Cell PRESS

Open ACCESS

Cell Reports

Report

The Transporter Spns2 Is Required for Secretion of Lymph but Not Plasma Sphingosine-1-Phosphate

Alejandra Mendoza,[1] Béatrice Bréart,[1] Willy D. Ramos-Perez,[1] Lauren A. Pitt,[1] Michael Gobert,[1] Manjula Sunkara,[2] Juan J. Lafaille,[1] Andrew J. Morris,[2] and Susan R. Schwab[1,*]

[1]Program in Molecular Pathogenesis and Department of Pathology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016, USA

[2]Division of Cardiovascular Medicine, Gill Heart Institute, University of Kentucky, Lexington, KY, 40536, USA

*Correspondence: Susan.Schwab@med.nyu.edu

http://dx.doi.org/10.1016/j.celrep.2012.09.021

SUMMARY

Plasma sphingosine-1-phosphate (S1P) regulates vascular permeability, and plasma and lymph S1P guide lymphocyte egress from lymphoid organs. S1P is made intracellularly, and little is known about how S1P is delivered into circulatory fluids. Here, we find that mice without the major facilitator superfamily transporter Spns2 have a profound reduction in lymph S1P, but only a minor decrease in plasma S1P. Spns2-deficient mice have a redistribution of lymphocytes from the spleen to lymph nodes and a loss of circulating lymphocytes, consistent with normal egress from the spleen directed by plasma S1P and blocked egress from lymph nodes directed by lymph S1P. Spns2 is needed in endothelial cells to supply lymph S1P and support lymphocyte circulation. As a differential requirement for lymph and blood S1P, Spns2 may be an attractive target for immune suppressive drugs.

INTRODUCTION

The concentration of sphingosine-1-phosphate (S1P) is high in circulatory fluids. Plasma S1P regulates vascular integrity; loss of plasma S1P causes increased vascular permeability, likely due to loss of signaling through S1P receptor 1 (S1PR1) on endothelial cells (Camerer et al., 2009; Lee et al., 1999). Plasma and lymph S1P guide lymphocyte egress from lymphoid organs into circulation; exit requires an S1P gradient, low in the organ parenchyma compared to the exit site, that is sensed by lymphocytes via S1PR1 (Schwab and Cyster, 2007). Although extracellular S1P in tissues is thought to be low in homeostasis, an influx of plasma S1P may be a powerful proinflammatory stimulus (Rivera et al., 2008). FTY720, a drug recently approved for treatment of multiple sclerosis, targets four of five S1P receptors (Brinkmann et al., 2010). By inhibiting S1PR1 signaling in lymphocytes, FTY720 traps activated cells in the draining lymph nodes and prevents them from reaching the central nervous system. FTY720 may, however, also target S1P signaling in endothelial cells and cardiomyocytes, contributing to side effects such as macular edema and bradycardia (Brinkmann et al., 2010). To our knowledge, no differential requirements for blood and lymph S1P have been reported to date. A major goal of therapies that manipulate S1P signaling is to achieve greater tissue selectivity.

Many questions remain about how S1P distribution is controlled; one outstanding problem is how S1P, made intracellularly by sphingosine kinases, is exported to the extracellular space where it can signal through cell surface receptors. This question is particularly interesting because S1P is thought to be made by all cells as an intermediate in sphingolipid metabolism; hence, export capacity may be a determinant of whether this S1P is further metabolized, acts on intracellular targets, or is used for cell-cell communication (Saba and Hla, 2004). Several ABC family transporters have been implicated in S1P secretion in vitro. Knockdown or pharmacological inhibition of ABCC1 inhibits S1P export from vascular endothelial cells, mast cells, MCF-7 breast cancer cells, skin fibroblasts, and rat myometrial cells; knockdown or pharmacological inhibition of ABCG2 further decreases S1P export from MCF-7 cells; deletion or pharmacological inhibition of ABCA1 limits S1P release from vascular endothelial cells and astrocytes (Lee et al., 2007; Mitra et al., 2006; Nieuwenhuis et al., 2009; Sato et al., 2007; Takabe et al., 2010; Tanfin et al., 2011). However, neither *Abcc1*$^{-/-}$ nor *Abca1*$^{-/-}$ mice have decreased plasma S1P (Lee et al., 2007). The major facilitator superfamily member Spns2 enables extracellular delivery of S1P, and zebrafish lacking this transporter develop cardia bifida similar to animals lacking S1PR2; Spns2 may transport S1P from the yolk sac into the embryo body to promote myocardial precursor migration to the midline (Kawahara et al., 2009). Spns2 expression by endothelial cells has recently been reported to promote egress of mature T cells from the thymus, likely due to local secretion of S1P at the exit site (Fukuhara et al., 2012; Hisano et al., 2012; Nijnik et al., 2012).

Here, we find that Spns2-deficient mice have a profound reduction in lymph S1P, with only a minor change in plasma S1P. Spns2-deficient mice have a redistribution of lymphocytes from the spleen to lymph nodes and loss of lymphocytes in circulation, consistent with normal egress from the spleen directed by plasma S1P and blocked egress from lymph nodes directed by lymph S1P. Spns2 is required in endothelial cells to supply lymph S1P and support lymphocyte circulation.

RESULTS AND DISCUSSION

After confirming that murine Spns2 can export S1P (Figure S1), we asked whether Spns2 is expressed in the cells that supply

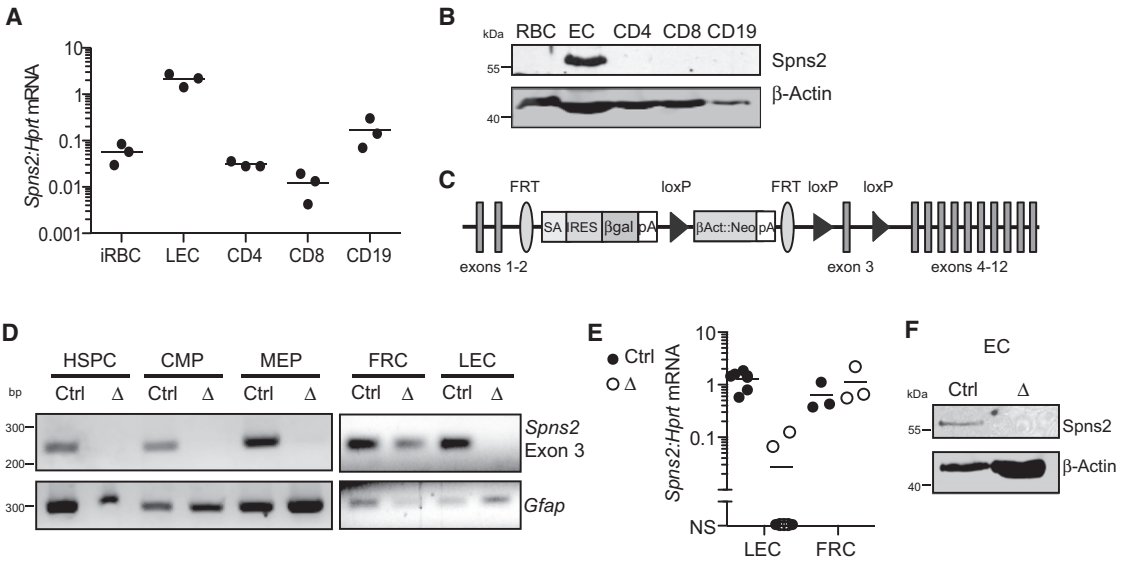

Figure 1. Murine Spns2 Is Expressed by Endothelial Cells but Not RBC

(A) Expression of *Spns2* mRNA by the indicated cell populations, expressed relative to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) transcript, assessed by RT-qPCR. Ter119$^+$CD71$^+$ immature RBC (iRBC) were sorted from bone marrow; CD31$^+$gp38$^+$ lymphatic endothelial cells (LEC), CD4$^+$CD62L^{hi} T cells (CD4), CD8$^+$CD62L^{hi} T cells (CD8), and CD19$^+$CD62L^{hi} B cells (CD19) were sorted from lymph nodes. Data compile three experiments with mice on a B6 background.

(B) Expression of Spns2 protein by the indicated cell populations, assessed by western blot. RBC were isolated from blood by differential centrifugation; CD31$^+$ endothelial cells (EC) were isolated from heart and lung by magnetic bead enrichment; and CD4$^+$ T cells, CD8$^+$ T cells, and CD19$^+$ B cells were isolated from lymph nodes by magnetic bead enrichment. Data are representative of three experiments with mice on a B6 background.

(C) *Spns2*-targeted allele. SA, splice acceptor; pA, polyadenylation signal.

(D–F) Efficiency of *Spns2* deletion in *Spns2$^{f/f}$Tie2-Cre$^+$* mice. PCR for *Spns2* exon 3 in genomic DNA from sorted *Spns2$^{f/f}$Tie2Cre$^+$*(Δ) or littermate control (Ctrl) cells (D). Littermate controls maintained one or two intact alleles of *Spns2*. RBC progenitors were isolated from bone marrow. Hematopoietic stem and progenitor cells (HSPC) were defined as Lin$^-$IL7Rα$^-$c-Kit$^+$Sca1$^+$; common myeloid progenitors (CMP) as Lin$^-$IL7Rα$^-$c-Kit$^+$Sca1$^-$FcγR^{lo}CD34$^+$; and megakaryocyte erythroid progenitors (MEP) as Lin$^-$IL7Rα$^-$c-Kit$^+$Sca1$^-$FcγR^{lo}CD34$^-$. Lymphatic endothelial cells (LEC), defined as CD45$^-$CD31$^+$gp38$^+$, and fibroblastic reticular cells (FRC), defined as CD45$^-$CD31$^-$gp38$^+$ were isolated from lymph nodes. Data are representative of at least two experiments. *Spns2* mRNA assessed by RT-qPCR of transcripts from sorted LEC and FRC (E). Data compile seven pairs of mice analyzed in seven experiments for LEC and three pairs of mice analyzed in three experiments for FRC. NS, no signal. Spns2 protein assessed by western blot of mixed heart and lung endothelial cells (F). Data are representative of two experiments. EC, endothelial cells.

See also Figure S1.

circulatory S1P. These cells have been identified primarily by lineage-specific deletion of the sphingosine kinases. Hematopoietic cells are the main source of plasma S1P, with red blood cells (RBC) being a major contributor (Pappu et al., 2007). RBC have lost most of their mRNA, but *Spns2* transcript is scarce in their immediate precursor reticulocytes (Figure 1A). Spns2 protein was undetectable in RBC (Figure 1B). Lymphatic endothelial cells are the main source of lymph S1P (Pham et al., 2010). *Spns2* mRNA is robustly expressed by lymph node lymphatic endothelial cells (Figure 1A), and Spns2 protein was readily detected in endothelial cells (Figure 1B).

Based on these results, we hypothesized that if Spns2 were important in secretion of circulatory S1P, it might have a selective role in supplying S1P to lymph. To test this, we obtained *Spns2* mutant mice from the NIH Knockout Mouse Project. A splice acceptor preceding exon 3 prematurely terminates the transcript, and we refer to this allele as *Spns2^{tr}*. After flippase-mediated recombination, the original sequence is restored except that exon 3 remains flanked by loxP sites, and we refer to this allele as *Spns2^{f}*. After Cre-mediated recombination, the loss of exon 3 results in a truncated nonfunctional protein (Figure 1C). We crossed *Spns2$^{f/f}$* animals with mice carrying Cre recombinase under the Tie2 promoter, which is expressed in hematopoietic and endothelial cells (Ficara et al., 2008; Kisanuki et al., 2001; Srinivasan et al., 2007). In *Spns2$^{f/f}$Tie2Cre$^+$* mice, exon 3 of *Spns2* was efficiently deleted in genomic DNA of hematopoietic stem cells, common myeloid progenitors, and megakaryocyte-erythrocyte progenitors (RBC and reticulocytes are anucleate), as well as lymph node lymphatic endothelial cells, but not lymph node fibroblastic reticular cells (Figure 1D). Furthermore, *Spns2* transcript was lost in *Spns2$^{f/f}$Tie2-Cre$^+$* lymph node lymphatic endothelial cells but not lymph node fibroblastic reticular cells (Figure 1E), and Spns2 protein was lost in *Spns2$^{f/f}$Tie2-Cre$^+$* endothelial cells (Figure 1F). The loss of *Spns2* mRNA and protein was substantial but not complete in *Spns2$^{tr/tr}$* mice (data not shown); hence, for most experiments we used *Spns2$^{f/f}$Tie2-Cre$^+$* animals.

We first assessed whether blood plasma S1P was altered in Spns2-deficient mice. Tandem mass spectrometry measurements revealed little reduction (~23%) of plasma S1P in *Spns2$^{f/f}$Tie2-Cre$^+$* mice compared to littermate controls (Figure 2A), similar to previous reports (Fukuhara et al., 2012; Hisano

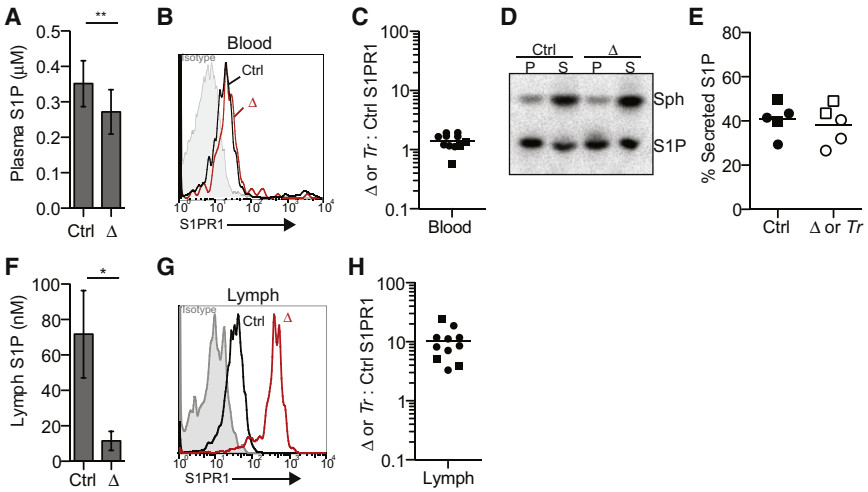

Figure 2. Spns2 Is Essential to Supply Lymph S1P, but Makes a Minor Contribution to Plasma S1P

(A–E) Spns2 makes a minor contribution to plasma S1P. (A) Plasma S1P of *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ (Δ) and littermate control mice quantified by mass spectrometry (n = 6, error bars show SD). (B) Surface S1PR1 on CD62L^{hi}CD4^{+} T cells circulating in the blood of a representative *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mouse (red) and its littermate control (black). Isotype control is shaded gray; note that based on staining of S1PR1 knockout animals, the isotype control staining may be artificially low (Green et al., 2011 and data not shown). (C) The ratio of surface S1PR1 MFI on CD62L^{hi}CD4^{+} T cells in the blood of a *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ or *Spns2*$^{tr/tr}$ (*Tr*) mouse to surface S1PR1 MFI on CD62L^{hi}CD4^{+} T cells in the blood of its littermate control. Circles indicate *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice and controls (eight pairs analyzed in seven experiments), and squares indicate *Spns2*$^{tr/tr}$ mice and controls (three pairs analyzed in three experiments). (D and E) *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ or *Spns2*$^{tr/tr}$ and littermate control RBC were incubated with [3-^{3}H]sphingosine (Sph), which crosses the plasma membrane into the cytosol where it can be phosphorylated. After 90 min, the cell pellet (P) and supernatant (S) were collected. Extracted lipids were separated by thin layer chromatography (TLC) to assess the distribution of [3-^{3}H]S1P. A representative TLC plate visualized by Phosphorimager (D). Data pooled from five experiments (E). Percent secreted S1P: 100 × [S1P in supernatant]/[S1P in pellet + S1P in supernatant]. Circles indicate *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice and controls, and squares indicate *Spns2*$^{tr/tr}$ mice and controls.

(F–H) Spns2 is essential to supply lymph S1P.

(F) Lymph S1P of *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice and littermate controls quantified by mass spectrometry (n = 2–3, error bars show SD).

(G) Surface S1PR1 on CD62L^{hi}CD4^{+} T cells circulating in the lymph of a representative *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mouse (red) and its littermate control (black). Isotype control is shaded gray.

(H) The ratio of surface S1PR1 MFI on CD62L^{hi}CD4^{+} T cells in the lymph of a *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ or *Spns2*$^{tr/tr}$ (*Tr*) mouse to surface S1PR1 MFI on CD62L^{hi}CD4^{+} T cells in the lymph of its littermate control. Circles indicate *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice and controls (eight pairs analyzed in seven experiments), and squares indicate *Spns2*$^{tr/tr}$ mice and controls (three pairs analyzed in three experiments). *p < 0.05, **p < 0.01

et al., 2012; Nijnik et al., 2012). As a complementary measure of S1P, we examined surface S1PR1 expression on naive T cells circulating in blood; S1PR1 is exquisitely sensitive to ligand-mediated internalization, and its surface expression has been used extensively as a measure of cell exposure to S1P (Liu et al., 1999; Pappu et al., 2007; Pham et al., 2010; Schwab et al., 2005). Naive T cells in the blood exhibited similarly low receptor levels in *Spns2*$^{tr/tr}$ mice, *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice, and littermate controls, suggesting that lymphocytes sense equivalent S1P (Figures 2B and 2C). Consistent with near wild-type plasma S1P levels in Spns2-deficient mice, Spns2-deficient RBC secrete S1P normally (Figures 2D and 2E).

We next measured lymph S1P in Spns2-deficient mice. Tandem mass spectrometry measurements revealed a dramatic loss of lymph S1P in *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice compared to littermate controls (Figure 2F). Again, as a complementary measure of S1P, we examined surface S1PR1 expression on naive T cells in lymph. T cells in the lymph of *Spns2*$^{tr/tr}$ and *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice expressed robust surface S1PR1 compared to littermate controls (Figures 2G and 2H), suggesting that the concentration of lymph S1P is very low in Spns2 mutant animals.

We then turned to the effects of Spns2 deletion on lymphocyte trafficking. Naive lymphocytes normally circulate among secondary lymphoid organs surveying for antigen. Exit from the spleen is guided by the high concentration of S1P in plasma compared to the spleen. One indication of this gradient is that surface S1PR1 is higher on lymphocytes in the spleen than in blood; this difference is maintained in *Spns2*$^{f/f}$*Tie2Cre*$^{+}$ mice (Figure 3A). Exit from lymph nodes is guided by the high concentration of S1P in lymph compared to lymph nodes. Again, one indication of this gradient is that surface S1PR1 is higher on lymphocytes in lymph nodes than in lymph; this difference is abolished in *Spns2*$^{f/f}$*Tie2Cre*$^{+}$ mice (Figure 3A).

We predicted that in the presence of normal plasma S1P, egress from the spleen would proceed as usual, but in the absence of lymph S1P, egress from lymph nodes would be blocked. Over time, this should lead to a redistribution of lymphocytes from spleen to lymph nodes, as any cell that left the spleen and entered a lymph node would be trapped in the lymph node. This would also lead to a loss of circulating lymphocytes in blood and lymph. *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice had a reduced percentage of total peripheral naive CD4 T cells in the spleen compared to littermate controls (15% versus 42%), and reciprocally an increased percentage of total peripheral CD4 T cells in the lymph nodes (85% versus 58%) (Figure 3B). Furthermore, *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice had a 24-fold reduction in CD4 T cells circulating in blood, and a 10-fold reduction in CD4 T cells circulating in lymph (Figure 3C). CD8 T cell redistribution in *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$ mice followed the same pattern (Figures 3D and 3E). Consistent with previous findings that B cells have higher tolerance for disruption of S1P gradients (Pham et al., 2010; Schwab et al., 2005), the effect on B cells was less dramatic, although it showed the same trend (Figures 3F and 3G). Layered on top of the lymphocyte redistribution in *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$mice, we saw an overall reduction in lymphocyte numbers in the periphery (Figure 3H). Part of this reduction may be due to inefficient

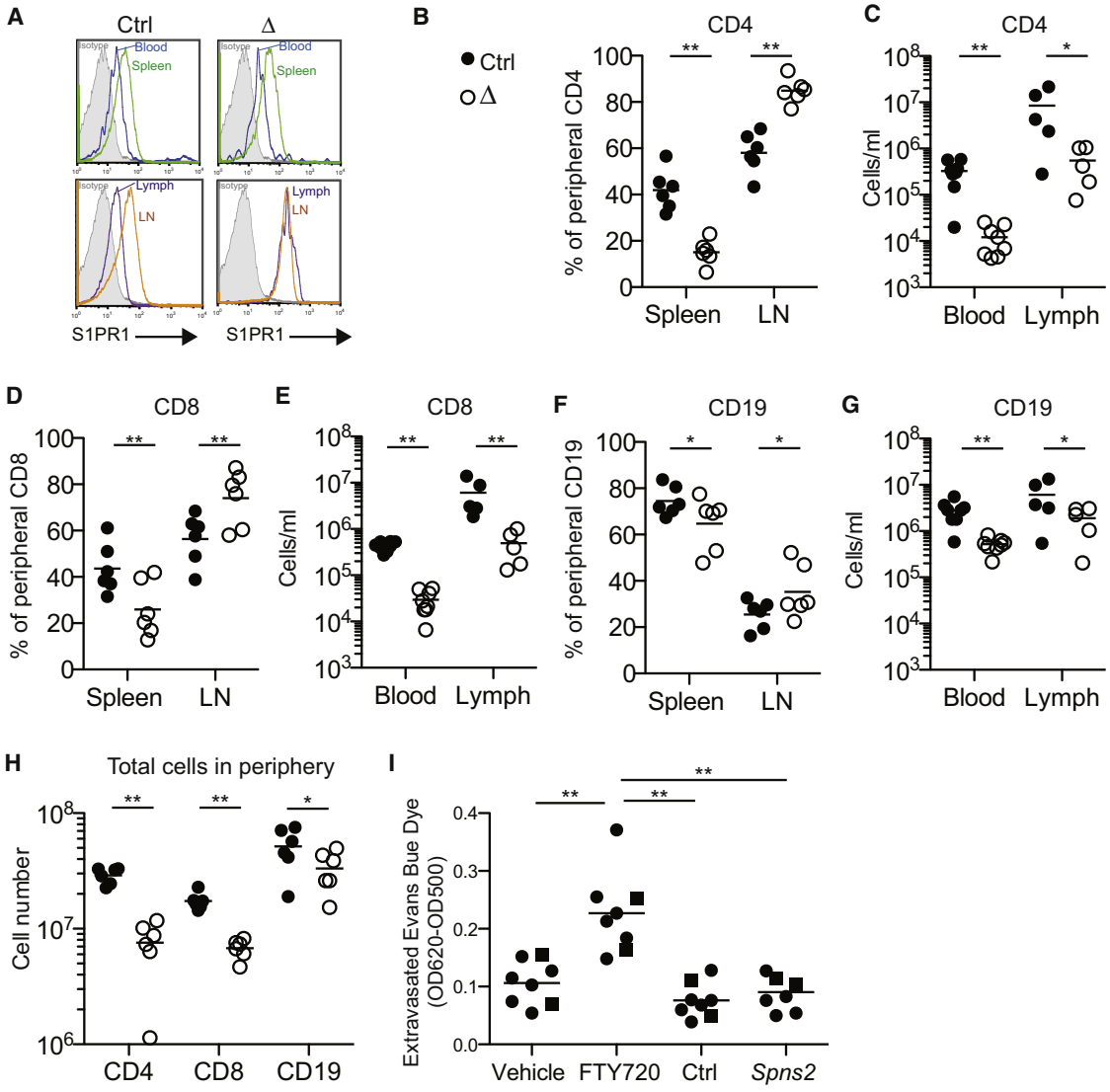

Figure 3. *Spns2* Is Required for Peripheral Lymphocyte Circulation

(A) Surface S1PR1 on CD62L^{hi}CD4^{+} T cells in the blood and spleen (top panels) and in the lymph and lymph nodes (bottom panels) of a representative *Spns2$^{f/f}$Tie2Cre^{+}* mouse (Δ) and its littermate control (Ctrl). Isotype control is shaded gray. Data are representative of eight pairs of mice analyzed in seven experiments.

(B–G) Lymphocyte distribution in *Spns2$^{f/f}$Tie2Cre^{+}* mice and littermate controls. Percent of total peripheral CD62L^{hi}CD4^{+} T cells (B), CD62L^{hi}CD8^{+} T cells (D), and CD62L^{hi}CD19^{+} B cells (F) in the spleen and lymph nodes (LN). Total peripheral lymphocytes are defined as those in spleen and a subset of LN (brachial, axillary, inguinal, and mesenteric); blood and lymph make a negligible contribution. For example, (B, spleen) shows 100 × (# CD62L^{hi}CD4^{+} T cells in spleen)/(# CD62L^{hi}CD4^{+} T cells in spleen + # CD62L^{hi}CD4^{+} T cells in LN). Data pool six pairs of mice analyzed in five experiments. Total number of CD62L^{hi}CD4^{+} T cells (C), CD62L^{hi}CD8^{+} T cells (E), and CD62L^{hi}CD19^{+} B cells (G) in blood and lymph. Data pool eight pairs of mice analyzed in seven experiments for blood, and five pairs of mice analyzed in four experiments for lymph.

(H) Total number of CD62L^{hi}CD4^{+} T cells, CD62L^{hi}CD8^{+} T cells, and CD62L^{hi}CD19^{+} B cells in the periphery. Data pool six pairs of mice analyzed in five experiments.

(I) Vascular permeability in *Spns2$^{f/f}$Tie2Cre^{+}* mice and littermate controls. Spns2-deficient mice and littermate controls, and C57BL6 mice treated with FTY720 or vehicle, were injected intravenously with Evans Blue dye. After 90 min, mice were perfused with PBS. Lungs were removed and extravasated Evans Blue dye was quantified by spectrophotometry. Circles indicate *Spns2$^{f/f}$Tie2-Cre^{+}* mice and controls (six groups analyzed in six experiments), and squares indicate *Spns2$^{tr/tr}$* mice and controls (two groups analyzed in two experiments).

$^{*}p < 0.05$, $^{**}p < 0.01$. See also Figure S2.

egress of mature T cells from the thymus in Spns2-deficient animals (Figure S2; Fukuhara et al., 2012; Hisano et al., 2012; Nijnik et al., 2012).

We next asked whether the requirement for Spns2 to supply lymph S1P and enable egress was intrinsic to endothelial cells. Tie2-Cre deletes in both hematopoietic cells and endothelial

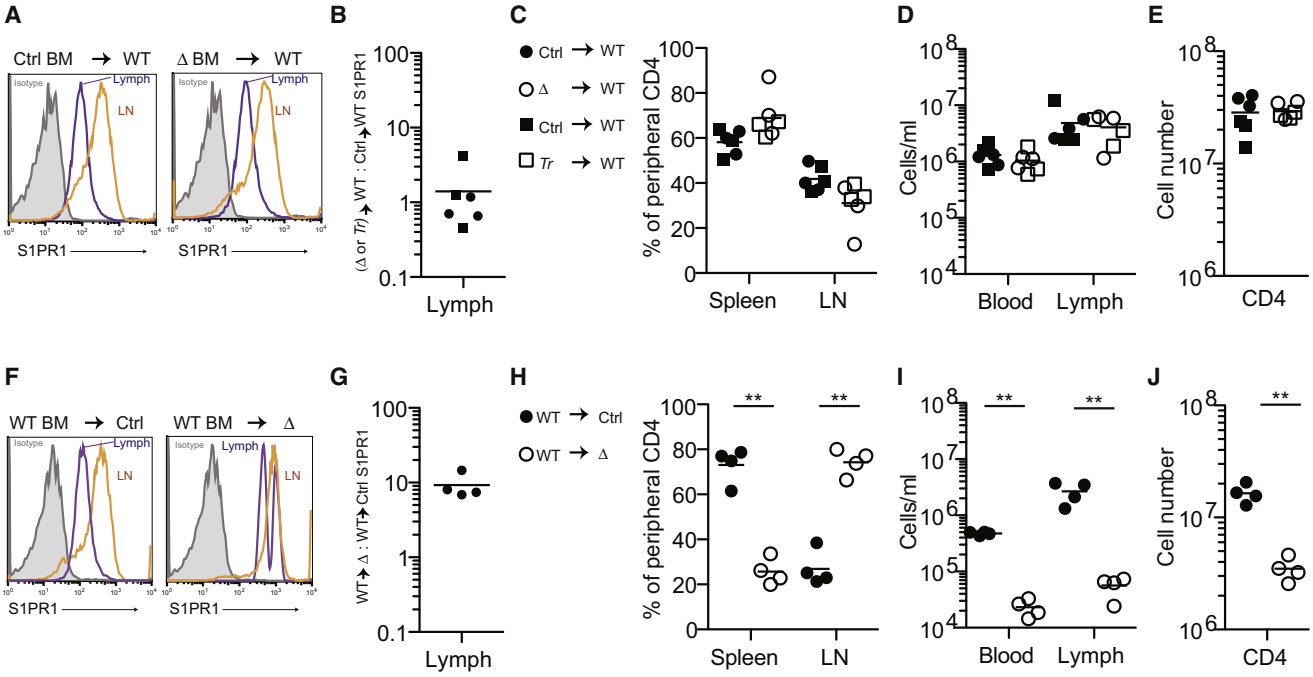

Figure 4. Spns2 Is Required in Endothelial Cells to Supply Lymph S1P and Support Lymphocyte Circulation

(A–E) Ubiquitin C:GFP$^+$ mice were lethally irradiated and reconstituted with BM from Spns2-deficient mice or littermate controls (GFP$^+$ mice were used to allow assessment of RBC chimerism). Mice were analyzed > 6 weeks after transplantation, when RBC and circulating lymphocytes were 97%–99% donor-derived. Deletion of Spns2 in *Spns2$^{f/f}$Tie2Cre$^+$* (Δ) donor hematopoietic stem and progenitor cells (HSPC) was confirmed by PCR. Data compile six pairs of recipients, with two pairs of *Spns2$^{f/f}$Tie2-Cre$^+$*/control BM donors (circles) and one pair of *Spns2$^{tr/tr}$ (Tr)*/control BM donors (squares), analyzed in six experiments. Representative histogram of surface S1PR1 on CD62L^{hi}CD4$^+$ T cells in the lymph and lymph nodes of the indicated chimeras (A). Isotype control is shaded gray. The ratio of surface S1PR1 MFI on CD62L^{hi}CD4$^+$ T cells in the lymph of a WT mouse with Spns2-deficient BM to surface S1PR1 MFI on CD62L^{hi}CD4$^+$ T cells in the lymph of a WT mouse with littermate control BM (B). Percent of total donor-derived peripheral CD62L^{hi}CD4$^+$ T cells in the spleen and lymph nodes (C). Total number of donor-derived CD62L^{hi}CD4$^+$ T cells in blood and lymph (D). Total number of donor-derived CD62L^{hi}CD4$^+$ T cells in the periphery (E).

(F–J) *Spns2$^{f/f}$Tie2-Cre$^+$* mice and littermate controls were lethally irradiated and reconstituted with BM from Ubiquitin C:GFP$^+$ mice. Mice were analyzed > 6 weeks after transplantation, when RBC were 88%–99% donor-derived and lymphocytes were 85%–98% donor-derived. Data compile four pairs of mice analyzed in four experiments. Representative histogram of surface S1PR1 on CD62L^{hi}CD4$^+$ T cells in the lymph and lymph nodes of the indicated chimeras (F). Isotype control is shaded gray. The ratio of surface S1PR1 MFI on CD62L^{hi}CD4$^+$ T cells in the lymph of a *Spns2$^{f/f}$Tie2-Cre$^+$* mouse with WT BM to surface S1PR1 MFI on CD62L^{hi}CD4$^+$ T cells in the lymph of its littermate control with WT BM (G). Percent of total donor-derived peripheral CD62L^{hi}CD4$^+$ T cells in the spleen and lymph nodes (H). Total number of donor-derived CD62L^{hi}CD4$^+$ T cells in blood and lymph (I). Total number of donor-derived CD62L^{hi}CD4$^+$ T cells in the periphery (J). *$p < 0.05$, **$p < 0.01$.

See also Figures S3 and S4.

cells, so to distinguish between the two we made bone marrow (BM) chimeras. First, lethally irradiated wild-type (WT) mice were reconstituted with Spns2-deficient or littermate control BM. We found no reduction in lymph S1P or defect in lymphocyte trafficking in WT mice with Spns2-deficient BM (Figures 4A–4E, S3, and S4). In complementary experiments, *Spns2$^{f/f}$Tie2Cre$^+$* mice and littermate controls were reconstituted with WT BM. In *Spns2$^{f/f}$Tie2Cre$^+$* mice with WT BM, lymph S1P remained low and lymphocyte circulation impaired (Figures 4F–4J, S3, and S4). These results are consistent with the requirement for the sphingosine kinases in lymphatic endothelial cells rather than hematopoietic cells to supply lymph S1P (Pham et al., 2010), and with weak-to-undetectable expression of Spns2 in lymphocytes (Figures 1A and 1B).

Taken together, these data demonstrate that Spns2 expression by endothelial cells is essential for secretion of lymph S1P, while Spns2 is dispensable for RBC secretion of plasma S1P. A major goal in improving therapies that disrupt S1P signaling is to achieve greater receptor and tissue specificity. Although FTY720 is remarkably effective in multiple sclerosis, at least in part because it inhibits exit of activated lymphocytes from lymphoid organs and hence prevents them from reaching the brain, FTY720 has serious vascular and cardiac side effects. To our knowledge, no distinct factors necessary to supply lymph and blood S1P have been identified to date. As a differential requirement for lymph and blood S1P, Spns2 may be an attractive target for immune suppressive drugs that inhibit lymphocyte egress while minimizing effects on vascular stability. In fact, we found that there is little difference in lung vascular permeability between Spns2-deficient and control animals, and Spns2-deficient mice show decreased lung vascular permeability compared to mice treated with FTY720 (Figure 3I). Future work will assess whether Spns2 plays a similarly minor role in other tissues and in inflammation.

As has been previously reported, we find that exit of mature T cells from the thymus of *Spns2*$^{f/f}$*Tie2-Cre*$^{+}$animals is inefficient (Fukuhara et al., 2012; Hisano et al., 2012; Nijnik et al., 2012). Although we cannot exclude a role of other Spns2 substrates in promoting thymic egress, the defect is likely due to disruption of the S1P gradient at the exit site, an interpretation supported by the slight increase in S1PR1 levels on thymocytes of Spns2-deficient mice (Figure S2). And although we cannot know whether Spns2 plays as dominant a role in S1P export from blood vessel endothelial cells in vivo as it does in lymphatic endothelial cells, it is likely a significant contributor. Endothelial cells have been reported to express several of the ABC transporters implicated in S1P export by cultured cells, but how this expression varies among subsets remains to be determined.

One fascinating question is why there is a loss of peripheral lymphocytes in Spns2-deficient mice. The thymic egress block may be a relatively small part of the explanation. Mice with a similar accumulation of mature T cells in the thymus due to disruption of S1P gradients, such as mice lacking lipid phosphate phosphatase 3 in thymic epithelial cells or mice lacking sphingosine kinases in neural crest-derived thymic pericytes, have a smaller reduction in peripheral T cell numbers than Spns2-deficient mice (Bréart et al., 2011; Zachariah and Cyster, 2010). By contrast, mice that lack lymph S1P due to ablation of sphingosine kinases in lymphatic endothelial cells, which does not affect thymic egress because mature T cells exit the thymus into blood, have a similar reduction in peripheral lymphocyte numbers to Spns2-deficient mice (Pham et al., 2010; Zachariah and Cyster, 2010). Future studies will address whether the decrease in overall lymphocyte number in the absence of lymph S1P may reflect a role of S1P itself, or another factor that cells receive during circulation, in promoting survival or homeostatic proliferation (Oskouian and Saba, 2010).

Much work remains to be done to identify the transporters that supply S1P in different tissues and disease states; they may be excellent candidates for local regulation of S1P. As a differential requirement for lymph and blood S1P, Spns2 is an attractive target for specific spatial modulation of S1P signaling.

EXPERIMENTAL PROCEDURES

Mice and Bone Marrow Chimeras

Spns2 mutant mice were obtained from the NIH Knockout Mouse Project. β-Actin-FLPe (Rodríguez et al., 2000), Tie2-Cre (Kisanuki et al., 2001), R26R-EYFP (Srinivas et al., 2001), and Ubiquitin C-GFP mice (Schaefer et al., 2001) were from Jackson laboratories. All mice were on a C57BL6 background. For bone marrow chimeras, recipients were irradiated with two 6.5 Gy doses of γ irradiation from a cesium source separated by 3 hr, and received 2-10 × 10^6 bone marrow cells by intravenous injection. Chimeras were analyzed at least 6 weeks after transplantation. Mice were housed in specific pathogen-free conditions at the Skirball Institute animal facility. All animal experiments were performed in accordance with protocols approved by the New York University Institutional Animal Care and Use Committee.

Cell Preparation and Analysis

Cells were enriched either by magnetic beads (Stem Cell Technologies, biotin selection kit, used according to the manufacturer's instructions), flow cytometry (Beckman Coulter MoFlo or BD Biosciences FACSAria), or differential centrifugation. S1P export assay was adapted from (Olivera and Spiegel, 1998), vascular permeability assay was adapted from (Camerer et al., 2009), and genomic PCR, reverse transcription followed by real-time quantitative PCR (RT-qPCR), western blot, mass spectrometry, confocal microscopy, and flow cytometry were performed using standard procedures. (Please see Extended Experimental Procedures for detailed protocols.)

Statistics

All comparisons are by Student's 2-tailed paired t test. When data are plotted on a log-scale, log-transformed data are compared.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.09.021.

LICENSING INFORMATION

ACKNOWLEDGMENTS

This work was supported by NIH grant R01AI085166 and an NYU Whitehead Fellowship (to S.R.S.), NIH grants R01GM50388 and P20RR021954 (to A.J.M.), NIH training grant T32-HD007520 (to A.M.), a Human Frontier Science Program Long-Term Fellowship (to B.B.), and NIH training grant T32-CA009161 (Levy, to W.D.R.P.). We thank Ken Cadwell, Jason Cyster, David Fooksman, Jason Hall, Sergei Koralov, MacLean Sellars, and Leslie Summers deLuca for critical comments on the manuscript.

Received: May 31, 2012
Revised: August 22, 2012
Accepted: September 14, 2012
Published: October 25, 2012

REFERENCES

Bréart, B., Ramos-Perez, W.D., Mendoza, A., Salous, A.K., Gobert, M., Huang, Y., Adams, R.H., Lafaille, J.J., Escalante-Alcalde, D., Morris, A.J., and Schwab, S.R. (2011). Lipid phosphate phosphatase 3 enables efficient thymic egress. J. Exp. Med. *208*, 1267–1278.

Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., Aradhye, S., and Burtin, P. (2010). Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat. Rev. Drug Discov. *9*, 883–897.

Camerer, E., Regard, J.B., Cornelissen, I., Srinivasan, Y., Duong, D.N., Palmer, D., Pham, T.H., Wong, J.S., Pappu, R., and Coughlin, S.R. (2009). Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. J. Clin. Invest. *119*, 1871–1879.

Ficara, F., Murphy, M.J., Lin, M., and Cleary, M.L. (2008). Pbx1 regulates self-renewal of long-term hematopoietic stem cells by maintaining their quiescence. Cell Stem Cell *2*, 484–496.

Fukuhara, S., Simmons, S., Kawamura, S., Inoue, A., Orba, Y., Tokudome, T., Sunden, Y., Arai, Y., Moriwaki, K., Ishida, J., et al. (2012). The sphingosine-1-phosphate transporter Spns2 expressed on endothelial cells regulates lymphocyte trafficking in mice. J. Clin. Invest. *122*, 1416–1426.

Green, J.A., Suzuki, K., Cho, B., Willison, L.D., Palmer, D., Allen, C.D.C., Schmidt, T.H., Xu, Y., Proia, R.L., Coughlin, S.R., and Cyster, J.G. (2011). The sphingosine 1-phosphate receptor $S1P_2$ maintains the homeostasis of germinal center B cells and promotes niche confinement. Nat. Immunol. *12*, 672–680.

Hisano, Y., Kobayashi, N., Yamaguchi, A., and Nishi, T. (2012). Mouse SPNS2 functions as a sphingosine-1-phosphate transporter in vascular endothelial cells. PLoS ONE *7*, e38941.

Kawahara, A., Nishi, T., Hisano, Y., Fukui, H., Yamaguchi, A., and Mochizuki, N. (2009). The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. Science *323*, 524–527.

Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev. Biol. *230*, 230–242.

Kobayashi, N., Yamaguchi, A., and Nishi, T. (2009). Characterization of the ATP-dependent sphingosine 1-phosphate transporter in rat erythrocytes. J. Biol. Chem. *284*, 21192–21200.

Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., and Hla, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell *99*, 301–312.

Lee, Y.M., Venkataraman, K., Hwang, S.I., Han, D.K., and Hla, T. (2007). A novel method to quantify sphingosine 1-phosphate by immobilized metal affinity chromatography (IMAC). Prostaglandins Other Lipid Mediat. *84*, 154–162.

Liu, C.H., Thangada, S., Lee, M.J., Van Brocklyn, J.R., Spiegel, S., and Hla, T. (1999). Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. Mol. Biol. Cell *10*, 1179–1190.

Lopez-Juarez, A., Morales-Lazaro, S., Sanchez-Sanchez, R., Sunkara, M., Lomeli, H., Velasco, I., Morris, A.J., and Escalante-Alcalde, D. (2011). Expression of LPP3 in Bergmann glia is required for proper cerebellar sphingosine-1-phosphate metabolism/signaling and development. Glia *59*, 577–589.

Mitra, P., Oskeritzian, C.A., Payne, S.G., Beaven, M.A., Milstien, S., and Spiegel, S. (2006). Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. Proc. Natl. Acad. Sci. USA *103*, 16394–16399.

Nieuwenhuis, B., Lüth, A., Chun, J., Huwiler, A., Pfeilschifter, J., Schäfer-Korting, M., and Kleuser, B. (2009). Involvement of the ABC-transporter ABCC1 and the sphingosine 1-phosphate receptor subtype S1P(3) in the cytoprotection of human fibroblasts by the glucocorticoid dexamethasone. J. Mol. Med. *87*, 645–657.

Nijnik, A., Clare, S., Hale, C., Chen, J., Raisen, C., Mottram, L., Lucas, M., Estabel, J., Ryder, E., Adissu, H., et al; Sanger Mouse Genetics Project. (2012). The role of sphingosine-1-phosphate transporter Spns2 in immune system function. J. Immunol. *189*, 102–111.

Olivera, A., and Spiegel, S. (1998). Sphingosine kinase. Assay and product analysis. Methods Mol. Biol. *105*, 233–242.

Oskouian, B., and Saba, J.D. (2010). Cancer treatment strategies targeting sphingolipid metabolism. Adv. Exp. Med. Biol. *688*, 185–205.

Pappu, R., Schwab, S.R., Cornelissen, I., Pereira, J.P., Regard, J.B., Xu, Y., Camerer, E., Zheng, Y.W., Huang, Y., Cyster, J.G., and Coughlin, S.R. (2007). Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science *316*, 295–298.

Pham, T.H., Baluk, P., Xu, Y., Grigorova, I., Bankovich, A.J., Pappu, R., Coughlin, S.R., McDonald, D.M., Schwab, S.R., and Cyster, J.G. (2010). Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. J. Exp. Med. *207*, 17–27, S11–S14.

Rivera, J., Proia, R.L., and Olivera, A. (2008). The alliance of sphingosine-1-phosphate and its receptors in immunity. Nat. Rev. Immunol. *8*, 753–763.

Rodríguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. *25*, 139–140.

Saba, J.D., and Hla, T. (2004). Point-counterpoint of sphingosine 1-phosphate metabolism. Circ. Res. *94*, 724–734.

Sato, K., Malchinkhuu, E., Horiuchi, Y., Mogi, C., Tomura, H., Tosaka, M., Yoshimoto, Y., Kuwabara, A., and Okajima, F. (2007). Critical role of ABCA1 transporter in sphingosine 1-phosphate release from astrocytes. J. Neurochem. *103*, 2610–2619.

Schaefer, B.C., Schaefer, M.L., Kappler, J.W., Marrack, P., and Kedl, R.M. (2001). Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions in vivo. Cell. Immunol. *214*, 110–122.

Schwab, S.R., and Cyster, J.G. (2007). Finding a way out: lymphocyte egress from lymphoid organs. Nat. Immunol. *8*, 1295–1301.

Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J.G. (2005). Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science *309*, 1735–1739.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Srinivasan, R.S., Dillard, M.E., Lagutin, O.V., Lin, F.J., Tsai, S., Tsai, M.J., Samokhvalov, I.M., and Oliver, G. (2007). Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes Dev. *21*, 2422–2432.

Takabe, K., Kim, R.H., Allegood, J.C., Mitra, P., Ramachandran, S., Nagahashi, M., Harikumar, K.B., Hait, N.C., Milstien, S., and Spiegel, S. (2010). Estradiol induces export of sphingosine 1-phosphate from breast cancer cells via ABCC1 and ABCG2. J. Biol. Chem. *285*, 10477–10486.

Tanfin, Z., Serrano-Sanchez, M., and Leiber, D. (2011). ATP-binding cassette ABCC1 is involved in the release of sphingosine 1-phosphate from rat uterine leiomyoma ELT3 cells and late pregnant rat myometrium. Cell. Signal. *23*, 1997–2004.

Zachariah, M.A., and Cyster, J.G. (2010). Neural crest-derived pericytes promote egress of mature thymocytes at the corticomedullary junction. Science *328*, 1129–1135.