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## The transporter Spns2 is required for secretion of lymph but not plasma sphingosine-1-phosphate

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### 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 the first differential requirement for lymph and blood S1P to our knowledge, 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 pro-inflammatory 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 including macular edema and bradycardia (Brinkmann et al., 2010). A major goal of therapies that manipulate S1P signaling is to achieve greater tissue selectivity.

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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*<sup>-/-</sup> nor *Abca1*<sup>-/-</sup> 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 (Fig. S1), we asked whether Spns2 is expressed in the cells that supply 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) a major contributor (Pappu et al., 2007). RBC have lost most of their mRNA, but *Spns2* transcript is scarce in their immediate precursor reticulocytes (Fig. 1A). Spns2 protein was undetectable in RBC (Fig. 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 (Fig. 1A), and Spns2 protein was readily detected in endothelial cells (Fig. 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<sup>tr</sup>*. 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<sup>f</sup>*. After Cre-mediated recombination, the loss of exon 3 results in a truncated non-functional protein (Fig. 1C). We crossed *Spns2<sup>tr</sup>* 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<sup>tr</sup>Tie2Cre<sup>+</sup>* mice, exon 3 of *Spns2* was efficiently deleted in genomic DNA of hematopoietic stem cells, common myeloid progenitors, and megakaryocyte-erythrocyte progenitors (red blood cells and reticulocytes are anucleate), as

well as lymph node lymphatic endothelial cells, but not lymph node fibroblastic reticular cells (Fig. 1D). Furthermore, *Spns2* transcript was lost in *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* lymph node lymphatic endothelial cells but not lymph node fibroblastic reticular cells (Fig. 1E), and *Spns2* protein was lost in *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* endothelial cells (Fig. 1F). The loss of *Spns2* mRNA and protein was substantial but not complete in *Spns2<sup>tr/tr</sup>* mice (data not shown), hence for most experiments we used *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* 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<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice compared to littermate controls (Fig. 2A), similar to previous reports (Fukuhara et al., 2012; Hisano et al., 2012; Nijnik et al., 2012). As a complementary measure of S1P, we examined surface S1PR1 expression on naïve 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). Naïve T cells in the blood exhibited similarly low receptor levels in *Spns2<sup>tr/tr</sup>* mice, *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice, and littermate controls, suggesting that lymphocytes sense equivalent S1P (Fig. 2B,C). Consistent with near wild-type plasma S1P levels in *Spns2*-deficient mice, *Spns2*-deficient RBC secrete S1P normally (Fig. 2D,E).

We next measured lymph S1P in *Spns2*-deficient mice. Tandem mass spectrometry measurements revealed a dramatic loss of lymph S1P in *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice compared to littermate controls (Fig. 2F). Again, as a complementary measure of S1P, we examined surface S1PR1 expression on naïve T cells in lymph. T cells in the lymph of *Spns2<sup>tr/tr</sup>* and *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice expressed robust surface S1PR1 compared to littermate controls (Fig. 2G,H), 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. Naïve 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<sup>fl/fl</sup>Tie2Cre<sup>+</sup>* mice (Fig. 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<sup>fl/fl</sup>Tie2Cre<sup>+</sup>* mice (Fig. 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<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice had a reduced percentage of total peripheral naïve CD4 T cells in the spleen compared to littermate controls (15% vs. 42%), and reciprocally an increased percentage of total peripheral CD4 T cells in the lymph nodes (85% vs 58%) (Fig. 3B). Furthermore, *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* 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 (Fig. 3C). CD8 T cell redistribution in *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice followed the same pattern (Fig. 3D,E). 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 (Fig. 3F,G). Layered on top of the lymphocyte redistribution in *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice, we saw an overall reduction in lymphocyte

numbers in the periphery (Fig. 3H). Part of this reduction may be due to inefficient egress of mature T cells from the thymus in *Spns2*-deficient animals (Fig. S2 and 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 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 (Fig. 4A-E, Fig. S3, and Fig. S4). In complementary experiments, *Spns2<sup>fl/fl</sup>Tie2Cre<sup>+</sup>* mice and littermate controls were reconstituted with WT BM. In *Spns2<sup>fl/fl</sup>Tie2Cre<sup>+</sup>* mice with WT BM, lymph S1P remained low and lymphocyte circulation impaired (Fig. 4F-J, Fig. S3, and Fig. 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 (Fig. 1A,B).

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. As the first differential requirement for lymph and blood S1P to our knowledge, *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 (Fig. 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<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* 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 (Fig. 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 (Breart 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 the first known 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.  $\beta$ -actin-FLPe (Rodriguez 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 Gray doses of  $\gamma$  irradiation from a cesium source separated by 3 hours, and received  $2-10 \times 10^6$  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, 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 is plotted on a log-scale, log-transformed data are compared.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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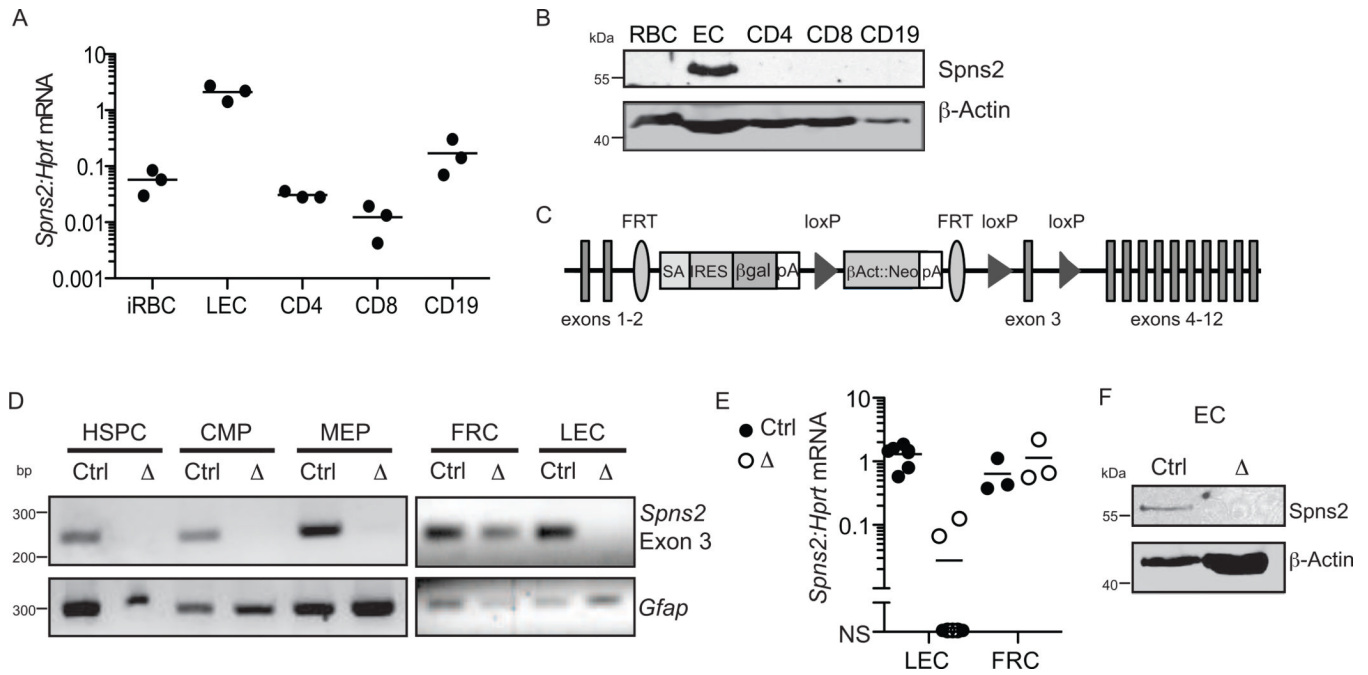
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**Highlights**

- The transporter Spns2 is required to supply lymph but not plasma S1P
- Spns2-deficient mice have disrupted peripheral lymphocyte circulation
- Spns2 is required in endothelial cells to secrete lymph S1P and support trafficking





### Figure 1. Murine *Spns2* is expressed by endothelial cells but not RBC

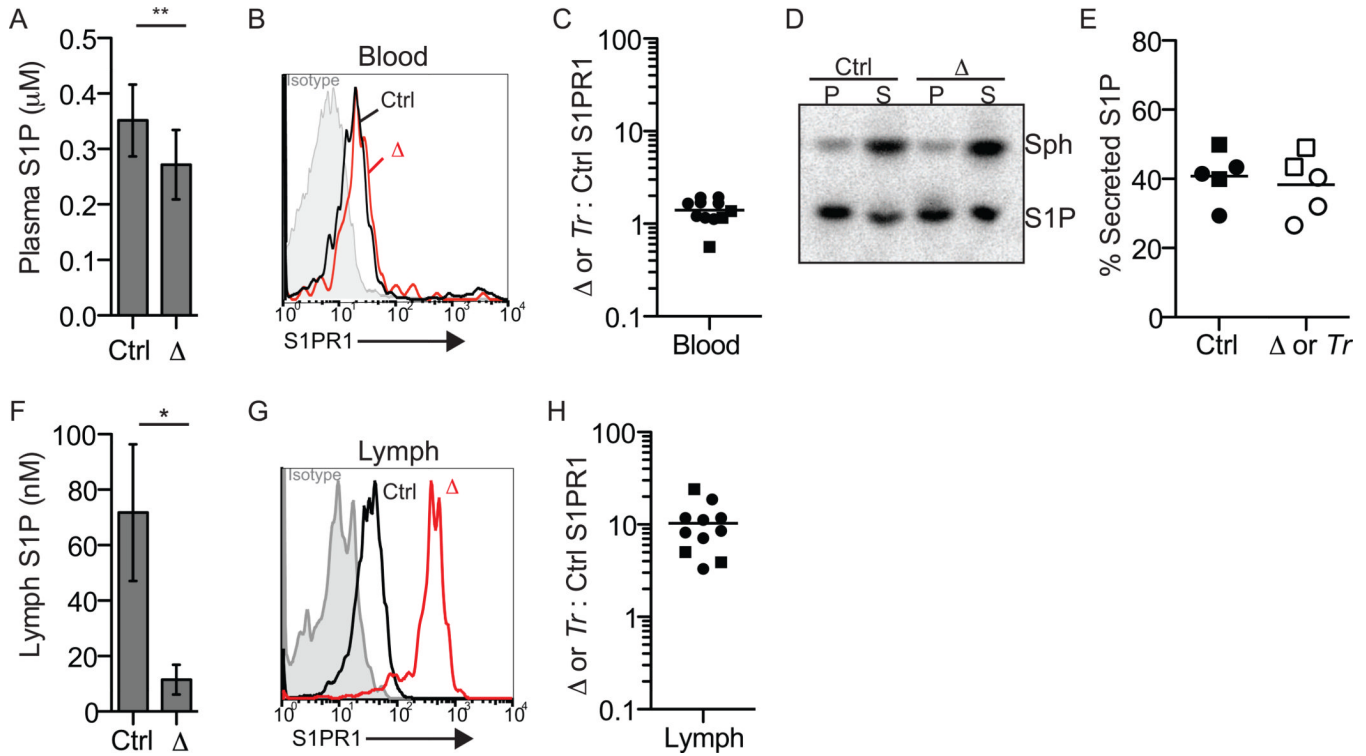
(A) Expression of *Spns2* mRNA by the indicated cell populations, expressed relative to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) transcript, assessed by RT-qPCR. Ter119<sup>+</sup>CD71<sup>+</sup> immature RBC (iRBC) were sorted from bone marrow; CD31<sup>+</sup>gp38<sup>+</sup> lymphatic endothelial cells (LEC), CD4<sup>+</sup>CD62L<sup>hi</sup> T cells (CD4), CD8<sup>+</sup>CD62L<sup>hi</sup> T cells (CD8), and CD19<sup>+</sup>CD62L<sup>hi</sup> B cells (CD19) were sorted from lymph nodes. Data compile 3 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<sup>+</sup> endothelial cells (EC) were isolated from heart and lung by magnetic bead enrichment; and CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD19<sup>+</sup> B cells were isolated from lymph nodes by magnetic bead enrichment. Data are representative of 3 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<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice.

(D) PCR for *Spns2* exon 3 in genomic DNA from sorted *Spns2<sup>fl/fl</sup>Tie2Cre<sup>+</sup>*(Δ) or littermate control (Ctrl) cells. 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<sup>-</sup>IL7Rα<sup>-c</sup>-Kit<sup>+</sup>Sca1<sup>+</sup>; common myeloid progenitors (CMP) as Lin<sup>-</sup>IL7Rα<sup>-c</sup> Kit<sup>+</sup>Sca1<sup>-</sup>FcγR<sup>lo</sup>CD34<sup>+</sup>; and megakaryocyte erythroid progenitors (MEP) as Lin<sup>-</sup>IL7Rα<sup>-c</sup>-Kit<sup>+</sup>Sca1<sup>-</sup>FcγR<sup>lo</sup>CD34<sup>-</sup>. Lymphatic endothelial cells (LEC), defined as CD45<sup>-</sup>CD31<sup>+</sup>gp38<sup>+</sup>, and fibroblastic reticular cells (FRC), defined as CD45<sup>-</sup>CD31<sup>-</sup>gp38<sup>+</sup> were isolated from lymph nodes. Data are representative of at least 2 experiments. (E) *Spns2* mRNA assessed by RT-qPCR of transcripts from sorted LEC and FRC. Data compile 7 pairs of mice analyzed in 7 experiments for LEC and 3 pairs of mice analyzed in 3 experiments for FRC. NS, no signal. (F) *Spns2* protein assessed by Western blot of mixed heart and lung endothelial cells (EC). Data are representative of 2 experiments. See also Fig. S1.



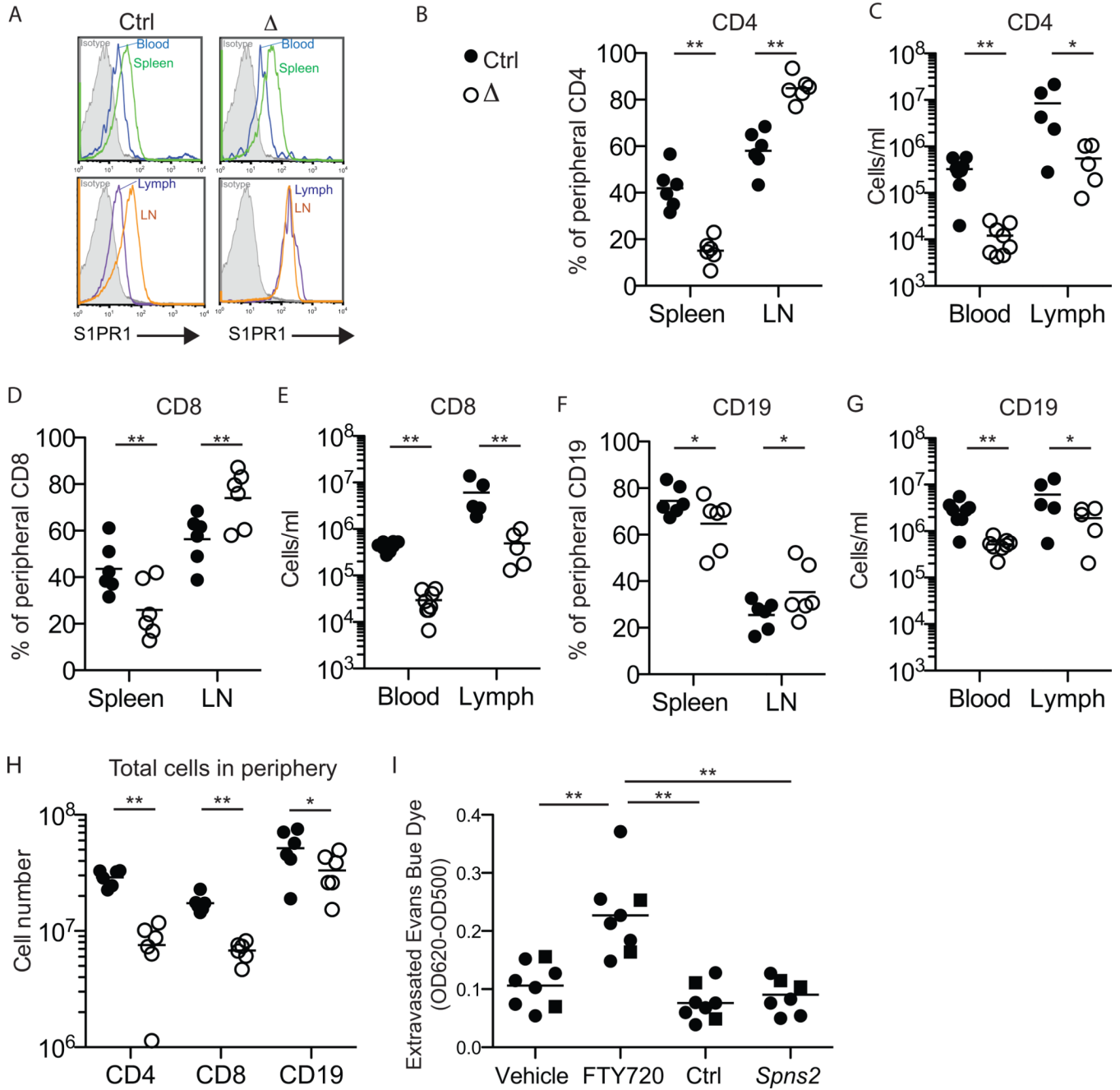
**Figure 2. Spns2 is essential to supply lymph S1P, but makes a minor contribution to plasma S1P** (A-E) Spns2 makes a minor contribution to plasma S1P.

(A) Plasma S1P of *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* ( $\Delta$ ) and littermate control mice quantified by mass spectrometry (n=6, error bars show standard deviation). (B) Surface S1PR1 on CD62L<sup>hi</sup>CD4<sup>+</sup>T cells circulating in the blood of a representative *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mouse (red) and its littermate control (black). Isotype control is shaded grey; 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<sup>hi</sup>CD4<sup>+</sup>T cells in the blood of a *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* or *Spns2<sup>tr/tr</sup>* (*Tr*) mouse to surface S1PR1 MFI on CD62L<sup>hi</sup>CD4<sup>+</sup>T cells in the blood of its littermate control. Circles indicate *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice and controls (8 pairs analyzed in 7 experiments), and squares indicate *Spns2<sup>tr/tr</sup>* mice and controls (3 pairs analyzed in 3 experiments). (D-E) *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* or *Spns2<sup>tr/tr</sup>* and littermate control RBC were incubated with [<sup>3</sup>-<sup>3</sup>H]sphingosine (Sph), which crosses the plasma membrane into the cytosol where it can be phosphorylated. After 90 minutes, the cell pellet (P) and supernatant (S) were collected. Extracted lipids were separated by thin layer chromatography (TLC) to assess the distribution of [<sup>3</sup>-<sup>3</sup>H]S1P. (D) A representative TLC plate visualized by Phosphorimager. (E) Data pooled from 5 experiments. % secreted S1P: 100×[S1P in supernatant]/[S1P in pellet + S1P in supernatant]. Circles indicate *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice and controls, and squares indicate *Spns2<sup>tr/tr</sup>* mice and controls.

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

(F) Lymph S1P of *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice and littermate controls quantified by mass spectrometry (n=2-3, error bars show standard deviation). (G) Surface S1PR1 on CD62L<sup>hi</sup>CD4<sup>+</sup>T cells circulating in the lymph of a representative *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mouse (red) and its littermate control (black). Isotype control is shaded grey. (H) The ratio of surface S1PR1 MFI on CD62L<sup>hi</sup>CD4<sup>+</sup>T cells in the lymph of a *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* or *Spns2<sup>tr/tr</sup>* (*Tr*) mouse to surface S1PR1 MFI on CD62L<sup>hi</sup>CD4<sup>+</sup>T cells in the lymph of its littermate control. Circles indicate *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice and controls (8 pairs analyzed in

7 experiments), and squares indicate *Spns2<sup>tr/tr</sup>* mice and controls (3 pairs analyzed in 3 experiments). \*p<0.05, \*\*p<0.01



**Figure 3. *Spns2* is required for peripheral lymphocyte circulation**

(A) Surface S1PR1 on CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the blood and spleen (top panels) and in the lymph and lymph nodes (bottom panels) of a representative *Spns2*<sup>fl/fl</sup>*Tie2Cre*<sup>+</sup> mouse (Δ) and its littermate control (Ctrl). Isotype control is shaded grey. Data are representative of 8 pairs of mice analyzed in 7 experiments.

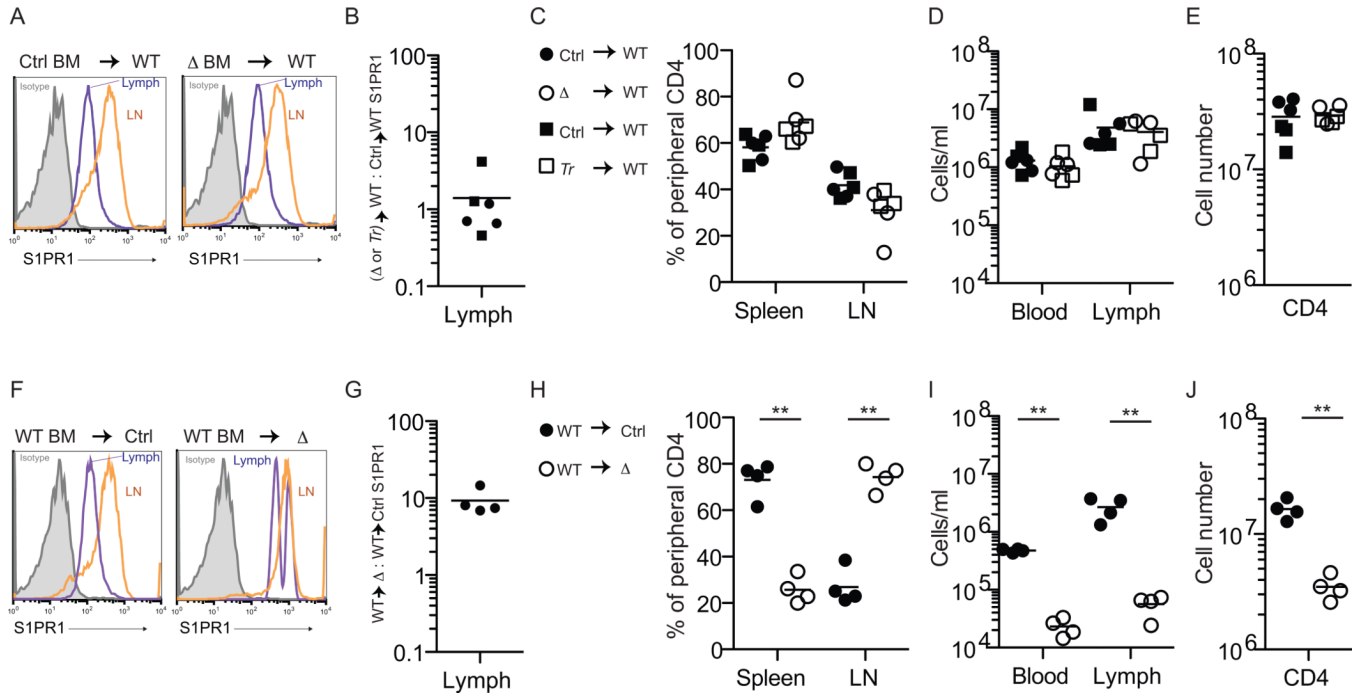
(B-G) Lymphocyte distribution in *Spns2*<sup>fl/fl</sup>*Tie2Cre*<sup>+</sup> mice and littermate controls. (B,D,F) Percent of total peripheral CD62L<sup>hi</sup>CD4<sup>+</sup> T cells (B), CD62L<sup>hi</sup>CD8<sup>+</sup> T cells (D), and CD62L<sup>hi</sup>CD19<sup>+</sup> B cells (F) in the spleen 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  $100 \times (\# \text{CD62L}^{\text{hi}}\text{CD4}^+ \text{ T cells in spleen}) / (\# \text{CD62L}^{\text{hi}}\text{CD4}^+ \text{ T cells in spleen} + \# \text{CD62L}^{\text{hi}}\text{CD4}^+ \text{ T cells in LN})$ .] Data pool 6 pairs of mice analyzed in 5 experiments. (C,E,G) Total number of  $\text{CD62L}^{\text{hi}}\text{CD4}^+$  T cells (C),  $\text{CD62L}^{\text{hi}}\text{CD8}^+$  T cells (E), and  $\text{CD62L}^{\text{hi}}\text{CD19}^+$  B cells (G) in blood and lymph. Data pool 8 pairs of mice analyzed in 7 experiments for blood, and 5 pairs of mice analyzed in 4 experiments for lymph. (H) Total number of  $\text{CD62L}^{\text{hi}}\text{CD4}^+$  T cells,  $\text{CD62L}^{\text{hi}}\text{CD8}^+$  T cells, and  $\text{CD62L}^{\text{hi}}\text{CD19}^+$  B cells in the periphery. Data pool 6 pairs of mice analyzed in 5 experiments.

(I) Vascular permeability in  $\text{Spns2}^{\text{fl/fl}}\text{Tie2Cre}^+$  mice and littermate controls.

$\text{Spns2}$ -deficient mice and littermate controls, and C57BL6 mice treated with FTY720 or vehicle, were injected intravenously with Evans Blue dye. After 90 minutes, mice were perfused with PBS. Lungs were removed and extravasated Evans Blue dye was quantified by spectrophotometry. Circles indicate  $\text{Spns2}^{\text{fl/fl}}\text{Tie2-Cre}^+$  mice and controls (6 groups analyzed in 6 experiments), and squares indicate  $\text{Spns2}^{\text{tr/tr}}$  mice and controls (2 groups analyzed in 2 experiments).

\* $p < 0.05$ , \*\* $p < 0.01$ . See also Fig. S2.



#### Figure 4. *Spns2* is required in endothelial cells to supply lymph S1P and support lymphocyte circulation

(A-E) Ubiquitin C:GFP<sup>+</sup> mice were lethally irradiated and reconstituted with BM from *Spns2*-deficient mice or littermate controls (GFP<sup>+</sup> 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<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* ( $\Delta$ ) donor hematopoietic stem and progenitor cells (HSPC) was confirmed by PCR. Data compile 6 pairs of recipients, with 2 pairs of *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>*/control BM donors (circles) and 1 pair of *Spns2<sup>tr/tr</sup>* (Tr)/control BM donors (squares), analyzed in 6 experiments. (A) Representative histogram of surface S1PR1 on CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the lymph and lymph nodes of the indicated chimeras. Isotype control is shaded grey. (B) The ratio of surface S1PR1 MFI on CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the lymph of a WT mouse with *Spns2*-deficient BM to surface S1PR1 MFI on CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the lymph of a WT mouse with littermate control BM. (C) Percent of total donor-derived peripheral CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the spleen and lymph nodes. (D) Total number of donor-derived CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in blood and lymph. (E) Total number of donor-derived CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the periphery.

(F-J) *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mice and littermate controls were lethally irradiated and reconstituted with BM from Ubiquitin C:GFP<sup>+</sup> mice. Mice were analyzed >6 weeks after transplantation, when RBC were 88–99% donor-derived and lymphocytes were 85–98% donor-derived. Data compile 4 pairs of mice analyzed in 4 experiments. (F) Representative histogram of surface S1PR1 on CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the lymph and lymph nodes of the indicated chimeras. Isotype control is shaded grey. (G) The ratio of surface S1PR1 MFI on CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the lymph of a *Spns2<sup>fl/fl</sup>Tie2-Cre<sup>+</sup>* mouse with WT BM to surface S1PR1 MFI on CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the lymph of its littermate control with WT BM. (H) Percent of total donor-derived peripheral CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the spleen and lymph nodes. (I) Total number of donor-derived CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in blood and lymph. (J) Total number of donor-derived CD62L<sup>hi</sup>CD4<sup>+</sup> T cells in the periphery. \*p<0.05, \*\*p<0.01. See also Fig. S3 and Fig. S4.