Arch. Immunol. Ther. Exp. (2014) 62:103–115
DOI 10.1007/s00005-013-0264-8

REVIEW

Sphingosine-1-Phosphate: a Master Regulator of Lymphocyte Egress and Immunity

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Received: 5 July 2013 / Accepted: 8 November 2013 / Published online: 26 November 2013

Abstract Sphingosine-1-phosphate (S1P) is a central factor responsible for lymphocyte distribution in the body. S1P is able to control the integrity of various effector cell populations within many lymphoid tissues by directing lymphocyte egress. In this review, we give an overview of the generation and degradation of S1P in specific lymphoid microenvironments. Furthermore, we discuss, sometimes contradictory, the functions of the five S1P receptors on different cells in diverse tissues and give an idea of additional counteracting chemotactic signals for lymphocyte immigration and emigration. We focus special attention to recent discoveries of S1P-specific transporters, like spinster-homolog-2 and the active secretion of S1P by endothelial cells, erythrocytes and platelets. In addition, we describe the microanatomical structures as well as entry and egress routes into lymphoid organs which lymphocytes use for efficient trafficking. Finally, we give an overview of open questions regarding the regulation of lymphocyte homing from primary lymphoid organs to secondary lymphoid organs and back again.

Keywords Sphingosine-1-phosphate · S1P receptors · Lymphocyte egress · Spinster-homolog-2

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Introduction

The immune system is in a continuous steady state equilibrium of developing cells and effector cells of the lymphoid and myeloid lineages. This highly orchestrated system is based on a spatiotemporal organization of development, selection and specialization of immune cells in lymphoid organs. Both innate and adaptive immune cells originate from hematopoietic stem and precursor cells (PCs) in the bone marrow or thymus and also develop and mature there. For further selection, specialization and activation, newly generated, i.e., naïve lymphocytes migrate from those primary lymphoid organs via blood to the central secondary lymphoid organs: spleen, lymph nodes (LNs) and Peyer's patches (PPs). Furthermore, naïve and effector lymphocytes use the circulatory systems, i.e., bloodstream and lymphatic vessels, to patrol through the body and provide adaptive immune responses in peripheral tissues. Blood and lymphatic vessels are used by lymphocytes as pipelines to be in the right place at the right time. Lymphocytes enter secondary lymphoid organs via the bloodstream and can then travel with the lymph to reach peripheral tissues. Once in the peripheral tissues, they can return to the bloodstream with extracellular fluid that drains into the lymphatic system and empties back into the blood.

The accessibility of the circulatory systems and the integrin-guided lymphocyte adhesion to the endothelial cells forming the vessel wall are the minimal requirements for the immune surveillance of peripheral tissues by lymphocytes. Initiated by the attraction of cytokines and chemokines, a combination of adhesive events with integrins, selectins and members of the immunoglobulin (Ig) superfamily captures rolling lymphocytes at the luminal surface of the endothelium of post-capillary venules and leads to transendothelial migration into the abluminal tissues (Ley et al. 2007). Here,

Table 1 Summary of the S1P/S1PR interactions on different hematopoietic PCs and lymphocytes with the resulting cellular actions in comparison to S1P-counteracting chemotaxis

S1PR subtype	Cellular expression	S1P-induced action	S1P-counteracting chemotaxis
S1PR1	Hematopoietic stem and PCs	Migration/egress from BM to high S1P concentration	CXCR4/CXCL12
	B cells:	Anti-apoptotic	
	Immature/mature B	Migration/egress from BM and LNs to high S1P concentration	CXCR4/CXCL12
	Follicular/marginal zone B	Shuttling between follicle and marginal zone	CXCR5/CXCL13
		Migration/egress from spleen to high S1P concentration	S1PR3/S1P
	IgG-secreting plasma cells	Migration/egress from spleen to high S1P concentration	
	T cells:	Migration/egress from thymus, spleen and LNs to high S1P concentration	CCR7/CCL19-CCL21
		Anti-apoptotic, anti-proliferative	
	T_{reg}	Differentiation	
		Migration/egress from thymus and LNs to high S1P concentration	
	$T_H1/2$	Differentiation (S1PR/mTOR dependent)	
		Migration/egress from BM and LNs to high S1P concentration	
	NKT	Migration/egress from BM to high S1P concentration (T-bet dependent)	
	IEL	Migration/egress from thymus and LNs to high S1P concentration	
	NK cells	Migration/egress from BM to high S1P concentration (T-bet dependent)	
	Eosinophils	Migration/egress from BM to high S1P concentration	
	Monocytes/osteoclast precursors	Activation, mobilization, migration to high S1P concentration in the BM parenchyma	S1PR2/S1P CXCR4/CXCL12 RANK/RANKL
S1PR2	B cells:		
	GC B cells	Migration	
		Rho/ROCK activation	
		Retention in the GC	
		Apoptosis	
	Monocytes/osteoclast precursors	Activation, migration to low S1P concentration, mobilization	S1PR1/S1P
		Chemorepulsion to high S1P concentrations	CCR2/CCL2
S1PR3	B cells:		
	Immature/mature B	Negative regulation of retention time in BM sinusoids	CXCR4/CXCL12
	Follicular/marginal zone B	Chemorepulsion to high S1P concentrations	
S1PR4	B cells	Anti-apoptotic	
	T cells	Anti-apoptotic, anti-proliferative	
S1PR5	NK cells	Migration/egress from BM and LNs to high S1P concentration (T-bet dependent)	

BM bone marrow, *PCs* precursor cells, *GC* germinal center

lymphocytes mature to effector cells, inspect the abluminal sites in search of cognate antigens and provide support to innate immune cells. If not needed, lymphocytes egress from the spleen into the blood and from LNs and PPs into the lymph and migrate to different peripheral lymphoid tissues to provide immune function at other locations.

The driving force that mediates egress of lymphocytes from thymus and secondary lymphoid organs was not understood for a long time until the small lipid mediator sphingosine-1-phosphate (S1P) came to light in experiments that reveal an S1P gradient between luminal and abluminal compartments. Since then, our image of the directional egress of lymphocytes from the thymus and the secondary lymphoid organs into the circulatory system has been constantly growing. In this review, we would like to give an overview of the cellular sources of S1P and the secretory and enzymatic processes that form an S1P gradient between luminal and abluminal compartments as well as the intrinsic role of S1P receptors (S1PRs) in lymphoid cells (Table 1).

The S1P Cycle: a Matter of Composition and Decomposition

Sphingolipids were recognized as structural components of cellular membranes and it was not expected that their

metabolites were important for the regulation of numerous physiological processes. However, intense work on the sphingolipid metabolites ceramide, sphingosine and S1P characterized them as critical regulators of cell fate. Despite their structural relationship, sphingolipids can regulate opposing cellular effects: while ceramide and sphingosine are proapoptotic (Ogretmen and Hannun 2004; Spiegel and Milstien 2003), S1P promotes cell proliferation and viability, angiogenesis, cell motility and migration and lymphocyte trafficking (Alvarez et al. 2007). Furthermore, the discovery of the pathological roles of sphingolipids in inflammatory diseases, cancer, osteoporosis and atherosclerosis proved the importance of sphingolipid metabolism for cellular homeostasis (Maceyka et al. 2012). S1P derives from ceramide which, in turn, is generated by hydrolyzation of sphingomyelin by sphingomyelinases. However, ceramide can be formed alternatively by the de novo pathway: serine-palmitoyl-transferase condenses serine and palmitoyl-CoA to generate 3-keto-dihydrosphingosine, which is then reduced to dihydro-sphingosine (Bartke and Hannun 2009). *N*-acetylation of dihydrosphingosine by dihydro-ceramide-synthases leads to the release of dihydro-ceramide. Subsequent desaturation of dihydro-ceramide through dihydro-ceramide-desaturase results finally in the release of ceramide (Bartke and Hannun 2009). Ceramide is further processed by ceramidases to release sphingosine that undergoes phosphorylation by one of two sphingosine-kinases (Sphk1 or Sphk2) at the plasma membrane (Sphk1) or in mitochondria, endoplasmic reticulum (ER) and nucleus (Sphk2) to form S1P (Spiegel and Milstien 2007). The equilibrium between sphingosine and S1P is furthermore controlled by S1P-phosphatase that catalyzes the reverse reaction, or is irreversibly degraded by S1P lyase at the ER into phosphoethanolamine and 2-hexadecanal (Saba and Hla 2004; Schwab et al. 2005; Serra and Saba 2010; Spiegel and Milstien 2011). Although S1P is believed to be generated intracellularly by all cell types during sphingolipid degradation (Spiegel and Milstien 2003), the bioactive S1P concentration is cell and tissue specific (Cyster and Schwab 2012).

Total lymphoid tissues (thymus, spleen, LNs) and mouse ears show micromolar concentrations of S1P, which is in the range of the plasma-S1P concentration (Ledgerwood et al. 2008; Schwab et al. 2005). Contradictorily, in most lymphoid tissues, S1P concentration in their interstitial fluid is low (subnanomolar) due to the S1P-degrading activity of S1P lyase in the interstitium (Schwab et al. 2005). Furthermore, in erythrocytes and platelets, which do not express S1P-phosphatase or S1P lyase, intracellular S1P concentrations are high (micromolar) (Ito et al. 2007; Kobayashi et al. 2009).These findings provide evidence that there is a large difference in S1P abundance between the circulatory fluids and the lymphoid organs. This is supported by indirect measurements of the inverse relationship between lymphocyte S1PR1 surface deposition and extracellular S1P (Schwab et al. 2005). S1PR1 is sensitive to S1P-mediated internalization as concluded from lymphocytes that lose S1PR1 surface expression if S1P concentration is $\geq$100 nM (Lo et al. 2005). In addition, mass spectrometry data of plasma and lymph revealed high (micromolar) concentrations of S1P (Pappu et al. 2007). Consequently, lymphocytes isolated from blood or lymph exhibit undetectable surface expression of S1PR1, whereas lymphocytes isolated from total lymphoid organs express S1PR1 on the surface.

Upon generation, S1P is secreted by specific transporters (as discussed below) to interact with the five heterotrimeric G-protein-coupled S1PR1, 2, 3, 4 and 5, in an autocrine or paracrine manner (Rosen and Goetzl 2005) (Table 1). All S1P receptors show high affinities for S1P (nanomolar) and are distributed on multiple cell lineages (Chun et al. 2010) (Table 1). The S1P–S1PR interaction first came to attention in pharmacological tests of the chemical myriocin derivate FTY720 that causes lymphopenia in blood and lymph of mice. Histological analyses revealed that in the medullary sinuses of LNs in FTY720-treated mice, a very low number of lymphocytes could be found, indicating an effect of the drug on lymphocyte egress from the LN tissue to efferent lymphatics (Mandala et al. 2002). In further analyses the pharmacological action of FTY720 could be explained by the fact that it shares structural similarities with sphingosine and can be phosphorylated by Sphk2 in vivo (Kharel et al. 2005; Zemann et al. 2006). Furthermore, phosphorylated FTY720 is a potent agonist of S1P on four of the five S1PRs: S1PR1, 3, 4 and 5 (Brinkmann et al. 2002).

Egress from Primary Lymphoid Organs

Bone Marrow

In adult mice and humans, hematopoietic development takes place in primary lymphatic organs. Those sites create an environment that, according to need, stimulates multilineage differentiation, proliferation and maturation of hematopoietic stem cells (HSCs) and PCs into immunocompetent cells. Mature leukocytes (myeloid cells, B cells, NK cells), erythrocytes and rarely HSCs and hematopoietic PCs are released from the bone marrow sinusoids to the blood to home to the peripheral lymphoid tissues to carry out their function. However, the thymus is the exclusive primary lymphoid organ for T cell differentiation and maturation of early hematopoietic and lymphoid bone marrow-derived PCs. Although mature lymphocytes

acquire immunocompetence during their differentiation and maturation in primary lymphoid organs, they do not show any immune response. Since interaction with antigens is necessary for naïve lymphocytes to finally differentiate into effector cells that drive immune responses, cells have to egress the primary lymphoid organs to migrate into the peripheral secondary lymphoid organs to undergo antigen-driven selection, activation and maturation.

The major factor retaining HSCs and PCs in the bone marrow is the chemokine CXCL12 (Ding and Morrison 2013; Greenbaum et al. 2013). CXCL12 is secreted by the mesenchymal and vascular cells of the hematopoietic microenvironment and induces chemoattraction of hematopoietic progenitor cells that mostly express its corresponding receptor CXCR4 (Sugiyama et al. 2006). Hence, CXCR4-deficient mice show high hematopoietic stem and progenitor cell mobilization in comparison to their wild-type littermates (Foudi et al. 2006). Furthermore, treatment with the CXCR4-antagonist AMD3100 prompted increased migration of mouse and human HSC and PC numbers into the circulatory fluids (Broxmeyer et al. 2005). Interestingly, a comparable effect was observed if the S1PR-agonist FTY720 was administered, or the S1P gradient was disrupted by inhibiting S1P lyase activity, which, in fact, led to a reduction of circulating HSCs and PCs (Massberg et al. 2007). These experiments provided evidence that S1P could attract hematopoietic progenitors into the blood (Table 1). Nevertheless, enhancing S1PR1 signaling by the S1PR1-specific agonist SEW2871 failed to increase progenitor mobilization into the periphery (Juarez et al. 2012). Finally, the retaining power of CXCR4/CXCL12 chemotaxis over the S1PR1/S1P chemoattraction became apparent, if additional treatment of AMD3100 was performed, as increased HSC and PC mobilization was observed (Juarez et al. 2012) (Table 1).

In our studies, we also focused on the highly organized migration of monocytoid osteoclast precursors between the bone marrow and blood vessels. We could show that S1P controls the migratory behavior of osteoclast precursor in concert with various chemokines. The administration of SEW2871 led to an activation and mobilization of $CSF1R^+$ or CX_3CR1^+ osteoclast precursors in vitro and in vivo. Furthermore, conditional deletion of S1PR1 in the monocytoid lineage led to an enhanced number of bone resorbing osteoclasts (Ishii et al. 2009). Thus, the enhanced motility of monocytoid osteoclast precursors in the bone marrow parenchyma, together with an enhanced bone resorption in S1PR1-deficient animals, may be evidence for an S1PR1/S1P-regulated access control of osteoclast precursor to the bone surface (Ishii et al. 2009) (Table 1). However, regulation of osteoclast precursor migration turned out to be more sophisticated, as we confirmed by the observation of a mobilizing effect of the S1PR2-specific antagonist JTE013 on CX_3CR1^+ monocytoid osteoclast precursors (Ishii et al. 2010). It was previously reported that S1PR2-expressing cells show reduced chemotaxis to S1P in vitro (Okamoto et al. 2000). Furthermore, we observed a reduced bone resorption in S1PR2-deficient animals in comparison to the control mice (Ishii et al. 2010). From those observations and the fact that high S1P concentration leads to a rapid internalization of S1PR1, we propose that S1PR2 mediates chemorepulsion of osteoclast precursors to regions with high S1P concentrations (Ishii et al. 2010) (Table 1). Hence, osteoclast PCs can be recruited by chemokines, i.e., CXCL12 and RANKL, from the bone marrow parenchyma to the bone surface. However, low S1P concentration in the interstitial bone marrow cavities results in surface presentation of S1PR1, counteracting chemorepulsion of S1PR2-signaling by S1P-driven chemotaxis back to the bone marrow parenchyma (Ishii et al. 2011) (Table 1). In summary, S1P contributes to the regulation of hematopoietic PC egress from the bone marrow. However, S1P is not the only player in this game and acts in a spatiotemporal cooperation with multiple chemoattractants on different precursors of different lineages, as shown, for example, for inflammatory monocytes by the egress-promoting CCR2/CCL2 signaling pathway (Shi et al. 2011) (Table 1).

However, do mature lymphocytes respond to S1P chemoattraction in the bone marrow?

In the bone marrow, lymphoid PCs differentiate into IgM-positive immature B cells, which undergo transition into the periphery. Upon successful selection of their B cell receptor, immature B cells accumulate between the bone marrow parenchyma and sinusoids (Osmond and Batten 1984; Pereira et al. 2009). The concept of S1P as the driving force behind the egress of immature B cells from parenchyma to sinusoids was not likely, as hematopoietic reconstitution of mice with total fetal liver cells of S1PR1-deficient animals revealed an almost complete absence of peripheral T cells, but not B cells. In fact, B cells in spleen, LNs and PPs of S1PR1-deficient fetal liver chimeras were altered in their proportions, but in total showed normal numbers when compared with wild-type controls (Matloubian et al. 2004). In addition, precursor and immature B cells were present in the bone marrow at their usual frequency (Matloubian et al. 2004). To overcome the embryonic lethality of S1PR1 deficiency and to prove the role of S1P and S1PR1 in immature B cell emigration from mouse bone marrow into the blood and peripheral lymphoid organs, B cell-specific conditional S1PR1-knockout animals were developed. Interestingly, B cell development was not affected in those mice either, but transitional immature B cells in the blood and spleen were significantly reduced (Allende et al. 2010) (Table 1). Forced expression

of CD69, a negative regulator of S1PR1 expression, induced comparable reduction of transitional immature B cells in the blood (Allende et al. 2010), while transgenic expression of S1PR1 in the B-lineage caused enforced transmigration of progenitor and immature B cells from the bone marrow parenchyma into the sinusoids (Pereira et al. 2010) (Table 1). In addition to S1PR1, immature B cells express S1PR3 and 4 as well. Although the role of S1PR4 on B cell migration is not revealed yet, and deficiency of S1PR3 does not affect the migratory response of immature B cells to S1P, S1PR3-deficient immunoglobulin μH-chain$^+$ precursor and immature B cells show a reduced retention time in bone marrow sinusoids (Donovan et al. 2010) (Table 1). Hence, these experiments indicate, again, a chemorepulsive effect of S1PR3 on sinusoidal B cells, as we have described for S1PR2 on osteoclast precursors in the bone marrow, emphasizing the importance of a fine-tuning of migratory responses of PCs and mature lymphocytes by the different S1PRs (Table 1).

The natural killer (NK) cell lineage represents another lymphoid lineage that develops in the bone marrow and emigrates from the bone marrow in response to S1P by using S1PR1 and S1PR5. In contrast to B cells and monocytoid precursors, NK cells show accumulation upon S1PR5 deficiency in bone marrow and LNs in a T-bet dependent manner (Jenne et al. 2009) (Table 1). The involvement of the inflammatory transcription factor T-bet in accomplishment of chemotactic responses to S1P by S1PR5 rather than S1PR1 of NK cells (Jenne et al. 2009) and the resulting mobilization of NK cells to inflamed tissues (Walzer et al. 2007) give insights into the dimensions of S1P orchestration of the cellular innate and adaptive immunity. Furthermore, inflammation-related eosinophilia in blood and skin can be abolished by inhibiting S1P chemotaxis of eosinophils from the bone marrow parenchyma to the sinusoids by FTY720 or SEW2871 treatment (Sugita et al. 2010). Moreover, FTY720 or SEW2871 treatment leads to T_H cell sequestration in the bone marrow (Fujii et al. 2008; Maeda et al. 2010) showing the importance of the S1PR1/S1P signaling pathway in the regulation of recirculation of effector immune cells (Table 1).

Thymus

The thymus recruits HSC-derived T cell-prone lymphoid PCs from the bone marrow and provides an environment which gives support for differentiation, selection, maturation and temporally coordinated release of mature T effector cells into the periphery (Nitta et al. 2008). Similar to developing B cells in the bone marrow, thymocytes rearrange their variable T cell receptor (TCR) genes α and β or γ and δ to form two lineages of T cells expressing TCRαβ or TCRγδ during their differentiation into CD4 or CD8 single-positive (SP) T cells. Passing the CD4 and CD8 double-negative (DN) thymocyte differentiation stages (with distinct CD44 and/or CD25 surface expression), thymocytes develop into double-positive (DP) T cells that give rise to SP stages of development. The developing T cells assemble in the cortico-medullary junction and migrate as DPs into the cortex to undergo negative and positive selection by proving their TCR avidity to peptide–MHC complexes provided by the cortical microenvironment. Subsequently, low avidity T cells are allowed to relocate by CCR7-directed migration into the medullary regions and differentiate to self-tolerant CD4 or CD8 SP semi-mature T cells with a diverse TCR repertoire (Kwan and Killeen 2004; Ueno et al. 2004; Weinreich and Hogquist 2008). However, semi-mature SP T cells are unable to leave the thymus before the transcription factor Krüppel-like factor-2 (KLF2) acts by inducing upregulation of S1PR1 and CD62L, and thereby directs the final differentiation into mature egress-competent SP T cells (Bai et al. 2007; Carlson et al. 2006). Consequently, accumulation of CD62L$^+$ SP T cells could be observed in mice which lack T-cell emigration inducing S1P gradient between thymic interstitium and blood vessel lumen because of their sphingosine-kinase deficiency (Pappu et al. 2007). Furthermore, the importance of the S1PR1/S1P chemoattraction for T cell egress was shown by loss-of-function approaches of S1PR1: fetal liver chimera of S1PR1-deficient mice and conditional deletion of S1PR1 in the T lineage resulted in T cell accumulation of mature CD62L$^+$ SP T cells (Allende et al. 2004; Matloubian et al. 2004) (Table 1).

These studies focused mainly on the most prominent conventional αβ T lineage, without giving much attention to specific T effector cell populations. Increasing interest in the role of T effector cells in immunity in the past decade has thrown light onto the regulation of migration of specific T cell subsets through the body. S1PR1 is expressed on CD4$^+$/CD25$^+$/FoxP3$^+$ regulatory T cells (T_{reg}) (Sawicka et al. 2005; Wang et al. 2004) (Table 1), but FTY720-induced sequestration to lymphoid organs has not been addressed. Consequently, its direct functional importance on thymus emigration is also unclear (Sawicka et al. 2005). Nevertheless, conditional deletion of S1PR1 in CD4$^+$ SP T cells leads to an accumulation of T_{reg} cells in the thymus, and gain-of-function experiments of S1PR1 resulted in a decreased thymic T_{reg} population (Liu et al. 2009). Strikingly, the S1P/S1PR1 interaction not only regulates the emigration capacity of T_{reg} cells but also inhibits T_{reg} generation and reciprocally emphasizes helper T cell type 1 (T_H1) differentiation by an S1PR1/mTOR pathway (Liu et al. 2009, 2010) (Table 1). Likewise, natural killer T cells (NKT) significantly increase in the thymus upon

conditional deletion of S1PR1 in T cells (Allende et al. 2008) (Table 1). Interestingly, KLF2 but not S1PR1 deletion resulted in an exceptional expansion of γδ-NKT cells in the thymus, whereas the KLF2 knockout altered S1PR1-dependent emigration of conventional γδ-T cells (Odumade et al. 2010). Furthermore, conventional CD4 or CD8αβ intraepithelial T lymphocytes (IEL) egress from the thymus in a S1PR1/S1P-dependent way (Table 1), whereas the unconventional DN precursors IEL are not S1P responsive (Kunisawa et al. 2007a, 2007b).

Although it is evident that S1P and S1PR1 are the main regulators of thymic T cell egress, transcriptional regulation of chemokine receptor expression and microenvironment-specific S1P degradation provide further dimensions in thymic T cell egress control.

Lipid phosphate phosphatase-3 (LPP3) is a S1P-degrading enzyme which controls S1P distribution between the thymic parenchyma and blood vessel. Importantly, lipid phosphate phosphatases are localized next to the plasma membrane with their metabolic domain exposed on the cell surface. Hence, LPP3 dominates extracellular S1P degradation in the thymus, whereas intracellular S1P-degrading enzymes such as S1P lyase or S1P phosphatase 1 or 2 have a minor contribution to the low S1P concentration in the thymus (Breart et al. 2011). Indeed, endothelial or epithelial cell-specific LPP3 deficiency impairs thymic egress of T cells (Breart et al. 2011). Thus, LPP3 facilitates efficient thymic T cell egress by maintaining low S1P concentrations in the thymic interstitium.

S1PR1-, CD62L- and β7-integrin expression did not occur in mice with a targeted mutation of KLF2 (Carlson et al. 2006). As a result, mature T cells were again unable to leave the thymus and move into the peripheral lymphoid organs (Carlson et al. 2006), but, when KLF2-deficient T cells were induced to express the KLF2-target S1PR1 again, T cells emigrated from the thymus normally (Zachariah and Cyster 2010). These results supported the hypothesis that KLF2-activated S1PR1 expression and surface deposition are essential for efficient T cell egress from the cortico-medullary junction into the blood. Conditional deletion of KLF2 in $CD4^+$ T cells endorsed these results, and revealed a negative regulative function of KLF2 on interleukin 4 production (Weinreich et al. 2009), emphasizing a regulative role for KLF2 on T cell homeostasis not only by regulation of T cell emigration, but also on cell-intrinsic fate decisions.

Post-translational regulation of S1PR1 is controlled by the C-type lectin CD69. In T cells, expression of CD69 can be detected from the DP T-cell stage onwards and forced overexpression inhibited mature T-cell egress (Feng et al. 2002; Nakayama et al. 2002). In addition to the role of CD69 in positive selection of thymocytes (which we will not discuss here), it binds to S1PR1, which leads to internalization and S1PR1 degradation (Bankovich et al. 2010; Shiow et al. 2006). Thus, in semi-mature T cells CD69 negatively regulates S1P-mediated T cell egress from the thymus. However, in the transition from semi-mature to mature T cells, CD69 surface expression becomes less abundant and S1PR1 surface deposition is prominent once more. However, the process of downregulation of CD69 expression on mature T cells and the resulting increase of S1PR1 surface deposition need to be clarified in future studies.

Egress from Secondary Lymphoid Organs

Secondary lymphoid organs provide the environment for naïve immunocompetent T and B cells to recognize an appropriate antigen, allowing selective maturation and expansion of specific T and B cells within a large lymphocyte pool which presents a diverse antigen-receptor repertoire (Neuberger 2008; von Boehmer and Melchers 2010). Secondary lymphoid organs serve as concentration points to which antigens are transported by circulatory fluids. Effectively, LNs funnel lymph and collecting antigens from skin and mucosal tissues, while the spleen filters blood-derived antigens. Lymphocytes home from the primary lymphoid organs with the blood stream to LNs and immigrate into the paracortex by specialized post-capillary venules, called high endothelial venules (HEVs) (Bai et al. 2013; von Andrian and Mempel 2003). The microanatomical areas of lymphocyte immigration are much more clearly described for LNs, and are much less accurately understood for the spleen.

Spleen

The spleen can be divided into two main compartments, the white pulp (WP), including the marginal zone, and the red pulp (RP). The blood constantly passes the RP in a complex network of arterioles, which are surrounded by lymphoid tissue. Most of the finest capillaries terminate at the marginal sinus between the WP and the marginal zone. The WP is further subdivided into the T cell-rich periarteriolar lymphoid sheath (PALS) forming the T cell zone, and the B-cell follicles, both surrounded by the marginal zone at the junction between WP and RP. Spleens do not have HEVs for lymphocytes to enter the T cell or B cell zones and accordingly transit in the marginal zone to the WP (Lyons and Parish 1995). Imaging studies helped to define the entry route for T cells into spleen, which could be observed at the junction between the marginal zone and WP when focusing on fibroblastic reticular cells-enriched areas of the PALS (Bajenoff et al. 2008). However, lymphocyte exit routes from the spleen are poorly

characterized. If specialized entry gates exist in the marginal zone, it is assumed that specialized exit paths should be present as well. One indication was the discovery of bridging channels that link the PALS of the WP with the RP and by which activated antigen-challenged $CD8^+$ T cells egress from the WP (Dauner et al. 2008; Khanna et al. 2008) into the RP.

So far, the chemotactic signals that promote lymphocyte egress from the spleen are not well defined, but S1PR/S1P signaling seems to have an influence on splenic lymphocyte emigration. Mice deficient in Sphk1 and Sphk2 respectively do not have detectable amounts of S1P in blood or lymph, and lymphocytes are trapped in spleen and LNs (Pappu et al. 2007) (Table 1). The significant lymphopenia in the blood of such mice could be rescued with S1P restoration by wild-type bone marrow reconstitution, which, in fact, drained the lymphocytes from the spleen and accumulated them in the LNs (Pappu et al. 2007). In addition, S1PR1-deficient T cells are unable to emigrate into either lymph or blood and accumulate in LNs and spleen upon intravenous transplantation (Matloubian et al. 2004) (Table 1). Moreover, IgG-secreting plasma cells isolated from spleen give a chemotactic response to S1P in vitro and fail to egress from the spleen if they lack S1PR1 (Kabashima et al. 2006) (Table 1).

Lymphocytes immigrate from the marginal zone into the WP and may egress from the WP by specialized bridging channels that drain into the RP. In consideration of this immigration–emigration route we would like to direct our attention to a possible egress-related phenomenon within the splenic B cell pool.

B cells are situated in the follicle of the spleen. To capture blood-borne antigens, a specialized B-cell population with a unique surface expression pattern of the complement receptors CD21 and CD35 and the non-classical major histocompatibility complex molecule CD1d can be detected in the marginal zone and is distinguishable from follicular B cells by expression of the C-type lectin CD23 (Waldschmidt et al. 1992). This unique and static B cell population is restricted to the spleen and supposed to be unable to recirculate (Martin and Kearney 2002). However, the adjacency of marginal zone B cells to the blood stream and the resulting exposure to S1P indicate a possible responsiveness to S1P chemotaxis. Interestingly, marginal zone B cells express S1PR1 and S1PR3 and show rapid, reversible delocalization into the follicle upon FTY720 treatment (Cinamon et al. 2004; Vora et al. 2005). Accordingly, S1PR1 deficiency results in follicular accumulation of marginal zone B cells, but S1PR3-deficient marginal zone B cells do not show misguided localization in the follicle (Cinamon et al. 2004; Girkontaite et al. 2004). The displacement of marginal zone B cells into the follicle is CXCL13 driven, as FTY720 treatment of CXCL13-deficient mice did not lead to misplacement of marginal zone B cells in the follicle (Cinamon et al. 2004). Effectively, marginal zone B cells continuously shuttle between the marginal zone and the follicle to present captured antigens to FDCs (Cinamon et al. 2008). In fact, the reverse competing S1PR1/S1P- and CXCR5/CXCL13-chemotactic pathways are extrinsically regulated by S1P-induced S1PR1 internalization and surface deposition (Arnon et al. 2011). S1P attraction to the marginal zone dominates the counteracting CXCL13 signal and leads to accumulation of marginal zone B cells in the marginal zone (Table 1). The exposure of marginal zone B cells to high S1P concentration induces S1PR1 internalization and CXCR5/CXCL13 chemoattraction recaptures displacement of marginal zone B cells to the follicle (Table 1). Furthermore, the low S1P concentration in the follicle allows surface deposition of S1PR1 again and a new cycle of the S1P-dominated recruitment of marginal zone B cells in the marginal zone is initialized (Arnon et al. 2011; Cinamon et al. 2008).

Lymph Nodes

B cells also follow the CXCL13-chemotactic signal to enter the LN follicle upon transendothelial migration across HEVs (Cinamon et al. 2004). Even T cells step into the lymph node PALS from HEVs and are recruited in the T cell zone by the CCR7-CCL19/CCL21signaling pathway (Baekkevold et al. 2001; Forster et al. 1999; Okada and Cyster 2007) (Table 1). However, HEVs serve only as entry portals, but not as an egress gate for B and T cells, to migrate from the LNs through the lymphatic sinus into the lymphatic vasculature. Afferent lymphatics stream lymph into the subcapsular sinus that encompasses the LN cortex and connects to the macrophage-rich medullary sinus. The cortical sinus branches through the cortex and, again, ends in the medullary sinus that drains, finally, into the efferent lymphatics (Cyster and Schwab 2012; Grigorova et al. 2009, 2010). Interestingly, lymphocyte immigration and emigration sites in the LN are in close proximity as revealed by serial electron-micrographic sectioning and intravital 2-photon imaging of LNs (He 1985; Ohtani et al. 2003). In fact, lymphocytes enter the lymphatic network through the cortical sinus, which can be found in close relationship to HEVs in the cortical parenchyma (Grigorova et al. 2010; He 1985; Ohtani et al. 2003). However, lymphocytes do not transmigrate directly from HEVs into cortical sinuses and are retained in the parenchyma for at least 30 min, whereas their median residency is up to 24 h (Grigorova et al. 2010; Tomura et al. 2008).

S1P provides the chemotactic force for lymphocyte egress into lymphatic sinuses and efferent lymphatics, as concluded from FTY720-induced depletion from LNs

(Brinkmann et al. 2002; Mandala et al. 2002). This hypothesis was supported by the absence of S1P in measurements of lymph from Sphk1- and Sphk2-deficient animals which displayed normal numbers of T and B lymphocytes in the LNs, but dramatically decreased frequencies in lymphatic vessels (Pappu et al. 2007). Interestingly, it was revealed that S1PR1 signals on T cells have the capacity to dominate CCR7-retaining signals in the LNs and promote egress via cortical sinusoids detected next to follicles (Pham et al. 2008) (Table 1). Finally, intravital 2-photon imaging experiments proved that significant numbers of T cells entered the cortical sinus from the cortical region of the LN and failed to egress, if they were S1PR1 deficient (Grigorova et al. 2009) (Table 1). Furthermore, B cells were shown to egress the LNs by the cortical sinus as well, and FTY720 treatment blocked transendothelial migration of B cells to the lymphatic lumen (Sinha et al. 2009). In addition, conditional deletion of the sphingosine-kinases in Lyve1$^+$ lymphatic endothelium reduced B- and T-cell egress into lymphatic sinuses (Pham et al. 2010). Hence, the cortical sinuses provide preferred egress routes for B and T cells from the LN parenchyma into the lymph in an S1PR1/S1P-dependent way. However, lymphocytes also leave the LNs by subcapsular and medullary sinuses, while the close localization of cortical sinuses to areas of B- and T-cell activation in the follicle predestines them as the "first choice" of circulating lymphocytes for emigration. Furthermore, it should be mentioned again that egress signals may be independent of S1P in rare lymphocyte populations (Grigorova et al. 2009; Sinha et al. 2009; Wei et al. 2005), something which needs to be clarified in future studies.

The dual role of S1PR1/S1P signaling to antagonize chemotactic signals that retain and organize lymphocytes in the follicle, on the one hand, and to actively induce directed migration across the endothelial border into the lymphatic vasculature, on the other, is clearly described (Table 1). However, the detailed cellular processes of the transendothelial migration through the lymphoid sinuses from the lymphocyte and endothelial point of view remain to be clarified.

Do lymphocytes emigrate randomly across the borderline of the sinus or do B and T cells use specific areas, possibly flanked by specialized egress supporting endothelial or stromal cells?

Interestingly, lymphatics are encapsulated in a basement membrane through which blood cells fail to penetrate. However, blind-ending initial skin lymphatics, which share morphological similarities with cortical sinuses, display a perforated basement membrane across which dendritic cells are able to enter the lymphatic vasculature (Pflicke and Sixt 2009). Furthermore, intra-endothelial channels in lymphatics of Peyer's patches were described that allow transmigration of lymphocytes (Azzali 2003). Whether these egress routes also exist in cortical, subcapsular or medullary sinuses will need to be determined in future studies.

The interaction of lymphatic endothelium with homing receptors expressed by leukocytes is another aspect of facilitating transendothelial migration. Recently, the Lyve1-receptor CLEC2 was described, which induces morphodynamic actin changes in dendritic cells mediating entry into lymphatics (Acton et al. 2012). Interestingly, B cells in LNs express CLEC2 and interact with Lyve1-expressing lymphatic endothelial and stromal cells (Acton et al. 2012). Furthermore, morphodynamic modulators such as RAC-GTPases have been described as essential for T-cell egress from lymph nodes (Faroudi et al. 2010). Additionally, a crucial role for the guanine exchange factor for RAC, DOCK2, has been noted to be involved in supporting interstitial lymphocyte motility and S1P-mediated B and T cell egress from peripheral LNs (Nombela-Arrieta et al. 2007).

Importantly, a link between S1P and intracellular actin modifying Rho-GTPases in germinal center (GC) B cells was found by focusing on S1PR2 signaling (Green et al. 2011). In fact, upon S1PR2 stimulation, Gα12, Gα13 and p115RhoGEF antagonized Akt signaling by triggering Rho and ROCK (Green et al. 2011). Consequently, homeostasis of chronically stimulated GCs was affected through S1PR2 signaling by counteracting chemokine-driven B-cell migration to the follicular mantle zone and by negatively regulating cell viability (Green et al. 2011). Both processes were found to be important for three-dimensional GC organization, size restriction and confinement (Green and Cyster 2012; Green et al. 2011).

Lymphocytes Caught Before the Act: How Spinster-Homolog-2 Controls S1P-Driven Lymphocyte Egress from Lymphoid Organs

With the discovery and description of the bio-functional consequences of S1PR/S1P interactions of multiple cell lineages, it became evident that many cell lineages produced and secreted S1P in specific environments. As a consequence, particular attention has been focused on the chemoattraction inducing S1P gradient. How the S1P gradient is maintained, what the cellular source of S1P is, and, how the process of S1P secretion is regulated are points for consideration. As discussed before, the enzymatic processes and steps of S1P generation, S1P bio-activation and S1P degradation are well understood.

Along with the description of the biological processing of sphingolipids, S1P and S1P metabolites, it has been described that platelets produce sphingosine by

sphingomyelin degradation (Tani et al. 2005) and secrete S1P in response to shear stress and thrombin-induced coagulation (Aoki et al. 2007; Kobayashi et al. 2006). Platelets were believed to be responsible for keeping plasma S1P levels at a constantly high level, because they do not express S1P lyase and can transform sphingosine into S1P by Sphk1 (Yatomi 2008). However, platelet-deficient NF-E2-knockout animals displayed normal plasma-S1P levels (Pappu et al. 2007). Furthermore, experiments with plasma and lymph S1P lacking Sphk1/Sphk2-deficient animals discovered that erythrocytes and radio-resistant endothelial cells were a source for plasma and lymph S1P (Pappu et al. 2007; Venkataraman et al. 2008). In addition, mononuclear cells, neutrophils and mast cells are able to metabolize sphingosine into bioactive S1P and secrete S1P in their microenvironment (Jolly et al. 2002; Olivera et al. 2007; Yang et al. 1999).

Interestingly, mast cells actively transport and secrete S1P through the ATP-binding cassette (ABC) family of transporters (Mitra et al. 2006). In humans, 49 different ABC transporters can be classified into seven groups, ABC-A to ABC-G. Sphingolipid transport was reported for ABC-C1, which secretes ceramide and sphingomyelin in gain-of-function experiments (Raggers et al. 1999) and S1P from mast cells (Mitra et al. 2006). Furthermore, ABC-A1-mediated S1P release from platelets can be inhibited by the ABC-A1-specific inhibitor glyburide (Kobayashi et al. 2006). Both ABC transporters, ABC-A1 and ABC-C1, were also suggested to be involved in S1P release from HUVECs in vitro (Lee et al. 2007), whereas ABC-A1 secretes S1P from astrocytes (Sato et al. 2007). In conclusion, some ABC transporters have the capacity to actively contribute to S1P secretion from multiple cell lineages. However, ABC transporters are maybe only in part responsible for secreting S1P from blood or endothelial cells into plasma and lymph, since plasma S1P is mainly supplied by the plural number of erythrocytes, which secrete S1P through a novel ATP-dependent transporter (Kobayashi et al. 2009).

The hypothesis that alternative transporters may be involved in S1P secretion was supported by a study of a point mutation in the zebrafish homolog of the human Spinster-homolog-2 (Spns2) gene that resulted in a defective migration of myocardial precursors, leading to cardia bifida (two hearts) formation (Kawahara et al. 2009). Importantly, this study characterized Spns2 as an active and specific S1P transporter, which upon mutation caused similar phenotype to a zebrafish mutant of the S1PR2 homolog (Kupperman et al. 2000). Furthermore, phosphorylated FTY720 was released from Spns2-transgenic CHO cells which express ABC transporters (ABC-A1, ABC-C1 and others) and supports the specificity of Spns2 for sphingolipid transport (Hisano et al. 2011). Finally, we could show by targeted mutation of Spns2 in mice that Spns2 does not secrete sphingosine and glycerolysophospholipids into plasma, but is responsible for S1P secretion in mammals, as shown in Fig. 1 (Fukuhara et al. 2012). Consequently, peripheral T and B cell populations in blood and spleen were dramatically decreased and mature $CD69^{low}/CD62L^{+}/S1PR1^{+}$ T cells accumulated significantly in the thymus (Fukuhara et al. 2012). Furthermore, Spns2-deficient animals have a reduced number of recirculating B cell populations in the bone marrow, another indication for impaired trafficking of mature lymphocyte populations (Fukuhara et al. 2012). In fact, Spns2-deficient mice displayed a strong reduction of B and T cell populations in LNs as well, further supporting the hypothesis that S1P secretion by Spns2 is not performed by hematopoietic cells (Fukuhara et al. 2012). Bone marrow reconstitution experiments and conditional deletion of Spns2 in endothelial cells confirmed this assumption since total wild-type bone marrow cells were unable to rescue the Spns2-knockout phenotype, and endothelial cell-specific deletion resulted in a comparable phenotype (Fukuhara et al. 2012). Furthermore, total blood cells from Spns2-deficient animals do not show impaired S1P secretion in vitro (Fukuhara et al. 2012). Analyses of alternatively generated conventional Spns2-knockout mice confirmed our observation of impaired lymphocyte egress (Hisano et al. 2012; Nagahashi et al. 2013; Nijnik et al. 2012). Furthermore, the analysis of a second conditional Tie2-CRE/$Spns2^{fl/fl}$-knockout mouse strain endorsed our conclusion that Spns2 secretes S1P specifically from endothelial cells (Mendoza et al. 2012). Taken together, all these studies reveal the importance of Spns2 in controlling S1P-driven lymphocyte egress from primary lymphoid organs (Fig. 1).

However, in comparison to mouse models which display a disrupted S1P gradient as a result of global Sphk1/Sphk2 deficiency (Pappu et al. 2007), specific ablation of those enzymes in neural-crest derived thymic pericytes (Zachariah and Cyster 2010) or LPP3-knockout in thymic epithelial cells (Breart et al. 2011), most recirculating lymphocyte populations are affected in Spns2-deficient animals. Furthermore, animals that conditionally lack Sphk1/Sphk2 in $Lyve1^{+}$ lymphatic endothelium (Pham et al. 2010) show a comparable reduction of lymphocytes in the LNs of Spns2-deficient mice. This Spns2-unique power of controlling the recirculation of almost all lymphocyte populations in traffic between different tissues will be the basis of further intense studies, and elucidate whether or not S1P is the only factor that is secreted via Spns2 and solely controls the recirculation of interstitial lymphocytes in both lymph and blood, respectively. Another fascinating question to answer will be how Spns2 expression and secretion is regulated.

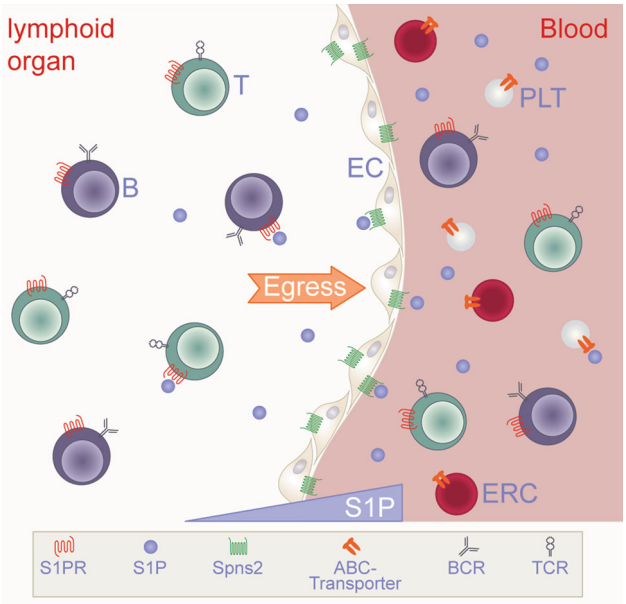

Fig. 1 The putative role of Spns2 in facilitating lymphocyte egress from lymphoid organs into the vasculature: Spns2 is expressed on endothelial cells (EC) and releases S1P into the vessel lumen and the abluminal lymphoid organ. In the blood vessel, platelets (PLT) and erythrocytes (ERC) additionally contribute to a relatively high S1P concentration in the plasma by S1P secretion through ATP-dependent transporters. Hence, B and T lymphocytes (B or T) expressing S1PR follow high S1P concentration along the S1P gradient between the abluminal and luminal side to egress into the vasculature

Are cell-intrinsic or -extrinsic signals responsible for modifying the amount of locally Spns2-secreted factors such as S1P in specific tissues?

In this regard, it will be interesting to clarify if Spns2-expression and the amount of S1P secretion changes in an inflammatory environment, and if negative or positive feedback loops exist that regulate the Spns2 action. Furthermore, the development of the conditional Spns2-deficient animals allows the detailed analysis of the role of Spns2-regulated S1P secretion in many different cells and tissues.

Since S1P is involved in many pathological processes that lead to inflammation and the development of, e.g., autoimmune diseases or cancer, the discovery of the impact of Spns2 on S1P secretion and, in fact, effector cell organization initiated the search for therapeutic strategies that use Spns2 as a potential target. The near future will show if therapeutic antibodies or chemical compounds can locally control S1P secretion in vivo and contribute to the cure of S1P-caused pathological processes.

Acknowledgments We thank Dr. Fritz Melchers (Max Planck Institute for Infection Biology, Berlin, Germany) and Dr. Alison Hobro (WPI-Immunology Frontier Research Center, Osaka, Japan) for critically reading the manuscript. This work was supported by Grants-in-Aid for Scientific Research (A) (25253070) and for Scientific Research on Innovative Areas (22113007), by the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) from the Ministry of Education, Science, Sports and Culture of Japan and by the grant from the International Human Frontier Science Program (RGY0077/2011) and by the Kishimoto Foundation, Osaka, Japan.

Conflict of interest The authors declare no competing financial or commercial conflict of interest.

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