Sphingosine Kinase 2 Is Required for Modulation of Lymphocyte Traffic by FTY720*

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Immunotherapeutic drugs that mimic sphingosine 1-phosphate (S1P) disrupt lymphocyte trafficking and cause T helper and T effector cells to be retained in secondary lymphoid tissue and away from sites of inflammation. The prototypical therapeutic agent, 2-alkyl-2-amino-1,3-propanediol (FTY720), stimulates S1P signaling pathways only after it is phosphorylated by one or more unknown kinases. We generated sphingosine kinase 2 (SPHK2) null mice to demonstrate that this kinase is responsible for FTY720 phosphorylation and thereby its subsequent actions on the immune system. Both systemic and lymphocyte-localized sources of SPHK2 contributed to FTY720 induced lymphopenia. Although FTY720 was selectively activated in vivo by SPHK2, other S1P pro-drugs can be phosphorylated to cause lymphopenia through the action of additional sphingosine kinases. Our results emphasize the importance of SPHK2 expression in both lymphocytes and other tissues for immune modulation and drug metabolism.

Sphingosine 1-phosphate (S1P) receptor agonists are likely to be the next generation of pharmacologic agents used to modulate immune system function. The prototype drug of this class is FTY720, which is highly efficacious in prolonging allograft survival and in ameliorating autoimmune disease in a variety of animal models (1–4). FTY720 is being tested in human trials for the indications renal transplantation and multiple sclerosis (5). Further, there is mounting evidence that S1P agonists are efficacious in animal models of atherosclerosis (6), renal ischemia-reperfusion injury (7), and acute lung injury (8).

FTY720 is a sphingosine analog that, after activation by phosphorylation (to FTY720-P), disrupts lymphocyte trafficking by decreasing lymphocyte egress from lymph nodes and the thymus (9, 10). Although the precise mechanisms that underlie this phenomenon are uncertain, the profound lymphopenia that is the index of FTY720 action is dependent on agonist activity at lymphocyte S1P1 receptors. Since FTY720-P is also known whether the multiple therapeutic benefits of the drug correlate with agonist activity at the S1P1 receptor. The propensity for S1P1 receptor responses to desensitize (13) and the similar behaviors of S1P1 receptor null thymocytes and FTY720-treated mouse lymphocytes have led to the suggestion that FTY720-P is a functional antagonist (14). In this scenario, the drug exaggerates S1P tone to the extent that the lymphocyte S1P1 receptor signaling is chronically down-regulated.

The kinase(s) responsible for FTY720 activation is the gateway whereby S1P signaling can be accessed readily with a therapeutic agent. Knowledge of this enzyme is important specifically to guide S1P prodrug design and generally to gain insight into the normal role of S1P in immune function. The identity of the kinase is not known currently; two candidates are sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2). These enzymes, which are expressed widely, catalyze the phosphorylation of d-erythro sphingosine to yield S1P. Both recombinant enzymes phosphorylated FTY720 in vitro (15–17). However, SPHK1 null mice become lymphopenic on administration of FTY720 (18), indicating that this isoform is not primarily responsible for activation of FTY720 in vivo. Here, we described the use of SPHK2 null mice to demonstrate that SPHK2 is the kinase activating FTY720, which emphasizes the importance of this enzyme as a target for immunotherapeutic agents.

EXPERIMENTAL PROCEDURES

Generation of SPHK2 Null Mice—We searched the BayGenomics Consortium data base for embryonic stem cell lines wherein an allele of the Sphk2 gene, which is on chromosome 7, was trapped. We found three such embryonic stem cell lines (129/Sv strain) associated with Sphk2. One of these, NPX-039, was obtained from the Mutant Mouse Regional Resource Center (University of California at Davis), cultured, and introduced into C57BL/6 mouse blastocysts to generate male chimera founder mice. The chimeric mice were mated with C57BL/6 females, and their agouti offspring were tested for transmission of the trapped allele. Heterozygous (+/tr) offspring were intercrossed to obtain homozygous (tr/tr) mice. The mutant Sphk2 alleles were maintained on a 129/Sv × C57BL/6 mixed background. Because the Sphk2+/tr females have diminished fertility, Sphk2tr/tr animals were obtained by crossing Sphk2tr/tr males with heterozygous females. Genotyping was accomplished by PCR of tail biopsies using the following primers: F1, 5′-TTTTCCTGGGATGTTAATCTCATTTC-3′; R1, 5′-CAGGCCCTCCGGAAGTCTGCACTA-3′; R2, 5′-CTCTCTTCTT- TGTTTTCGGGACCTGGGAC3′ (reaction conditions: 94 °C, 0.5 min; 57 °C, 1 min; 72 °C, 3 min). The wild type allele generated a band of 629 bp (F1 × R1), whereas the trapped allele generated a band of 924 bp (F1 × R2) (see Fig. 1, a and b). The 5′ junction of the trap element was amplified and subjected to sequence analysis; the nucleotide sequence

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2The abbreviations used are: S1P, sphingosine 1-phosphate; SPHK, sphingosine kinase; Sphk, gene encoding sphingosine kinase; tr, (exon) trap; FTY720, 2-alkyl-2-amino-1,3-propanediol; FTY720-P, phosphorylated FTY720; CFSE, 5-carboxyfluorescein diacetate succinimidyl ester; HPLC, high performance liquid chromatography; PLN, peripheral lymph nodes; RT, reverse transcription; NK, natural killer; WT, wild type; FAM, 5,6-carboxyfluorescein; BHQ1, black hole quencher 1.
of the junction is 5’gctgctgtggcgcgcccgcagttgctgc/TTGCCGGTCCATGCCAGGGTTCTTCTCATCCTCCTACCTACGCCCTCTC-3’, where the lowercase signifies mouse genomic DNA and the uppercase signifies the trap element DNA. The trap element inserted 1.7 kbp downstream of the second exon of Spfh2; the third exon encodes the AUG translation initiation codon.

Measurement of Plasma S1P Levels—S1P concentrations in mouse plasma were determined by high performance liquid chromatography (HPLC) with detection by electrospray ionization mass spectrometry (LCQ-Classic, ThermoFinnigan) similar to a published procedure (18). In summary, plasma samples were purified using a Strata X solid phase extraction cartridge (Phenomenex) that had been pre-equilibrated with methanol and ultrapure water (Barnsted). The plasma samples and internal standard C17-S1P (Avanti Polar Lipids, Alabaster, AL) were methanol and ultrapure water (Barnsted). The plasma samples and internal standard C17-S1P (Avanti Polar Lipids, Alabaster, AL) were loaded and washed with ultrapure water. The analytes of interest were eluted with 50:50 methanol:acetonitrile under vacuum and concentrated by lyophilization. Partially purified samples were reconstituted in 30 μl of acetonitrile and injected into a Phenomenex phenyl-hexyl column (5 μm, 502.00 mm) using a Waters 2695 HPLC system and separated using a gradient method of 0.02% trifluoroacetic acid in water and acetonitrile. Initial composition consisted of 0.02% trifluoroacetic acid in water, and the concentration of acetonitrile was increased to 100% over 25 min. Samples were analyzed in positive ion mode with S1P cations monitored at a mass to charge ratio of 380.

FFTY720 Treatments—Mice were dosed by oral gavage with FTY720 (0.3 or 3.0 mg/kg), CA6 (10.0 mg/kg), or CA5 (20 mg/kg) dissolved in 2% hydroxypropyl β-cyclodextrin. FTY720-P was dissolved in acidified Me₂SO at 20 mM, diluted 1:20 into 3% fatty acid-free bovine serum albumin, and injected intraperitoneally (0.3 mg/kg). Peripheral blood lymphocytes were counted using a Hemavet blood analyzer (Drew Scientific, Oxford, CT).

SPHK Assay—SPHK activity was measured in kinase buffer that consisted of (in mM): 20 Tris-Cl (pH 7.4), 1 2-mercaptopethanol, 1 EDTA, 5 sodium orthovanadate, 40 β-glycerophosphate, 15 NaF, 1 phenylmethlysulfonfl fluoride, 10 MgCl₂, 0.5 4-deoxyxypuridine, 10% glyceral, and 0.01 mg/ml each leupeptin, apro tin, and soybean trypsin inhibitor. To determine the fractional activity of SPHK1 versus SPHK2, the buffer was supplemented with either 0.5% Triton X-100 or 1% KCl, respectively. The buffer was supplemented with substrate (d-erythro-sphingosine, 5 μM FTY720, CA-5, CA-6, 50 μM), [γ-32P]ATP (10 μM, specific activity = 8.3 Ci/mmole), and cell or tissue extract (0.02–0.03 mg of total protein). After 30 min at 37°C, the reaction mixture was extracted with 2 volumes of chloroform/methanol/HCl (100:200:1), and the components in the organic phase were separated by thin layer chromatography using a 1-butanol/acetic acid/water (3:1:1) solvent system. Radiolabeled enzyme products were detected by autoradiography and identified by migration relative to authentic standards. For quantification, the silica gel containing radiolabeled lipid was scraped into a scintillation vial and counted.

Preparation of Mouse Tissues for SPHK Activity Measurements—Tissue homogenates were prepared in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethlysulfonfl fluoride, and complete protease inhibitors (Sigma). Homogenates were clarified by centrifugation at 15,000 rpm in a microcentrifuge and were assayed for SPHK activity immediately after preparation.

Preparation of Recombinant Mouse SPHK1 and Mouse SPHK2—Mouse SPHK1 or SPHK2 were expressed in HEK293T cells by transfection of the cognate plasmid DNAs. Two days after transfection, cells were harvested by scraping into the kinase buffer and disrupted with a Dounce homogenizer, and the homogenate was clarified by centrifugation at 15,000 rpm in a microcentrifuge, aliquoted, and stored frozen until use. The kinase buffer was supplemented with 0.05% Triton X-100 or 200 mM KCl for assessment of SPHK1 and SPHK2 activity, respectively.

Tissue Harvesting for Lymphocyte Analyses—Wild type littermates and heterozygous- and homozygous-trapped SPHK2 mice were euthanized, and the following organs were harvested from at least four animals of each type: spleen, peripheral lymph nodes (PLN), mesenteric lymph nodes, bone marrow, thymus, Peyer’s patches, peripheral blood leukocytes, liver, and lung. Harvested peripheral lymph nodes include the inguinal, axillary, brachial, and cervical nodes. Bone marrow was obtained from both femurs and tibias of each mouse. With the exception of blood, liver, and lungs, single cell suspensions of all tissues were made in 5 ml of phosphate-buffered saline. Cell counts were determined from these suspensions, yielding cell counts in thousands of cells per microliter. For total cell counts from organs that were not harvested in full (i.e. bone marrow and liver), total numbers were extrapolated based on the percentage of cells removed. For example, cell numbers flushed from femurs and tibias were estimated to be 20% of the total bone marrow cellularity based on previous published data (19). The bone marrow counts from cells harvested were then multiplied by 5 to yield the total number of bone marrow cells expected in each mouse. The liver cells harvested were approximately half of the total liver cells. The total blood volume was estimated to be 2.5 ml/mouse. We estimated that the cells from PLN harvested were half of the total PLN cellularity for each mouse based on available data (20). The total number of lymphocytes harvested from each mouse was determined to be the “total pooled lymphocytes.” Liver and lung fragments were digested in 5 ml of phosphate-buffered saline containing collagenase type XI (125 units/ml), deoxyribonuclease (60 units/ml), and hyaluronidase (60 units/ml) (all from Sigma) for 30 min at 37°C. Blood obtained for analysis by flow cytometry was harvested via heart puncture from anesthetized mice. Blood acquired for Hemavet cell counts (see below) was obtained by bleeding unanesthetized mice via the lateral tail vein using capillary tubes coated with EDTA.

Cell Counts—Aliquots (0.04 ml) were obtained from each thoroughly mixed cell suspension. With the exception of liver samples, cell counts of the aliquots were recorded via a Hemavet (Drew Scientific). The Hemavet quantified total white blood cells, neutrophils, monocytes, lymphocytes, eosinophils, and basophils in a sample. The accuracy of the Hemavet was verified by manually counting cells in a hemocytometer (data not shown). Aliquots (10 μl) from mixed liver cell suspensions were mixed with 90 μl of Kimura stain (11 ml of toluidine blue, 0.8 ml of 0.03% light green SF yellowish (Sigma), 0.5 ml of saturated saponin in 50% ethanol, and 5 ml of 0.067 M phosphate buffer, pH 6.4). Cell counts of lymphocytes, granulocytes, and total leukocytes were obtained by analyzing the solution in a hemocytometer. Total cell numbers for each compartment were determined either by estimation for larger organs (i.e. liver, bone marrow, blood, and PLN, as described above) or by multiplying total suspension volume by cell concentration (i.e. spleen, thymus, mesenteric lymph nodes, and lungs).

Flow Cytometry Preparation and Analysis—To identify and quantify lymphocyte subsets, cell suspensions were analyzed by flow cytometry. Following red blood cell lysis, cells were stained with anti-mouse monoclonal antibodies against CD3, CD4, CD8, CD19, and NK1.1 (BD Biosciences). Cells were analyzed via four-color flow cytometry on a FACSCaliber (BD Biosciences) in the University of Virginia Cancer Center Core Facility. Lymphocyte subsets, including B cells, total T cells, CD4 T cells, CD8 T cells, double positive thymocytes, double negative thymocytes, NK cells, and NK/T cells, were analyzed. The size of each cell population was calculated as the product of the total lymphocyte count recorded by the Hemavet or hemocytometer and the
percentage of positive lymphocytes recorded by the flow cytometer. All data were analyzed with BD Biosciences CellQuest analysis software.

**Real-time RT-PCR**—Total RNA was isolated using an RNaseasy Mini kit (Qiagen) with optional DNase treatment (RNase-free DNase kit, Qiagen). Reverse transcription was performed using an Omniscript RT kit (Qiagen). Real-time PCR was performed on an iCycler iQ real-time detection system (Bio-Rad) using sequence-specific primers and TaqMan probes designed on Beacon Designer 4.0 software. Values were determined using iCycler iQ real-time detection system software v3.0a (Qiagen). The corresponding values were normalized to glyceraldehyde-3-phosphate dehydrogenase forward, 5'-GGCTCATGACCAAGTCCAT-3'; reverse, 5'-GCCTGCTTCACCACCTTCT-3'; probe, 5'-CCTGGAGAAACCTGCCAAGTATGAC-3'.

**Adoptive Transfer of Lymphocytes**—Single cell suspensions were prepared from spleen and pooled lymph nodes of Sphk2+/tr and Sphk22/2/2 mice. Cells from Sphk22/2/2 mice were labeled with 0.5 μM CFSE. Differently labeled Sphk2+/tr and Sphk22/2/2 cells were mixed in a 1:1 ratio and washed twice, and 20 × 10⁶ cells were injected intravenously into the dorsal tail vein of host Sphk2+/tr and Sphk22/2/2 animals. Animals were bled 12–23 h after adoptive transfer to measure CFSE-labeled Sphk2+/tr and Sphk22/2/2 lymphocyte subsets in blood prior to treatment. Host animals were then administered either 0.3 mg/kg of FTY720 or water vehicle, via oral gavage, 24 h after adoptive transfer. Blood from host mice was collected 24 h after FTY720 or water administration. Lymphocyte numbers in blood were determined with a Hemavet blood analyzer (Drew Scientific). Lymphocyte subsets were identified using anti-B220-PE, anti-CD8-APC (eBioscience), and anti-CD4-PerCP-Cy5.5 (BD Biosciences). Samples were collected on a FACSCaliber (BD Biosciences) and analyzed using FlowJo software. Statistical analysis was performed using Student’s t test (GraphPad software).

**Histology and Immunostaining**—Lymph nodes were fixed in formalin, and 5-μm sections were stained by hematoxylin and eosin. To study splenic marginal zone alterations, Sphk22/2/2 mice and their wild type littermates were given FTY720 (0.3 mg/kg) by oral gavage. After 24 h, frozen spleen sections were fixed in acetone at 4 °C for 10 min and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgM (diluted 1:50) (Pharmingen) and rat anti-mouse IgG (diluted 1:50) (Pharmingen). The sections were incubated subsequently with biotinylated sheep anti-rat IgG (preabsorbed with mouse IgG) (diluted 1:100) followed by avidin-Texas red (diluted 1:200). The tissue sections were examined and photographed by fluorescence microscopy.

**Statistical Analysis**—Statistical analyses (see TABLE ONE) were completed using the student’s t test (+/tr and tr/tr were both compared with wild type) and showed no statistically significant differences between wild type and +/tr or tr/tr mice in their lymphoid compartments.

**Materials**—FTY720 and FTY720-P were gifts from Novartis Pharma AG (Basel, Switzerland). C17 S1P and sphingosine were purchased from Avanti Polar Lipids (Alabaster, AL). Hydroxypyrrol β-cyclodextrin was purchased from Cargill, Inc. (Cedar Rapids, IA).
SPHK2 Activates FTY720 in Vivo

RESULTS

Generation of SPHK2 Null Mice—To determine whether SPHK2 is necessary for phosphorylating S1P receptor agonist pro-drugs such as FTY720, we developed a SPHK2 null mouse. Beginning with an embryonic stem cell line with one allele of the Sphk2 gene trapped, we generated a colony of mice wherein both alleles of Sphk2 contained the exon trap. The trap inserted between exons 2 and 3 of Sphk2 and Southern blotting documented that a single trap element was present in the haploid genome (Fig. 1, a–c). SPHK2 mRNA measurements by RT-PCR did not detect a signal in Sphk2\(^{tr/tr}\) mouse kidney (Fig. 1d, and see also Fig. 4). From these data, we concluded that expression of the Sphk2 gene is disrupted in Sphk2\(^{tr/tr}\) mice.

Both male and female Sphk2\(^{tr/tr}\) mice are fertile and can survive to at least 12 months of age. However, the number of tr/tr animals found at weaning was significantly less than predicted by simple Mendelian inheritance. For example, one heterozygous breeding pair produced 77 pups surviving until weaning (47 male, 30 female; +/+:+/tr:tr/tr, 21:47:9), whereas a second breeding pair produced 34 pups (18 male, 16 female; +/+:+/tr:tr/tr, 7:24:3). Likewise, crosses of male Sphk2\(^{tr/tr}\) mice with heterozygous females produced fewer than expected tr/tr pups at weaning. When compared with heterozygous or wild type animals, Sphk2\(^{tr/tr}\) dams were less apt to carry a pregnancy to term and give birth to litters with fewer pups. This collective phenotype is the subject of ongoing investigations.

S1P Tone and Lymphocyte Distribution in SPHK2 Null Mice—Because SPHK2 is one of only two enzymes known to mediate the phosphorylation of sphingosine, we were interested to determine how its absence affected S1P tone and characteristics of the mice. Plasma S1P concentrations in adult Sphk2\(^{tr/tr}\) mice were reduced by about 25% when compared with wild type and heterozygous mice (in nM ± S.D.: Sphk2\(^{+/+}\) 473 ± 66 (n = 8), Sphk2\(^{+/tr}\) 548 ± 90 (n = 8), Sphk2\(^{tr/tr}\) 393 ± 52 (n = 7)), demonstrating that SPHK2 is a significant, but not major, determinant of S1P tone. In addition, the cellularity and fractional distribution of total lymphocytes did not differ significantly in lymphoid tissues, blood, liver, or lung from wild type, heterozygous, or homozygous genotypes.

### TABLE ONE

| Organ  | +/+ | +/tr | tr/tr |
|--------|-----|------|------|
| Spleen | 161 ± 25 | 148 ± 20 | 154 ± 8 |
| PLN    | 105 ± 12 | 91 ± 19 | 114 ± 22 |
| MLN    | 14 ± 5 | 11 ± 4 | 11 ± 2 |
| BM     | 351 ± 93 | 287 ± 63 | 270 ± 34 |
| Thymus | 67 ± 10 | 98 ± 24 | 92 ± 1 |
| PP     | 14 ± 2 | 9 ± 2 | 19 ± 4 |
| PB     | 28 ± 6 | 34 ± 6 | 34 ± 8 |
| Liver  | 32 ± 5 | 33 ± 5 | 30 ± 1 |
| Lung   | 17 ± 2 | 19 ± 2 | 24 ± 3 |

Data were averaged from six Sphk2\(^{+/+}\) and seven of both Sphk2\(^{+/tr}\) and Sphk2\(^{tr/tr}\) mice. The abbreviations are as follows: MLN, mesenteric lymph nodes; BM, bone marrow; PP, Peyer’s patches; PB, peripheral blood.

**FIGURE 2. FTY720 and lymphopenia in Sphk2\(^{tr/tr}\) mice.** a, groups of three mice (8–9 weeks old) of the indicated genotype were dosed by oral gavage with vehicle or FTY720 (0.3 mg/kg of body weight), blood was drawn after 24 h, and total lymphocyte counts were determined. K/μl, thousands of cells per microliter. b, homogenates of kidneys from 8–9-week-old Sphk2\(^{+/+}\), Sphk2\(^{+/tr}\), and Sphk2\(^{tr/tr}\) mice were prepared and assayed for SPHK activity as described under “Experimental Procedures.” c, groups of three mice (8–9 weeks old) of the indicated genotype were bled, dosed with vehicle alone or FTY720-P (0.3 mg/kg), and bled again at 5 and 24 h; total lymphocytes were counted in each blood sample. Error bars represent S.D.
gous mutant Spkh2 mouse (TABLE ONE). Further, the fractional distribution of CD3, CD4, CD8, CD19, CD45, NK1.1, or Gr-1 cells in these compartments was not influenced by the Spkh2 genotype (data not shown). Thus, this reduction in S1P levels does not have a significant effect on the cellular organization of the immune system. Examination at necropsy revealed no pathologic abnormalities in organ appearance, size, or histology. Overall, adult Spkh2+/− mice were phenotypically unremarkable.

**FTY720 Activation in Vivo Requires SPHK2**—The primary evidence that phosphorylated FTY720 is required to induce lymphopenia comes from the use of FTY720 analogs that either are enantiomers (12) or contain a non-hydrolyzable phosphonate (11). A mouse lacking the activating kinase provides the opportunity to test further the concept that FTY720 action in vivo requires phosphorylation. Therefore, we treated Spkh2+/− mice as well as heterozygous and wild type littermate mice with an ED95 dose of FTY720 (0.3 mg/kg of body weight). As expected, both wild type and Spkh2−/− mice showed an 80% reduction in total lymphocytes in the bloodstream at 24 h after dosing (Fig. 2a). In contrast, the blood lymphocyte counts in Spkh2−/− mice were not significantly decreased from those of vehicle-treated animals. The experience in vivo was recapitulated ex vivo using kidney extracts in which FTY720 was phosphorylated by +/+ mice, less so by +/-tr mice, but not detectably by tr/tr mice (Fig. 2b). When the active form of the drug (FTY720-P) was administered to mice (0.3 mg/kg), lymphopenia was observed at 5 h and disappeared by 24 h. These data documented that SPHK2 is required for activation of FTY720 and further that FTY720 and FTY720-P must participate in a cycle of phosphorylation/dephosphorylation for the drug effect to persist.

**Splenic Marginal Zone B Cell Migration in Spkh2 Null Mice**—FTY720 causes rapid migration of marginal zone B-cells from the marginal zone to lymphoid follicles (21). To learn whether this FTY720-evoked response was observable in the Spkh2 null mice, we examined the marginal zone in the spleen by immunofluorescence 24 h following FTY720 treatment. We confirmed the observation of FTY720-driven ablation of the marginal zone in wild type (and heterozygous) mice treated with FTY720 (0.3 mg/kg); in contrast, we found the marginal zone architecture unperturbed in FTY720-treated Spkh2+/− mice (Fig. 3, a, b, d, and e). These data indicate that dislodging B cells from the marginal zone niche requires phosphorylation of FTY720 by SPHK2. In FTY720-treated mice, the retention of lymphocytes in lymph nodes was accompanied by depletion of lymphocytes in their efferent sinuses. Indeed, empty efferent sinuses were observed in the lymph node of normal mice but not of Spkh2+/− mice treated with FTY720 (Fig. 3, c and f).

**SPHK2 RNA Expression**—Because SPHK2 mediates phosphorylation of FTY720 in vivo, its distribution might influence egress of lymphocytes from thymus and lymph nodes by creating locally high concentrations of FTY720-P inside these organs. Therefore, we examined the distribution of SPHK2 mRNA levels in several tissues, including lymphoid organs. Quantitative RT-PCR (Fig. 4) revealed that SPHK2 RNA was most abundant in kidney, liver, salivary glands, bone marrow, and lung, which confirms and extends data reported using Northern blot techniques (22). SPHK2 mRNA was detected also in isolated CD4+, CD8+, and CD19+ splenocyte populations from Spkh2−/− mice (Fig. 4) but not in comparable populations from Spkh2+/− animals (Fig. 4 and not shown).

**SPHK2 Expression in Lymphocytes Influences Their Depletion from Blood**—Based on the expression of SPHK2 in lymphocytes, we tested the hypothesis that FTY720-P generated by lymphocytes could act in an
autocrine manner to induce self-sequestration from blood. We differentially labeled Sphk2<sup>-/-</sup> and Sphk2<sup>+/+</sup> cells with low and high concentrations of CFSE, respectively, to enable the two cell populations to be distinguished by flow cytometry. Equal numbers of both populations were adoptively transferred into Sphk2<sup>-/-</sup> or Sphk2<sup>+/+</sup> recipients, and a pretreatment blood sample was taken 12–23 h later. Mice were then treated by oral gavage with 0.3 mg/kg of FTY720 or water vehicle alone 24 h after adoptive transfer, and a post-treatment blood sample was taken 24 h later. Representative data for two individual animals are presented in Fig. 5c. As anticipated, the numbers of Sphk2<sup>-/-</sup> T and B lymphocytes remaining in a Sphk2<sup>+/+</sup> recipient (Fig. 5b). Further, Sphk2<sup>-/-</sup> T and B cells were not significantly reduced in Sphk2<sup>+/+</sup> hosts after FTY720 treatment, indicating that any FTY720-P generated by these adoptively transferred cells was not adequate to induce self-sequestration from blood. Conversely, adoptively transferred Sphk2<sup>-/-</sup> T and B lymphocytes were significantly reduced in Sphk2<sup>+/+</sup> recipients 24 h after FTY720 treatment (Fig. 5b). This result indicated that FTY720-P generated outside the adoptively transferred cells in Sphk2<sup>+/+</sup> recipients sufficed to induce Sphk2<sup>-/-</sup> lymphocyte depletion from blood; endogenous SPHK2 expression was not required. However, when the extent of depletion of Sphk2<sup>-/-</sup> cells and Sphk2<sup>+/+</sup> cells in FTY720-treated Sphk2<sup>-/-</sup> recipients was compared by evaluating the ratios of these populations to one another in individual animals, there were relatively more Sphk2<sup>-/-</sup> T and B lymphocytes in blood (Fig. 5c). Thus, Sphk2<sup>-/-</sup> cells were depleted from blood more efficiently than Sphk2<sup>+/+</sup> cells. Collectively, these results demonstrated that endogenous SPHK2 expression in T and B lymphocytes was neither absolutely required nor alone sufficient for lymphocyte depletion from blood.
However, FTY720-P produced by SPHK2 in lymphocytes acts in an autocrine manner to augment the extent of sequestration from blood.

S1P Pro-drug Activation by Another Kinase—Administration of FTY720 to SPHK1 null mice was reported to result in levels of FTY720-P that were significantly lower than in wild type littermates, although sufficient active drug was formed to evoke the same degree of lymphopenia in animals of either genotype (18). Furthermore, FTY720 is phosphorylated in vitro by recombinant SPHK1, albeit far less efficiently than by recombinant SPHK2 (15–17) (Fig. 6a). Therefore, it is likely that at high doses, FTY720 is metabolized to some extent by SPHK1. Indeed, we found that a 10-fold increase in FTY720 dose to 3.0 mg/kg showed a 35% reduction in total bloodstream lymphocytes of Sphk2tr/tr mice (not shown). The SPHK2 null mouse allowed us to test directly whether a sphingosine-like pro-drug that is an efficient substrate for SPHK1 can evoke lymphopenia in the absence of SPHK2. In the course of structure activity relationship studies of SPHK substrates, we have synthesized numerous molecules that, like FTY720, are S1P1 agonists when phosphorylated. One compound, CA6, has the unusual property of being an equally good substrate for SPHK1 and SPHK2 (15–17) (Fig. 6a). Therefore, it is likely that at high doses, FTY720 is metabolized to some extent by SPHK1. Indeed, we found that a 10-fold increase in FTY720 dose to 3.0 mg/kg showed a 35% reduction in total bloodstream lymphocytes of Sphk2tr/tr mice (not shown). The SPHK2 null mouse allowed us to test directly whether a sphingosine-like pro-drug that is an efficient substrate for SPHK1 can evoke lymphopenia in the absence of SPHK2. In the course of structure activity relationship studies of SPHK substrates, we have synthesized numerous molecules that, like FTY720, are S1P1 agonists when phosphorylated. One compound, CA6, has the unusual property of being an equally good substrate for SPHK1 and SPHK2 (Fig. 6a). At a dose of 10 mg/kg (=ED95), CA6 evokes marked lymphopenia in wild type as well as SPHK2 null mice (Fig. 6b). In contrast, another compound, CA5, is a substrate in vitro only for SPHK2 (Fig. 6a) and, like FTY720, does not cause lymphopenia when administered to Sphk2 null mice (Fig. 6c). These data demonstrated that a kinase other than SPHK2, presumably SPHK1, can serve to activate S1P pro-drugs.

DISCUSSION

The SPHK2 null mouse, although viable, does not respond normally to the immunomodulatory pro-drug FTY720, indicating that SPHK2 is the predominant enzyme in activating FTY720 to FTY720-P in vivo. Nevertheless, other S1P-like pro-drugs can be activated via another kinase (probably SPHK1) instead of, or in addition to, SPHK2. Further, SPHK2 exerts a local effect on T and B cell trafficking because lymphocytes lacking SPHK2 are cleared from blood less efficiently in response to FTY720 treatment.

Unlike the SPHK1 null mice, female SPHK2 null mice do have the phenotype of variable subfertility. Further characterization of this phenotype is underway. S1P1 receptor null mice die in utero from a failure in vascular maturation (23). The absence of the ligand, S1P, would thus likewise be expected to be embryonic lethal. That SPHK2 null mice are viable is probably because the essential S1P is provided by the action of SPHK1. Indeed, the plasma concentration of S1P in our SPHK2 null animals was only about 25% less than age-matched wild type control mice. Similarly, SPHK1 null mice were reported to have reductions in plasma S1P levels of about 50% (18). However, the plasma S1P levels we measured in control mice (~500 nM) were significantly less than the plasma levels reported by Allende et al. (~1500 nM, Ref. 18); we do not know the reason for this difference.

The structure activity relationship studies that led to FTY720 from the original compound, myriocin, were performed entirely in vivo (24). This unusual path was necessitated by the pro-drug nature of FTY720, which was not recognized initially. In retrospect, the choice of FTY720...
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was fortunate because the active drug, FTY720-P, is not orally available and is less tractable synthetically and because its physiochemical properties make solubility problematic. Thus, it is important to characterize the kinases responsible for the metabolism of this drug class.

Our data demonstrated that SPHK2 is the enzyme primarily responsible for activating FTY720. However, the route through SPHK2 is dictated by the substrate and not by cellular or tissue localization of the enzyme because S1P pro-drugs that are efficient substrates for SPHK1 (e.g. compound CA6, Fig. 6, a and b) are active in evoking lymphopenia, which is the index of S1P$_1$ receptor agonist action. In principle, a variety of sphingosine-like pro-drugs that are substrates for either or both sphingosine kinases could be designed to manipulate subsets of the five S1P receptors as agonists or antagonists. It is also conceivable that the related lysophosphatidic acid receptors could be reached via this route.

Our SPHK2 null mice have provided the opportunity to make several measurements that were heretofore not possible. For example, the decay rate constant for the metabolism (via dephosphorylation) of active S1P-like drugs phosphorylated by SPHK2 can be calculated. We found in the Sphk2$^{+/+}$ mice that FTY720-P was fully efficacious 5 h after administration, but the lymphopenia had disappeared at 24 h. Testing the hypothesis that S1P is synthesized solely via SPHK1 or SPHK2 is also possible. If true, crossing SPHK2 null mice with SPHK1 null mice will produce animals lacking both SPHK isoforms that will die in utero, mimicking the phenotype of S1P$_1$ receptor null mice.

In conclusion, the immunomodulatory pro-drug, FTY720, was not metabolized efficiently in SPHK2 null mice. Their viability, albeit reduced, allowed us to determine that SPHK1 alone is sufficient to synthesize S1P at near physiologic levels in adult animals. Certain other sphingosine analogs can be activated by another sphingosine kinase isotype, probably SPHK1. Further, T and B lymphocytes without SPHK2 were not depleted from blood as efficiently as wild type lymphocytes in a wild type mouse. SPHK2 null mice will be valuable tools in S1P pro-drug discovery as well as in exploring the role of SPHK2 in biologic processes including immune system and vascular function.

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