $S1P_1$ signaling and mechanotransduction systems.

### Bidirectional Roles of $S1P_2$ and $S1P_3$ in Vascular Tone Regulation

The role of $S1P_2$ on vascular tone regulation seems to be either vasoconstrictive or vasodilatory, depending on the vascular beds or segments. A study with *S1pr2*-knockout mice elucidated a role of $S1P_2$ in the homeostatic vascular tone (131). *S1pr2* knockout mice display normal systolic blood pressure; however, they showed regional (renal and mesenteric artery) elevated blood flow and a decrease in vascular resistance (131). Vascular contractile response to α-adrenergic stimulation was also blunted in *S1pr2* knockout mice both in vivo

and ex vivo (131). Exogenous application of S1P also in duced vasoconstriction in perfused mouse lung models, which was blunted in *S1pr2* knockout mice (139); $S1P_2$ can also function as a vasodilatory receptor. In an isolated cerebral artery, S1P induced an exaggerated vasoconstriction in *S1pr2* knockout mice compared with wild-type mice (140). Together, $S1P_2$ function in vascular tone is bidirectional depending on the vascular beds. Notably, in renal microcirculation, S1P induced an $S1P_2$-dependent vasoconstriction in afferent but not efferent arterioles (141). This observation indicates that S1P can regulate vascular tone in both a vascular bed-dependent and a segment-specific manner. *S1pr2* is preferentially expressed in VSMCs in the vessel wall. However, some ECs in a variety of organs also express *S1pr2* in vivo (76). Thus, the bidirectional or locus-dependent role of $S1P_2$ would be explained by an expression balance between ECs and VSMCs among different vascular beds or segments. Further detailed $S1P_2$ expression analysis or experiments using tissue-specific *S1pr2* knockout mice will likely elucidate the context-dependent role of $S1P_2$ in vascular tone regulation.

$S1P_2$ is essential for proper maintenance of the auditory and vestibular systems by controlling blood flow in the cochlea. *S1pr2* knockout mice exhibited profound hearing loss early in life with the progressive degeneration of auditory and vestibular organs (142–144). Notably, the earliest cellular lesions in the cochlea were found in the stria vascularis, a compartment that harbors the main vasculature of the inner ear (144). *S1pr2* mRNA is abundantly expressed in stria vascu-laris (144), and *S1pr2* knockout stria vascularis showed morphologic abnormalities of capillary dilation and distortion (144). In addition, S1P induced vasoconstriction in gerbil spiral modiolar arteries that directly supply blood to the vessels of the stria vascularis (145). Remarkably, the S1P– induced vasoconstriction was blunted by an $S1P_2$ antagonist (144). Hence, the $S1P_2$-mediated F egress was suggested by the observation vasoconstriction of modiolar arteries might be essential to protect strial capillary beds from high pressure. Importantly, *Spns2* knockout mice clearly phenocopied the hearing loss and stria vas-cularis phenotypes of *S1pr2* knockout mice (146). The hearing loss phenotype was observed in mice with neural crest-specific deletion of *Spns2* but not in mice with EC-, LEC-, RBC-, or platelet-specific *Spns2* deletion (146). Therefore, the local secretion of S1P rather than circulatory S1P seems to be important for maintenance of the auditory and vestibular systems. A recent report shows that *S1PR2* is a causative gene for autosomal-recessive hearing impairment in consanguineous Pakistani families (147). A pharmacological agent that controls $S1P_2$ activity may have a therapeutic benefit to prevent and/or treat hearing disorders not only by gene mutation but also by otoxic drugs, noise, and aging.

As is the case in $S1P_2$, $S1P_3$ activation induces either vasoconstriction or vasodilation, depending on the vascular beds. In a preconstructed thoracic artery, S1P induced vasodilation, which was blunted in *S1pr3* knockout mice (148). In the same model, *S1pr3* knockout thoracic artery showed a 60% reduction in vasorelaxation responses to HDL, suggesting that the vasoactive role of HDL is partially preserved by $S1P_3$ (148). These effects of S1P or HDL were absent in *Nos3* (the gene coding eNOS)-knockout mice, indicating the involvement of eNOS-dependent NO production (148). Therefore, $S1P_3$ seems to work as a vasorelaxant receptor presumably cooperating with $S1P_1$, although $S1P_3$ activation induced vasoconstriction in murine isolated cerebral artery (140, 149). $S1P_3$ may

serve vasoconstrictive functions in humans. FTY720 administration is associated with an increase in blood pressure in human patients (83, 84). Remarkably, siponimod, an $S1P_3$-sparing $S1P_{1/5}$ selective agonist under clinical testing (150), had no significant effect on rat or human blood pressure (151). Thus, FTY720–induced hypertension is likely from the activation of $S1P_3$.

## TRAFFICKING OF IMMUNE CELLS AT THE TISSUE-CIRCULATORY INTERFACE

Enrichment of S1P in blood and lymph circulation is critical for immune cell trafficking. In lymphoid organs, tissue S1P levels are kept low by S1P lyase-dependent degradation (4, 53–55). Lymphocytes egress into blood or lymph circulation at egress portals by $S1P_1$-dependent sensing of S1P gradients at the tissue-circulatory interface (89, 152). Indeed, lymphocyte egress was severely inhibited when circulatory S1P was depleted (9, 33, 70) or when S1P levels in lymphoid tissue were elevated (4, 53–55). As mentioned above, FTY720-P induces internalization/degradation of $S1P_1$ in lymphocytes and thereby inhibits lymphocyte egress (89). The $S1P_1$ receptor on circulating lymphocytes is mostly internalized and desensitized due to the high S1P concentration (153). The internalization/desensitization of $S1P_1$ is critical for circulating lymphocytes to overcome the S1P–dependent attraction to blood and to home again to lymphoid tissues via other chemoattractant cues. Indeed, blood lymphocytes that lack GRK2 displayed a reduced ability to enter LNs, an ability that was restored in *S1pr1*-deficient mice (154). This S1P gradient-mediated lymphocyte trafficking strategy is also utilized for splenic B cell shuttling (155) and trafficking of the other immune cells, including dendritic cells (156), natural killer cells (157, 158), and hematopoietic stem cells (159) by utilizing $S1P_1$ or other S1P receptor(s).

### Microenvironmental Distribution of S1P at the Thymic-Vascular Interface

In addition to the steep S1P gradients at the tissue-vascular interface, local perivascular S1P heterogeneity may also be important for the efficient thymic egress of mature T lymphocytes. The contribution of blood S1P–independent thymic egress was suggested by the observation that the restoration of blood S1P failed to achieve full rescue of thymic egress in pS1Pless mice (9). Therefore, nonhematopoietic cell-intrinsic factors may facilitate $S1P_1$-dependent thymocyte trafficking into the vascular system.

Neural crest-derived pericytes that ensheath blood vessels are proposed to be contributors. This hypothesis originated from the observation that overexpression of $S1P_1$ in thymocytes leads to their perivascular accumulation in the thymus. Thus, it is likely that thymocyte $S1P_1$ activation was ongoing at the region between the basement membrane of the blood vessels and the adjacent pericytes. This scenario was further supported by the analysis using neural crest-specific *Sphk1/Sphk2* knockout mice (80). These mice had normal S1P concentration in blood and tissues. Nevertheless, they showed a significantly increased retention of mature thymocytes in the thymus (80). Consistently, $S1P_1$ cell surface expression was slightly elevated in these mature thymocytes, indicating their reduced exposure to S1P (80). These results suggest that local S1P supply in the perivascular microenvironment might support the recruitment of thymocytes to the thymic-vascular interface.

In addition, ECs also might contribute to the perivascular control of S1P levels by supplying S1P to the abluminal side of thymic vasculature. This hypothesis originated from the analyses of EC-specific *Spns2* knockout mice, which showed severe defects in thymic egress of lymphocytes (31). As discussed above, *Spns2* knockout mice displayed a 50% reduction of blood S1P. However, this partial reduction cannot fully explain the total impairment of thymic egress. First, that amount of blood S1P was enough to stimulate lymphocyte S1P1 in vitro. Furthermore, normal thymic egress was observed in *Sphk1* knockout mice showing a 50% reduction of blood S1P. Therefore, thymocytes can sense the S1P gradients at the thymic-vascular interface even if blood S1P level decreased ~50%. If so, how can their thymic egress defects be explained? One possible explanation is that ECs might release S1P to the abluminal side of thymic vasculature. As a result, thymocytes can be efficiently recruited to the thymic-vascular exit portals.

Moreover, epithelial and endothelial LPP3 may be important to generate and maintain proper S1P distribution in the perivascular microenvironment of the thymus. Global *Ppap2b* (the gene coding LPP3) knockout mice showed impaired thymic egress of lymphocytes (59). Importantly, the S1P levels in blood and whole thymic tissue were unchanged in *Ppap2b* knockout mice. Nevertheless, the thymocytes showed decreased $S1P_1$ cell surface expression, indicating their exposure to high S1P levels. These data suggested that local but not global S1P levels might be elevated in the *Ppap2b* knockout thymus. The impaired thymic egress and decreased thymocyte $S1P_1$ cell surface expression were also observed in epithelial cell-specific *Ppap2b* knockout mice. Because epithelial cells form a network throughout the thymus, LPP3-dependent S1P clearance around epithelial cells might modify perivascular S1P distribution. EC-specific *Ppap2b* knockout mice also displayed impaired thymic egress. Notably, LPP3 showed polarized localization toward the basolateral side of ECs (59). Therefore, endothelial LPP3 might maintain proper perivascular S1P distribution by finely tuning the S1P levels just at the thymus-blood interface, where thymocytes need a sharp gradient to egress into blood circulation.

Taken together, it is likely that a fine microenvironmental distribution of S1P is generated at the perivascular space by the cooperation of multiple cell types, such as ECs, pericytes, and epithelial cells. This S1P distribution pattern would facilitate efficient recruitment of thymocytes to the thymic-vascular interface and ultimately into the blood stream (Figure 3). However, the precise shape of the S1P distribution pattern is not yet elucidated. A related question is whether a similar strategy is applied in other vascular beds. The abluminal release of S1P by SPNS2 might provide specific egress cues for vascular bed- or segment-specific perivascular resident cells. This may also provide unique modulation of barrier integrity and vascular tone in vasculatures of different regions. In addition, given that S1P release is regulated by shear stress, region-specific differences in mechanical stimuli might facilitate flow-mediated fine-tuning of vascular function. The findings of microenvironmental S1P distribution also have highlighted the limitation of traditional S1P quantification systems. Novel reporter mice systems for in vivo mapping of $S1P_1$ activation or S1P distribution will be needed to answer these questions.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Recent studies have provided us with new insights into the generation and maintenance of circulatory S1P. With this in mind, we have now begun to appreciate the physiological significance of this simple lipid mediator in the closed circulatory system of vertebrates, such as the establishment and maintenance of stable vasculature and precise hematopoietic cell trafficking. Furthermore, the primordial role of circulatory S1P is highlighted by the tight link between S1P and blood flow. Therefore, we speculate that circulatory S1P confinement is a strategy of vertebrates to adapt to the closed circulatory system. Moreover, recent data on perivascular microenvironmental S1P distribution have led to a deeper understanding of S1P physiology in and around the tissue barriers. These findings generate new questions. For example, does S1P bound to different chaperones or from different sources have unique roles in vivo? What is the molecular basis for overlapping functional roles of S1P and shear stress signaling? Do different vascular beds have unique perivas-cular S1P distribution? To answer these questions, future studies are needed that combine genetic knockout models, as well as experiments that integrate biochemistry, biophysics, and biophotonics approaches. For example, novel in vivo reporter tools should help us by providing both spatial and temporal information on S1P distribution or S1P receptor activation. These studies are likely to provide significant physiological insights with potential implications for novel diagnostics and therapeutics.

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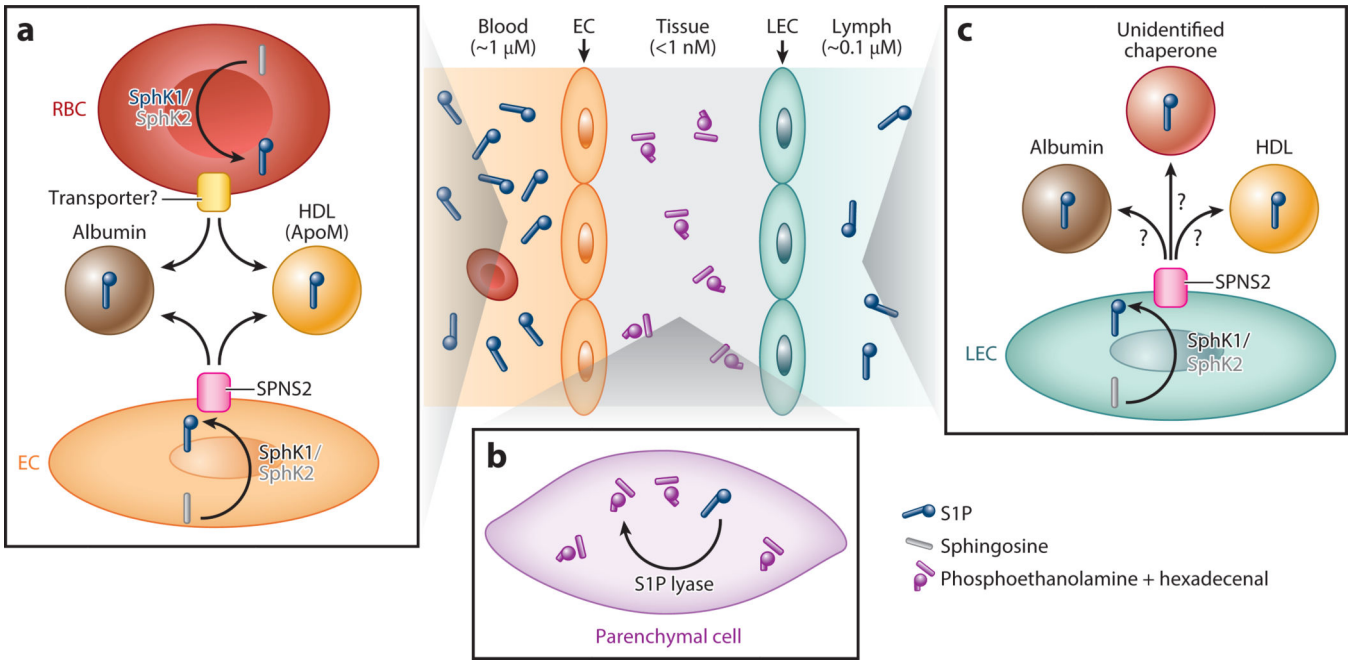


**Figure 1.**

Circulatory compartmentalization of S1P. (*a*) RBCs and ECs are the major sources of plasma S1P. In RBCs, S1P is synthesized from sphingosine mainly by SphK1. Intracellular S1P is secreted by S1P transporter(s). In ECs, S1P is produced mainly by SphK1 and secreted by SPNS2. In plasma, more than half of S1P is found in the ApoM-positive HDL fraction, and 30–40% is found in the albumin fraction. (*b*) In tissues, high S1P lyase activity and irreversible degradation of S1P facilitate their low S1P levels. (*c*) LECs are major sources of lymphatic S1P. S1P is synthesized mainly by SphK1 and secreted through SPNS2. S1P carriers in lymphatic circulation are not yet elucidated. Abbreviations: ApoM, apolipoprotein M; EC, endothelial cell; HDL, high-density lipoprotein; LEC, lymphatic endothelial cell; RBC, red blood cell; S1P, sphingosine 1-phosphate; SphK1, sphingosine kinase; SPNS2, spinster homolog 2.

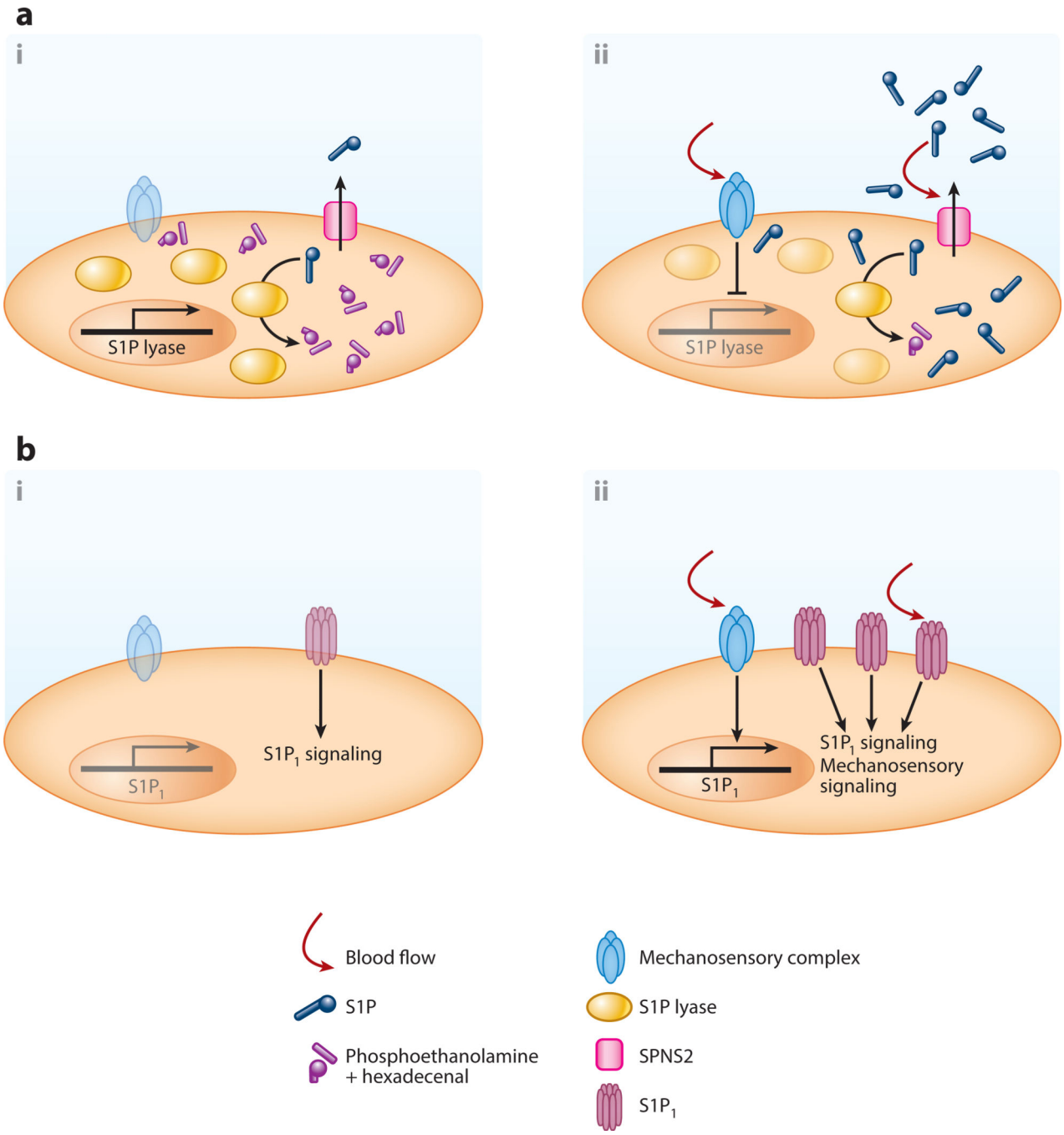


**Figure 2.**

Blood flow-dependent regulation of endothelial sphingosine 1-phosphate (S1P) degradation/secretion and the receptor $S1P_1$ expression/signaling. (*a*) Blood flow enhances endothelial S1P secretion. At the static state, S1P lyase transcription is highly active, which would limit endothelial S1P production and secretion (*i*). Shear stress substantially downregulates S1P lyase expression, which results in an increase of endothelial S1P levels (*ii*). In addition, blood flow rapidly enhances S1P secretion via spinster homolog 2 (SPNS2); however, the precise molecular mechanisms are still unknown (*ii*). (*b*) Flow-dependent regulation of $S1P_1$

transcription and signaling. Without blood flow, $S1P_1$ transcription is less active, which leads to less $S1P_1$ activity (*i*). Shear stress induces $S1P_1$ expression presumably by flow-regulated transcription factors (*ii*). Furthermore, shear stress enhances S1P–dependent $S1P_1$ signaling or even directly activates $S1P_1$ in an S1P–independent manner (*ii*).

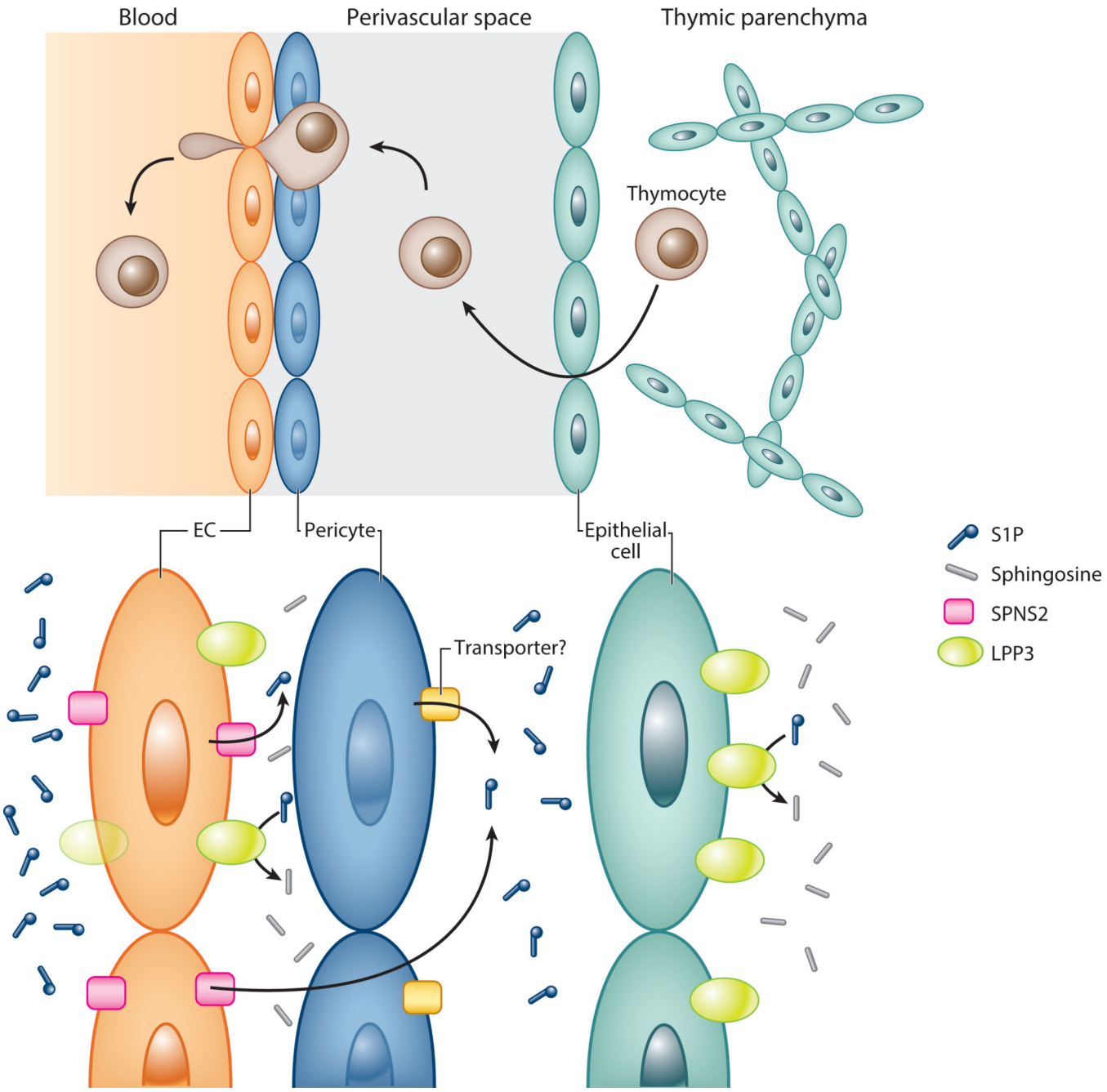


**Figure 3.**

The thymic egress strategy and microenvironmental distribution of S1P. Newly matured thymocytes exit thymic parenchyma into perivascular spaces. Double basement membrane, one located in ECs and the other in epithelial cells, forms the unique space. After migrating to the perivascular spaces, thymocytes exit into the blood by sensing the sharp S1P gradient at the thymic-vascular interface. Pericytes are proposed to provide S1P in the perivascular space to support the recruitment of thymocytes. The pericytic S1P transporter is not yet determined. ECs might also contribute to S1P distribution at the perivascular space by secreting S1P to the abluminal side of thymic vasculature. Conversely, LPP3 localizes

toward the basolateral side of ECs and finely tunes S1P levels at the thymus-blood interface. LPP3 is also expressed in epithelial cells, which would highlight the perivascular S1P compartmentalization or gradients. Abbreviations: EC, endothelial cell; LPP3, lipid phosphate phosphatase 3; S1P, sphingosine 1-phosphate; SPNS2, spinster homolog 2.