Sphingosine 1-Phosphate (S1P) Receptor Subtypes S1P₁ and S1P₃, Respectively, Regulate Lymphocyte Recirculation and Heart Rate*

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Sphingosine 1-phosphate (S1P) influences heart rate, coronary artery caliber, endothelial integrity, and lymphocyte recirculation through five related high affinity G-protein-coupled receptors. Inhibition of lymphocyte recirculation by non-selective S1P receptor agonists produces clinical immunosuppression preventing transplant rejection but is associated with transient bradycardia. Understanding the contribution of individual receptors has been limited by the embryonic lethality of the S1P₁ knock-out and the unavailability of selective agonists or antagonists. A potent, S1P₁ receptor selective agonist structurally unrelated to S1P was found to activate multiple signals triggered by S1P, including guanosine 5'-3-O-(thio)triphosphate binding, calcium flux, Akt and ERK1/2 phosphorylation, and stimulation of migration of S1P₁ receptors but not S1P₃-expressing cells in vitro. The agonist also alters lymphocyte trafficking in vivo. Use of selective agonist together with delenat mice lacking S1P₁ receptor reveals that agonism of S1P₁ receptor alone is sufficient to control lymphocyte recirculation. Moreover, S1P₁ receptor agonist plasma levels are causally associated with induction and maintenance of lymphopenia. S1P₃, and not S1P₁, is directly implicated in sinus bradycardia. The sustained bradycardia induced by S1P₁ receptor non-selective immunosuppressive agonists in wild-type mice is abolished in S1P₃⁻/⁻ mice, whereas S1P₃-selective agonist does not produce bradycardia. Separation of receptor subtype usage for control of lymphocyte recirculation and heart rate may allow the identification of selective immunosuppressive S1P₁ receptor agonists with an enhanced therapeutic window. S1P₁-selective agonists will be of broad utility in understanding cell functions in vitro, and vascular physiology in vivo, and the success of the chemical approach for S1P₁ suggests that selective tools for the resolution of function across this broad lipid receptor family are now possible.

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† The abbreviations used are: S1P, sphingosine 1-phosphate; hS1P, human S1P; GTP-S, guanosine 5'-3-O-(thio)triphosphate; SEW2871, 5-(4-phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylpheno-yl)-(1,2,4)-oxadiazole; CHO, Chinese hamster ovary; BSA, bovine serum albumin; FLIPR, Fluorescence Imaging Plate Reader; FACS, fluorescence-activated cell sorting; CHAPS, 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonic acid; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; GC-MS, gas chromatography-mass spectrometry; AFD-(R), phosphate ester of AAL; AAL-(R), 2-amino-4-(4-heptyloxyphenyl)-2-methyl butanol.
well as from thymus into blood (11, 23). Inhibition of lymphocyte egress is associated with clinically useful immunosuppression in both transplantation and autoimmune disease models (24–26). Pleiotropic responses at low nanomolar plasma concentrations is seen in this system, because FTY727 mediates both lymphopenia and a transient dose-dependent bradycardia on initial dosing in humans (27). The therapeutic window for S1P receptor agonists may therefore depend on the association of single receptors with critical functions.

We have combined a chemical approach with the use of S1P receptor null mice to help define receptor selectivity. We chose a chemical approach for S1P4, because of the absence of the knock-out. Published data on FTY727 phosphonate (respective IC50 values for human S1P1 (8.2 nM), S1P2 (>10,000 nM), S1P3 (151 nM), S1P4 (33 nM), and S1P5 (178 nM)) suggested that S1P4 is responsible for inhibition of lymphocyte egress (9), a fact that was subsequently strengthened by structure-activity correlations among a collection of semi-selective S1PR agonists (43, 44). We now show that the discovery of selective S1P receptor agonists is useful in demonstrating that selective biochemical signals can regulate complex in vivo biology.

EXPERIMENTAL PROCEDURES

SIP Receptor Agonists

AFD-R (it was the kind gift of Novartis Pharma (Basel, Switzerland, Volker Brinkmann). 5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)4,1,2,4-oxidazole was purchased from Maybridge (Tintagel, Cornwall).

Cells and Plasmids

CHO cells stably expressing human S1P receptors (hS1P1, hS1P2, hS1P3, hS1P4, and hS1P5), were kindly provided by Danilo Guerini (Novartis Pharma).

Membrane Preparations

Membranes were prepared from CHO cells expressing human or murine S1P1, S1P2, S1P3, S1P4, and S1P5, for use in ligand and [35S]GTPγS binding studies as described previously (9) and suspended in Buffer B with 15% glycerol and stored at −80 °C.

Agonist Assays

Measurements of [35S]GTPγS Binding—Serial dilutions of S1P (diluted in 4% BSA) or SEW2871 (diluted in 80 mM NaCl) were added to membranes (1–10 μg of protein/well) and assayed as described (9).

Measurements of Ca2+ Flux—Calcium flux assays in the Fluorescence Imaging Plate Reader (FLIPR, Molecular Devices) format were performed as described (9). The assay was initiated by transferring an equal volume of ligand to the cell plate, and calcium flux was recorded over a 3-min interval. Cellular response was quantitated as maximal response relative to S1P activation without pretreatment. Spleen cells, upon laser excitation. Flow cytometric measurement of calcium flux Imaging Plate Reader (FLIPR; Molecular Devices) format were performed as described (9).

Calcium flux assays were performed in a temperature-controlled FACSCalibur flow cytometer (BD Bioscience, Mountain View, CA). FACScan events were collected using CELLQUEST software (BD Bioscience), and then analyzed with FLOWJO (Treestar, San Carlos, CA). FACScan events were collected for 30 s, and then immunoycin, S1P, or SEW2871 were added and events were collected for an additional 10 min. The ratio fluorescence at 420 nm to that at 510 nm was used to measure calcium flux of propidium iodide-negative cells.

Western Blotting of S1P-activated Kinases

Control CHO cells and the CHO cells stably transduced with human S1P1 or S1P2, were cultured to 50% confluence on 6-well plate in complete RPMI 1640 supplemented with 10% fetal bovine serum. Cells were serum-starved for 16 h and stimulated with SEW2871 diluted to various concentrations in the serum-free medium with 0.1% fatty acid-free BSA. At 5 min, cells were lysed in 50 mM Tris, pH 8.0, 125 mM NaCl, 20 mM CHAPS, 2 mM dithiothreitol, 1 mM EDTA, 2 mM NaN3, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. Cell lysates were analyzed by Western blotting after separation on 10% SDS-PAGE using mouse monoclonal anti-phospho-ERK1/2 antibody (sc-7383, Santa Cruz Biotechnology) and rabbit polyclonal anti-phospho-Akt antibody (BD Biosciences). Total ERK1 and ERK2 were detected using a rabbit affinity-purified polyclonal anti-ERK antibody (sc-94, Santa Cruz Biotechnology), and total Akt was detected using a rabbit affinity-purified polyclonal anti-Akt1 antibody (BD Biosciences). Band intensities corresponding to pERK1, pERK2, and pAkt were quantitated by imaging (Kodak 1D Scientific Imaging System). Amounts of pERK1/2 and pAkt were normalized for the total amounts of ERK1/2 and Akt. Primary lymph node lymphocytes were teased from the peripheral and mesenteric nodes of C57BL6 mice kept rigorously at 4 °C. Cells were warmed to 37 °C, stimulated for 5 min with S1P or SEW2871 (50 nM and 500 nM), or 50 ng/ml phorbol myristate acetate and vehicle, and Akt and ERK phosphorylation was determined as above.

Assay of S1P Receptors-dependent Cell Migration

Cell adhesion and migration assays were performed as follows. Cells expressing CHO, CHO-S1P1, or CHO-S1P2, were starved overnight in regular medium without fetal bovine serum prior to migration assay. Cell migration assays were performed using modified Boyden chambers (tissue culture-treated, 6.5-mm diameter, 10-μm thickness, 8-μm pores, Transwell®; Costar Corp., Cambridge, MA) containing polycarbonate membranes coated on the underside of the membrane with 5 μg/ml fibronectin in PBS for 2 h at 37 °C, rinsed once with PBS, and then placed into the lower chamber containing 500 μl of migration buffer (RPMI with 0.5% BSA; Invitrogen, San Diego, CA). Serum-starved cells were removed from culture dishes with Hanks’ balanced salt solution containing 5 mM EDTA and 25 mM Hepes, pH 7.2, and 0.01% trypsin, washed twice with migration buffer, and then resuspended in Migration buffer (106 cells/ml). 75,000 cells were then added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for 3 h in the presence or absence of either S1P or SEW2871 (1 μM, which had been added to the medium). The non-migratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane were fixed with 4% paraformaldehyde and stained with propidium iodide (1 μg/ml) in PBS for 20 min at room temperature. The number of migratory cells per membrane was evaluated by looking at five random fields with an inverted microscope using a 40× objective. Each determination represents the average of three individual wells. In control experiments, cell migration on vehicle control was less than 0.01% of the total cell population.

Pharmacokinetic Analysis

All samples were analyzed after CHCl3 extraction, evaporation to dryness, and redissolution in 0.1 ml of CHCl3, followed by splitless injection on an Agilent 6890N gas chromatograph. Sample detection was carried out by using a 5973 mass-selective detector with single ion monitoring at 440 m/z for SEW2871 and 372 m/z for a spiked and structurally related internal standard, SEW2898. Limit of quantitation was 0.4 ng/ml in plasma, based on spikes into human serum. Sample amounts were determined by comparison to a standard curve, R2 = 0.99. Non-compartmental pharmacokinetic analysis of plasma levels was performed by using PK Solutions 2.0 software (Pharmacia Reserch Services, Montrose, CO).

Induction of Lymphopenia in Mice

C57BL6 or S1P3−/− mice (16) or their S1P1−/+ litter mate controls were challenged with increasing doses of SEW2871 or vehicle (10% MeSO25%) twice 20 h prior to blood collection into EDTA tubes (BD Biosciences). Full blood counts were determined by veterinary automanalyzer calibrated for mouse blood (H2000, CARESIDE, Culver City, CA) at times stated as described previously (9). All animal studies were approved by the Institutional Animal Care and Use Committee.

a S. Mandala, J. Hale, R. Hajdu, and H. Rosen, unpublished results.
Histology
C57Bl/6 mice were gavaged with 0.1 ml of vehicle or SEW 2871 (10 mg/kg). Sixteen hours later, mesenteric and inguinal lymph nodes were fixed in 10% formalin in PBS and paraffin-embedded, and 5-µm sections were stained with hematoxylin and eosin. Images were acquired by Metamorph software on an Olympus AX70 microscope.

Measurement of Heart Rate in Conscious Mice
Effects on heart rate in S1P3−/− or wild type littermates or C57BL6 controls were measured by ECG analysis in conscious mice using the ANONYmouse ECG screening system (MouseSpecifics, Boston, MA), before and after injection of the non-selective S1P receptor agonist AFD-(R) or vehicle control. No difference between WT littermates and C57BL6 mice were seen.

RESULTS
High Throughput Screening Identifies S1P1-selective Agonists—Published binding studies on hS1P1 with FTY720 and FTY-P (9), as well as mutagenesis and modeling with natural ligand S1P (28, 29), suggested a two-site binding model. The hydrophobic-aromatic residues bind within receptor transmembrane domains and the ligand headgroups form salt bridges with glutamate and arginine side chains. Specifically, FTY720 has a measurable IC50 (300 nM) for S1P1 that is enhanced 1000-fold by the enantioselective addition of the phosphate ester (9). FTY720 binding implies that G-protein-coupled receptor privileged structures, structurally unrelated to S1P, could likely access the transmembrane site as agonists, with sequence differences between receptor subtypes making the discovery of selective agonists probable (30). Indeed, such agents, including the featured compound in this report, have previously been identified and characterized (43).

SEW2871 Activates Signals and Responses through S1P1 Alone Comparable to S1P in GTPγS Activation, Calcium Flux, Kinase Phosphorylation, and Cell Migration—5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl) (1,2,4)-oxadiazole (SEW2871) (Fig. 1A) is a novel selective agonist for hS1P1 structurally unrelated to S1P. Unlike S1P, it has no solubilizing or charged headgroups. S1P showed 50% maximal receptor activation in the GTPγS binding assays (EC50 of 0.4 ± 0.24 nM (mean ± S.D.; n = 6) on human S1P1 (hS1P1), whereas EC50 values for SEW2871 on hS1P1 were 13 ± 8.58 nM (mean ± S.D.; n = 3) (Fig. 1B). Like S1P, SEW2871 was a full agonist with levels of receptor activation comparable to S1P (Fig. 1B). Although S1P is a non-selective agonist with EC50 values (mean ± S.D.) of 3.8 ± 3.5 nM (hS1P2; n = 4), 0.6 ± 0.35 nM (hS1P3; n = 6), 67 ± 13 nM (hS1P4; n = 4), 0.5 ± 0.39 nM (hS1P5; n = 3) on the respective human receptors, SEW2871 was inactive at 10,000 nM on hS1P2, hS1P3, hS1P4, and hS1P5 (Fig. 1C).
We confirmed full selective agonism for hS1P1 alone in the ligand-dependent calcium flux assay (Fig. 1D) for SEW2871 in stably transfected CHO cell lines, with no significant activation of hS1P2–5 (Fig. 1D) up to 10 μM. We found evidence for selective but full agonism of murine S1P1 (mS1P1) with EC50 = 20.7 nM (Fig. 2A), with no activity at 10 μM on mS1P2–5 (Fig. 2B). EC50 values for S1P on the transiently transfected murine receptors S1P1–5 were 1.4 nM (mS1P1), 2.0 nM (mS1P2), 2.3 nM (mS1P3), 75 nM (mS1P4), and 16 nM (mS1P5), respectively. Identical selectivity was seen both in membranes of CHO cells transiently transfected with the respective murine receptors and assayed for agonism in GTPγS binding assays, as well as in intact cells in calcium flux assays (Fig. 2C). Pretreatment with 30 ng/ml pertussis toxin in the GTPγS assay fully inhibited GTPγS binding induced by either S1P or SEW2871, confirming that SEW2871 is also acting through the Gc-coupled receptor.

We also compared kinase phosphorylation in response to S1P and SEW2871 stimulation in both S1P1 and S1P3 CHO cell lines (Table I). Substantial ligand concentration-dependent pAKT and pERK1 signals were induced by SEW2871 in S1P1 but not S1P3 CHO cells, whereas modest phosphorylation of pERK2 was also seen. In contrast, S1P activated kinases in both cell lines equally (not shown).

The multiple signals induced by SEW2871 are sufficient to replicate complex functional responses of S1P through S1P1. In a Transwell migration assay, SEW2871 (Fig. 3B) and S1P (Fig. 3E) induced equivalent cell migration in vivo in S1P1-CHO cells with obvious morphology for stimulation of cytoskeletal rearrangements. Minimal cell migration or cytoskeletal reorganization occurred in response to S1P or SEW2871 in untransfected CHO cells (<0.01% of cell migrated) (Fig. 3A, A and D), whereas S1P3 CHO cells migrated and changed shape in response to S1P (Fig. 3C) but not SEW2871 (Fig. 3F), confirming the selectivity of SEW2871. Despite its structural similarities to S1P, and lack of headgroups, SEW2871 is a selective low nanomolar full agonist of S1P1 in all biochemical parameters and one complex cellular behavior tested, and could potentially be usefully studied in vivo.
A lent cell migration in vivo A affected CHO cells (0.01% of cell migrated) (Fig. 4A). These data were confirmed by flow cytometry, where freshly isolated CD4+ (shown) or CD8+ (data not shown) T lymphocytes from spleen, lymph node, or thymus responded to SEW2871 (Fig. 5B). There was a dose-response relationship between plasma levels of SEW2871 and the number of blood lymphocytes. We showed the relationship between plasma levels of S1P1 receptor agonist and the maintenance of circulating blood lymphocyte numbers in the duration of action study (Fig. 4D), where 20 mg/kg SEW2871 was gavaged and blood lymphocyte numbers and compound plasma concentrations were measured for the first 42 h. The curves for plasma levels of SEW2871 and the induction and maintenance of lymphopenia were mirror images of each other. Induction of lymphopenia was as rapid for SEW2871, as for non-selective S1P receptor agonists such as AAL(R) (9–11), and full lymphopenia was maintained for more than the first 12 h. As SEW2871 concentrations in plasma decline, the lymphopenia reverses, suggesting that the continuous presence of S1P1 receptor agonist is necessary for the maintenance of lymphopenia.

SEW2871 Inhibits Lymphocyte Migration into Murine Lymphatic Sinuses—S1P receptor agonists inhibit egress of lymphocytes into lymphatic sinuses in peripheral and mesenteric lymph nodes and Peyer’s patch but not spleen (7, 9). Effects are easily seen histologically within 6–15 h of a single dose of agonist. SEW2871 (Fig. 5B) but not vehicle (Fig. 5A) induced clearing of lymphatic sinuses (arrows) and the log-jamming of lymphocytes immediately subjacent to sinus-lining endothelium in lymph nodes. SEW2871 histological changes were indistinguishable from those seen with the non-selective S1P receptor agonist control AAL-(R) (not shown) and those published for FTY720 (9). Both SEW2871 and the non-selective S1P agonists inhibit the egress of lymphocytes across sinus lining endothelium supporting the conclusion that activation of S1P1 alone is sufficient to shut down entry of lymphocytes into lymph.

Freshly Isolated Lymphocytes from Spleen or Lymph Node Do Not Respond to SEW2871 with a Ligand-evoked Calcium Flux—SEW2871 has rapid effects upon the bulk trafficking of lymphocytes in vivo, although these effects are confined to lymph node and thymus but not spleen, despite the facts that naïve lymphocyte populations in lymph node and spleen show no distinguishing characteristics. Inhibition of lymphocyte egress from lymph node and thymus but not spleen suggests that this mechanism may therefore depend upon non-lymphocytic stromal cell effects in addition perhaps to direct effects upon lymphocytes. To assess whether SEW2871 mediated its effects upon lymphocytes directly or indirectly, we looked for evidence of S1P1 activation and expression on freshly isolated murine lymphocytes that had not been cultured at all.

Spleen adherent cells, but not lymphocytes freshly isolated from spleen, lymph node, or thymus responded to SEW2871 with a ligand-induced calcium flux in FLIPR format assays (Fig. 6A). These data were confirmed by flow cytometry, where freshly isolated CD4+ (shown) or CD8+ (data not shown) T cells passing through the membranes are stained with propidium iodide.
lymphocytes did not undergo a calcium flux in response to either SEW2871 (at concentrations up to 10 μM) or S1P (1 μM), but did respond to ionomycin (Fig. 6B).

We also examined phosphorylation of Akt and ERK1 in freshly isolated lymph node lymphocytes. Neither S1P nor SEW2871 at both 50 and 500 nM induced phosphorylation of Akt and ERK1 in lymphocytes, whereas stimulation with PMA induced a 9.2-fold increase in ERK phosphorylation of the same cells (data not shown).

In addition, we were not able to identify the presence of mS1P1 upon freshly isolated lymphocytes by Western blotting or immunohistology, whereas both transfected cells and lymphoid organ stroma can be shown to express S1P1 by these methods.3 Further work to identify functional S1P receptors on lymphocytes in vivo is required.

S1P3 Regulates Heart Rate and Is Not Required for the Induction of Lymphopenia—S1P1 and S1P3 are coexpressed in some cells, especially endothelium (7, 9). The association of a dose-dependent bradycardia with administration of the relatively non-selective receptor FTY720 in humans (27) led us to study the lymphopenic and heart rate responses that associated with S1P1 and S1P3. Induction of lymphopenia (Fig. 7A) in homozygous S1P3−/− mice was indistinguishable from wild-type mice, with no statistically significant difference in the depth of lymphopenia at 5 h between the S1P1-selective agonist SEW2871 and the S1P1, S1P3, S1P4, and S1P5 active prodrug AAL-(R), which is phosphorylated to its active form AFD-(R) (10, 11). Deletion of S1P3 therefore did not affect the S1P receptor agonist-induced inhibition of lymphocyte recirculation.

Consistent with previous observations that S1P3-active compounds were associated with toxicity and heart rate changes in rodents (44), we observed that the acute heart rate changes...
Discrete Functions for S1P₁ and S1P₃ Receptors

S. Pan, H. Rosen, and N. Gray, unpublished observation.

We tested the S1P₁-selective agonist SEW2871 at a dose of vehicle alone in wild-type mice, and no bradycardia was seen. We therefore tested the ability of the non-selective S1P receptor agonist AFD-(R) (10, 11) for the induction of heart rate changes in conscious mice by electrocardiogram analysis. Wild-type mice showed a significant maximal sinus bradycardia (−41.5 ± 2.0%) sustained for over 5 h in response to the administration of AFD-(R) (Fig. 7B) or a structurally unrelated non-selective S1P₃ agonist.⁴ AFD administration in S1P₃-deletant mice was statistically equivalent to administration of vehicle alone in wild-type mice, and no bradycardia was seen. We therefore tested the S1P₁-selective agonist SEW2871 at a dose of 10 mg/kg that induced full lymphopenia for bradycardia (Fig. 7C) and found no induction of bradycardia in either wild-type or S1P₃−/− mice, and it was indistinguishable from vehicle alone. Non-selective S1P receptor agonists therefore have effects upon both lymphocyte recirculation and heart rate. The use of SEW2871 together with the S1P₃-deletant mice shows that S1P₁ and S1P₃ appear to have mutually exclusive roles:

activation of S1P₁ is sufficient to control lymphocyte numbers and plays no discernable role in control of sinus rhythm, whereas S1P₃ regulates sinus rhythm and not lymphocyte recirculation.

DISCUSSION

Establishing the relationships between receptor subtype usage and discrete physiological processes mediated by the same physiological ligand is of general importance, especially when the ligand is associated with complex physiological effects that can be both advantageous and highly deleterious, as with S1P. Resolution of specific receptor contribution within the broad family of lipid G-protein-coupled receptors, of which S1P₁,₅ are a subset (30), have been limited by the lack of selective agonist or antagonists, and by the essential role of receptors such as S1P₁ in embryogenesis (31). The need to understand selective receptor contributions becomes more acute with a relatively nonspecific S1P receptor agonist (FTY720) in phase III clinical trials showing significant immunosuppression and transient dose-dependent bradycardia (27, 32).

The discovery of receptor-selective agonists in this receptor family, as exemplified by SEW2871, shows that this general approach is now, and will be increasingly, important to the field. SEW2871 is a potent receptor-selective agonist that allows the specific roles of S1P₁ to be elucidated. We have done this initially in lymphocyte recirculation, but the reagent can now be usefully pursued in broad functions ranging from the study of endothelial integrity in vivo (6), to the detailed understanding of S1P₁’s transduction pathways and cytoskeletal reorganization in primary cells in vitro (33), where the activation of related S1P receptors can now be excluded.

Multiple lines of evidence are presented to show that SEW2871 is a selective S1P₁ agonist. SEW2871 is a full agonist on S1P₁ alone on both human (Fig. 1) and murine (Fig. 2) receptors for induced GTPγS binding, calcium flux, kinase activation, and cell migration; yet it is not active on the related receptors S1P₂,₅ in either species. SEW2871 is structurally unrelated to S1P. It is highly hydrophobic and lacks any solubilizing or head groups. Despite this, it is an effective full agonist of S1P₁ suggesting that full agonism does not require the headgroup interactions, and can be achieved by hydrophobic-aromatic interactions alone. SEW2871 may be a less efficient full agonist than AFD-(R). Our published data (11) showed that total plasma concentrations at the ED₁₀₀ for lymphopenia for AFD-(R) were 20.6 ± 16-fold the EC₅₀ in vitro, whereas the same ratio calculated for SEW2871 from the plasma pharmacokinetics required ~300-fold the EC₅₀ to reach the ED₁₀₀ despite the fact that SEW2871 is only 65% bound in plasma, compared with AFD-(R), which is 96.8% plasma-bound (11). This could be explained in part by the 15-fold loss of potency of SEW2871 compared with AFD-(R), although it remains possible that the headgroup interactions contribute both potency and agonist efficiency (28). Quantitative evaluation of analogs of SEW2871 with headgroups attached may provide the answer. In addition, SEW2871 is not a unique data point in establishing the link between the S1P₁ activation and control of lymphocyte egress. A chemically distinct S1P₁-selective agonist that reproduces the findings shown here with SEW2871 has also been discovered.⁵ The chemical approach is thus broadly applicable.

S1P₁ activation alone is sufficient for the control of blood lymphocyte numbers, because the induction of agonist-dependent lymphopenia is as deep and fast in onset for SEW2871 as that described for non-selective S1P receptor agonists (7, 9–11) (Fig. 4, B and D). Maintenance of lymphopenia (Fig. 4D) re-

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⁴ S. Lefebvre, N. Gray, and H. Rosen, data not shown.

⁵ S. Pan, H. Rosen, and N. Gray, unpublished observation.
quires the continuous presence of SEW2871 in plasma, with lymphopenia maximal at high plasma concentrations of ligand, and the degree of lymphopenia progressively declining as plasma concentrations decline. There is histological evidence (Fig. 5) for the inhibition of lymphocyte egress from lymph node, where SEW2871 induces logjamming of lymphocytes subjacent to sinus-lining endothelium and the clearance of lymphocytes from lymphatic sinuses.

Although selective agonism of S1P1 is sufficient to regulate lymphocyte recirculation, additional studies are needed to show whether it is S1P1 alone, or whether other S1P receptors can still play a role in the control of lymphocyte egress, and which of these effects are directly upon lymphocytes themselves. The roles of S1P2 and S1P4 can be ruled out, because FTY-P is inactive on S1P2, and lymphopenia is complete in the S1P3−/− mice (Fig. 7). The roles of S1P4 and S1P5 in lymphocyte recirculation remain to be convincingly clarified. In addition, a series of yet unresolved mechanistic questions remain to be answered. S1P1 expression at the mRNA and protein level is well established upon the endothelium in vivo (18). Expression studies using the lacZ-targeted S1P1 gene were performed up to embryonic day 9.5 (31), where expression in endothelium was established but lymphoid organ development was too early to be usefully studied. Although cultured lymphocytes can express S1P1 protein (34–36), the role of S1P1 on lymphocytes in vivo remains uncertain, and there is evidence for the expression of S1P1 after culture. S1P1/P2 binding has been difficult to demonstrate on freshly isolated lymphocytes, despite an easy ability to show its expression on other murine primary and transfected cells. SEW2871 does not induce a calcium flux in freshly isolated lymphocytes, nor does it induce kinase phosphorylation. In addition, lymphocyte egress of naïve cells that are phenotypically and functionally indistinguishable from each other is inhibited from thymus to blood and lymph node to lymph but not from spleen to blood (9, 7), suggesting a tissue-specific heterogeneity not accounted for in the naïve lymphocyte populations. The effects of SEW2871 on lymphocyte trafficking may thus be indirect, perhaps via endothelial cells. This has precedent for S1P1, where vascular smooth muscle migration is dependent upon S1P1 activation in endothelial cells (18, 31, 37, 38).

The cellular and molecular mechanisms underlying this rapid and significant alteration in lymphocyte recirculation requires further study, particularly in the light of what already is known about S1P1 (39, 40). The selection of S1P1 for the control of immune cell recirculation is an example of exploitation by the immune system of a receptor whose earliest and primary function is vascular maturation (18, 31, 37). We do not yet understand what selective evolutionary advantage accrued to the immune system by this choice of control of lymphocyte recirculation.

The separation of control of lymphocyte recirculation from the control of bradycardia sheds some light into the tissue-dependent usage of S1P receptors. Circumstantial evidence

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6 M. G. Sanna, C. Alfonso, and H. Rosen, unpublished observation.
largely from cultured atrial myocytes and using suramin inhibition as a measure of S1P₃ function has postulated a role for S1P₃ in the activation of an J_{Kach} channel-inducing bradycardia (41). The demonstration in vivo that a non-selective S1P receptor agonist active on S1P₃ induces bradycardia in wild-type mice that is abolished in S1P₁⁻/⁻ mice provides further support for the role of S1P₃ in the heart. Both S1P₁ and S1P₃ are expressed on cardiac endothelium and perhaps myocar-
dium (42), yet deletion of S1P₃ alone abolishes the bradycardia induced by non-selective S1P receptor agonists, and an S1P₃-selective agonist does not induce bradycardia. These data provide evidence that the pleiotropism of S1P function has, at least in the specific instances of lymphocyte recirculation and bradycardia, a basis in differential receptor subtype usage in the cardiac and lymphatic systems, respectively.

The general approach of identifying receptor-selective pharmacological tools should allow the contributions of other S1P receptors to be clarified, and the hierarchy and signaling mechanisms of receptor usage in different physiological systems will become clear and potentially therapeutically useful over time.

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