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Amelioration of Collagen-Induced Arthritis by a Novel S1P1 Antagonist with Immunomodulatory Activities

Yasuyuki Fujii,* Takehiro Hirayama,† Hidenori Ohtake,* Naoya Ono,* Tomoyuki Inoue,* Takanobu Sakurai,* Tetsuo Takayama,* Kayo Matsumoto,* Narutoshi Tsukahara,† Shinya Hidano,† Nobue Harima,* Kiyoshi Nakazawa,* Yasuyuki Igarashi,‡ and Ryo Goitsuka‡

Sphingosine 1-phosphate (S1P) regulates lymphocyte trafficking through the type 1 sphingosine 1-phosphate receptor (S1P1) and participates in many pathological conditions, including autoimmune diseases. We developed a novel S1P1-selective antagonist, TASP0277308, which is structurally unrelated to S1P. This antagonist competitively inhibited S1P-induced cellular responses, such as chemotaxis and receptor internalization. Furthermore, differing from previously reported S1P1 antagonists, TASP0277308 demonstrated in vivo activities to induce lymphopenia, a block in T cell egress from the thymus, displacement of marginal zone B cells, and upregulation of CD69 expression on both T and B cells, all of which recapitulate phenotypes of S1P1-deficient lymphocytes. In a mouse collagen-induced arthritis model, TASP0277308 significantly suppressed the development of arthritis, even after the onset of disease. These findings provide the first chemical evidence to our knowledge that S1P1 antagonism is responsible for immunosuppression in the treatment of autoimmune diseases and also resolve the discrepancies between genetic and chemical studies on the functions of S1P1 in lymphocytes.

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Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite that mediates a wide variety of cellular responses including lymphocyte trafficking (1), regulation of vascular permeability, and angiogenesis by interacting with five members of the G protein-coupled receptors, including S1P1 to S1P5 (2–6). Of these five receptors, much attention has recently been focused on type 1 sphingosine 1-phosphate receptor (S1P1) because of its involvement in immune-mediated diseases such as rheumatoid arthritis and multiple sclerosis (7, 8). Elevated levels of S1P and S1P1 have been demonstrated in the synovium of patients with rheumatoid arthritis (9, 10), and S1P signaling through S1P1 was also found to promote synovioctyes proliferation (10) and inflammatory cytokine-induced cyclooxygenase-2 and PGE2 production (10). Furthermore, S1P1 signaling has been shown to augment Th17 differentiation, one of the major T cell populations involved in autoimmune diseases (11, 12).

Studies with S1P1-deficient mice have revealed a necessary role of S1P1 in lymphocyte egress from lymphoid tissues (13–15). However, experiments with S1P1-neutralizing Abs and S1P1 antagonists did not inhibit this process (16–18). By contrast, synthetic S1P1 agonists have been reported to block lymphocyte trafficking and are in clinical development due to their immunosuppressive properties (8, 19, 20). For example, the first discovered S1P1 agonist, FTY720, has recently been approved by the U.S. Food and Drug Administration for the treatment of multiple sclerosis (7) and has been shown to induce rapid lymphopenia by inhibiting the egress of lymphocytes from the lymph nodes into the lymph and from the thymus into the blood (8). Because speculation from genetic studies predicts the potential for S1P1 antagonists as immunosuppressive agents, the utility of S1P1 agonists in immunotherapy is unexpected. Thus, these phenotypic discrepancies between genetic and pharmacological approaches obscure the understanding of the physiological and pathological roles of the S1P–S1P1 axis in immune regulation.

In the current study, we developed a highly S1P1-selective antagonistic compound, TASP0277308, and demonstrated that this S1P1 antagonist possesses immunomodulatory activities, including lymphopenia, a block in T cell egress from the thymus, marginal zone B cell displacement, and the upregulation on CD69 expression on lymphocytes. Furthermore, TASP0277308 effectively suppresses the development of collagen-induced arthritis in mice, providing chemical evidence for the efficacy of S1P1 antagonism in the treatment of autoimmune diseases through its in vivo activities. These findings help to solve long-lasting discrepancies between genetic and chemical approaches to the pathophysiological roles of the S1P–S1P1 axis.

Materials and Methods

Chemicals

(D)-Boc-alanine methyl ester with hydrazide was used as the starting compound for TASP0277308 synthesis. The resulting hydrazide was reacted with ethyl isoiosihoxycyanate, and the adduct produced was then subjected to base-catalyzed cyclization to produce 1,2,4-triazole-3-thion, which was readily S-methylated using iodomethane. The resulting 3-methylthio-1,2,4-triazole was oxidized using m-chloroperbenzoic acid to produce the corresponding sulfone, which underwent deprotection of amino group using trifluoroacetic acid. The resulting trifluoroacetic acid salt of sulfone was treated with 3-(4-methyl-piperazin-1-yl)-phenol and cesium carbonate at...
200°C to provide the ipso arlyoxy substituted product, which was reacted with 3,4-dichlorobenzensalicyl chloride to produce TASP0277308. SEW2871 and S1P were purchased from Cayman Chemical Company and Biomol, respectively.

Cell lines
293 cell lines stably expressing Flag epitope-tagged human S1P1, hS1P1, hS1P2, hS1P3, hS1P4, or hS1P5 were generated by transfecting 293 cells with the pcMV-tag2 plasmid containing full-length cDNAs for hS1P1, hS1P2, hS1P3, or hS1P5 using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen), followed by selection of drug-resistant clones in the presence of 0.5 mg/ml G418. Expression of human S1P receptors on these clones was confirmed by flow cytometry analysis with an anti-Flag M2 mAb using the flow cytometer EPICS XL-MCL (Coulter Co.). CHO-K1 cells stably expressing mouse S1P receptors, including S1P1, S1P2, S1P3, and S1P5, were generated as previously described (21).

Competitive ligand binding assays
Membranes prepared from 293 cells and CHO cells expressing S1P receptors were incubated with 32P-labeled S1P in the presence or absence of various concentrations of TASP0277308 for 60 min at room temperature. The reaction was terminated by collecting the membranes onto GF/C filter plates using a Packard Filtermate Universal Harvester. The filter-bound radioactivity was counted using the TopCount NXT Microplate Scintillation and Luminescence Counter C384V01J (PerkinElmer Life Science). Specific binding was calculated by subtracting radioactivity that remained in the presence of 1000-fold excess of unlabeled S1P.

Functional assays
Female BALB/c mice (7–9 wk of age), purchased from Charles River (Kanagawa, Japan), were orally administered the indicated doses of TASP0277308 or vehicle, and blood samples were collected at given times after administration. Full blood cell counts were determined using the ADVIA120 hematology system calibrated for mouse blood (Siemens Health Diagnostics). Plasma concentrations of TASP0277308 were analyzed by liquid chromatography–tandem mass spectrometry with negative ion electrospray ionization. Pharmacokinetic parameters of TASP0277308 were evaluated by a noncompartmental analysis using a WinNonlin (version 4.1) pharmacokinetic software package.

Flow cytometry and immunofluorescence
Single-cell suspensions from the indicated organs were stained with a combination of FITC-, PE-, allophycocyanin-Pacific blue- and biotin-conjugated Abs, followed by streptavidin–PE–Cy7 (BD Biosciences). Conjugated and unconjugated Abs specific to the following Ags were purchased from BD Biosciences and eBioscience: CD4, CD8a, CD26, CD69, CD20, CD24, CCR7, CD25, CD80, CD86, MHC class II, CD21, CD23, CD16, TCRβ, CD3e, B20, IgM, IgD, and MadCAM-1. Rat anti-mouse S1P mAb was obtained as a pre-release reagent from R&D Systems. Data were collected on a FACSCountII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Intrathymic injection of FITC was performed as previously described (22, 23). For immunofluorescence analyses, frozen sections (7 μm) fixed with cold acetone were stained with anti-IgD–FITC and anti-IgM–PE or anti-CD21–FITC and anti-MadCAM-1, followed by anti-rat IgG–Fluor555. Images were acquired with a fluorescence microscope equipped with an Olympus DML cooled video camera (Olympus) and were processed with Photoshop software (Adobe Systems).

Statistical analysis
Statistical analyses were performed using parametric Dunnett’s and Steel’s’ tests. For the acute collagen-induced arthritis tests, a value of p < 0.05 was considered to be statistically significant.

Results
TASP0277308 antagonizes S1P binding to S1P1 by competitively inhibiting their interactions
Potentially antagonistic compounds selectively binding to S1P1 were first screened using a competitive receptor binding assay of the 293 cell line stably expressing hS1P1 with 32P-labeled S1P. The selected compounds were then used to screen optimized antago-
nistic compounds. Of these compounds, several showed IC$_{50}$ values under 1 nM and appeared to belong to the same structural family. The most potent compound from this series was TASP0277308 (Fig. 1A), which strongly inhibited the binding of $^{33}$P-labeled S1P to the hS1P$_1$ receptor (IC$_{50}$ = 4.2 ± 0.6 nM) (Table I). TASP0277308 exhibited no binding affinity for S1P$_3$ or S1P$_4$ and showed only a very weak affinity for S1P$_5$ that is 2000-fold lower than that for S1P$_1$. TASP0277308 also selectively inhibited S1P binding to mouse S1P$_1$, but not to S1P$_2$, S1P$_3$, or S1P$_4$ (Table I). These results indicate that TASP0277308 is highly selective for both human and mouse S1P$_1$. TASP0277308 itself displayed no agonistic activity to S1P$_1$ in the $[^{35}]$GTP$_\gamma$S binding assay (data not shown) but inhibited S1P$_1$-induced $[^{35}]$GTP$_\gamma$S binding to S1P$_1$ with IC$_{50}$ values of 7.8 ± 0.7 nM (Fig. 1B). TASP0277308 also attenuated S1P$_1$-induced inhibition of cAMP formation in S1P$_1$-expressing 293 cells with IC$_{50}$ values of 7.8 ± 0.7 nM (Fig. 1C), which is in good agreement with the receptor-binding affinity. Furthermore, S1P$_1$-induced chemotactic migration of CHO cells expressing S1P$_1$ was dose-dependently inhibited by TASP0277308 with IC$_{50}$ values of 18 ± 4 nM (Fig. 1D).

Given that S1P$_1$ agonists, such as FTY720 and SEW2871, exert their effects by downregulating S1P$_1$ on cell surfaces (24–26), we examined whether TASP0277308 affects S1P$_1$ internalization using Flag epitope-tagged S1P$_1$-expressing 293 cells by flow cytometry. The natural ligand S1P and the S1P$_1$-specific agonist SEW2871 rapidly induced receptor internalization of S1P$_1$ (Fig. 1E). By contrast, TASP0277308 itself did not induce S1P$_1$ internalization, and it completely inhibited receptor internalization induced by S1P or SEW2871 in a dose-dependent manner (Fig. 1E). Taken together, these results indicate that TASP0277308 is a selective antagonist for S1P$_1$ and that its antagonistic activity against S1P$_1$-induced cellular responses results from competitive inhibition of S1P binding to S1P$_1$, but not from S1P$_1$ internalization.

TASP0277308 induces and maintains lymphopenia, which correlates with its plasma levels

S1P$_1$ agonists, such as FTY720 and SEW2871, induce rapid lymphopenia in peripheral blood (24, 27), whereas compound 2a, an S1P$_1$ antagonist, fails to cause lymphopenia but inhibits S1P$_1$ agonist-induced lymphocyte sequestration (17). To clarify whether S1P$_1$ agonism or antagonism causes lymphocyte sequestration, we examined the efficacy of TASP0277308 to induce and maintain

Table I. Binding affinities of TASP0277308 to S1P receptors

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<th>S1P$_1$</th>
<th>S1P$_2$</th>
<th>S1P$_3$</th>
<th>S1P$_4$</th>
<th>S1P$_5$</th>
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<td>Human</td>
<td>4.2 ± 3.2</td>
<td>n.d.</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>8,756</td>
</tr>
<tr>
<td>Mouse</td>
<td>2.7 ± 0.5</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>n.d.</td>
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IC$_{50}$ values (nM) of TASP0277308 determined by competition of 0.1 nM $[^{33}$P$]$S1P binding with membranes prepared from 293 cells and CHO cells expressing the indicated S1P receptors. Data represented as mean ± SEM obtained from three separate experiments.

n.d., not determined.

FIGURE 1. Antagonistic activities of TASP0277308 against S1P$_1$. A, Chemical structures of TASP0277308 and S1P. B, Antagonistic activity was measured by GTP$_\gamma$S binding to membranes prepared from 293 cells expressing S1P$_1$ in the presence of 50 nM S1P. Results normalized to the percentage of GTP$_\gamma$S induced at maximal S1P response without TASP0277308 are shown. IC$_{50}$ values (mean ± SD) are 7.8 ± 0.7 nM (n = 3). C, Antagonistic activity was measured by inhibiting the S1P$_1$-induced decrease in cAMP formation in hS1P$_1$-expressing 293 cells. The IC$_{50}$ value (mean ± SD) was 3.5 ± 1.2 nM (n = 3). D, Chemotaxis assays were performed using S1P$_1$-expressing CHO cells in the presence of the indicated concentrations of TASP0277308. The IC$_{50}$ value (mean ± SD) was 18 ± 4 nM (n = 3). Results are mean ± SD from three independent experiments performed in triplicate. E, Inhibition of S1P$_1$- and agonist-induced S1P$_1$ internalization by TASP0277308. Flag-tagged S1P$_1$-expressing 293 cells were incubated for 15 min with saline (black shaded), 100 nM S1P (upper panels), or 1 µM SEW2871 (lower panels) in the presence of the indicated concentrations of TASP0277308 (solid lines). Surface expression of S1P$_1$ was determined by flow cytometry. Control-stained 293 cells are also shown (dashed lines). Data are representative of three independent experiments.
lymphopenia in vivo. A single oral administration of 100 mg/kg TASP0277308 reduced the number of lymphocytes significantly to ~10% of control values as early as 2 h after administration and maintained lymphopenic states until 12 h (Fig. 2A). By contrast, administration of 10 mg/kg TASP0277308 induced a temporary decrease in lymphocytes 2 h after administration; however, after 8 h, the number of lymphocytes started to increase and reached control levels until 12 h (Fig. 2A). The plasma half-life of TASP0277308 after the oral administration was estimated to be 1.9 h, with the EC50 value of TASP0277308 for inducing and maintaining lymphopenia as 200 nM, which is ~10 times higher than the in vitro IC50 effective antagonistic activities (Fig. 2B). These findings suggest that S1P1 antagonism, but not S1P1 agonism, is responsible for inducing lymphopenia in the blood and also that the continuous presence of high levels of S1P1 antagonists is necessary for the maintenance of the lymphopenic states.

TASP0277308 upregulates CD69 expression on thymocytes and blocks their egress from the thymus

We next examined the effect of TASP0277308 on thymocyte CD69 expression, because phenotypic differences have previously been observed between the genetic deletion of S1P1 and the treatment of S1P1 agonists (23, 28–30). When we analyzed thymocyte populations 24 h after the treatment with TASP0277308 or SEW2871, total cell numbers and the distribution of major thymocyte populations were not significantly altered (Fig. 3A, 3B). However, as previously reported (29), SEW2871 treatment reduced CD69 expression on β7 integrin+ CD69int CD4 single-positive (SP) and β7 integrin+ CD69int CD8 SP thymocytes in the thymus (Fig. 3C). In sharp contrast, TASP0277308 enhanced CD69 expression on the same CD69int SP populations in the thymus (Fig. 3C), a similar phenotype observed in S1P1-deficient thymocytes (28). Because CD69 expression is downregulated during maturation of SP thymocytes from a semimature CD69hiCD24lo phenotype to a mature CD69loCD24hi phenotype, we compared the effect of TASP0277308 on CD69 expression between semimature CD24lo and mature CD24hi SP populations (Fig. 3D). As shown in Fig. 3F, both TASP0277308 and SEW2871 did not affect the expression level of CD69 on the CD24hi semimature CD4 or CD8 SP population, whereas CD69 expression levels on mature CD24lo SP populations of both CD4 and CD8 thymocytes were downregulated and upregulated by SEW2871 and TASP0277308, respectively. Because it has been reported that surface expression of S1P1 and CD69 on lymphocytes is reciprocally regulated by each other (31), we examined S1P1 expression levels on thymocytes treated with TASP0277308. SEW2871 treatment caused downregulation of S1P1 expression on mature Qu2+ CD4 SP thymocytes, whereas S1P1 expression on the same thymocyte population was upregulated upon TASP0277308 treatments (Fig. 3G). The expression levels of CD62L and CCR7 on CD4 and CD8 SP thymocytes were unaltered by the treatment of TASP0277308 or SEW2871 (data not shown). Thus, these findings indicate that the S1P1 antagonist and agonist appeared to selectively modulate CD69 and S1P1 expression on mature SP thymocytes in opposite ways.

As we observed that the frequency and total cell numbers of CD24lo mature SP thymocytes in the thymus were slightly but significantly increased in mice treated with TASP0277308 when compared with those in controls treated with vehicles (Fig. 3D, 3E), we further examined whether TASP0277308 affects T cell egress from the thymus using in vivo labeling of the thymocytes by intrathyMIC injection of FITC (22). The FITC-positive CD4 and CD8 T cells that emigrated from the thymus were significantly reduced in the spleen 24 h after TASP0277308 treatment compared with those treated with vehicles (Fig. 4A, 4B), a similar observation reported after FTY720 treatment (23). Taken together, these findings indicate that S1P1 antagonism is responsible for the inhibition of thymic egress and also that S1P1 antagonism and agonism lead to the opposite outcomes in terms of S1P1 and CD69 expression on mature CD4 SP and CD8 SP thymocytes.

TASP0277308 relocates marginal zone B cells into the follicles and induces CD69 expression on peripheral B and T cells

It has previously been reported that S1P–S1P1 interaction is required for the retention of B cells in the splenic marginal zone (32, 33), and FTY720 has been shown to induce the displacement of B cells from the marginal zone to the splenic follicles (34). To understand whether S1P1 antagonism or agonism causes this migration of marginal zone B cells into the follicle, we compared the effect of TASP0277308 with SEW2871 on marginal zone B cells. Similarly to the reported effect of FTY720 on marginal zone B cells (33), TASP0277308 caused complete displacement of marginal zone B cells (CD21hi or IgM+ IgD+) from the outside of the marginal zone sinus (MAdCAM-1+) into the follicles where follicular B cells (CD21iso or IgM+ IgD+) reside (Fig. 5A). Flow cytometry analysis revealed that TASP0277308 treatment did not alter the frequency of marginal zone B cells in the spleen, as defined by high CD21 and low CD23 expression (Fig. 5B). Similar phenotypes were observed after the treatment of SEW2871 (Fig. 5A, 5B), with one exception in the expression level of CD69; TASP0277308 enhanced CD69 expression on marginal zone B cells and follicular B cells, whereas SEW2871 failed to upregulate CD69 expression on B cells (Fig. 5C). We also observed that TASP0277308, but not SEW2871, induces CD69 expression on CD4 and CD8 T cells, in addition to B220+ B cells, in the

![FIGURE 2. TASP0277308 induces and maintains lymphopenia in the blood. A. Circulating PBLs were determined at the indicated time intervals after oral administration of TASP0277308 (10 or 100 mg/kg). Data are represented as mean ± SEM (n = 5). ***p < 0.001 versus vehicle-treated group (parametric Dunnett’s test). B. The plasma half-life of TASP0277308 after the oral administration. T1/2 was 1.9 h.]
spleen and lymph nodes, without affecting other activation markers, including CD25, CD80, CD86, and MHC class II (Fig. 5D, 5E). As similar to the effect of TASP0277308 on the mature SP thymocytes (Fig. 3G), the level of surface S1P1 expressed on splenic CD19\(^+\) B cells and CD3\(^+\) T cells was upregulated by TASP0277308 treatment (Fig. 5F). Thus, the observed effects of TASP0277308 on the distribution of marginal zone B cells and their CD69 expression recapitulate the phenotypes of S1P\(_1\)-deficient B cells (32, 33), supporting the notion that S1P\(_1\) antagonism is responsible for the relocalization of marginal zone B cells into the follicles.

**TASP0277308 suppresses the progression and ongoing collagen-induced arthritis**

To assess the therapeutic impact of the S1P\(_1\) blockade by TASP0277308, we next investigated the effect of TASP0277308 on the development of collagen-induced arthritis in DBA/1 mice, a surrogate model of human rheumatoid arthritis (35, 36). In this

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**FIGURE 3.** TASP0277308 upregulates CD69 expression on mature thymocytes. Mice were orally treated twice at 12-h intervals with vehicle control, 10 mg/kg SEW2871, or 100 mg/kg TASP0277308, and surface phenotypes of thymocytes were analyzed by flow cytometry 24 h after the first treatment. A. Dot plot profiles of CD4 and CD8 expression gated on B220/propidium iodide-negative thymocytes. Numbers in dot plots indicate the percentages of indicated cell populations in the outlined gates. B. The total cell numbers of thymocytes from mice treated with vehicle (white bar), SEW2871 (gray bar), and TASP0277308 (black bar). Data are the mean ± SEM (n = 3). C. Counterplot profiles of CD69 and \(\beta7\) integrin expression on CD4 SP and CD8 SP thymocytes. Numbers indicate percentage of cells in the outlined gates. D. Counterplot profiles of CD24 and \(\beta7\) integrin expression on CD4 SP and CD8 SP thymocytes. Numbers indicate percentage of cells in the outlined gates (I and II). E and F. For each of the gated populations, the total cell numbers (E) and histograms for expression levels of CD69 (F) were generated. *p < 0.05 (versus vehicle-treated group; Student t test). G. Histograms for S1P1 expression were generated on mature Qa2\(^+\) CD4 SP thymocytes. Data are the mean ± SEM (n = 3). Expression of CD69 and S1P1 on the indicated cell populations from mice treated with vehicle (red lines), SEW2871 (blue lines), and TASP0277308 (green lines). Shaded histograms indicate unstained controls. Data are representative of three independent experiments.
rapidly and aggressively developed after the booster immunization using the acute collagen-induced arthritis model, in which arthritis treated with 100 mg/kg TASP0277308 (Fig. 6E) was suppressed significantly in mice treated with vehicle (Fig. 6F).

Infiltration into the joint compartment, which were all evident in synovial hyperplasia, proteoglycan content, and inflammatory TASP0277308 treatment ameliorated cartilage and bone erosion, indicating that TASP0277308 must be continuously present at a certain dosage that is unable to maintain lymphopenia. Three weeks after the first sensitization with CII, DBA/1 mice received a booster immunization with CII to induce arthritis. TASP0277308 was administered 1 h and 13 h after intrathyMIC injection of FITC. CD4 and CD8 SP emigrants (FITC-positive) in the spleen (upper panels) and thymic residents (lower panels) were analyzed by flow cytometry 24 h after intrathyMIC FITC labeling. Representative profiles are shown. B, The number of FITC-labeled thymic emigrants in the spleen. The number of FITC-positive cells in the spleen was estimated after normalization of the percentages of FITC-positive thymocytes in the relevant mice. Data are the mean ± SEM (n = 4). *p < 0.05, **p < 0.001 (versus vehicle-treated group; Student t test).

We further assessed the therapeutic efficacy of TASP0277308 using the acute collagen-induced arthritis model, in which arthritis rapidly and aggressively developed after the booster immunization with CII and LPS. Administration of 100 mg/kg, but not 30 mg/kg, TASP0277308 significantly suppressed the development of arthritis and anti-CII Ab response, even when the treatment was started from the day of the booster immunization (Fig. 7A, 7B). These findings indicate that the S1P1 antagonism is responsible for suppression of progression and also ongoing collagen-induced arthritis and also reveal the therapeutic efficacy of TASP0277308 in the treatment of ongoing arthritis.

**Discussion**

The S1P–S1P1 axis has become a promising molecular target for ameliorating autoimmune diseases, and a representative S1P1 agonist FTY720 has recently been approved by the U.S. Food and Drug Administration for the treatment of multiple sclerosis (7). However, whether FTY720 acts as an agonist or a functional antagonist and whether the critical effect of the drug is on lymphocytes or endothelial cells remains controversial. In the functional antagonism model, S1P1 agonists, such as phosphorylated FTY720, bind directly to S1P1 on lymphocytes and cause receptor internalization, creating an S1P1-null state on lymphocytes, which in turn prevents lymphocyte trafficking (14). This model is supported by a series of studies using genetically modified mice where S1P1 is deleted from lymphocytes (14, 15) or S1P1 gradients are disrupted (37). An alternative explanation known as the stromal gate model, proposes that lymphocyte egress is blocked by agonism of S1P1 on endothelial cells, resulting in the tightening of the endothelial barrier and therefore the prevention of lymphocyte egress. This model is supported by the findings that S1P1 agonists block lymphocyte egress and by the reported failure of S1P1 antagonism to induce lymphopenia (16, 17). Using a newly developed competitive S1P1 antagonist, TASP0277308, we demonstrated support for the functional antagonism model for the action of S1P1 agonists on lymphocytes. This S1P1 selective antagonist has the ability to induce lymphopenia, block thymic egress, relocalize marginal zone B cells from marginal zones to follicles, and upregulate CD69 on lymphocytes, all of which recapitulate the effect of deleting S1P1 from lymphocytes (14).

A key determinant for lymphocyte trafficking is cell surface expression of S1P1 on lymphocytes, which is primarily regulated by the local concentrations of S1P in vivo (38); S1P1 is undetectable on the cell surface of circulating T cells in lymph or blood where high levels of S1P are present, however, the receptor is readily detectable on the surface of T cells in the spleen and lymph nodes where S1P levels are estimated to be extremely low. S1P-mediated regulation of S1P1 surface expression has been supported by two findings: S1P lyase inhibition increases the concentration of S1P in the lymphoid organs resulting in a disappearance of S1P1 from the cell surface (37), and decreased concentrations of S1P due to deletion of sphingosine kinases cause an upregulation of lymphocyte cell surface S1P1 in lymphoid tissues (39). Because an S1P gradient and lymphocyte S1P1 cell surface expression are both required for lymphocytes to exit lymphoid tissues and enter the circulation, it is likely that low levels of S1P in lymphoid tissue weakly activate S1P1-mediated signaling, which may induce steady-state recycling, but not complete internalization of cell surface S1P1 under homeostatic conditions. With TASP0277308 treatment, there is competitive blocking of endogenous S1P–S1P1 interaction, thus inhibiting weak S1P1-mediated signaling-induced receptor recycling and upregulated S1P1 lymphocyte cell surface levels.

In addition to the local S1P concentration in vivo, there is another regulatory mechanism for S1P1 expression and function in lymphocytes: the interaction with CD69. S1P1 has been demonstrated
to form a complex with CD69 and negatively regulates CD69 expression, and reciprocally CD69 expression downmodulates S1P1 expression (28). Although CD69 is transcriptionally induced by many activation stimuli, such as TCR signaling and IFN-α/β, naive surface CD69-negative lymphocytes express detectable amounts of intracellular CD69 protein (40), and also CD69 is found on the cell surface of S1P1-deficient T and B cells (14), which leads to the interpretation that the small amounts of CD69 that are expressed in naive T and B cells might be prevented from being expressed on the cell surface by S1P1. In this regard, we found that TASP0277308 selectively causes upregulation of CD69 on mature SP thymocytes and peripheral T and B cells without diminishing cell surface S1P1 expression, and conversely S1P1 agonist SEW2871 suppresses CD69 expression on mature SP thymocytes and downregulates cell surface S1P1. Combined with previous observations that both the G protein-coupling motif and the ligand binding domain of S1P1 are needed for CD69 downregulation (31) and that S1P1 agonists, such as FTY720, induce persistent S1P1 signaling even after internalization of the receptor (41), our findings can be interpreted as S1P1-mediated signaling, but not cell surface residency of S1P1 itself, suppresses cell surface transport of CD69 in naive lymphocytes. In support of this notion, it has recently been reported that the loss of S1P production in perivascular pericytes causes upregulation of CD69 and S1P1 on mature CD4 SP thymocytes (42), suggesting a requirement of S1P1 signaling for CD69 downregulation on mature SP thymocytes. An alternative, but not mutually exclusive possibility could be that binding of TASP0277308 to S1P1 may also induce S1P1 conformational changes that allow cell surface CD69 expression in the presence of S1P1, as CD69 expression was demonstrated to stabilize a high-affinity conformation of S1P1 despite its inhibitory effect on S1P1 expression levels (31).

In contrast to TASP0277308, other S1P1 antagonists, such as VPC44116 and W146, have been demonstrated to induce pulmonary vascular leakage by inhibiting S1P1 function on the vascular endothelial cells but failed to induce lymphocyte sequestration (17, 43). Because TASP0277308 is able to cause pulmonary vascular leakage, in addition to lymphocyte sequestration, and
also inhibits S1P-induced vascular endothelial cell migration and proliferation in vitro (Y. Fujii, unpublished observations). TASP0277308 appears to function as an S1P₁ antagonist on both lymphocyte and endothelial cells. One potential reason that might explain why previously described S1P₁ antagonists lack the ability to cause lymphopenia could be their lower antagonistic activity relative to endogenous S1P and/or lower stability in vivo. TASP0277308, with an IC₅₀ value of less than 100 nM, competitively inhibits S1P binding to S1P₁, S1P₁-induced signaling downstream of S1P₁, including GTPᵦS-binding and cAMP formation, and S1P₁-induced cellular responses, such as chemotaxis and receptor internalization. In comparison, 10 μM W146 is required for inhibition of agonist-induced S1P₁ internalization (17), and 1 μM VPC23019 inhibits S1P₁-induced chemotaxis (44). When in vivo pharmacokinetic stability was compared between these antagonists, the plasma concentration of W146 was reported to be 268 ± 98 nM at 5 h after a 10 mg/kg i.v. injection with a half-life of 73 ± 3 min (17), while the plasma concentration of TASP0277308 4 h after oral administration at a concentration of 10 mg/kg was estimated to be 239 ± 60 nM with a plasma half-life of 1.9 h. Although it is difficult accurately to evaluate differences in antagonistic activities and in vivo stabilities between TASP0277308 and previously reported antagonists because of differences in experimental conditions, including cell lines used and the route of compound administration, both antagonistic activity and in vivo stability of TASP0277308 appear to be higher than those of other antagonists, which may enable TASP0277308 to be accessible to lymphocyte S1P₁. In this regard, recent reevaluation of W146 activities revealed that W146 is a potent inducer of early and short-lasting peripheral blood lymphopenia when high plasma levels are produced by i.p. administration (45), supporting the notion that maintenance of high in vivo S1P₁ antagonistic activities is required for inducing lymphocyte sequestration.

An alternative, but not mutually exclusive, possibility is the structural differences between S1P₁ antagonists: VPC44116 and W146 are alkyl phenyl amide phosphonates that have been generated as a series of structural analogues of FTY720 phosphate (17, 43), whereas TASP0277308 does not contain the alkyl side chain and is structurally unrelated to S1P and FTY720 phosphate. Thus, the mode of binding to S1P₁ and/or sensitivity to inhibitors, such as lipid phosphate phosphatases expressed on the cell surface, might be different between TASP0277308 and the alkyl phenyl amide phosphate group of S1P₁ antagonists. This may lead to the different in vivo outcomes of these compounds, especially with regard to lymphocyte sequestration. In relation to the structural differences between these antagonists, TASP0277308 treatment upregulates CD69 expression without downregulating S1P₁ expression on the lymphocyte cell surface, whereas W146 itself exerts no effect on CD69 expression on CD4 SP thymocytes but is able to cancel the effect of SEW2871 (17). Thus, it is reasonable to speculate that TASP0277308 has dual antagonistic actions on S1P₁, competitively inhibiting ligand binding and also inhibiting S1P₁-mediated signaling via the induction of inactive

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**FIGURE 6.** Suppression of clinical and histological signs of collagen-induced arthritis by TASP0277308. Collagen-primed DBA/1 mice (n = 5 mice/group) were treated orally with vehicle or the indicated dosages of TASP0277308 twice daily from days 20 to 63 and monitored for disease progression. A, Changes in arthritis severity scores. Data are represented as mean ± SEM (n = 5). *p < 0.05 (versus vehicle-treated group; parametric Dunnett’s test). B, Changes in body weight at the indicated time periods. C, Serum Ab levels against CII. IgG Ab levels in the indicated groups were measured on day 63. **p < 0.01 (versus vehicle-treated group; parametric Dunnett’s test). D, H&E staining of ankle joint from mice with collagen-induced arthritis. Typical histopathological lesions of the model, including synovial inflammation, extra-articular inflammation, and bone erosion, were observed in the ankle joint treated with vehicle (upper photograph), but not in joints treated with 100 mg/kg TASP0277308 (lower photograph). Scale bar, 1 mm. E, Semi-quantitative histopathological scores of arthritis. *p < 0.05, **p < 0.01 (versus vehicle-treated group; parametric Steel’s test).

**FIGURE 7.** Suppression of the development of acute collagen-induced arthritis by TASP0277308. Collagen-primed DBA/1 mice (n = 5 mice/group) were treated orally with vehicle or the indicated dosages of TASP0277308 twice daily from days 26 to 35 and monitored for disease progression. A, Changes in arthritis scores. Data are represented as mean ± SEM (n = 5). **p < 0.01 (versus vehicle-treated group; parametric Steel’s test). B, Serum Ab levels against CII. IgG Ab levels in the indicated groups were measured on day 35. Data are the mean ± SEM (n = 5). *p < 0.05 (versus vehicle-treated group; parametric Dunnett’s test).
conformational changes in S1P1. Further investigation of these possibilities would help to understand the mechanism of action of S1P1 antagonists on lymphocyte S1P1 as well as physiological function of S1P1 in relation to CD69.

With regard to the therapeutic activity of TASP0277308 on the development of collagen-induced arthritis, TASP0277308, like FTY720 (46, 47), significantly suppressed development of arthritis, including reduction of cartilage and bone erosion, synovial hyperplasia, cellular infiltration into the joints, and anti-CD8 Ab responses. Furthermore, TASP0277308 effectively suppressed ongoing acute collagen-induced arthritis. This suggests that the immunosuppressive effects of FTY720 on collagen-induced arthritis are a result of its antigenic effects, although it does not exclude possible agonistic effects of FTY720 on endothelial cells for immunosuppression. CD69 expression is induced after activation of mature lymphocytes at inflammatory sites (48–53) and is required for the production of the anti-inflammatory cytokine, TGF-β, from lymphocytes (54). Furthermore, an exacerbation of collagen-induced arthritis due to defective TGF-β production from lymphocytes in the synovium was observed in CD69-deficient mice (55).

Based on our finding that TASP0277308, but not SEW2871, induces CD69 expression on mature CD4 and CD8 T cells and B220+ B cells in the spleen and lymph nodes, TASP0277308 may have a therapeutic advantage over S1P agonists in the treatment of immunemediated arthritis, although further studies are needed fully to understand the molecular action of TASP0277308 on immune effector functions associated with immune-mediated diseases.

In conclusion, the discovery of an effective in vivo S1P1-selective antagonist, TASP0277308, in combination with other S1P1-selective agonists, has enabled detailed dissection of the S1P–S1P1 axis in physiological as well as pathological conditions. Continued efforts further to improve this class of compounds will also be highly beneficial in the development of immunosuppressive drugs for the treatment of various immunemediated disorders.

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