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Inhibition of Th1- and Th2-Mediated Airway Inflammation by the Sphingosine 1-Phosphate Receptor Agonist FTY720

Elzbieta Sawicka,^{*‡} Claudia Zuany-Amorim,^{1*} Corinne Manlius,^{*} Alexandre Trifilieff,^{*} Volker Brinkmann,[†] David M. Kemeny,[‡] and Christoph Walker^{2*}

The sphingosine 1-phosphate receptor agonist FTY720 is a novel immunomodulator that sequesters lymphocytes in secondary lymphoid organs and thereby prevents their migration to sites of inflammation. However, there is currently no information available on whether this drug affects Th1 or Th2 cell-mediated lung-inflammatory responses. The effect of FTY720 was therefore investigated in a murine airway inflammation model using OVA-specific, *in vitro* differentiated, and adoptively transferred Th1 and Th2 cells. Both Th1 and Th2 cells express a similar pattern of FTY720-targeted sphingosine 1-phosphate receptors. The OVA-induced Th1-mediated airway inflammation characterized by increased numbers of lymphocytes and neutrophils in bronchoalveolar lavage fluid was significantly inhibited by oral FTY720 treatment. Similarly, FTY720 suppressed the Th2 cell-induced bronchoalveolar lavage fluid eosinophilia and the infiltration of T lymphocytes and eosinophils into the bronchial tissue. Moreover, the Ag-induced bronchial hyperresponsiveness to inhaled metacholine was almost completely blocked. The inhibitory effect of FTY720 on airway inflammation, induction of bronchial hyperresponsiveness, and goblet cell hyperplasia could be confirmed in an actively Ag-sensitized murine asthma model, clearly indicating that Th2 cell-driven allergic diseases such as asthma could benefit from such treatment. *The Journal of Immunology*, 2003, 171: 6206–6214.

The novel immunomodulator FTY720 is a chemical derivative of myriocin, a metabolite of the ascomycete *Isaria sinclairii* (1, 2). The drug is highly effective in animal models of transplantation and autoimmunity and has recently been shown to be effective in human kidney transplantation (3–7). In contrast to classical immunosuppressants such as cyclosporine A or FK506, FTY720 selectively and reversibly sequesters lymphocytes but not monocytes or granulocytes from blood and spleen into secondary lymphoid organs, thereby preventing their migration toward sites of inflammation and allograft rejection (8–11). Moreover, FTY720 does not impair T cell activation, expansion, and memory to systemic viral infections or induce T cell apoptosis at clinically relevant concentrations (12, 13).

The FTY720-induced lymphocyte homing has been shown to be pertussis toxin sensitive, suggesting that the molecular targets of the drug belong to the family of G-protein-coupled receptors (9, 14). In this regard, the chemokine receptor CCR7 and its ligands CC chemokine ligand (CCL)³ 19 and CCL21 are known to play a decisive role in the migration of T and B cells through high endothelial venules into lymph nodes (15). However, a recent study has demonstrated that FTY720 treatment of CCR7^{-/-} mice as

well as CCL19- and CCL21-deficient *plt* mice restores the lymphocyte homing defect in a pertussis toxin-sensitive manner, thus pointing to an alternative, CCR7-CCL19/CCL21-independent mechanisms for the FTY720-induced lymphocyte migration into secondary lymphoid tissue (14). Because FTY720 is a structural analog of sphingosine and because sphingosine 1-phosphate (S1P) receptors mediate cell motility, it is reasonable to assume that it may act as an agonist for S1P receptors. Recent data have indeed shown that FTY720 is effectively phosphorylated *in vivo* and that phosphorylated FTY720 is an agonist at four S1P receptors (16, 17). Stimulation of these receptors is most likely the mechanism by which this drug leads to sequestration of lymphocytes in secondary lymphatic tissues and thus away from inflammatory lesions.

In all experimental models described to date, FTY720 has been shown to affect the migration of naive and activated CD4 and CD8 T cells and B cells (5, 8, 16, 18). However, to date only a few studies have focused on the role of FTY720 in altering the trafficking of effector T cell populations (12, 19), and little is known about the effect of this compound on fully differentiated and functionally different T cell subsets such as Th1 or Th2 cells (19). Moreover, the expression of S1P receptors on these cell types has not been studied thus far. Furthermore, the effectiveness of FTY720 has been demonstrated almost exclusively in predominantly Th1 cell-driven disease models, and it is currently not known whether Th2 cell-mediated diseases could also benefit from such treatment. The aim of the present study was therefore to investigate the effect of FTY720 in Th1- and Th2-driven inflammatory responses with a particular focus on airway inflammation given that no information for a potential therapeutic benefit of this drug in respiratory diseases is published to date. The results presented in this study demonstrate that FTY720 blocks both Th1- and Th2-driven inflammatory responses. Moreover, the inhibition of Th2 cell-mediated allergic airway inflammation, suppression of bronchial hyperresponsiveness (BHR), and goblet cell hyperplasia suggest that allergic diseases such as asthma could also benefit from treatment with FTY720.

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³ Abbreviations used in this paper: CCL, CC chemokine ligand; BAL, bronchoalveolar lavage; S1P, sphingosine 1-phosphate; BHR, bronchial hyperresponsiveness; Penh, enhanced pause.

Materials and Methods

Animals

C57BL/6 mice (5–8 wk old) were obtained from Harlan (Oxon, U.K.). OVA peptide-specific MHC class II-restricted TCR-transgenic mice (OT-II) (20) were obtained from Iffa Credo, France. All intergroup and inter-experiment groups were age, sex, and weight matched. All experimental protocols complied with the Home Office 1986 Animals Scientific Act and were approved by the Novartis Horsham Research Centre Animal Welfare Committee.

FTY720 administration

FTY720 was obtained from Novartis Pharmaceuticals (Basel, Switzerland) and was dissolved in distilled water containing 10% neoral placebo as vehicle. A dose of 0.1, 0.03, or 0.01 mg/kg was administered by gavage in a volume of 200 μ l/mouse at the time points indicated in *Results*. Control mice were given vehicle only.

Th1-Th2 cell polarization

OVA-specific TCR-transgenic CD4⁺ T cells were isolated from the lymph nodes and spleens by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, a single-cell suspension was incubated with CD4 (L3T4) microbeads, and the desired cells were separated by positive selection according to the manufacturer's instruction. The resulting cell populations were >95% pure as determined by FACS. CD4⁺ cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 μ g/ml streptomycin, and 100 U/ml penicillin (all from Life Technologies, Paisley, U.K.) with OVA_{323–339} (1 μ g/ml) and irradiated (4000 rad) splenocytes (1:2 ratio). For Th1 phenotype development, recombinant murine IL-12 (10 ng/ml; BD PharMingen, Oxford, U.K.) and neutralizing anti-IL-4 Ab (11 B 11, 5 μ g/ml; BD PharMingen) were added, and for Th2 phenotype development recombinant murine IL-4 (10 ng/ml; BD PharMingen) and anti-IL-12 (C17.8, 5 μ g/ml; BD PharMingen) were used. IL-2 was added from day 3 onward at 20 U/ml. CD4⁺ T cells were cultured with peptide-pulsed APCs that were given weekly for three rounds of antigenic stimulations under polarizing conditions. To determine the cytokine profile of polarized T cells, 1 \times 10⁶ cells were restimulated with PMA (10 ng/ml) and ionomycin (400 ng/ml) for 5 h. Cells were then washed with PBS and stained for intracellular cytokines: IFN- γ , IL-4, IL-5, and IL-10 (BD PharMingen), using a Fix&Perm kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer's instructions. Th1 cells produced high levels of IFN- γ (60–80%) but little IL-4 and IL-5 (<2%), whereas Th2 cells produced high levels of IL-4 (40–70%) and IL-5 (15–30%) but little IFN- γ (<5%). The viability of Th1 and Th2 cells was >95%. Subsequently, these cells were used for adoptive transfer experiments.

T cell transfer model

Recipient C57BL/6 mice were given 5 \times 10⁶ Th1 or Th2 cells in 50 μ l of PBS i.v. Twenty-four hours later, mice were exposed to an aerosol of OVA (50 mg/ml) for 20 min on 3 consecutive days. Control groups received cells but were challenged with aerosolized PBS. Mice were sacrificed 24 or 48 h after the last Ag exposure.

Where indicated, Th2 cells were incubated before transfer with 0.5 μ M CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C followed by two washes with RPMI containing 10% FCS. At the indicated time points, the number of fluorescent cells in bronchoalveolar lavage (BAL) fluid or lymph nodes were analyzed by flow cytometry and converted into absolute cell numbers based on the determined percentage of CD4- and CFSE-positive cells and total cell counts.

OVA sensitization and challenge model

C57BL/6 mice were immunized i.p. on days 0 and 14 with 10 μ g of chicken OVA (Grade V; Sigma-Aldrich, St. Louis, MO) in 0.2 ml of alum (Serva, Heidelberg, Germany). On day 21, animals were exposed for 20 min to an aerosol of OVA in sterile PBS (50 mg/ml) or PBS alone.

Determination of BHR

BHR was assessed 48 h after the last Ag challenge. BHR was determined by metacholine (Sigma-Aldrich)-induced airflow obstruction using a whole body plethysmograph (Buxco, Electronics, Troy, NY), as previously described (21). Unanesthetized mice were placed in a plethysmograph and allowed to acclimate. Baseline averages of breathing frequency, tidal volume, inspiratory and expiratory times, and airway resistance of all breaths over a 1-min period were recorded. Following baseline measurements, animals were exposed for 1 min to an aerosol generated from PBS. Immediately after the aerosolization period, the pulmonary function measure-

ments of the animal were taken during a 2-min period. Animals were then exposed to aerosol generated from acetyl- β -methylcholine bromide (metacholine) solutions at 0.1 M or 0.3 M (optimal concentrations as determined in pilot experiments, data not shown) for 1 min followed by a 4-min recording after exposure. In some experiments, BHR was recorded for a range of metacholine concentrations (0.01, 0.03, 0.1, 0.3, and 0.6 M).

BHR was expressed as enhanced pause (Penh), a calculated value, which correlates with measurement of airway resistance, impedance, and intrapleural pressure in the same mouse: Penh = $[(T_e/T_r - 1) \times (P_{ef}/P_{if})]$, where T_e is the expiration time, T_r is the relaxation time, P_{ef} is the peak expiratory flow, and P_{if} is the peak inspiratory flow \times 0.67 coefficient). The relaxation time is the time it takes for the box pressure to change from a maximum to a user-defined percentage of the maximum. Here, T_r measurement begins at the maximum box pressure and ends at 40%. Values were expressed as the percentage shift from baseline, which was measured by comparing the maximum Penh value of mice before and after stimulation with metacholine.

Assessment of inflammation

At the specified time point, animals were anesthetized i.p. with 60 mg/kg pentobarbitone sodium. The trachea was cannulated, and BAL was collected by three injections of 0.4 ml of PBS into the lung. Total cell counts were determined, and cytospin preparation (Shandon Scientific, Cheshire, U.K.) was performed. Cells were stained with Diff-Quik (Baxter Dade, Duding, Switzerland), and a differential count of 200 cells was performed.

For histological examination, lungs were inflated with optimum cutting temperature compound, snap-frozen in liquid nitrogen, and stored at -80°C or perfused with 10% buffered formalin, fixed overnight with formaldehyde before embedding in paraffin. Sections were stained with H&E to examine infiltrating cells. For analysis of mucous producing cells, paraffin-embedded and sectioned lungs were stained with periodic acid-Schiff. Nuclei were counterstained with hematoxylin. The histological analyses were conducted with a Zeiss AxioCam HRC Cooled Color Digital Camera (Zeiss, Oberkochen, Germany).

Cytokine levels (IL-4, IL-5, IL-13, and IFN- γ) were determined in BAL fluid of Th1 and Th2 recipient mice 24 h after the last OVA challenge or 24 h after a single challenge in the actively immunized and challenged mice using commercially available ELISA kits (R&D Systems, Oxon, U.K.) following the manufacturer's instructions. The detection limit of all the ELISAs used was \sim 3 pg/ml.

Peripheral blood was collected from the abdominal aorta into tubes containing heparin. Erythrocytes were removed by hypotonic lysis. Total cell counts were determined, and cytospin preparation (Shandon Scientific) was performed. Cells were stained with Diff-Quik (Baxter Dade), and a differential count of 200 cells was performed.

Flow cytometry

To determine the numbers of effector (transgenic, V β 5.2-positive) and naive T lymphocytes in BAL of FTY720-treated and untreated mice, leukocytes were stained with anti-CD3-FITC and anti-V β 5.2-PE murine mAbs (BD PharMingen). Isotype-matched mAb were used as negative controls. Flow cytometry was conducted with a FACScalibur (BD Biosciences, Oxford, U.K.) and the data was analyzed with Cellquest software (BD Biosciences).

RT-PCR

Total RNA was extracted from Th1 and Th2 cultures using the guanidine isothiocyanate method (22). Reverse transcription was performed with 10 μ g of total RNA and primed with 1 μ g of oligo(dT)₁₅ (Boehringer Mannheim, Mannheim, Germany) in the presence of 8 U RNAsin (Promega, Madison, WI). This mixture was first heated to 70°C for 5 min, and the reaction was then conducted in a mixture (final volume, 50 μ l) including 400 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies) and 4 mM DTT in a buffer containing 50 mM Tris-HCl (pH8.3), 7.5 mM KCl, 3 mM MgCl₂, 8 U RNAsin and 2 mM concentrations each of dATP, dGTP, dCTP, and dTTP (dNTP mix; Life Technologies). This mixture was incubated at 37°C for 45 min, and the reaction was terminated by heating at 100°C for 5 min. After cooling on ice, reverse transcriptase was added again, and the mixture was incubated for an additional 30 min at 37°C before heating at 100°C. cDNA samples were amplified by PCR with sense and antisense primers designed for specific detection of gene sequences for mouse SIP₁ (Edg-1), SIP₂ (Edg-5), SIP₃ (Edg-3), SIP₄ (Edg-6), and SIP₅ (Edg-8) receptors. Primers were selected through computer analysis using Gene Works software (IntelliGenetics, Mountain View, CA): EDG1 (PubMed NM_007901) forward TCCATCGTCATCCTC

TACTGC and reverse AGGATGTCACAGGTCTCCGC; EDG3 (PubMed NM_010101) forward TCACTGGTTCATCATGCTGG and reverse CAG GTCTTCCTTGACCTTCC; EDG5 (PubMed AF 108020) forward TAT CGTGGCTCTGTACGTCC and reverse CGCCACGTATAGATGAC AGG; EDG6 (PubMed NM_010102) forward AAGACCAGCCGTGTGT ATGG and reverse TCAGCAGGTGTTGAGTAGC; EDG8 (PubMed NM_053190) forward CTTGCTATTACTGGATGTCGC and reverse GTT GGAGGAGTCTTGGTTGC. PCR experiments were performed in a mixture (20 μ l) containing 1.5 U of *Taq* DNA polymerase (Life Technologies) and 1 μ M concentrations of each primer in a buffer containing 20 mM Tris-HCl (pH8.4), MgCl₂ (1.5 mM), 50 mM KCl, and 0.2 mM concentrations each of dATP, dGTP, dCTP, and dTTP. PCR experiments were run in a PerkinElmerCetus (Norwalk, CT) DNA Thermal Cycler programmed for the following steps: initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 92°C for 10 s; annealing at 55°C for 10 s; and a primer extension step at 72°C for 20 s. Specific amplified DNA fragments were purified by electrophoresis on 2% (w/v) agarose gel, stained with ethidium bromide, and photographed.

Statistical analysis

Results are representative of at least three independent experiments. Data were analyzed by standard statistical packages for one-way ANOVA followed by unpaired Student's *t* test for unpaired values. *p* < 0.05 was considered significant. Results are expressed as means \pm SEM.

Results

Airway inflammation induced by adoptive transfer of Th1 or Th2 cells

To answer the question whether FTY720 treatment differentially affects Th1- or Th2-mediated airway inflammation, a murine adoptive transfer model using Ag-specific, in vitro differentiated Th1 and Th2 cells was established. For that purpose, CD4⁺ T cells purified from spleens of TCR-transgenic OT-II mice recognizing a specific immunogenic peptide sequence of OVA were cultured in vitro under polarizing condition to produce Ag-specific Th1 or Th2 cells. After stimulation, these in vitro differentiated Th1 and Th2 cells produced the characteristic cytokine pattern with high numbers of IFN- γ - and no IL-4-producing cells in the Th1 population, but a high level of IL-4 but no IFN- γ production in Th2 cells as measured by intracellular cytokine staining and FACS (Fig. 1A). Quantification of the cytokine levels in culture supernatants confirmed the highly polarized status of Th1 and Th2 cells (data not shown).

To investigate whether FTY720 affects both Th1- and Th2-driven airway inflammation, 5×10^6 of in vitro differentiated Ag-specific Th1 and Th2 cells (optimal cell concentration as determined in pilot experiments; data not shown) were adoptively transferred into naive C57BL/6 mice, followed by three consecutive daily aerosol Ag challenges. The exposure to Ag induced a significant and comparable increase in the number of lymphocytes present in BAL fluid 24 h after the last challenge in both Th1 and Th2 cell transfer models (Fig. 1B). However, whereas the Th1 cell transfer induced a massive increase of the number of neutrophils but not eosinophils, a characteristic eosinophilic airway inflammation was found after the injection of Th2 cells. Moreover, the Ag challenge of mice pretreated with Th2 cells resulted in a characteristic and significant metacholine-induced airflow obstruction, whereas no induction of BHR was observed after the transfer of Th1 cells.

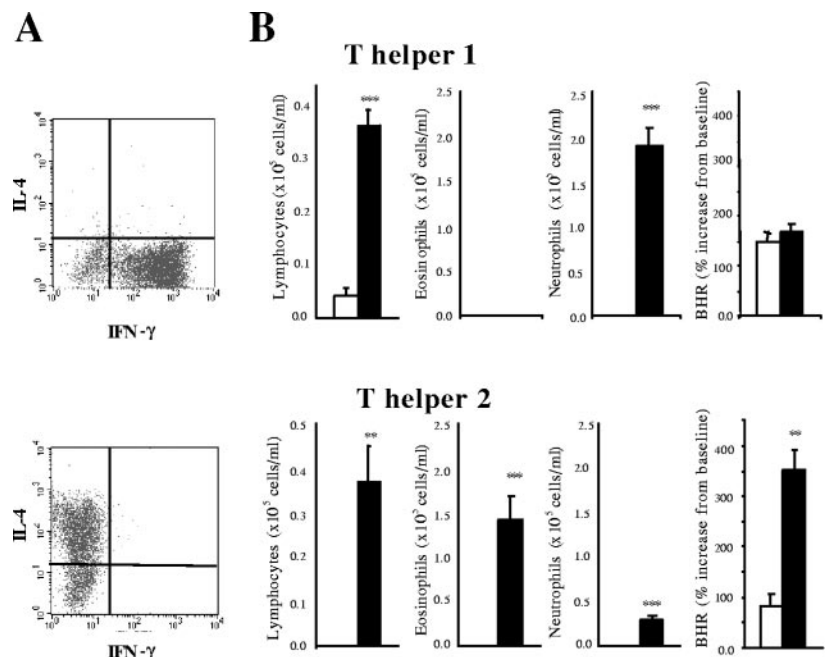
Treatment with FTY720 suppresses Th1-driven lung inflammation

To study the effect of FTY720 on Th1-induced lung inflammation, C57BL/6 mice were injected i.v. with 5×10^6 of Th1 cells. Oral administration of FTY720 (0.01, 0.3, or 0.1 mg/kg) 3 h before the cell transfer and every antigenic challenge not only inhibited the number of blood lymphocytes ($11.68 \pm 1.31 \times 10^5$ in control compared with $1.68 \pm 0.18 \times 10^5$ in 0.1 mg/kg FTY720-treated mice with no effect on monocytes or granulocyte numbers) but also significantly and dose dependently reduced the number of lymphocytes present in BAL fluid of Ag-challenged animals (Fig. 2A). Most likely as a result of the reduced lymphocyte infiltration, a parallel decrease of neutrophils was observed, demonstrating the anti-inflammatory potential of FTY720 in Th1-mediated airway inflammation.

FTY720 is equally effective at suppressing Th2-driven airway inflammation

To determine whether FTY720 could also down-regulate Th2-mediated airway inflammation, the compound was tested in the above described Th2 cell transfer model. As shown in Fig. 2B, oral

FIGURE 1. Airway inflammation induced by adoptive transfer of in vitro polarized Th1 and Th2 cells. **A**, Representative dot blot from in vitro differentiated Th1 and Th2 cells, stimulated with PMA and ionomycin in the presence of monensin for 5 h and tested for IL-4 and IFN- γ production using intracellular staining with anti-IL-4-PE (*y*-axis) and anti-IFN- γ -FITC (*x*-axis) Abs and flow cytometry. **B**, Analysis of airway inflammation and BHR in C57BL/6 mice injected i.v. with 5×10^6 polarized OVA-specific Th1 or Th2 cells and challenged with aerosolized OVA (■) or PBS (□) on three consecutive days. BAL fluid lymphocytes, eosinophils, and neutrophil numbers ($\times 10^5$ /ml) were determined 24 h after the last Ag challenge. BHR to inhaled metacholine (0.1 M) was measured as an increase in Penh value at 24 h after the last Ag exposure. Data are presented as mean \pm SEM. *n* = 8 in each group. **, *p* < 0.005; ***, *p* < 0.0005.



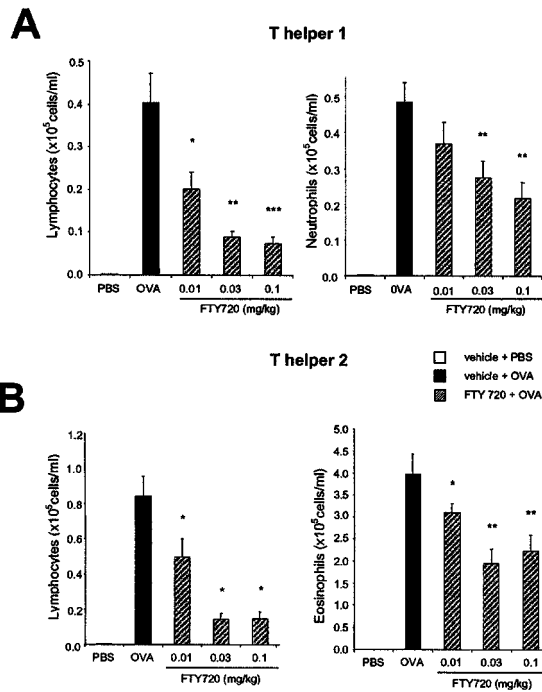


FIGURE 2. FTY720 suppresses Th1- and Th2-induced airway inflammation. *A*, Analysis of airway inflammation in vehicle or FTY720 (0.01, 0.03, or 0.1 mg/kg) pretreated (▨) C57BL/6 mice injected i.v. with 5×10^6 polarized OVA-specific Th1 cells and challenged with aerosolized OVA (OVA- and FTY720-treated mice) or PBS on three consecutive days. BAL fluid lymphocyte (*left*) and neutrophil (*right*) numbers ($\times 10^5$ /ml) were determined 24 h after the last Ag challenge. *B*, Same experimental procedure as described in *A*, using i.v. injection of highly polarized OVA-specific Th2 cells (5×10^6 cells/mouse). BAL fluid lymphocyte (*left*) and eosinophil (*right*) numbers are presented as mean \pm SEM. $n = 8$ in each group, *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

FTY720 treatment significantly reduced the Ag-induced infiltration of lymphocytes into the airways. Both CD4⁺ and CD8⁺ T cells were similarly affected by the treatment (data not shown). The dose required and the extent of inhibition was comparable with the effects seen in the Th1 transfer model. A significant reduction of the eosinophilic inflammation was observed after FTY720 treatment, suggesting that the reduction in numbers of Th2 cells and the associated decrease in Th2 cell-derived cytokines may affect the eosinophil recruitment process. Indeed, the OVA challenge of mice after adoptive transfer of Th2 cells resulted in a substantial increase in BAL fluid IL-4, IL-13, and IL-5 but not IFN- γ levels (Table I). Treatment with FTY720 significantly reduced the levels of all Th2 cell-derived cytokines to a similar degree. In contrast, the exposure to allergen after adoptive transfer of Th1 cells led to a substantial increase in IFN- γ but not IL-4, IL-13,

or IL-5 levels. Again, treatment with FTY720 significantly reduced the production of IFN- γ without affecting the levels of the Th2 cell-derived cytokines, suggesting that the reduced infiltration of neutrophils in the Th1 model or eosinophils in the Th2 model is most likely due to the reduced number of lymphocytes and consequently diminished levels of granulocyte active cytokines and chemokines present in the airways.

Th1 and Th2 cells express similar pattern of S1P receptors

Because FTY720 acts as a potent agonist of multiple S1P receptors (16, 17), the expression of S1P₁ (Edg-1), S1P₂ (Edg-5), S1P₃ (Edg-3), S1P₄ (Edg-6), and S1P₅ (Edg-8) on both Th1 and Th2 cell subsets was analyzed using RT-PCR (Fig. 3). The results demonstrate the expression of mRNA for S1P₁, S1P₃, S1P₄, and S1P₅ but not S1P₂ on both Th1 and Th2 cell subsets. S1P₁ and S1P₄ are expressed at higher levels than S1P₃ and S1P₅ with no apparent difference between the two cell populations, explaining the similar response to FTY720 treatment in both Th1 and Th2 cell-induced airway inflammation models.

FTY720 suppresses the infiltration of both Ag-specific and bystander T cells

To further characterize the anti-inflammatory effect of FTY720 in the Th2 cell transfer model, histological sections of the airways were analyzed to determine whether the infiltration of proinflammatory cells into the lung tissue was also diminished. As shown in Fig. 4, the exposure of Th2-treated C57BL/6 mice to Ag resulted in a characteristic eosinophil- and T lymphocyte-dominated tissue infiltrate. Confirming the data obtained in BAL fluid, treatment with FTY720 significantly reduced the numbers of both T cells and eosinophils within the bronchial tissue.

To analyze the effect of FTY720 treatment on the infiltration of Ag-specific Th2 cells, the numbers of TCR-transgenic T cell recovered in BAL fluid 24 h after the last OVA challenge were determined. The TCR of the transgenic T cells from OT-II mice consists of V α 2 and V β 5.2 chains. Therefore, BAL fluid T cells were double-stained with a FITC-conjugated anti-CD3 and a PE-conjugated anti-V β 5.2 Ab and analyzed by flow cytometry. As shown in Fig. 5, the massive infiltration of lymphocytes into the BAL fluid after the transfer of Th2 cells and exposure to Ag was mainly due to the recruitment of non-Ag-specific lymphocytes. The Ag-specific T cell population accounted for only ~10–20% of the total T cell pool present in the BAL fluid of these mice. However, treatment with FTY720 not only reduced the number of bystander T cells but also significantly suppressed the number of infiltrating, V β 5.2-expressing cells, demonstrating that the compound directly affects the migration of the Ag-specific Th2 cell population into the airway after Ag exposure. Similar results were found by analyzing the number of CFSE-prelabeled and adoptively transferred Ag-specific Th2 cells in BAL fluid by flow cytometry (data not shown).

Table I. Effect of FTY720 treatment on cytokine levels in BAL fluid

	Th1 Cell Transfer Model			Th2 Cell Transfer Model		
	PBS ^a	OVA	OVA/FTY	PBS	OVA	OVA/FTY
IL-4	<3 ^b	10.8 \pm 2.4	12.3 \pm 2.2	<3	32.7 \pm 4.8	20.8 \pm 4.3
IL-5	<3	<3	<3	<3	57.8 \pm 12.6	24.8 \pm 12.7
IL-13	<3	5.3 \pm 0.9	9.1 \pm 2.1	<3	116.3 \pm 15.0	66.7 \pm 13.9
IFN- γ	23.9 \pm 15.9	1454 \pm 272	711 \pm 128	<3	14.5 \pm 1.1	6.6 \pm 1.5

^a PBS, vehicle treated and PBS challenged; OVA, vehicle treated and OVA challenged; OVA/FTY, FTY720 (0.03 mg/kg) treated and OVA challenged.

^b Picograms per milliliter; mean \pm SEM from eight individual animals.

^c Italicized values significantly different from OVA-challenged controls ($p < 0.05$).

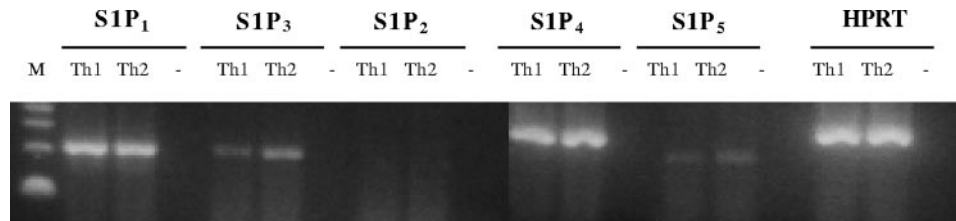


FIGURE 3. S1P receptor mRNA expression in Th1 and Th2 cells. RT-PCR experiments were performed using total RNA isolated from Th1 and Th2 cells and specific primers for mouse S1P₁₋₅ (Edg-1, -5, -3, -6, and -8) genes. Controls were run using cDNA from Th1 or Th2 cells and primers for HPRT. RT-PCR products were separated by electrophoresis on 2% agarose gel and revealed by ethidium bromide fluorescence. Data are representative results obtained from three different experiments. M, 100 bp markers.

Because FTY720 acts by selectively sequestering lymphocytes from blood and spleen into secondary lymphoid organs, the number of Ag-specific Th2 cells in draining and non-draining lymph nodes was analyzed. In vitro cultured OVA-specific Th2 cells were labeled with CFSE and adoptively transferred into naive animals, and the number of fluorescently labeled cells in draining and non-draining lymph nodes was analyzed by flow cytometry. As shown in Fig. 5C, the exposure to OVA induced a massive increase in transferred Th2 cells present in lung-draining lymph nodes. Significantly fewer Ag-specific Th2 cells were found in draining lymph nodes of FTY720-treated mice, most likely due to a reduced recirculation and subsequent expansion of these cells in response to the local allergen exposure. In contrast, a much less pronounced increase of Th2 cells were found in non-draining lymph nodes after the challenge with OVA. These numbers were, however, significantly increased in FTY720-treated animals, demonstrating that lymph node sequestration of these Ag-specific T cells is the most likely mechanisms by which FTY720 induces its anti-inflammatory effect.

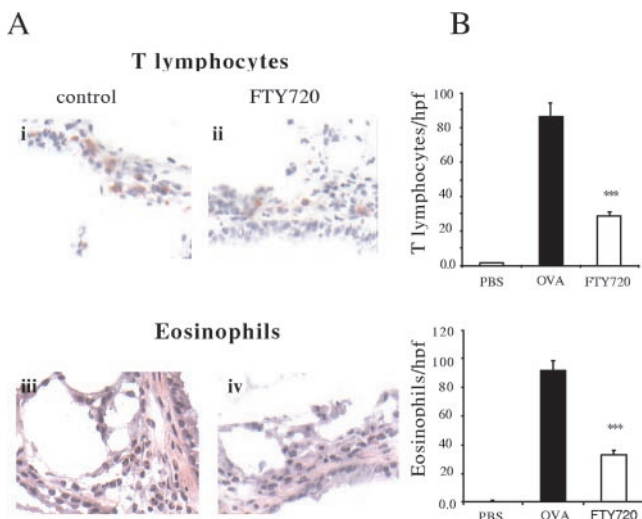


FIGURE 4. Effect of FTY720 on lymphocyte and eosinophil accumulation in the peribronchial wall. Histological analysis of airway inflammation in vehicle- or FTY720 (0.1 mg/kg)-pretreated C57BL/6 mice injected i.v. with 5×10^6 polarized OVA-specific Th2 cells and challenged with aerosolized OVA or PBS on three consecutive days. Lungs were collected 48 h after the last aerosol challenge for histological examination. A, Representative pictures of lung sections from control and FTY720-treated mice challenged with OVA and stained with anti-CD3 Abs (*i* and *ii*, T lymphocytes) or H&E (*iii* and *iv*, eosinophils). B, Total numbers of T lymphocytes and eosinophils obtained from 10 high power fields (hpv; $\times 1000$ magnification) in histological sections from each lung of PBS- or OVA (OVA- and FTY720-treated mice)-challenged animals. Data are mean \pm SEM; $n = 8$ mice in each group; ***, $p < 0.0005$.

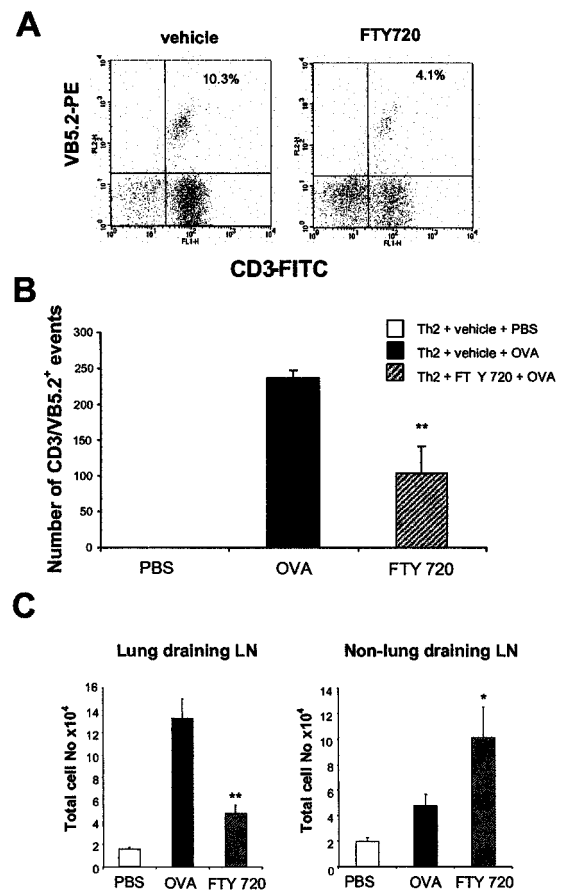


FIGURE 5. FTY720 blocks the lung infiltration of Ag-specific T cells by sequestration of cells in lymph nodes (LN). A, Representative dot plot of lymphocytes recovered in BAL fluid of vehicle- or FTY720 (0.1 mg/kg)-treated, OVA-challenged mice. Cells were stained with anti-CD3 (CD3-FITC) and anti-V β 5.2 (V β 5.2-PE) Abs and analyzed on the electronically gated lymphocyte population. B, Total numbers of OVA-specific T cells in BAL; 1×10^4 events were acquired from every sample; lymphocyte population was gated using the forward and side scatter characteristics and total numbers of OVA-specific T lymphocytes (CD3⁺V β 5.2⁺) in PBS- or OVA-challenged and FTY720-treated animals were calculated as: $[(\%CD3^+ \times \text{total lymphocyte number}/100) \times (\%CD3^+ \text{ and } V\beta 5.2^+)/100]$. Data are mean \pm SEM, $n = 8$ mice in each group. **, $p < 0.005$. C, Number of CFSE-stained and transferred Th2 cells in lung draining (*left*) and non-draining (*right*) lymph nodes as determined by flow cytometry following vehicle treatment and PBS challenge, vehicle treatment and OVA challenge, or 0.1 mg/kg FTY720-treated and OVA-challenged (FTY720) mice. Data are presented as mean \pm SEM. $n = 8$ in each group. *, $p < 0.05$; **, $p < 0.005$.

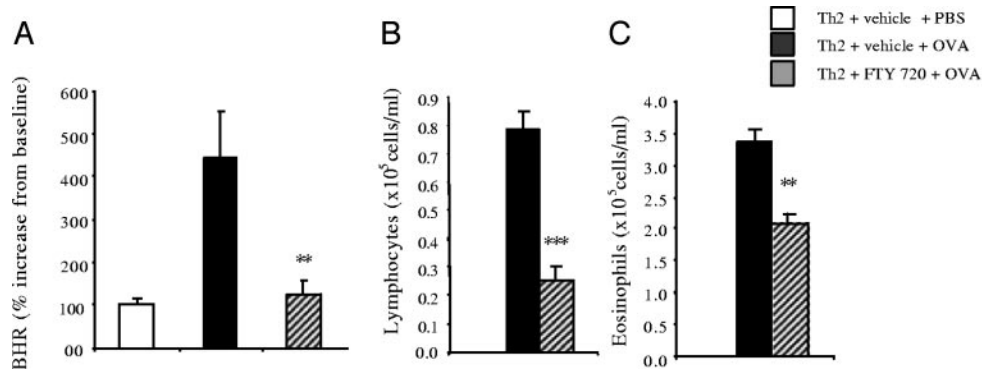


FIGURE 6. FTY720 inhibits the induction of BHR. *A*, BHR to inhaled metacholine in vehicle- or FTY720 (0.1 mg/kg)-pretreated (▨) C57BL/6 mice injected i.v. with 5×10^6 polarized OVA-specific Th2 cells and challenged with aerosolized OVA (OVA- and FTY720-treated mice) or PBS on 3 consecutive days. BHR was measured as increase in Penh values (% increase from baseline) 48 h after the last Ag exposure. Data are mean \pm SEM; $n = 8$ mice in each group. **, $p < 0.005$. Numbers of BAL fluid lymphocytes (*B*) and eosinophils (*C*) obtained from the same animals 48 h after the last allergen challenge.

FTY720 blocks the Th2 cell-induced increase in BHR

As shown in Fig. 1*B*, the OVA challenge of mice injected with Ag-specific Th2 cells not only resulted in the infiltration of lymphocytes and eosinophils into the airways but also induced a characteristic increase in BHR to metacholine. To analyze the effect of FTY720 on BHR, the metacholine-induced airflow obstruction in conscious Th2 recipient mice was determined 48 h after the last OVA challenge using whole body plethysmography. The allergen challenge induced a significant increase in BHR to metacholine (Fig. 6). Treatment with 0.1 mg/kg FTY720 almost completely abolished this Ag-induced change in BHR, which was paralleled by a reduction in lymphocyte and eosinophil numbers present in the BAL fluid of these mice at the same time point. Thus, FTY720 not only reduced the infiltration of proinflammatory cells into the lung tissue but also suppressed more pathophysiological readouts such as BHR.

FTY720 suppresses the inflammation in an animal model of asthma

The adoptive transfer models are very powerful tools for investigating the role of specific T cell populations or compounds that act on T cells in airway inflammation. However, these models may represent only a small part of the inflammatory response seen in more complex disease models, e.g., known to be also dependent on the production of allergen-specific IgE and mast cell degranulation events. Therefore, the effect of FTY720 was analyzed in a well-established model of allergen-induced airway inflammation (23). Similar to the Th2 cell transfer model, the OVA challenge of mice actively sensitized with Ag induced a significant increase in both lymphocytes and eosinophils (Fig. 7*A*). Treatment with FTY720 24 h and 3 h before the OVA challenge resulted in a significant and dose-dependent reduction of the lymphocyte and eosinophil infiltrate, accompanied by significantly reduced levels of IL-4 and IL-13 (5.1 ± 2.1 pg/ml IL-4 in OVA challenged vs <3 pg/ml IL-4 in FTY720 treated animals and 24.9 ± 4.4 pg/ml IL-13 in OVA challenged vs 13.9 ± 2.7 pg/ml IL-13 in FTY720-treated animals) but without changes in the concentration of IFN- γ (data not shown). As compared with vehicle-treated and OVA-challenged mice, FTY720 effectively suppressed the migration of lymphocytes to the BAL by $>70\%$ and markedly reduced eosinophilia by $>60\%$. Both CD4⁺ and CD8⁺ T cell population were similarly affected (reduction in CD4⁺ T cells by 89% and CD8⁺ T cells by 60%). Similar results were obtained by analyzing histological sections of lungs for the number of infiltrating eosinophils (Fig. 7*B*),

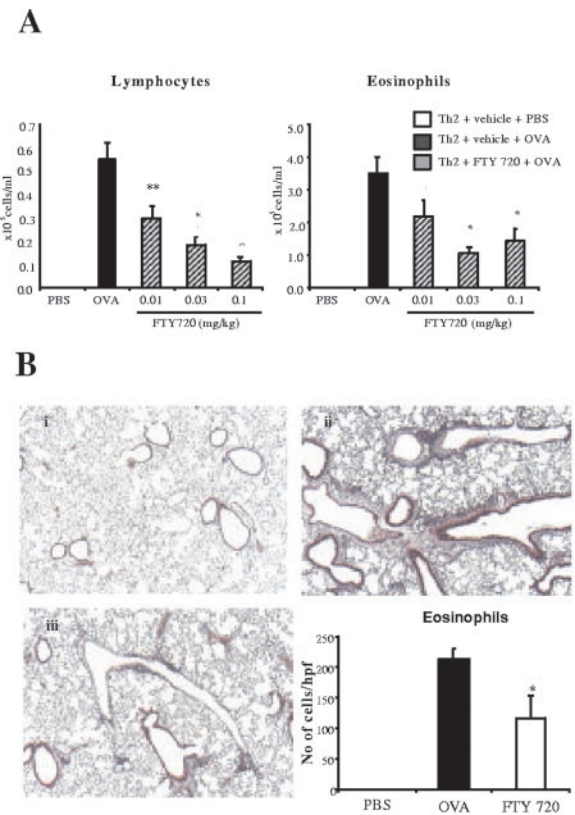


FIGURE 7. FTY720 inhibits the accumulation of lymphocytes and eosinophils in actively OVA-immunized and -challenged mice. *A*, OVA-immunized C57BL/6 mice were treated orally with 0.01, 0.03, or 0.1 mg/kg FTY720 or control vehicle 24 and 3 h before single aerosol OVA challenge (OVA- and FTY720-treated mice). Control mice received a PBS challenge alone. Animals were sacrificed 24 h after the Ag challenge and BAL fluid lymphocytes (*left*) and eosinophils (*right*) determined. Data are presented as mean \pm SEM; $n = 8$ in each group. *, $p < 0.05$. *B*, Representative pictures of lung sections from control and FTY720 (0.1 mg/kg)-treated mice challenged with OVA and stained with H&E. *i*, vehicle treated and PBS challenged; *ii*, vehicle treated and OVA challenged; *iii*, FTY720 treated and OVA challenged. Also shown are the total numbers of eosinophils obtained from 10 high power fields ($\times 1000$ magnification) from these histological sections. Data are mean \pm SEM; $n = 8$ mice in each group. *, $p < 0.05$.

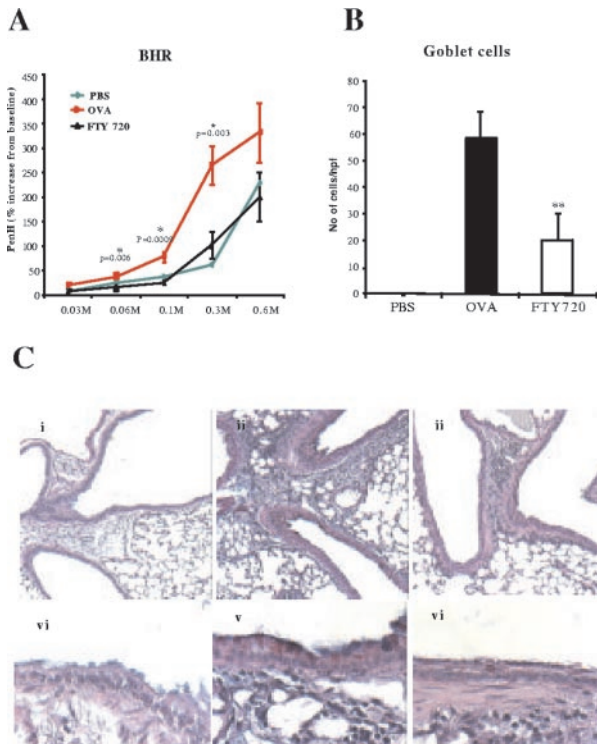


FIGURE 8. FTY720 blocks the induction of BHR and goblet cell hyperplasia in actively OVA-sensitized and -challenged mice. **A**, BHR to various doses of inhaled metacholine in actively OVA-sensitized and vehicle-treated and PBS-challenged, vehicle-treated and OVA-challenged (OVA), or FTY720 (0.1 mg/kg)-treated and OVA-challenged C57BL/6 mice. BHR was measured as increase in Penh values (% increase from baseline) 48 h after the Ag exposure. Data are mean \pm SEM; $n = 8$ mice in each group. **B**, Numbers of goblet cells counted in 10 different fields per section; mean \pm SEM; $n = 8$ mice in each group. **, $p < 0.005$. **C**, Representative pictures of lung sections (two different magnifications) from control and FTY720 (0.1 mg/kg)-treated mice challenged with OVA and stained with PAS for goblet cell numbers. *i* and *iv*, vehicle treated and PBS challenged; *ii* and *v*, vehicle treated and OVA challenged; *iii* and *vi*, FTY720 treated and OVA challenged. hpf, High powered field.

clearly demonstrating that FTY720 not only suppresses the Th2-mediated airway inflammation in a Th2 adoptive transfer model but is also effective as an anti-inflammatory compound in a more complex disease model of asthma. Moreover, and again similar to the Th2 cell model, allergen exposure of actively immunized mice induced a significant change in BHR, which was almost completely suppressed by treatment with FTY720 (Fig. 8A). In addition, the histological evaluation of lung sections revealed that exposure to Ag induced a significant increase in goblet cell numbers, which was profoundly reduced after treatment with FTY720 (Fig. 8, B and C).

Discussion

This study demonstrates that the S1P receptor agonist FTY720 equally affects inflammatory responses induced by Ag-specific Th1 and Th2 cells. More specifically, we could demonstrate that 1) both Th1 and Th2 cells express a similar pattern of S1P receptors, 2) both Th1-induced neutrophilic and the Th2-induced eosinophilic airway inflammation was inhibited by oral treatment with FTY720, and 3) that the FTY720-induced suppression of Ag-specific T cell infiltration into the airways also affected more pathophysiological readouts such as BHR and goblet cell hyperplasia.

FTY720 is a structural analog of sphingosine and has recently been shown to be a substrate for sphingosine-metabolizing en-

zymes (16, 17). Like sphingosine, FTY720 is effectively phosphorylated by sphingosine kinase, and phosphorylation is essential for its biological activity. Phosphorylated FTY720 is considerably more potent than S1P itself as agonist at four of five S1P receptors (S1P₁, S1P₃, S1P₄, and S1P₅, formerly Edg-1, -3, -6, and -8, respectively) and only the S1P₂ (Edg-5) receptor was not activated by the compound (17). The data presented in this study demonstrate that both Th1 and Th2 cells express mRNA for all four FTY720 targeted S1P receptors with no apparent difference between the two cell populations. Higher mRNA expressions of S1P₁ and S1P₄ than with S1P₃ and S1P₅ were found in both T cell subsets. However, whether this also translates into higher expression of membrane receptors and functional responses remains to be demonstrated. Similar results were recently published for S1P receptor expression on mouse spleen CD4⁺ and CD8⁺ T cells, which express predominantly S1P₁ and S1P₄ with only minimal representation of S1P₂, S1P₃, and S1P₅ (24). Moreover, the same study demonstrated that TCR-mediated activation of CD4⁺ T cells suppresses the expression of S1P₁ and S1P₄ mRNA and eliminates their chemotactic response to S1P. FTY720 had no direct chemotactic activity but competitively inhibited the T cell chemotactic response to S1P in vitro and in vivo. These data contradict other reports that demonstrated that phosphorylated FTY720 mimics S1P as an S1P receptor agonist and lymph node homing factor for lymphocytes (16, 17). However, there is currently no explanation for these controversial findings, and more detailed investigations are required to answer this question.

We could demonstrate that FTY720 equally inhibits both Th1-induced neutrophilic and Th2-induced eosinophilic inflammation, raising the question whether the compound directly interferes with the infiltration of neutrophils and eosinophils. It has been demonstrated that administration of FTY720 and S1P produced rapid peripheral blood lymphopenia in mice and rats, affecting T cells (both CD4⁺ and CD8⁺ T cells) and B cells, whereas other leukocyte subpopulations such as monocytes and granulocytes were unaltered (8–11, 16). Similar data were found in our studies; it is therefore very unlikely that the inhibition of the neutrophil and eosinophil infiltration is linked to a direct inhibitory effect of FTY720 on these two proinflammatory cell types. Moreover, the reduction of both cell populations was less pronounced than the suppression of infiltrating lymphocytes. Because it has been shown that FTY720 does not impair activation, expansion, and memory function of T cells and its main function is to effectively reduce the recirculation of effector T cells and their recruitment to peripheral lesions (12, 13), the difference in the inhibitory capacity of FTY720 on airway-infiltrating lymphocytes opposed to neutrophils or eosinophils is best explained by reducing the threshold levels of neutrophil- or eosinophil-active cytokines and/or chemoattractants as a result of the reduced numbers of Ag-specific T cells present in the lung. A recent study examined the effect of FTY720 on T cell activation and expansion in the draining lymph nodes and subsequent release of activated T cells to the peripheral blood compartment in a local Ag-challenged mouse model (19). Similar to our data, the number of Ag-activated CD4⁺ T cells in the draining lymph nodes was significantly reduced after FTY720 treatment. However, T cells responded and proliferated to the Ag stimulus in both nontreated and treated animals to a similar extent, suggesting that the reduced efficiency of T cell responses in the draining lymph nodes to a local Ag is due to a defective recirculation of Ag-specific T cells caused by FTY720 treatment which subsequently prevents activated T cells from homing to the lung. In contrast, we cannot rule out the possibility that other mechanisms, such as altering migration through endothelial cells, may play a role in the reduced tissue infiltration of eosinophils and

neutrophils. S1P receptors are also expressed on endothelial cells and activation of these receptors have been shown to alter junctional properties of endothelium, which may subsequently affect the tissue infiltration of various proinflammatory cells (25).

Of particular interest for us with regard to identifying novel antiasthma treatments was the finding that Th2 cell-mediated inflammatory responses were also affected by treatment with FTY720. Allergic asthma is a chronic inflammatory disease of the airways, characterized by reversible airway obstruction and airway hyperresponsiveness (26). The inflammatory response in asthma is associated with a characteristic infiltration of eosinophils and T lymphocytes into the airway submucosa (27). There is strong evidence based on experimental animal models as well as clinical studies that allergen-specific Th2 lymphocytes and their products are the central mediators of asthma (28–31). Th2 cytokines can account directly or indirectly for the majority of pathophysiological manifestations. For example, IL-4 and IL-13 are the switch factors for B cell production of IgE; IL-5 is a growth and differentiation factor for eosinophils (32, 33). The present study clearly demonstrates that FTY720 not only inhibits the Th2 cell-mediated eosinophilic inflammation but is also active in a relevant disease model using active sensitization and challenge to allergen, which besides allergen-specific Th2 cells also involves allergen-specific IgE and subsequent allergen-mediated mast cell degranulation events. Moreover, FTY720 inhibited the infiltration of both Th2 cells and eosinophils into bronchial tissue, reduced the levels of Th2-related cytokines and goblet cell hyperplasia, and almost completely blocked the hyperresponsiveness to metacholine, all characteristic features of clinical asthma, suggesting that FTY720 might have therapeutic benefits in this disease.

In contrast, recent findings point toward a pathophysiological role of S1P in bronchial inflammation and airway remodeling in patients with asthma, suggesting that S1P antagonists rather than agonists might be of therapeutic value in this disease (34, 35). S1P levels are strongly enhanced in the airways of asthmatics but not control patients after segmental allergen challenge (36). In mast cells, FcεRI cross-linking leads to activation of sphingosine kinase, and conversion of sphingosine to S1P and S1P is able to mobilize calcium and induce degranulation in these cells (37, 38). Moreover, S1P was shown to stimulate cell growth and airway hypercontractility and to modulate secretion of cytokines such as IL-6 and RANTES in airway smooth muscle cells (36). Similarly, S1P treatment of mature dendritic cells altered the cytokine release in these cells which was associated with a Th1 to Th2 switch of in vitro primed T cell responses (39). Thus, the capacity of S1P to activate mast cells, airway smooth muscle cells, and dendritic cells in addition to its effect on lymphocyte infiltration implies S1P to augment and not to inhibit the allergic inflammation on a broad scale. This apparent discrepancy might be explained by the distribution and G-protein-coupled receptor subclass of FTY720-targeted S1P receptors on different cell types. For example, the activation of S1P₂ appears to be responsible for the induction of degranulation in mast cells in a pertussis toxin-insensitive manner (34). However, FTY720 does not activate S1P₂ and therefore is not expected to directly induce mast cell degranulation. Moreover, the concentration of S1P receptor agonists and the affinity for the different receptor subtypes in relation to the concentration of sphingosine might be an important factor that determines the functional response in various cell types. It is for example well documented that the dynamic balance between intracellular S1P vs sphingosine and ceramide and the consequent regulation of opposing signaling pathways is an important factor that determines the functional response to S1P receptor activation (37, 38). Preliminary results indeed indicate that the inhibitory effect of FTY720 on the Th2-

mediated eosinophilic inflammation was lost by increasing the concentration of the drug by >10-fold over the highest concentration used in our study (E. Sawicka, unpublished observation). Similarly, S1P has been demonstrated to both stimulate and inhibit chemotactic responses in different cell types dependent on the concentrations used (40).

In conclusion, the present study provides a first insight into how treatment with the S1P agonist FTY720 might therapeutically affect Th1 or Th2 cell-mediated airway inflammation, and more specifically, Th2-driven inflammatory responses as seen in diseases such as asthma. However, further studies are required to find out whether the immunosuppressive effect of FTY720 is due solely to the sequestration of lymphocytes or whether it interferes with subsequent lymphocyte activation pathways. Additional chemical entities and genetically modified mice lacking one or more S1P receptor genes or sphingosine kinase genes are needed to define the FTY720 target and the functional consequences in various disease models more precisely.

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