The Role of Sphingosine-1-phosphate Transporter *Spns2* in Immune System Function

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Abstract

Sphingosine-1-phosphate (S1P) is lipid messenger involved in the regulation of embryonic development, immune system functions, and many other physiological processes. However the mechanisms of S1P transport across cellular membranes remain poorly understood with several ATP-binding cassette family members and the spinster 2 (Spns2) member of the major facilitator superfamily known to mediate S1P transport in cell culture. Spns2 was also shown to control S1P activities in zebrafish *in vivo* and to play a critical role in zebrafish cardiovascular development. However the *in vivo* roles of Spns2 in mammals and its involvement in the different S1P-dependent physiological processes have not been investigated. Here we characterized *Spns2*-null mouse line carrying the *Spns2*[tm1a(KOMP)Wtsi] allele (*Spns2*[tm1a]). The *Spns2*[tm1a/tm1a] animals were viable, indicating a divergence in Spns2 function from its zebrafish orthologue. However the immunological phenotype of the *Spns2*[tm1a/tm1a] mice closely mimicked the phenotypes of partial S1P deficiency and impaired S1P-dependent lymphocyte trafficking, with a depletion of lymphocytes in circulation, an increase in mature single-positive T cells in the thymus, and a selective reduction in mature B cells in the spleen and bone marrow. Spns2 activity in the non-hematopoietic cells was critical for normal lymphocyte development and localization. Overall *Spns2*[tm1a/tm1a] resulted in impaired humoral immune responses to immunization. This work thus demonstrated a physiological role for Spns2 in mammalian immune system functions but not in cardiovascular development. Other components of the S1P signaling network are investigated as drug targets for immunosuppressive therapy, but the selective action of Spns2 may present an advantage in this regard.

Keywords

Spns2 (spinster 2); sphingosine-1-phosphate (S1P); lymphocyte egress

Introduction

Lipid messenger sphingosine-1-phosphate (S1P) is essential for normal embryonic development and the functions of the cardiovascular and immune systems (1); and components of the S1P signaling network are widely investigated as drug targets for suppression of transplant rejection, autoimmunity and sepsis (2). In the extracellular environment S1P acts through five G-protein coupled receptors ($S1P_{1-5}$) expressed on a variety of cell types (reviewed in (3, 4)). In particular the loss of S1P receptor 1 ($S1P_1$) results in embryonic lethality with abnormal development of the cardiovascular system, while a lymphocyte-specific loss of $S1P_1$ causes impaired exit of mature T cells out of the thymus (5, 6), B cells out of the bone marrow (7, 8), as well as a severe defect in lymphocyte egress from secondary lymphoid organs during their physiological recirculation (9, 10). Receptor $S1P_1$ is also required for normal localization of marginal zone B cells (11), B1 cells (12), plasma cells (13), and gut intra-epithelial T lymphocytes (14), while receptor $S1P_5$ controls NK cell migration (15). Intracellular activities of S1P as a second-messenger molecule are broadly linked to cell survival and growth (16), and in the immune system intracellular S1P promotes inflammatory and antimicrobial activities of mast cells, macrophages, and neutrophils (17-22). In particular S1P is produced downstream of FcεR receptor cross-linking and stimulates mast cell degranulation (23, 24). It is also produced downstream of TNFα-receptor (TNFR) signaling, required for TRAF2-dependent RIP1 activation, NFκB signaling (25) and inflammatory cytokine production (26). These activities of S1P have been implicated in the pathologies of allergic and inflammatory disorders (27, 28).

The activities of S1P as a chemokine and a second-messenger are critically dependent on the S1P concentrations in the different cellular compartments, the tissue environment and the circulation, and these are established through the controlled rates of S1P production, degradation, and transport. S1P is synthesized by the sphingosine kinases SphK1 and SphK2 (29-31), and degraded by the S1P lyase and S1P phosphatases (1, 32). Combined loss of SphK1 and SphK2 in mice results in embryonic lethality with abnormal cardiovascular development, while the loss of S1P lyase causes a milder phenotype with impaired lymphocyte egress from lymphoid organs. In contrast, the mechanisms of S1P transport across cellular membranes remain poorly understood. Several proteins of the ATP-binding cassette (ABC) transporter superfamily can mediate S1P secretion in cell culture, for example ABCC1 in mast cells (33), ABCA1 in red blood cells (34), and another family member in platelets (35). Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) on epithelial cell lines was also shown to contribute to S1P transport (36). Yet knockout mice for these proteins do not phenocopy the knockouts of known S1P signaling network components, and plasma S1P levels in the mice lacking ABCA1 and ABCC1 are unaltered (37). Some S1P is also produced extracellularly, by the extracellular SphK1 enzyme released from the vascular endothelium (38).

Spns2 (spinster homologue 2) is a member of the major facilitator superfamily of transmembrane proteins (39). In recent studies, zebrafish Spns2 was shown to mediate S1P secretion, and the loss of Spns2 activity resulted in lethal defects in cardiovascular development (40, 41), similar to the phenotypes of the $SphK1^{-/-}SphK2^{-/-}$ and $S1P_1^{-/-}$ mouse lines (31, 42). The human Spns2 protein was also shown to mediate the secretion of S1P as well as of S1P-receptor agonist and immunosuppressive drug phospho-FTY720 (43). Furthermore, human Spns2 expression could rescue the developmental defects in zebrafish embryos (40). However, the *in vivo* functions of Spns2 in the mammalian system have not been investigated.

In the current work we characterized an *Spns2*-targeted mouse line *Spns2*^{tm1a/tm1a} generated by the Wellcome Trust Sanger Institute as part of the International Knockout Mouse Consortium (IKMC), and demonstrate the requirement for Spns2 for the normal lymphocyte localization and mammalian immune system function but not the other S1P-mediated functions such as embryonic viability and cardiovascular development.

Materials and Methods

Gene Targeting and Mouse Production

The mouse strain carrying the *Spns2*^{tm1a(KOMP)Wtsi} allele was generated by blastocyst injection of ESC clone EPD0090_5_B04 obtained from the KOMP resource (44). Prior to microinjection, the identity of the targeted ES cells was verified by 5′ long-range PCR using a primer external to the targeting vector. Chimeric mice were bred to C57BL/6-*Tyr*^{c-Brd} and germline transmission was verified by quantitative PCR (qPCR) to detect the neo transgene included in the mutant allele (single insertion event), as well as by loss-of-wild type allele (LOA) qPCR (correct targeted locus) in the F1 heterozygous mice. The presence of the downstream loxP site was verified by PCR. The C57BL/6N-Hprt^{Tg(CMV-cre)Brd/Wtsi} and C57BL/6N-Gt(ROSA)26Sor^{tm1(FLP1)Dym/Wtsi} transgenic lines with systemic expression of Cre and Flp recombinases were previously described (45, 46). The *Spns2*^{tm1b(KOMP)Wtsi} allele was generated by crossing the tm1a allele to the C57BL/6N-*Hprt*^{Tg(CMV-Cre)Brd} allele to delete exon 3 and the neo cassette between the LoxP sites, and the Cre allele was bred out of the colony before study. The *Spns2*^{tm1c(KOMP)Wtsi} allele was generated by breeding to C57BL/6N-Gt(ROSA)26Sor^{tm1(FLP1)Dym/Wtsi} (*Rosa26*^{Fki}) allele expressing Flp recombinase ubiquitously, to delete the inserted cassette but retaining exon 3 flanked by LoxP sites. All the studies were performed on a C57BL/6 genetic background. The mice were maintained in specific pathogen-free conditions, and matched by age and sex within experiments. The care and use of all mice was in accordance with UK Home Office regulations, UK Animals Scientific Procedures Act 1986.

RNA isolation and qPCR

For the comparisons of *Spns2* transcript levels in wild type and *Spns2*^{tm1a/tm1a} tissues (Figure 1B) RT-qPCR was performed using an "RNA-to-Ct One Step" kit (Applied Biosystems) in a 10μl reaction with 1μl of total RNA (20-500ng depending on tissue type). A TaqMan assay (Mm01249325) spanning the exons flanking the splice acceptor site of the construct was used in a multiplex reaction with a GAPDH endogenous control to normalize for variations between the amounts of input RNA (Applied Biosystems), and amplified in triplicate using a Viia7 qPCR machine (Applied Biosystems). Analysis was performed using the Viia7 1.1 analysis software and the ΔΔCt relative quantification module. For the comparisons of *Spns2*-transcript levels across different tissues of wild type mice (Figure 5A), RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). qPCRs were performed using the QuantiTect SYBR Green PCR Kit (Qiagen), using Spns2 QuantiTect Primer Assay (Qiagen) and primers *Actb*_Fw CTAAGGCCAACCGTGAAAAG, *Actb*_Rv ACCAGAGGCATACAGGGACA (Sigma-Aldrich). The *Spns2* primers spanned the boundaries of exons 5 to 7 of the *Spns2* coding transcript ENSMUST00000045303. The data was acquired on the StepOnePlus™ Real-Time PCR system (Applied Biosystems), and analyzed using the ΔΔCt method.

Flow cytometry

Cell suspensions of mouse tissues were prepared in RPMI-1640 with 2% (v/v) fetal calf serum (Sigma-Aldrich), 100 μg/ml streptomycin, 100 U/ml penicillin (all from Invitrogen). Blood was collected into heparin-coated tubes (Kabe Labotechnik) by cardiac puncture and

erythrocytes lysed using PharmaLyse[TM] (BD Biosciences). The cells were stained in PBS with 2% fetal calf serum (Sigma-Aldrich) and 0.2% (w/v) sodium azide (Sigma-Aldrich) for 20 minutes on ice, with the following antibodies. Fluorescein-conjugated antibodies were against CD4 (clone L3T4), CD8 (53-6.7), CD11b (M1/70), CD21 (7G6), CD86 (GL1), and B220 (RA3-6B2, all from BD Pharmingen). Phycoerythrin-conjugated antibodies were against CD8 (clone 53-6.7), CD19 (1D3), CD69 (H1.2F3), CD80 (16-10A1), and IgM (R6-60.2, all from BD Pharmingen). Allophycocyanin conjugated antibodies were against CD4 (RM4-5), CD8 (53-6.7), and CD44 (IM7, all from BD Pharmingen). Allophycocyanin Cy7 antibodies were against CD8 (53-6.7), CD11b (M1/70, both from BioLegend), and B220 (RA3-6B2, from BD Pharmingen). Peridinin chlorophyll A protein (PerCP) conjugated anti-CD45.1 (A20, BioLegend), Alexa Fluor 647 conjugated anti-IgD (clone 11-26, eBioscience), and Phycoerythrin Cy7 anti-CD23 (B3B4, eBioscience) were also used. Flow cytometric measurements of β-galactosidase activity were performed using FluoReporter LacZ Flow Cytometry Kits (Invitrogen, Molecular Probes). The cells were stained for appropriate combinations of cell-surface lineage markers, before loading with fluorescein di-β-D-galactopyranoside (FDG) and analysis by flow cytometry. The data was acquired on BD FACS Aria or LSRII flow cytometers, and analyzed with FACS Diva Software.

ELISA

For the measurements of antibody levels, mouse blood was collected by tail-bleed or cardiac puncture, and serum prepared and stored at −20°C. For antigen-specific antibody measurements in mouse serum Nunc Maxisorp plates were coated overnight at 4°C with 2 mg/mL of tetanus toxoid C (TetC) in 0.1M Na_2HPO_4 pH 9.0, blocked with 3% (w/v) bovine serum albumin (BSA) in PBS for 1 hour, and incubated with 5-fold serial dilutions of mouse serum in PBS with 1% BSA for 1 hour. The plates were developed with anti-mouse IgG, IgG_1 or IgG_{2a} horseradish peroxidase conjugated antibodies (BD Pharmingen), followed by the OPD Substrate Tablets (o-phenylenediamine, Sigma-Aldrich) dissolved in water. Cytokine ELISA on cell culture supernatants was performed using anti-mouse TNF-α coating antibody clone 1F3F3D4 and biotin-conjugated detection antibody clone XT3/XT22, followed by avidin horseradish peroxidase (all from eBioscience), and the TMB Liquid Substrate System (Sigma-Aldrich). Absorbances were measured using the BioRad 680 MicroPlate Reader (BioRad).

Measurements of S1P levels and activity

For the measurements of S1P levels, mouse blood was collected from the retro-orbital sinus. S1P levels in the plasma were measured using the ELISA-based S1P assay kit (Echelon Biosciences) according to the manufacturer's protocol. Assays of S1P activity in mouse plasma used the $S1P_1$ Redistribution Assay (Thermo Scientific) according to the manufacturer's instructions. Briefly, the assay measured S1P-induced internalization of $S1P_1$ receptor, when mouse plasma is added at different dilutions to the U2OS cells stably expressing GFP-tagged $S1P_1$. Internalization of $S1P_1$-GFP was quantified using the spot detection algorithm and the 'spot total area per object' function on Cellomics ArrayScan VTI system. S1P (Cayman Chemicals) was used to prepare the positive controls.

Mouse immunization

Recipient mice were immunized by intranasal inhalation of 30μL volume of PBS containing 10mg Tet-c (tetanus-toxin fragment C recombinant protein, a gift from Omar Qazi, Imperial College) combined with 1mg heat-labile toxin (LT) of Escherichia coli (gift of Rino Rappuoli, Chiron) adjuvant. Mice were boosted on days 7, 21 and 37. Serum samples were collected on days 36 and 40. Detection of TetC specific antibodies from sera was performed by ELISA as described above.

Mouse bone marrow transfer experiments

Recipient animals were irradiated with 2 doses of 4.5 Gy, 3 hours apart, and injected intravenously with 3×10^6 donor bone marrow cells. The mice were maintained on clindamycin (250mg/l) in drinking water for 2 week, and analyzed 8 weeks after reconstitution. In one experiment *Spns2*$^{tm1a/tm1a}$ and *Spns2*$^{+/tm1a}$ recipients were reconstituted either with wild type CD45.1$^+$-marked or with *Spns2*$^{tm1a/tm1a}$ donor bone marrow. In a separate study *Spns2*$^{+/+}$*Rag1*$^{-/-}$ recipients were reconstituted with wild type CD45.1$^+$-marked bone marrow and *Spns2*$^{tm1a/tm1a}$ bone marrow, either separately or together mixed in 1:1 ratio. Using CD45.1$^+$-marked wild type donor bone marrow we confirmed that this protocol results in a complete replacement of the hematopoietic system of the recipients, so that >95% of the hematopoietic progenitors, bone marrow cells of all hematopoietic lineages, as well as cells in the thymus were donor-derived (data not shown).

Tissue culture

Bone marrow derived macrophages (BMDMs) were generated by culturing mouse bone marrow for 6 days in high-glucose DMEM (Invitrogen) supplemented with 20% (v/v) fetal calf serum (Sigma-Aldrich), 25% (v/v) L-conditioned media (supernatant of cell line L-929), 2mM L-glutamine, 1mM sodium pyruvate, 100 μg/ml streptomycin, 100 U/ml penicillin (all from Invitrogen). The cells were maintained at 37°C and 5% CO_2 in a humidified incubator. For stimulation the cells were re-plated at 1×10^6/ml, and treated with 100 ng/ml LPS (Sigma-Aldrich), 25 ng/ml IFNγ (R&D Systems), and/or 25 ng/ml TNFα (eBioscience) over a 48 hour time course.

Tissue staining for β-galactosidase activity

Mice were fixed by cardiac perfusion with 4% (w/v) paraformaldehyde. Following dissection, the tissues were fixed in 4% paraformaldehyde for further 30 minutes, rinsed in PBS, and stained in 0.1% (w/v) X-gal solution (bromo-chloro-indolyl-galactopyranoside, Invitrogen) for up to 48 hours. After an additional overnight fixation in 4% paraformaldehyde, the tissues were cleared with 50% (v/v) glycerol and transferred to 70% glycerol for long-term storage. Images were taken using a Leica MZ16A microscope and Imagic software.

Statistical analyses

Statistical comparisons were performed with Prism 4.0 Software (GraphPad Inc.), using two-tailed Student's t-test or non-parametric Mann Whitney test for comparisons of two data sets, and ANOVA for multiple comparisons.

Results

Spns2 gene targeting and mouse production

Spns2 gene targeting was carried out as part of the International Knockout Mouse Consortium, in the JM8 embryonic stem cell line on a C57BL/6N genetic background (44). The targeted *Spns2*$^{tm1a(KOMP)WTSI}$ allele carried a gene-trap DNA-cassette, inserted into the second intron of the gene, consisting of a splice acceptor site, an internal ribosome entry site (IRES) and a β-galactosidase reporter, followed by a neomycin resistance marker expressed from an independent β-actin promoter (Figure 1A, Genbank file available at www.knockoutmouse.org). The use of the splice acceptor site in the cassette is predicted to generate a truncated non-functional transcript encoding the first 145 out of 549 amino acids of the Spns2 protein, including only one out of the eleven predicted Spns2 transmembrane α-helices, (prediction using Swiss EMBNet www.ch.embnet.org/software/TMPRED_form.html). qRT-PCR analysis of *Spns2*$^{tm1a/tm1a}$ mouse tissues (liver, lung,

spleen, and mesenteric lymph nodes) confirmed that the targeted allele effectively disrupted the production of *Spns2*-coding mRNA, with 200-1000 fold reduction in the levels of the transcript containing the junction of exons 2-3 of *Spns2* mRNA ENSMUST00000045303 in the different tissues analyzed (Figure 1B). The locations of the *LoxP* and *Frt* sites are designed to allow the conversion of the allele to a conditional configuration in future studies (44).

The *Spns2*[tm1a/tm1a] mice were profiled using a series of high-throughput phenotype screens, under the scope of the Sanger Institute Mouse Genetics Project, with the full information available at http://www.sanger.ac.uk/mouseportal/search?query=spns2. The *Spns2*[tm1a/tm1a] animals showed no significant increase in the levels of embryonic mortality in strong contrast to the previously characterized embryonic lethal $SphK1^{-/-}SphK2^{-/-}$ and $S1P_1^{-/-}$ mouse lines (31, 42). The *Spns2*[tm1a/tm1a] mice also showed no gross dysmorphology, apart from abnormal eye pigmentation and opacity, and were able to breed normally. In particular these mice demonstrated no alterations in heart weight or histology at 16 weeks of age, in strong contrast to *Spns2*^{-/-} zebrafish that exhibited defects in embryonic heart development (*cardia bifida*) (40, 41), and $SphK1^{-/-}SphK2^{-/-}$ mice that demonstrated poor embryonic development of the dorsal aorta (31). Abnormalities in the immune phenotype of the *Spns2*[tm1a/tm1a] line were analyzed in this work, while the phenotypes of the ear and eye will be reported elsewhere (Chen J., Steel K.P., manuscript in preparation).

Abnormal T and B lymphocyte development and localization in Spns2[tm1a/tm1a] mice

To establish the impact of *Spns2*[tm1a/tm1a] on lymphocyte development and localization, the blood and lymphoid organs of *Spns2*[tm1a/tm1a] mice were analyzed. We observed a reduction in the overall leukocyte counts in *Spns2*[tm1a/tm1a] mouse blood ($3.38 \times 10^3/\mu l$ in *Spns2*[tm1a/tm1a] versus $6.63 \times 10^3/\mu l$ in the wild type mice, Supplementary Figure S1A), while erythrocyte and platelet counts were not affected (data not shown). The percentage of CD4 and CD8 lineage T cells in the blood, and the absolute numbers of CD4 and CD8 T cells in the spleen of *Spns2*[tm1a/tm1a] mice were significantly reduced (Figure 2A, ~ 4 fold). This was accompanied by a moderate increase in the proportion of mature CD4 and CD8 single-positive T cells in the thymus (Figure 2B-C), with the CD4+CD8− cells constituting 12±0.9% of thymocytes in *Spns2*[tm1a/tm1a] as compared with 7±0.5% in wild type mice (mean ± S.D.). The thymic CD4+CD8− and CD4−CD8+ *Spns2*[tm1a/tm1a] T cells expressed lower levels of CD24 and CD69, and higher levels of CD62L, indicating their more mature status (Figure 2D-E). These features closely resemble the phenotypes of other mouse lines with defects in S1P production, turnover, or sensing (5, 6, 32, 47), indicating an accumulation of mature T cells in the thymus and suggesting a defect in T cell recruitment from the thymus into the circulation. There were no abnormalities in the numbers of the earlier thymocyte subsets, including CD4−CD8− double negative thymocytes (DN1-DN4, differentiated by CD44 and CD25 expression) and CD4+CD8+ double-positive thymocytes (data not shown).

There was also a significant reduction in the number of re-circulating B cells in *Spns2*[tm1a/tm1a] mice (CD45+CD19+, Figure 3A). This was accompanied by a reduction in the follicular B cell population in the spleen (gated as B220+IgM+IgD+ or B220+CD23+CD21^{lo}, Figures 3B-D) and the mature B cell population in the bone marrow (B220+IgM+IgD+, Figure 3E). In contrast no abnormalities in earlier B cell developmental subsets were observed, with normal numbers of pro/pre-B cells (B220+IgM−IgD−) and immature B cells (B220+IgM+IgD−) in the bone marrow and transitional B cells (B220+CD23^{lo}CD21^{lo}) in the spleen of the *Spns2*[tm1a/tm1a] mice (Figure 3D-E). Overall, this phenotype resembles the loss of re-circulating and mature follicular B cell population previously reported in other mouse lines with impaired S1P production or sensing (5, 32, 47). We also observed a reduction in the numbers of B1 cells in the peritoneal cavity of

$Spns2^{tm1a/tm1a}$ mice (B220^{low}IgM^{high}IgD^{low}, data not shown), consistent with the role of S1P in the control of their trafficking (12).

Reduced antibody responses to immunization in Spns2$^{tm1a/tm1a}$ mice

To establish whether the defects in lymphocyte development and localization seen in the $Spns2^{tm1a/tm1a}$ mice led to impaired antibody responses to immune challenge, the mice were immunized intra-nasally with TetC (tetanus-toxin fragment C recombinant protein), boosted at days 7 and 21, and analyzed for antigen specific antibody titers in the serum at day 36. Significant reductions in antigen-specific total-IgG and IgG$_1$ were seen (Figure 4), indicating impaired humoral immunity in the $Spns2^{tm1a/tm1a}$ mouse line. The mice were subsequently given a tertiary boost, and serum antibody levels measured again three days later (day 40), confirming the reduction in antigen-specific total-IgG and IgG in $Spns2^{tm1a/tm1a}$ mice (data not shown). The levels of IgG$_{2a}$ were, however, not significantly reduced (Figure 4), and this might have been due to the low levels and high variation in IgG$_{2a}$ production in both the wild type and $Spns2^{tm1a/tm1a}$ groups.

Normal macrophage responses to inflammatory stimuli in Spns2$^{tm1a/tm1a}$ mice

S1P functions not only as an chemoattractant in the extracellular environment, but also as an intracellular messenger required for the normal signaling downstream of TNFR and Toll-like receptors (TLRs) (25); furthermore inhibition of S1P production is protective in animal models of endotoxemia and sepsis (27). To establish whether $Spns2^{tm1a/tm1a}$ disrupted normal cellular responses to TLR and TNFR stimulation, bone marrow derived macrophages (BMDM), generated from $Spns2^{tm1a/tm1a}$ and wild type control mice, were stimulated with lipopolysaccharide (LPS, 100 ng/ml), interferon γ (IFNγ, 25 ng/ml), and/or TNFα (25 ng/ml), and analyzed for induction of CD80 and CD86 activation markers and secretion of inflammatory cytokines. No differences in the responses of $Spns2^{tm1a/tm1a}$ and wild type cells were observed (Supplemental Figure S1B). This suggests that Spns2 does not affect intracellular S1P functions in macrophages downstream of TLR or TNFR stimulation. This is in contrast to SphK1, the knockdown of which in similar systems was previously shown to reduce monocyte and macrophage responses to TLR and TNFR stimulation (26).

Cell-intrinsic activity of Spns2 is dispensable for lymphocyte development and localization

Many cell types have been shown to produce and release S1P, including erythrocytes, lymphatic and vascular endothelial cells, platelets, and mast cells (33, 35, 47-51). To assess Spns2 expression in a broad range of mammalian cell types, a range of tissues and organs from $Spns2^{+/tm1a}$ mice were profiled for the activity of the β-galactosidase reporter expressed from the endogenous $Spns2$-promoter in mice carrying the $Spns2^{tm1a}$ allele (52). The studies were done as part of the Sanger Mouse Genetics Project high-throughput phenotyping, and the data on the β-galactosidase reporter activity in 39 organs and tissues of $Spns2^{+/tm1a}$ mice is summarized in Supplemental Table 1, with images available at: www.sanger.ac.uk/mouseportal/phenotyping/MBNZ/adult-lac-z-expression. The expression of the $Spns2$ gene was further confirmed in a selection of tissues using wild type tissues and qRT-PCR. Overall it was demonstrated that there were high $Spns2$ transcript levels in the liver and lung, lower levels in the lymph nodes, spleen, and bone marrow, and low but detectable levels in the thymus (Figure 5A).

To establish whether $Spns2$ was expressed in the lymphocyte populations affected by the $Spns2^{tm1a/tm1a}$ phenotype, $Spns2$-promoter-driven β-galactosidase reporter activity was analyzed in $Spns2^{+/tm1a}$ lymphocytes using flow cytometry with a fluorescent β-galactosidase substrate. The data showed no significant β-galactosidase activity over the background level in the different subpopulations of thymocytes or in splenic T cells (Figure 5B), indicating that $Spns2$ gene is not expressed in these cell types. The $Spns2$ gene was also

not expressed in splenic B cells (Figure 5B). As a positive control, high levels of β-galactosidase reporter activity were detected in hematopoietic cells of another mouse line $Mysm1^{+/tm1a}$ (53).

The requirement for Spns2 expression and activity in lymphocytes for their normal development and localization was further tested using bone marrow chimeras. Lethally-irradiated $Spns2^{+/+}Rag1^{-/-}$ recipients were reconstituted with a 50:50 mix of CD45.1$^+$-marked wild type and $Spns2^{tm1a/tm1a}$ bone marrow and analyzed at 8 weeks after reconstitution. No significant differences in the development and localization of wild type and $Spns2^{tm1a/tm1a}$ lymphocytes were observed in this study. For example, approximately 50% of CD4 and CD8 T cells in the blood and spleen of the chimeric mice were CD45.1^{-ve} $Spns2^{tm1a/tm1a}$ (Figure 5C). This demonstrated that $Spns2^{tm1a/tm1a}$ lymphocytes could develop and migrate normally when placed in a wild type environment, and therefore that Spns2 expression in lymphocytes was dispensable for their normal development and localization. Additionally, an adoptive intra-venous transfer of 10^7 $Spns2$-wild type GFP-expressing splenocytes into either wild type or $Spns2^{tm1a/tm1a}$ recipients, demonstrated a significant reduction in the transferred CD4 T cells in the blood of $Spns2^{tm1a/tm1a}$ as compared to wild type mice at 48 hours, suggesting the role of $Spns2^{tm1a/tm1a}$ environment in affecting CD4 T cell localization, however no reduction in CD8 T cells and B cells was observed (data not shown).

Spns2 activity in the non-hematopoietic stromal cells is essential for normal immune function

The requirement for $Spns2$ expression and activity on different cell types for normal lymphocyte development and trafficking was investigated further using bone marrow chimeras. Lethally-irradiated recipients of $Spns2^{tm1a/tm1a}$ and $Spns2^{+/tm1a}$ genotypes were reconstituted either with wild type (CD45.1$^+$) or with $Spns2^{tm1a/tm1a}$ (CD45.1$^-$) bone marrow, and analyzed by flow cytometry at 8 weeks after the reconstitution. The results indicated that the $Spns2$ genotype of the non-hematopoietic cells was of primary importance for normal lymphocyte development in the chimeras. Thus when the wild type hematopoietic system was reconstituted into $Spns2^{tm1a/tm1a}$ hosts, the numbers of T cells were depleted in the blood and spleen, to the same extent as in $Spns2^{tm1a/tm1a}$ mice reconstituted with $Spns2^{tm1a/tm1a}$ bone marrow (Figure 6 A-C). Similarly, mature B cells were depleted to an equal extent in both groups of mice, in the blood, spleen, as well as the bone marrow (Supplemental Figure S2). In contrast, in the chimeric mice with selective loss of Spns2-function in the hematopoietic compartment, there was a trend towards decreased lymphocyte numbers but this did not reach statistical significance (Figure 6, Figure S3), further indicating that Spns2 is primarily functional in the non-hematopoietic cells. Overall these data suggested that Spns2 expression and function on the cells of the non-hematopoietic stroma had a primary role in the maintenance of normal lymphocyte development and immune system function.

No significant reduction in the plasma S1P levels in Spns2$^{tm1a/tm1a}$ mice

S1P levels in the plasma of $Spns2^{tm1a/tm1a}$ and wild type mice were measured using two methods, an ELISA-based S1P assay (Echelon Biosciences, Figure 7A), and the S1P$_1$ Redistribution Assay that measures S1P-induced internalization of S1P$_1$ receptor (Thermo Scientific, Figure 7B-C). In the latter assay, the plasma of wild type and $Spns2^{tm1a/tm1a}$ mice was added at different dilutions to U2OS cells expressing GFP-tagged S1P$_1$ receptor and the internalization of S1P$_1$-GFP was quantified using a Cellomics Array-Scan VTI high-throughput cell imaging system. No significant differences in S1P concentrations or activity were observed between $Spns2^{tm1a/tm1a}$ and wild type mice in either assay (Figure 7A, C). This indicated that Spns2-independent mechanisms exist for maintaining overall S1P levels

in the blood of $Spns2^{tm1a/tm1a}$ mice, but did not rule out the possibility that S1P levels are reduced in certain localized environments and that this was responsible for the altered lymphocyte distribution and immune function in $Spns2^{tm1a/tm1a}$ mice. Normal viability and lack of gross developmental defects in the $Spns2^{tm1a/tm1a}$ mice are also consistent with localized rather than systemic defects in S1P export. S1P concentrations in the lysates of spleen and thymus tissues were below the limit of detection of the Echelon Biosciences ELISA assay for both wild type and $Spns2^{tm1a/tm1a}$ mice ($<0.06\mu$M, data not shown).

Characterization of Spns2-knockout Spns2$^{tm1b/tm1b}$ mice

To further confirm that the $Spns2^{tm1a/tm1a}$ mice were phenotypically equivalent to $Spns2$ knockout animals, the $Spns2^{tm1a/tm1a}$ line was crossed to the C57BL/6N-Hprt$^{Tg(CMV-cre)Brd/Wtsi}$ transgenic line with systemic expression of Cre-recombinase (45). This resulted in germline excision of asymmetric exon 3 of the $Spns2$ gene (Supplemental Figure S3A), causing a frame-shift in the $Spns2$-transcript, and is therefore predicted to result in a full loss Spns2 protein expression. Additionally the removal of the β-actin promoter, contained in the neomycin cassette, controlled for any possible side-effects of this cassette on the phenotype. The resulting allele structure was designated $Spns2^{tm1b(KOMP)Wtsi}$; and the homozygous $Spns2^{tm1b/tm1b}$ mice were viable with no significant increase in embryonic mortality. Flow cytometry analysis of lymphoid organs demonstrated a reduction in CD4 and CD8 T cells, and mature B cells in the spleen of $Spns2^{tm1b/tm1b}$ (Supplemental Figure S3B), comparable to $Spns2^{tm1a/tm1a}$ mice (Figures 1A, 2A). There was also an increase in the proportion of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells in the thymus of $Spns2^{tm1b/tm1b}$ mice, and these cells expressed higher levels of CD62L and lower levels of CD24 indicating their more mature status (Supplemental Figure S3B), as seen previously in $Spns2^{tm1a/tm1a}$ mice (Figure 1B-E). Overall this confirmed that $Spns2^{tm1a/tm1a}$ phenotype is equivalent to the Spns2-knockout $Spns2^{tm1b/tm1b}$.

Additionally, to further confirm that the $Spns2^{tm1a/tm1a}$ phenotype resulted from the gene-trap cassette in the $Spns2$-locus, the $Spns2^{tm1a/tm1a}$ animals were crossed to a transgenic line with systemic expression of Flp-recombinase C57BL/6N-Gt(ROSA)26Sor$^{tm1(FLP1)Dym/Wtsi}$ (46), causing germline excision of the gene-trap cassette (Supplemental Figure S3A). The resulting allele was designated $Spns2^{tm1c(KOMP)Wtsi}$, and the $Spns2^{tm1c/tm1c}$ mice showed a rescue of lymphocyte numbers in the spleen and bone marrow and thymus (Supplemental Figure S3C and data not shown), confirming that the immune phenotype of the $Spns2^{tm1a/tm1a}$ line was caused by the $Spns2^{tm1a}$-gene trap cassette.

Discussion

In this study we have characterized an $Spns2$-targeted mouse line and demonstrated that Spns2 is required for normal lymphocyte development and localization, and for normal humoral immune response to immunization. Overall the changes in lymphocyte subpopulations in $Spns2^{tm1a/tm1a}$ and $Spns2^{tm1b/tm1b}$ mice closely mimicked the phenotypes of partial S1P deficiency and impaired S1P-dependent lymphocyte trafficking, including the depletion of lymphocytes in circulation, increase in the mature single-positive T cells in the thymus, and a selective reduction in the mature B cell population in the spleen and bone marrow (5, 6, 10, 32, 47). Although we did not detect a reduction in S1P levels in $Spns2^{tm1a/tma1}$ mouse plasma, the phenotypic data presented here together with the previous *in vitro* demonstrations that human Spns2 can transport S1P and S1P-mimic FTY720 (40, 43), suggest that the $Spns2^{tm1a/tm1a}$ phenotype may arise from localized disruptions in S1P concentrations at certain restricted physiological locations.

Critically this work indicates that Spns2 functions are limiting for lymphocyte trafficking with some degree of specificity, as the viability and lack of developmental defects in

Spns2[tm1a/tm1a] mice contrasts with lethality and defects in cardiovascular and neural development in *Spns2*-mutant zebrafish (40, 41). Importantly this is not due to different requirements for S1P-production between the two species, as knockout mice lacking sphingosine kinases *SphK1*^{−/−} *SphK2*^{−/−} or S1P-receptor *S1P₁*[−/−] are also embryonic lethal with abnormal cardiovascular development (31, 42). In comparison, *Spns2*[tm1a/tm1a] mice more closely mimic the phenotypes of partially reduced S1P production, such as the single-null knockouts for *SphK1* or *SphK2* (54-56), or the knockout for S1P lyase with normal S1P production but disrupted S1P concentration gradients (57). Overall this indicates that sufficient S1P levels are maintained in correct anatomical and cellular locations in the *Spns2*[tm1a/tm1a] mice to allow normal embryonic development, and suggests significant divergence in the expression and functions of Spns2 between mouse and zebrafish. We can speculate that alternative mechanisms of S1P release may operate during embryonic development in mouse but not in zebrafish species. These may include other transporters that have been shown to pump S1P *in vitro* in mammalian cells, such as ABCC1 and ABCA1 (33, 34), or extracellular S1P production by secreted SphK1 enzyme (38), or even the two Spns2 paralogs Spns1 and Spns3.

This work further demonstrated that Spns2 activity in the non-hematopoietic cells of the stroma is of key importance for normal immune system function. In contrast, previous studies showed that plasma S1P levels in the mouse are maintained by hematopoietic cells such as erythrocytes (47), and ABC-family transporters have been implicated in mediating S1P export from erythrocytes (33) and platelets (35). This suggests that the primary role of Spns2 is to maintain appropriate S1P concentrations at other *in vivo* locations, consistent with the unaltered S1P levels in the plasma of *Spns2*[tm1a/tm1a] mice. Lymph is one of the sites were analysis of S1P concentrations would be particularly interesting (47), as S1P in the lymph was shown to be derived from non-hematopoietic cells, in particular the lymphatic endothelium (48). Overall this warrants further investigation of Spns2 expression in different non-hematopoietic cell types and tissues, including lymphatic endothelium (47, 48).

S1P also acts as an intracellular messenger; and in mast cells and antigen presenting cells increased S1P production is associated with cell activation, degranulation, and inflammatory cytokine production (18, 20, 23, 24). However, the current work indicates that in contrast to the *SphK1*-knockdown cells , *Spns2*[tm1a/tm1a] macrophages respond normally to TNFR and TLR stimulation. This indicates that Spns2 likely does not impact on intracellular S1P levels, at least in this cell type. Whether the activity of Spns2 and other S1P transporters can affect intracellular S1P levels in other cell types, by altering S1P secretion or uptake, remains to be addressed in future research.

Components of the S1P signaling pathways are targets for therapies aimed at treating autoimmunity, transplant rejection, inflammatory diseases, and cancer (2). S1P receptor agonist FTY720 is the most advanced of such therapies and was recently approved for the treatment of multiple sclerosis (58), while other pharmaceutical agents are under development and have shown efficacy in animal models of inflammatory diseases, sepsis, and cancer (28, 59, 60). Knowledge of the mechanisms regulating S1P concentrations *in vivo* in a mammal is essential for the future development of such pharmaceutical agents and may lead to better targeted therapies. For example, S1P-targeting therapies for the treatment of autoimmunity and transplant rejection aim to achieve immunosuppression without inhibiting other S1P activities. S1P-targeting cancer therapies suppress vascularization, cell growth and migration, but ideally aim to maintain full immune system function. In contrast therapies for systemic inflammatory disorders primarily aim to suppress inflammation and intravascular coagulation, while retaining the protective activities of S1P on endothelial barrier integrity (61, 62). Given the diverse roles of S1P in many physiological processes,

understanding of the mechanisms regulating its bioavailability in different tissues and conditions is essential for the development of such therapies. Transporter proteins have proven highly effective drug targets in other areas, particularly neuropharmacology (63). The demonstration that *Spns2*-deficiency selectively impaired lymphocyte functions and antigen-specific immune responses, without affecting vascular and neural development, highlights Spns2 as a possible drug target with potential for the treatment of autoimmunity and transplant rejection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

|	
ABC	ATP-binding cassette
BMDM	bone marrow derived macrophage
CFTR	cystic fibrosis transmembrane conductance regulator
FTY720	2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol, hydrochloride
TetC	tetanus-toxin fragment C recombinant protein
S1P	sphingosine-1-phosphate
S1P$_1$	S1P receptor 1
SphK	sphingosine kinase
Spns2	spinster homologue 2

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A

B

Tissue	Liver	Spleen	Mesenteric lymph node	Lung
Fold Reduction	400	243	>1000	>1000
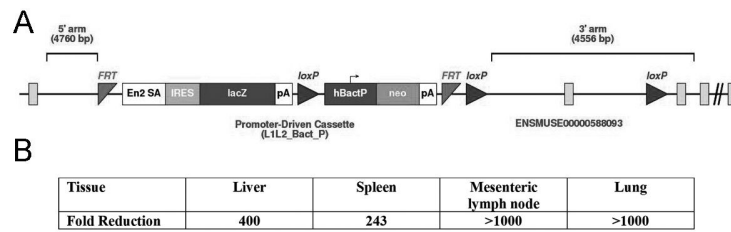
**Figure 1. *Spns2* gene targeting**
(A) Structure of the *Spns2*[m1a(KOMP)WTSI] (*Spns2*[tm1a]) allele. (B) Average fold reduction in *Spns2*-transcript levels in *Spns2*[tm1a/tm1a] relative to wild type tissues (liver, spleen, lymph nodes and lung), analyzed by qRT-PCR using primers spanning the junction of exons 2-3 of the *Spns2* coding transcript ENSMUST00000045303.
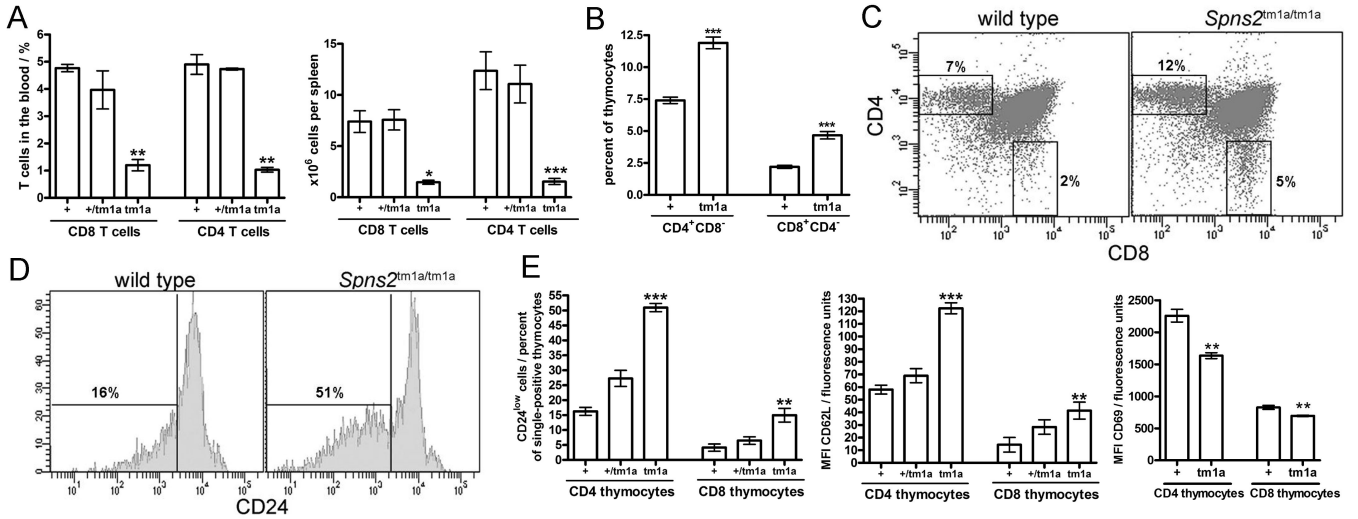
Figure 2. T cell abnormalities in the *Spns2*^{tm1a/tm1a} mice

(A) Percentage of CD4 and CD8 T cells in the blood, and numbers of CD4 and CD8 T cells in the spleen of wild type, *Spns2*$^{+/tm1a}$, and *Spns2*$^{tm1a/tm1a}$ mice (+, +/tm1a, and tm1a, respectively). (B) Percentage of CD4$^+$ and CD8$^+$ single-positive cells in the thymus of wild type, *Spns2*$^{+/tm1a}$, and *Spns2*$^{tm1a/tm1a}$ mice (+, +/tm1a, and tm1a, respectively). (C) Representative flow cytometry plots of wild type and *Spns2*$^{tm1a/tm1a}$ thymocytes stained for CD4 and CD8; average percentage of cells within the CD4$^+$ and CD8$^+$ single-positive gates is indicated. (D) Representative flow cytometry histograms indicating CD24 expression on CD4$^+$ single-positive thymocytes in wild type and *Spns2*$^{tm1a/tm1a}$ mice. (E) Percentage of CD24^{low} cells within the CD4$^+$ and CD8$^+$ single-positive thymocyte gates; and the expression of CD62L and CD69 on CD4$^+$ and CD8$^+$ single-positive thymocytes in wild type (+), *Spns2*$^{+/tm1a}$ (+/tm1a) and *Spns2*$^{tm1a/tm1a}$ (tm1a) mice. Bars represent means ± SEM; MFI, mean fluorescence intensity; *p<0.05, **p<0.01, *** p<0.001 using ANOVA with Bonferroni's post-hoc test or t-test; data from 3-4 mice per group and reproducible in two independent experiments.
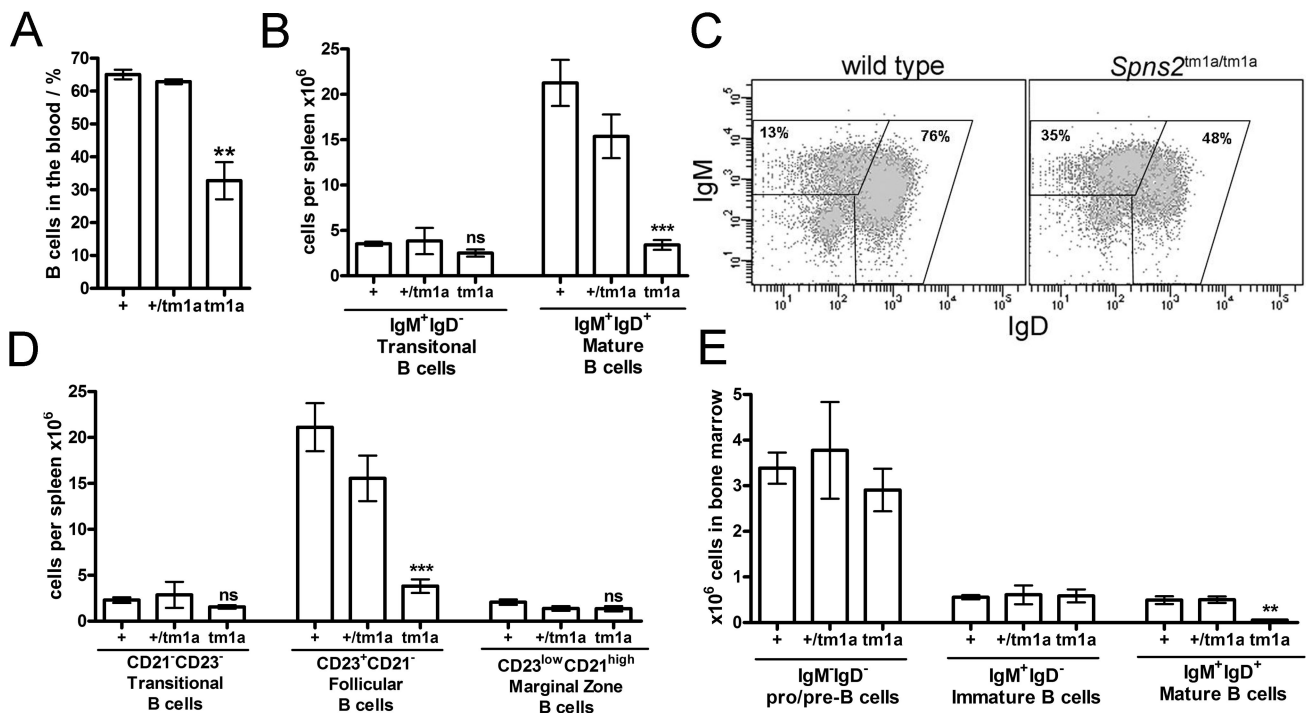
Figure 3. Reduction in the numbers of re-circulating B cells in the blood and mature B cells in the spleen and bone marrow of *Spns2*^{tm1a/tm1a} mice

(A) Reduction in the percentage of B cells (gated as $CD45^+CD19^+$) in the blood of *Spns2*^{tm1a/tm1a} mice. (B) Reduction in the absolute number of mature B cells (gated as $B220^+IgM^+IgD^+$) in the spleen of *Spns2*^{tm1a/tm1a} mice. (C) Representative flow cytometry plots of splenocytes, stained for B220, IgM and IgD, and gated on the $B220^+$ B cell population. Gates indicate IgM^+IgD^- transitional and IgM^+IgD^+ mature B cells; the average percentage of cells within each gate for all mice in the group is indicated. (D) Reduction in the numbers of mature follicular B cells in the spleen of *Spns2*^{tm1a/tm1a} mice confirmed by $B220^+CD21^-CD23^+$ staining. (E) Reduction in mature B cells ($B220^+IgM^+IgD^+$) in the bone marrow of *Spns2*^{tm1a/tm1a} mice. All bars represent means ± SEM; **$p<0.01$, *** $p<0.001$ using ANOVA with Bonferroni's post-hoc test; data from 3 mice per group and reproducible in two independent experiments; bone marrow cell counts are per one tibia and femur.
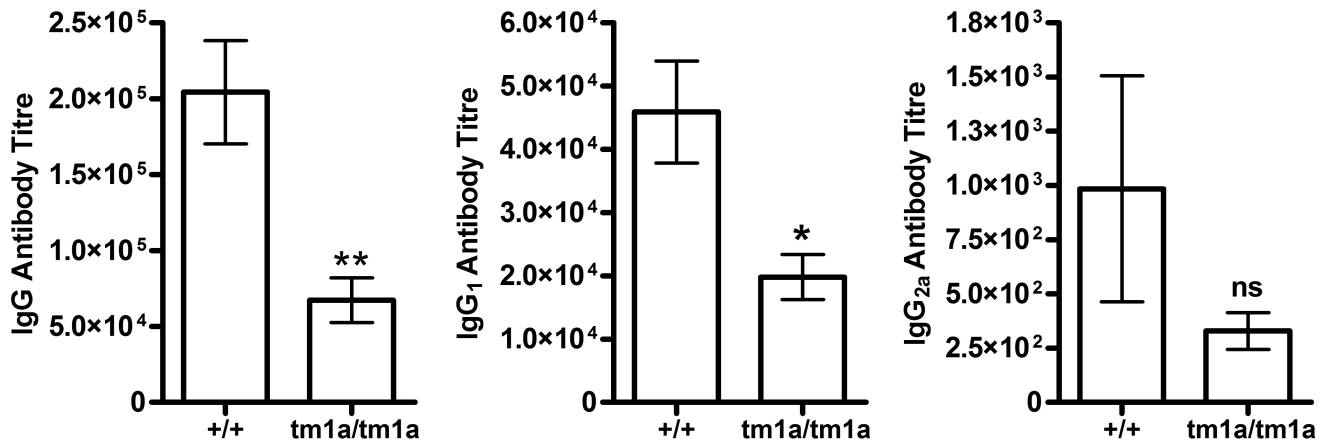
Figure 4. Impaired humoral immune response in the *Spns2*^{tm1a/tm1a} mouse line

Wild type (+/+) and *Spns2*^{tm1a/tm1a} (tm1a/tm1a) mice were immunized intra-nasally with TetC, boosted at days 7 and 21, and analyzed for antigen specific antibody titers in the serum at day 36. Bars represent means ± SEM; *p<0.05, ** p<0.01, ns- non-significant, using Student's t-test; data from 6 mice per group.
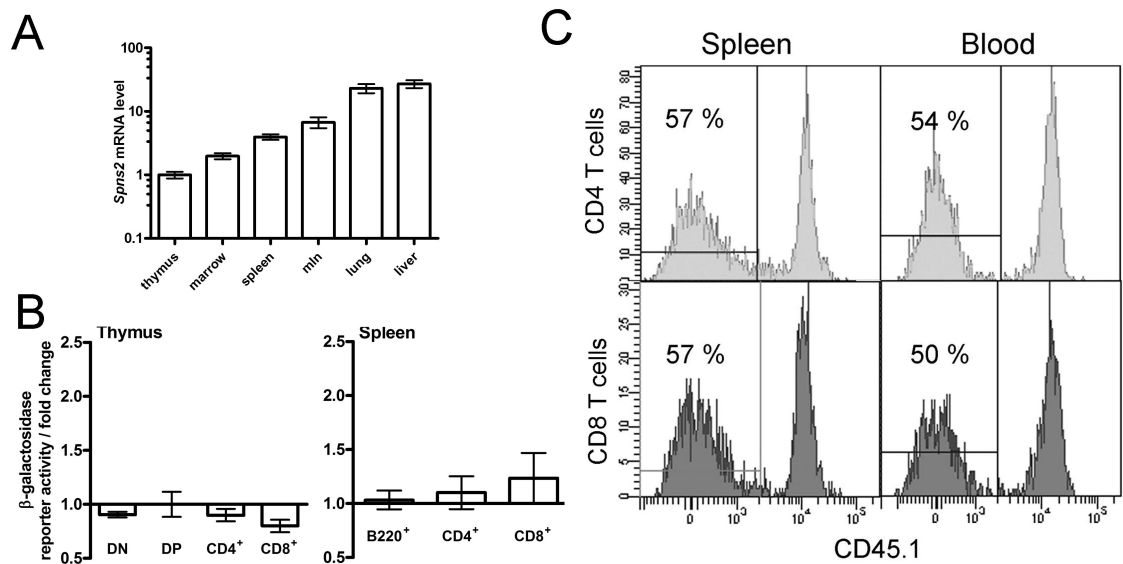
Figure 5. Analysis of Spns2 expression
(A) qRT-PCR analysis of the *Spns2* transcript levels in the tissues of wild type mice. Analysis using primers spanning exon junctions 5-7 of *Spns2* coding transcript ENSMUST00000045303, data analyzed using the ΔΔC method with β-actin as the housekeeping control; *Spns2* transcript levels in the thymus were assigned the arbitrary value of one and the levels in other tissues expressed relative to it. (B) β-galactosidase reporter activity in thymocytes, and splenic B and T cells, as a measure *Spns2* gene expression. The measurements were done in *Spns2*[+/tm1a] cells and are presented as fold change relative to the background β-galactosidase activity in the same cell type in wild type mice. The cells were gated as: CD4^{−}CD8^{−} for double negative thymocytes (DN), CD4^{+}CD8^{+} for double positive thymocytes (DP), and B220^{+} for splenic B cells. Bars represent means ± SEM from 3 mice per group. (C) Mixed bone marrow chimeras, demonstrating that the defects in the development and localization of *Spns2*[tm1a/tm1a] T cells are not due to a cell-intrinsic requirement for Spns2. Lethally-irradiated *Spns2*^{+/+}*Rag1*^{−/−} recipients were reconstituted with a 50:50 mix of wild type CD45.1[+]-marked and *Spns2*[tm1a/tm1a] bone marrow. Flow cytometry histograms of CD4 and CD8 T cells in the spleen and blood at 8 weeks following reconstitution are shown, percentage of C45.1[-] *Spns2*[tm1a/tm1a] T cells in each plot is indicated.
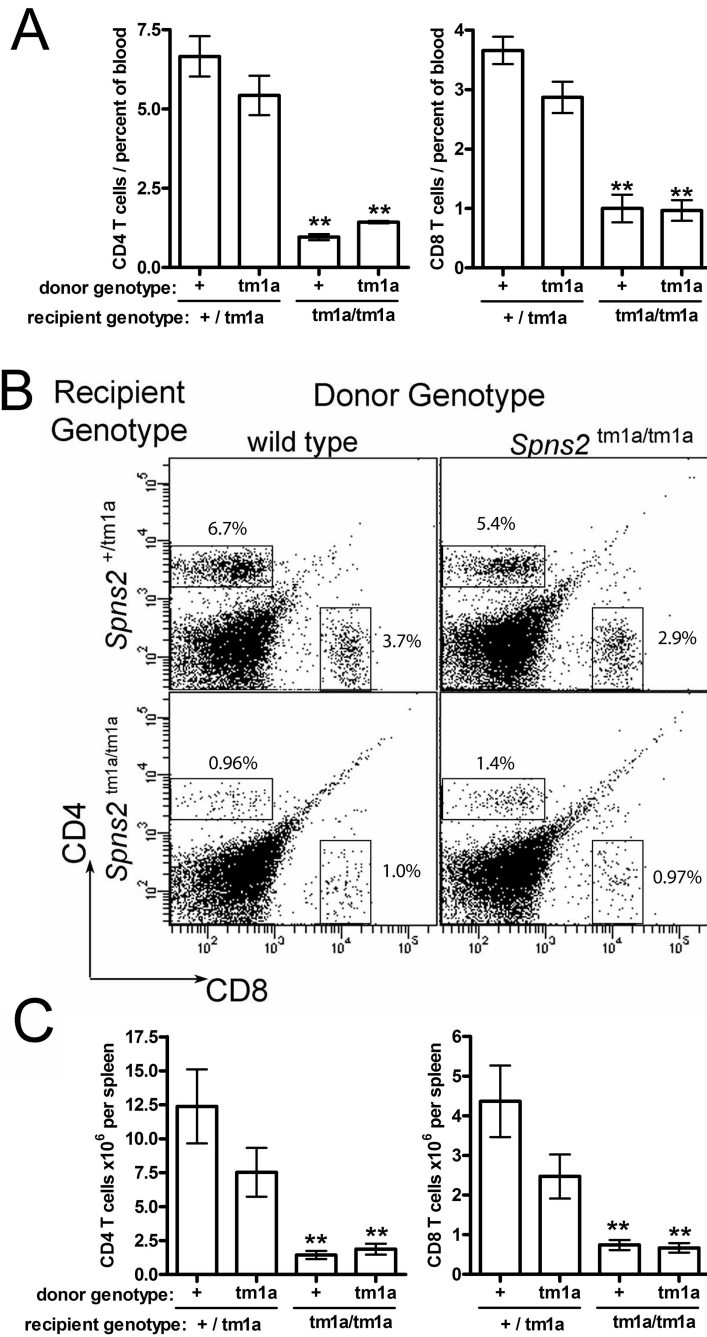
Figure 6. Bone marrow chimeras experiments indicate that Spns2 expression and function in the non-hematopoietic cells of the stoma is required for normal lymphocyte development

Lethally irradiated (2×4.5Gy) recipients of $Spns2^{tm1a/tm1a}$ and $Spns2^{+/tm1a}$ genotypes were reconstituted either with wild type (+) or with $Spns2^{tm1a/tm1a}$ (tm1a) donor bone marrow, and the numbers of lymphocyte subsets were analyzed by flow cytometry at 8 weeks following reconstitution. (A-B) Percentage of CD4 and CD8 T cells in the blood of the chimeric mice of the four groups. (B) Representative flow cytometry plots of the blood of the chimeric mice, stained for CD4 and CD8; average percentages of cells within the gated populations are indicated. (C) Absolute numbers of CD4 and CD8 T cells in the spleen of the chimeric mice of the four groups. Bars represent means ± SEM from 3 mice per group;

statistical comparisons using ANOVA with Bonferroni's post-hoc test to compare each dataset to the control group; **p<0.01.
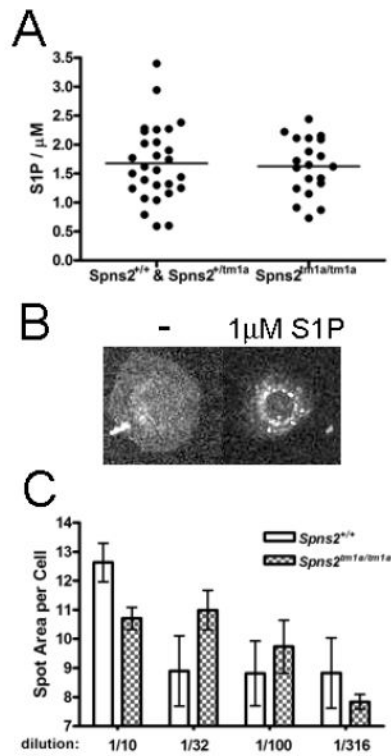
Figure 7. No significant alterations in the levels of S1P in the plasma of *Spns2*tm1a/tm1a mice
(A) S1P concentration in plasma measured using the ELISA-based S1P assay kit (from Echelon Biosciences). (B) Sample images of U2OS cells expressing GFP-tagged S1P$_1$ receptor (Thermo Scientific) following exposure to media containing either no S1P or 1μM S1P. The data was acquired using the Cellomics cell imaging system, and shows internalization of S1P$_1$ receptor in the cells exposed to S1P. (C) Cellomics-system based quantification of S1P$_1$-GFP receptor internalization in U2OS cells exposed to wild type or *Spns2*tm1a/tm1a plasma at different dilutions; quantification performed using the spot detection algorithm and the 'spot total area per object' function. Bars represent means ± SEM from 4 mice per group; the differences between wild type and *Spns2*tm1a/tm1a samples are not statistically significant.