Sphingosine-1-Phosphate Agonists Increase Macrophage Homing, Lymphocyte Contacts, and Endothelial Junctional Complex Formation in Murine Lymph Nodes

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The sphingosine-1-phosphate (SIP) receptor agonist, phosphorylated FTY720 (FTY-P), causes lymphopenia, lymphocyte sequestration in mesenteric lymph nodes (MLNs), and immunosuppression. Using multiple techniques to analyze MLN cells harvested from mice treated with SIP receptor agonists, we saw a redistribution of lymphocytes out of nodal sinuses and an expansion of follicles. Although changes in circulating monocytes were not observed with overnight exposure to FTY720, we saw a significant increase in SIP receptor 1 (SIP1)-expressing CD68+ macrophages in subcapsular sinuses of FTY-P-treated MLNs. This was confirmed by quantitative analysis of F4/80+ cells in MLN suspensions. The sinus volume and number of SIP1-positive cells within sinuses were also increased by FTY-P. High endothelial venules and lymphatic endothelium expressed high levels of SIP1, and treatment with FTY-P resulted in intense staining and colocalization of CD31, β-catenin, and zona occludens 1 in junctions between sinus cells. Transmission electron microscopy showed that FTY-P greatly reduced lymphocyte microvilli and increased cell-cell contacts in the parenchyma. Immunoelectron microscopy revealed that intranodal lymphocytes lacked surface expression of SIP1, whereas SIP1 was evident on the surface and within the cytoplasm of macrophages, endothelial cells, and stromal cells. This subcellular pattern of intranodal receptor distribution was unchanged by treatment with FTY-P. We conclude that SIP1 agonists have profound effects on macrophages and endothelial cells, in addition to inducing lymphopenia. The Journal of Immunology, 2005, 175: 7151–7161.

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in lymphocyte migration from thymus and SLO, suggesting that FTY-P may behave as a functional antagonist (12, 13).

Many G protein-coupled receptors undergo ligand-dependent receptor internalization, and protein kinase C-dependent and S1P-dependent phosphorylation sites on the C terminus of S1P1 have been shown to regulate phosphorylation and internalization (15). Recently, it has been shown that FTY720 binding to S1P1 transfected into CD4+ T cells causes internalization and degradation of the receptor through a protein kinase C-dependent mechanism (16). Thus, sustained desensitization and down-regulation of S1P1 on lymphocytes is proposed to be the mechanism by which FTY720 and other potent agonists alter lymphocyte recirculation. The molecular components downstream of S1P1 that are involved in lymphocyte retention have not yet been characterized, and plausible mechanisms include decreased motility, increased adhesion, or failure to interact with a putative ligand on sinus-lining endothelium required for egress.

Lymphocyte sequestration alone is unlikely to account for the ability of FTY720 to prevent graft rejection, since rejection of allogeneic skin in mouse models can occur with as few as 1 x 10^3 donor-specific CD8+ T cells (17), and circulating lymphocytes are not fully depleted by the drug. The function and identity of the residual lymphocytes that escape depletion is unknown, but they may be long-lived effector memory T cells. These cells lack surface receptors needed for constitutive homing to SLOs and, thus, they should not be sequestered by S1P receptor agonists. Additionally, FTY-P has been shown to cause potent and rapid improvement in disease symptoms in rodent models of autoimmune encephalitis that occur before depletion of PBLs (18). Thus, other cells may contribute to the immunosuppressive activities of FTY720. ECs are known to express high levels of S1P1, and important functions for the receptor on ECs have been well characterized, including enhanced chemotaxis, survival, and barrier function.

The formation of specialized junctional complexes at endothelial cell-cell contacts, termed tight junctions and adherens junctions (AJs), is thought to be critical to endothelial barrier regulation (19). CD31 (PECAM), cadherins, β-catenin, and zona occludin-1 (ZO-1) are membrane-associated adhesion molecules that are located in junctional complexes formed between ECs (19–21). Junctional complexes also function as signaling structures that regulate cell position and growth. Phosphorylated-FTY720 and S1P promote adherens junction assembly by inducing the translocation of vascular endothelial-cadherin (VE-cadherin) to the contact sites between ECs.
(22, 23). These compounds also promote the translocation of β-catenin to focal contact sites, where it associates with the cytoskeletal elements to form cortical actin rings that help stabilize the endothelium (24). In addition, S1P-mediated phosphorylation of N-cadherin and subsequent formation of cadherin-catenin-actin complexes strengthens nascent endothelial cell adhesion to surrounding mural cells, a process known as vascular stabilization (25).

Maintaining the integrity of the barrier prevents vascular leak, which is an important component of inflammatory states such as adult respiratory distress syndrome and ischemia-reperfusion injury (24). FTY720 and S1P protected mice from vascular leak and inflammation in a murine model of acute lung injury (26). The barrier enhancing effect of FTY-P on endothelium in vivo was demonstrated by studies showing that vascular endothelial growth factor-induced permeability of murine ear vessels could be blocked by oral administration of FTY720 (23). Likewise, S1P was shown to rapidly increase the electrical resistance across monolayers of human and bovine ECs, as well as block vascular leakage induced by thrombin (27). The observation that treatment of endothelium by either FTY-P or S1P leads to a similar enhancement in barrier integrity suggests that desensitization through S1P₁ internalization does not occur with endothelium, as it does with lymphocytes.

To further elucidate the structural changes occurring within lymph nodes upon FTY720 treatment and to characterize the cellular location of S1P₁, we have undertaken immunofluorescent and immunoelectron microscopic studies of MLNs. In this study, we show that three cell types important for immune function display dramatically different levels of S1P₁ receptor expression and respond differentially to FTY-P treatment. S1P₁ expression on lymphocytes is nearly undetectable and almost exclusively intracellular, even in the absence of agonist treatment. Lymphocyte clustering seen at the light microscopy level in FTY-P-treated MLNs is due to increased contact between intranodal cells, including homotypic (lymphocyte:lymphocyte) and heterotypic (lymphocyte:macrophage or lymphocyte:endothelium) interactions within the parenchyma. HEVs and lymphatic endothelium were intensely labeled by S1P₁ Abs, and cell surface expression remained high even after FTY-P treatment. At the same time, there was a marked increase in coexpression of CD31, β-catenin, and ZO-1 in ECs by FTY-P. Macrophage functions have not previously been found to be altered by FTY720. We report here the novel observation that the subcapsular sinuses appeared to be expanded in size and contained increased numbers of S1P₁-positive, CD68⁺ macrophages.

**Materials and Methods**

**Animals**

C57BL/6 female mice (8–10 wk of age) were purchased from Taconic Farms and housed in a specific pathogen-free animal facility. Experiments were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee.

**Immunofluorescent staining (IFS) and quantitation of MLN cells**

C57BL/6 female mice were administered FTY-P (3 mg/kg/day; i.p.) or an equivalent volume of vehicle, and MLNs were harvested 18 h later. In some studies, FTY-P or vehicle was given daily for 14 days. Animals were euthanized by CO₂ inhalation. Single-cell suspensions of pooled MLNs were prepared by passage through a 40-μm sieve, following 1 h of collagenase treatment (30 U/ml; Sigma-Aldrich) at 37°C. Cells were washed in PBS containing 0.01% sodium azide and 0.1% BSA (FACS buffer). All samples were adjusted to 1 ml with FACS buffer and 100 μl was removed for quantitation of unstained cells. The rest of the sample was used for IFS. One hundred microliters of 4% paraformaldehyde was added to the 100-μl aliquot of unstained cells, and each sample was counted for 30 s on a FACScan (BD Biosciences).

**Immunofluorescence microscopy (IFM)**

MLNs were dissected from control and compound-treated mice immediately following euthanasia. MLNs were fixed in 4% formaldehyde (generated from paraformaldehyde; Fisher Scientific) in 0.1 M phosphate buffer (pH 7.4) at 4°C for a total of 2 h. Fixed MLNs were infused stepwise to permeabilize, as shown by the rightward shift in the negative control sample (histogram a) in B.

The total number of cells in each sample was determined using CellQuest software (BD Biosciences), based upon forward and side scatter characteristics. Blank particles (Spherotech; ACBP-100-10) were used as an internal standard to control for flow differences from sample to sample. For IFS, cells were incubated for 30 min on ice with 10 μg/ml anti-mouse CD16/CD32 (anti-FcγRIIb receptor; BD Biosciences) and subsequently with 10 μg/ml FITC-conjugated anti-murine IgG secondary Ab; b, vehicle-treated MLN cells stained with rabbit anti-mouse S1P₁ Ab and FITC-conjugated goat anti-rabbit IgG secondary Ab; and c, FTY-P-treated MLN cells stained with rabbit anti-mouse S1P₁ Ab and FITC-conjugated secondary Ab. A high degree of autofluorescence occurs with permeabilization, as shown by the rightward shift in the negative control sample (histogram a) in B.

FIGURE 2. Quantitation of cell subsets and S1P₁ expression in MLNs treated with FTY-P. A, MLNs were harvested 18 h after exposure to a single dose of FTY-P or vehicle, and the sequestration index was calculated for the total nodal cell population, CD3⁺ T cells, B220⁺ B cells, and F4/80⁺ macrophages, as described in Materials and Methods. Each circle represents the sequestration index calculated per experiment, and the average sequestration index was 2.3 (for total cells), 4.6 (for macrophages), 2.0 for CD3⁺ cells, and 2.7 for B220⁺ cells. B, MLN cells were harvested after overnight exposure to a single dose of FTY-P (1 mg/kg), permeabilized, and stained for S1P₁ as described in Materials and Methods. Histograms represent: a, vehicle-treated MLN cells stained with nonimmune rabbit Ig and FITC-conjugated goat anti-rabbit IgG secondary Ab; b, vehicle-treated MLN cells stained with rabbit anti-mouse S1P₁ Ab, and FITC-conjugated secondary Ab; and c, FTY-P-treated MLN cells stained with rabbit anti-mouse S1P₁ Ab, and FITC-conjugated secondary Ab. A high degree of autofluorescence occurs with permeabilization, as shown by the rightward shift in the negative control sample (histogram a) in B.
FIGURE 3. Higher resolution immunofluorescent micrographs showing the distribution of S1P1 in murine MLNs. Semithin cryosections (0.5 μm) of MLNs from vehicle (A, C, E, and F) or FTY-P-treated mice (B and D) were labeled with anti-S1P1n either alone (A and B) or in combination with anti-B220 (C–E) or anti-CD68 (F). A, S1P1n+ cells and processes (red) are present in the narrow SCS of a control MLN. B, Following administration of FTY720, the expanded SCS is filled with many closely packed S1P1n+ cells. C, Scattered B220+ cells (red; arrowheads) are present in the S1P1n+ (green) narrow SCS of vehicle-treated MLNs. D, After FTY-P treatment, the enlarged SCS is devoid of lymphocytes but contains numerous cells expressing S1P1n (green). E, The parenchyma of a vehicle-treated MLN contains S1P1n+ macrophages (red; arrowheads) scattered among B220+ cells. F, SCS of a vehicle-treated MLN contains CD68+ (red) macrophages expressing S1P1n (green; arrowheads). Bar for A and B, 50 μm; bar for C–F, 25 μm; nuclei were counterstained with DAPI (blue).

Results

IFM of S1P agonist-treated MLNs

Sequestration of lymphocytes and macrophages. FTY720 and its phosphorylated active metabolite FTY-P have been shown to induce lymphopenia by inhibiting the egress of lymphocytes from the lymph node parenchyma into the sinuses, thereby blocking...
lymphocyte recirculation (9). We have now extended these observations by conducting IFM experiments on MLNs of mice treated with FTY-P, FTY720 or vehicle. Blood obtained by cardiac puncture was routinely checked for complete blood counts, and the expected degree of lymphopenia (80% depleted) was found in all animals dosed with FTY-P (data not shown). B cells were cleared from the subcapsular sinuses (SCS) and appeared to be highly aggregated within the MLN parenchyma following FTY-P administration (Fig. 1, A and B). Surprisingly, FTY-P treatment was also accompanied by numerous CD68+ macrophages concentrated in the SCS (Fig. 1, C and D). This observation was confirmed by quantitative immunofluorescent cell staining experiments demonstrating a 4.6-fold increase, on average, in macrophage accumulation in MLNs of FTY-P treated mice (Fig. 2A). Lymphocytes have previously been shown to accumulate in lymph nodes treated with FTY720 (3, 7) and, in our experiments, CD3+ and B220+ cells were also increased in MLNs after FTY-P treatment by 2.0- and 2.7-fold, respectively (Fig. 2A). Furthermore, the intracellular expression of S1P1 was unchanged following FTY-P treatment (Fig. 2B), strongly suggesting that this compound does not cause S1P1 degradation or up-regulation.

**Localization of S1P1 in MLNs.** FTY-P is a nonselective agonist on four S1P receptors, but recent evidence from conditional knock-out mice and more S1P1-selective compounds have indicated that lymphocyte sequestration is due to down-regulation of S1P1. Therefore, expression of S1P1 in MLNs was studied using affinity-purified, highly specific N- or C-terminal peptide Abs that have been shown previously to bind specifically to S1P1 in the endothelium of rodent cardiac tissue (10, 31). High endothelial venules and lymphatic endothelium were heavily labeled for S1P1 in both vehicle and treated MLNs using S1P1c IgGs. In contrast, weaker S1P1c staining was observed in the lymphocyte-laden parenchyma of vehicle- or FTY-P-treated nodes (Fig. 1, E and F). Furthermore, FTY-P treatment did not change the mean fluorescent intensity of S1P1 in permeabilized MLN cells (Fig. 2B).

Other cell types in control MLNs showed low to moderate amounts of S1P1 labeling, while increased S1P1 expression was seen in the SCS following FTY-P treatment (Fig. 1, E and F). To obtain higher resolution immunofluorescent images, semithin sections (0.5-µm thickness) of MLNs fixed and processed for IEM were analyzed (Fig. 3). The SCS of vehicle-treated MLNs contained moderate numbers of elongated cells and fibers that were strongly S1P1+ (Fig. 3A). Following treatment with FTY720, the SCS appeared to be greatly enlarged and was filled with numerous S1P1+ cells and cellular processes (Fig. 3B). In double-labeling experiments with B220 and S1P1c Abs, B lymphocytes were readily detected among the S1P1c+ cells of the control SCS, but were conspicuously depleted from the expanded S1P1c-positive SCS of FTY-P-treated MLNs (Fig. 3, C and D). S1P1c was also heavily expressed in macrophages distributed among B lymphocytes in the parenchyma and within the SCS (Fig. 3, E and F). However, no S1P1c labeling was detected on B220+ lymphocytes in the SCS or the parenchyma (Fig. 3, C–E). S1P1 immunolabeling

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**FIGURE 4.** FTY-P increases CD31 and β-catenin expression in the SCS endothelium of murine MLNs. MLN frozen sections (5 µm) from vehicle- or FTY-P-treated mice were double labeled with anti-CD31 and anti-β-catenin IgGs. A–C, Matching views of vehicle-treated MLNs showing CD31 labeling alone (A, red fluorescence), β-catenin alone (B, green fluorescence), and their codistribution (C, yellow fluorescence); nuclei were stained with DAPI (blue). A, SCS contains delicate CD31+ vessels (arrowheads), while an HEV (arrow) in the parenchyma (P) exhibits prominent heads, while an HEV (arrow) in the SCS (Fig. 1, 2B), strongly suggesting that this compound does not cause S1P1 expression of S1P1 in MLNs was studied using affinity-purified, highly specific N- or C-terminal peptide Abs that have been shown previously to bind specifically to S1P1 in the endothelium of rodent cardiac tissue (10, 31). High endothelial venules and lymphatic endothelium were heavily labeled for S1P1 in both vehicle and treated MLNs using S1P1c IgGs. In contrast, weaker S1P1c staining was observed in the lymphocyte-laden parenchyma of vehicle- or FTY-P-treated nodes (Fig. 1, E and F). Furthermore, FTY-P treatment did not change the mean fluorescent intensity of S1P1 in permeabilized MLN cells (Fig. 2B).

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was shown to be specific by the following criteria: 1) closely similar staining patterns were observed with both C-terminal and N-terminal peptide IgGs (see Figs. 1, 3, and 8), 2) the labeling was blocked by preincubation with relevant but not irrelevant peptides (data not shown) (10), 3) both S1P1 Abs labeled the proper proteins in immunobots (data not shown) (10), and 5) S1P1 Abs from control MLNs exhibited a very delicate pattern of CD31 labeling that was colocalized with enhanced ZO-1 expression in the parenchyma of control vs FTY-P-treated MLNs revealed a 67.5% increase in the number of close contacts between lymphocytes, as well as between lymphocytes and ECs (Fig. 7, inset). Following FTY-P treatment, there was a substantial increase in β-catenin expression in the SCS lymphatic endothelium that was colocalized with enhanced ZO-1 labeling (Fig. 5, D–F).

Effects of an S1P agonist on lymphatic endothelium
Since CD31 is a marker of vascular endothelium and, along with β-catenin, is an important structural component of endothelial junctional complexes (19, 20), we studied the effects of S1P agonists on the localization of CD31 and β-catenin in the MLN endothelium (Fig. 4). The SCS lymphatic endothelium of control MLNs exhibited a very delicate pattern of CD31 labeling that was coincident with a similar level of β-catenin expression (arrowheads in Fig. 4, A–C). The parenchyma showed very intense CD31 and β-catenin staining codistributed in HEVs (arrows in Fig. 4, A–C). In contrast, there was a marked increase in the coexpression of both CD31 and β-catenin in the SCS lymphatic endothelium following FTY-P treatment (matching arrows in Fig. 4, D–F). Interestingly, this increase was accompanied by the localization of prominent β-catenin labeling in the nuclei of many SCS cells (green arrowheads in Fig. 4E and corresponding arrowheads in Fig. 4D and F).
increase (Table I). After prolonged FTY-P treatment, lymphocytes in the parenchyma exhibited broad regions of cell surface membrane apposition (Fig. 7F).

**IEM localization of S1P₁.** Macrophages in the parenchyma of control MLNs exhibited considerable S1P₁ immunogold labeling on their cell surface membranes, while adjacent lymphocytes lacked S1P₁ expression on their surfaces, and showed relatively minor S1P₁ labeling in small cytoplasmic vesicles (Fig. 8A). Similarly, robust S1P₁ immunostaining was present on both cell surface and cytoplasmic membranes of EC processes in the SCS of FTY-P-treated and control MLNs (Fig. 8, B and C). Administration of FTY-P or FTY720 did not substantially alter the subcellular distribution of S1P₁ in parenchymal macrophages, and treatment with these compounds did not enhance the S1P₁ staining of lymphocytes in the MLN parenchyma (Fig. 8, D and E). Significantly, regions of close contact between the cell surface membranes of lymphocytes and macrophages did not exhibit S1P₁ labeling (arrows in Fig. 8, D and E).

**Discussion**

We have examined the effects of phosphorylated FTY720 on cell morphology and S1P₁ expression within MLNs by using IFM to map the distribution of leukocytes at the light microscopic level (32, 33) and by IEM to provide patterning information at supramolecular levels of organization (34).

When FTY-P was administered overnight, B220⁺ B cells in MLNs were increased (Fig. 1B), as was the total number of nodal cells (on average by 2.3-fold; Fig. 2). This compares favorably with previous studies (3) that reported a 1.8-fold rise in B220⁺ cells in the MLNs of rats treated for 24 h with FTY720. T cells also become sequestered in SLOs following FTY720 treatment (3, 7). This accumulation may be species, tissue, or cell type specific,
since S1P1-deficient lymphocytes used in short-term homing experiments had a reduced ability to undergo integrin-mediated firm arrest in HEVs of peripheral lymph nodes (35). By contrast, S1P1-deficient T cells, but not S1P1-deficient B cells, appeared to home normally to Peyer’s patches (PPs). FTY720 treatment enhanced integrin-mediated arrest of both S1P1-expressing and S1P1-deficient T cells in HEVs in PPs, suggesting that some of FTY720’s effects, at least on PP-homing T cells, are independent of S1P1.

Lymphocytes showed a loss of microvilli after FTY-P administration, strikingly similar to the significant decrease in microvilli observed in FTY720-treated breast cancer cells. This change was accompanied by reduced metastases (36). We also observed statistically significant increases in the number of contacts between lymphocytes (Table I). TEM imaging of contacts showed that they contain electron-dense material (Fig. 7E) and more surface area than contacts between lymphocytes of controls. After 14 days of treatment, some lymphocytes transformed into epithelial-like sheets (Fig. 7F). In other cell types, S1P also induces changes in cytoskeletal proteins, surface adhesion molecules, and extracellular matrix components (22, 24, 37–39). Unfortunately, the epitopes of many adhesion molecules were inactivated by fixation, precluding their IEM identification at contact sites.

Recent data using receptor-selective agonists and mice with lymphocyte-specific deletions revealed that S1P1 is the target receptor for lymphocyte trafficking defects and immunosuppression. Despite the abundant levels of S1P1 mRNA previously measured (16), we observed few S1P1 receptors in intranodal lymphocytes. S1P1 staining was weakest in the lymphocyte-rich regions of MLNs, and the few receptors detected were almost exclusively intracellular (Figs. 1, E and F, 3, and 8, A, D, and E). This suggests that relatively small numbers of S1P1 receptors regulate lymphocyte egress, making the recirculation pathway exquisitely sensitive

Table I. TEM frequency of close contacts between intranodal lymphocytes harvested from FTY-P-treated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Close Contacts</th>
<th>No. of Lymphocytes Counted</th>
<th>SEM</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-h vehicle</td>
<td>37.4</td>
<td>425</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>18-h FTY-P treated</td>
<td>62.0</td>
<td>467</td>
<td>0.03</td>
<td>67.5 (p = 0.0005)</td>
</tr>
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*MLNs of vehicle- or FTY-P-treated C57BL/6 mice (n = 3) were prepared for TEM, and the percentage of parenchymal lymphocytes with cell-cell contacts was determined blindly using 31 vehicle-treated and 26 FTY-P-treated electron micrographs magnified ×7500.

FIGURE 7. Treatment with S1P agonists induces a loss of lymphocyte microvilli and the generation of electron-dense contacts between lymphocytes and other cells in the lymph node parenchyma. A. The parenchyma of a vehicle-treated MLN contains many lymphocytes with numerous cell surface microvilli (arrowheads). B. Following 18 h of FTY-P administration, surface microvilli have disappeared, the pericellular space (arrowheads) has become narrow, and cells have developed close contacts. C. Example of close contacts between a lymphocyte (Lφ) and an EC after 18 h of FTY720 treatment. D. Close contacts between two lymphocytes following 18 h of FTY-P treatment. E. Another example of a cell-cell contact containing membrane-associated fibers (arrowhead) after 18 h of FTY-P administration. F. Lymphocytes in the parenchyma exhibit broad areas of cell surface membrane apposition (arrowheads) following 14 days of FTY-P treatment. Bars for A, B, and F, 1 μm; bars for C and E, 0.2 μm; and for D, 0.3 μm.
to modulation by agonists that sustain receptor down-modulation. However, we cannot rule out that S1P1 expression in intranodal lymphocytes may vary between cortical and medullary regions, where migratory behavior and stromal cell populations may differ significantly.

In contrast, ECs within MLNs expressed abundant levels of cell surface and intracellular S1P1 receptors (Figs. 1, 3, 5, and 8). We did not observe gross changes in S1P1 distribution in HEVs following FTY720 treatment. This is consistent with published reports that S1P and FTY720 act as agonists of endothelial barrier function (23, 24). In MLNs, many of the sinus resident cells had an endothelial morphology and were organized into capillaries; they expressed the endothelial cell Ag CD31 (PECAM; Fig. 4) and are presumably lymphatic ECs (20). These SCS ECs were still S1P1

FIGURE 8. S1P1 is heavily expressed by mesenteric node macrophages and SCS endothelial cells, but not by lymphocytes, as detected by high resolution IEM. Ultrathin cryosections of vehicle- or S1P1 agonist-treated MLNs were prepared for IEM and stained with Abs to S1P1, followed by immunogold-conjugated secondary Abs, as detailed in Materials and Methods. A, Parenchyma of a vehicle-treated MLN shows a tangential section of macrophage surface membrane exhibiting high levels of S1P1 expression (arrowheads; 5 nm gold), while the cell membrane (arrow) and contacting process (P) of an adjacent lymphocyte (L) lacks S1P1 labeling; V indicates a small S1P1+ cytoplasmic vesicle. B, SCS of an FTY720-treated MLN double labeled with anti-S1P1n (5 nm gold) and RFP (10 nm gold) Abs. The process of an EC exhibits intense expression of S1P1 on both cytoplasmic and cell surface membranes (arrowheads), while the pericellular connective tissue is positive for RFP (arrow). C, SCS of a vehicle-treated MLN immunostained with anti-S1P1c (5 nm gold). S1P1c is localized on cell surface (arrowheads) and on cytoplasmic membranes of an EC. D, Parenchyma of an FTY720-treated MLN labeled with S1P1n (5 nm gold). The macrophage exhibits S1P1n expression on the cell membrane (arrowheads) and in cytoplasmic vesicles (V), while the contacting lymphocyte is unlabeled (arrows depict intercellular contact sites). E, Ultrathin cryosection of an FTY720-treated MLN labeled with anti-S1P1n (5 nm gold). The macrophage process shows both cell surface (arrowheads) and cytoplasmic S1P1n expression, whereas the attached lymphocyte is unlabeled; arrow delineates a large cell surface contact. Mφ, Macrophage. Bars, 0.1 μm.
positive after FTY-P treatment (Fig. 5F, inset) and showed no evidence of S1P1 down-regulation (Fig. 8, B and C).

Similar to ECs, S1P1 was found at the cell surface and in the cytoplasm of macrophages, and treatment with FTY-P did not result in significant redistribution of this receptor (Fig. 8). S1P1 desensitization has been studied in transfected cells, but relatively little is known about regulation of endogenous receptors. Recent studies indicate that S1P1 internalization, recovery, or down-modulation are regulated by distinct posttranslational modification pathways that may be differentially expressed in various cells (15, 40–42).

S1P1, originally cloned from ECs (43), plays a major role in regulating EC survival, migration, and differentiation. Using a transgenic mouse designed to express the β-galactosidase reporter gene product from the S1P1 locus, the receptor was found to be expressed constitutively in selected vascular beds, e.g., in lung, heart, and liver ECs (44). It is also expressed strongly in newly formed vessels, e.g., in tumors (45). S1P promotes the assembly of β-catenin- and VE-cadherin-containing AJs (22) and increases endothelial: mural cell adhesion, which is important for vascular maturation (25). β-catenin in AJs promotes junctional stabilization by anchoring cadherins and CD31 to actin (21). The association of β-catenin with VE-cadherin may prevent its translocation to the nucleus, where β-catenin acts as a transcription factor for cyclin D1, leading to contact inhibition of growth (19, 46). ZO-1 is localized in tight junctions, which are frequently intermingled with AJs in EC junctions (19). Endothelial barrier function is enhanced by S1P-mediated cytoskeletal changes (24), thus we examined the effects of FTY-P on endothelial junctional proteins. We observed that CD31, β-catenin, and ZO-1 were up-regulated and colocalized in junctions between SCS ECs in FTY-P-treated MLNs (Figs. 4 and 5). The enhanced expression of these molecules may increase the integrity of the SCS endothelial barrier.

FTY-P increased the area and intensity of SCS staining with S1P1-specific Abs, in part due to morphological changes in lymphatic ECs or to changes in other stromal cells (Figs. 1, 2, and 5). The enhanced expression of these molecules may increase the integrity of the SCS endothelial barrier.

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Lipid mediators, such as platelet-activating factor, inhibit the migration of macrophages and dendritic cells from peripheral sites to SLOs (56). FTY-P may accelerate this migration, and macrophages may become trapped within SCS afferent lymphatics due to the increased integrity of EC junctional complexes in treated MLNs.

In summary, our data show that S1P1 expression and distribution varies in the different MLN cell types. FTY-P evokes differential responses, including increased focal contacts in lymphocytes, enhanced expression, and colocalization of CD31, S1P1, β-catenin, and ZO-1 in ECs and accumulation of macrophages in SCS. These findings strongly support the concept that multiple cell types are profoundly affected by treatment with S1P agonists and may contribute to the immunosuppressive effects of FTY720.

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Disclosures

The authors are employees of Merck & Company, Incorporated, and potentially own stock and/or hold stock in the company.

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