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The Sphingosine 1-Phosphate Receptor Agonist FTY720 Differentially Affects the Sequestration of CD4$^+$/CD25$^+$ T-Regulatory Cells and Enhances Their Functional Activity

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The sphingosine 1-phosphate (S1P) receptor agonist FTY720 is well known for its immunomodulatory activity, sequestering lymphocytes from blood and spleen into secondary lymphoid organs and thereby preventing their migration to sites of inflammation. Because inflammation is critically dependent on a balance between Ag-specific Th/effector cells and T-regulatory cells, we investigated the effect of FTY720 on T-regulatory cell trafficking and functional activity. An increased number of CD4$^+$/CD25$^+$ T cells was found in blood and spleens of FTY720-treated mice, and transfer of these cells resulted in a significantly more pronounced accumulation in spleens but not lymph nodes after treatment, suggesting that this compound differentially affects the homing properties of T-regulatory cells compared with other T cell subsets. Indeed, CD4$^+$/CD25$^+$ T cells express lower levels of S1P$_1$ and S1P$_4$ receptors and demonstrate a reduced chemotactic response to S1P. Moreover, analysis of the functional response of FTY720-treated CD4$^+$/CD25$^+$ T cells revealed an increased suppressive activity in an in vitro Ag-specific proliferation assay. This correlated with enhanced function in vivo, with T-regulatory cells obtained from FTY720-treated mice being able to suppress OVA-induced airway inflammation. Thus, FTY720 differentially affects the sequestration of T-regulatory cells and importantly, increases the functional activity of T-regulatory cells, suggesting that it may have disease-modifying potential in inflammatory disorders. ***The Journal of Immunology,*** **2005, 175: 7973–7980.**

The novel immunomodulator FTY720, a chemical derivative of myriocin, shares structural similarities with sphingosine 1-phosphate (S1P),[2] a natural lysophospholipid known to regulate a wide variety of fundamental functions including cell survival, cytoskeletal rearrangements, and cell motility (1, 2). FTY720 becomes phosphorylated by sphingosine kinases and the phosphorylated form (FTY-P) acts as an agonist on four of the five known S1P receptors, S1P$_1$, S1P$_3$, S1P$_4$, and S1P$_5$ (3, 4). In contrast to conventional immunosuppressants such as cyclosporine A or FK506, FTY720 does not impair T and B cell activation, proliferation, and effector function but interferes with cell trafficking between lymphoid organs and blood (5, 6). Currently two models are being proposed to explain the control of lymphocyte trafficking by FTY720 (7). One model proposes that FTY720 induces changes in the permeability of the endothelium of blood vessels and lymphoid vascular sinuses that result in altered egression of lymphocytes from lymph nodes. However, the more popular hypothesis is that site-specific suppression of S1P-mediated lymphocyte chemotaxis is the principle mechanism responsible for the reduced egression of lymphocytes from lymph nodes. Indeed, a recent study has demonstrated that FTY-P induces internalization of S1P$_1$ on lymphocytes and, therefore, abrogates S1P/S1P$_1$-dependent egression of these cells from lymphoid organs (8). This is supported by findings that S1P$_1$-deficient thymocytes do not egress from the thymus and that labeled S1P1-deficient T cells that are adoptively transferred to irradiated wild-type mice show strikingly diminished egression from the secondary lymphoid organs (8, 9). Thus, treatment with FTY720 results in selective and reversible sequestration of lymphocytes from blood and spleen into secondary lymphoid organs, thereby preventing the migration of inflammatory cells toward sites of inflammation and allograft rejection (10–12). Indeed, the drug is highly effective in experimental models of transplantation and autoimmunity and has recently been shown to be effective in human kidney transplantation (13–16). Moreover, our own studies using murine models of asthma demonstrated that FTY720 potently inhibits Th1- and Th2-mediated airway inflammation (17). The OVA-induced Th1-mediated airway inflammation characterized by increased numbers of lymphocytes and neutrophils in bronchoalveolar lavage (BAL) fluid was significantly inhibited by oral FTY720 treatment. Similarly, FTY720 also suppressed the Th2 cell-induced BAL fluid eosinophilia and the infiltration of T lymphocytes and eosinophils into the bronchial tissue. Furthermore, the Ag-induced bronchial hyperresponsiveness to inhaled methacholine was almost completely blocked, clearly indicating that Th2-cell driven allergic diseases such as asthma could benefit from such treatment.

We and others have demonstrated that the extent of airway inflammation critically depends on a balance between Ag-specific Th/effector and T-regulatory cells (18, 19). In addition, multiple studies have also shown that CD4$^+$/CD25$^+$ T-regulatory cells play a critical role in the maintenance of self tolerance, control of autoimmune diseases, transplant rejection, and cancer (20, 21). These

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[2] Abbreviations used in this paper: S1P, sphingosine 1-phosphate; FTY-P, phosphorylated FTY720; BAL, bronchoalveolar lavage.

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$CD4^+/CD25^+$ T-regulatory cells are typically anergic, unresponsive to TCR stimulation alone, and suppress the proliferation and activation of other $CD4^+$ or $CD8^+$ T cells either via cell-cell contact-dependent mechanisms or the production of suppressor cytokines such as IL-10 and/or TGF-β (20). Interestingly, a very recent study has demonstrated that S1P appears to be required for optimal suppression of effector T cell activities by $CD4^+/CD25^+$ T-regulatory cells, suggesting that FTY720 may not only affect the traffic of mature lymphocytes but may also interfere with the function of T-regulatory cells (22). Therefore, we investigated the effect of FTY720 on the sequestration and activity of $CD4^+/CD25^+$ T-regulatory cells. The results presented in this study clearly demonstrate that FTY720 differentially affects the recirculation of $CD4^+/CD25^+$ T-regulatory cells and alters their functional activity and thus may have long-term disease modifying potential in inflammatory diseases such as asthma.

Materials and Methods

Animals

C57BL/6 mice (5–8 wk old) were obtained from Charles River Laboratories. All experimental 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.

Compounds and cell culture medium

FTY720, obtained from Novartis Pharmaceuticals, was dissolved in distilled water containing 10% of neoral placebo as vehicle. A dose of 0.1 mg/kg was administered by gavage in a volume of 200 μl per mouse. Control mice were given vehicle alone.

Two chiral analogs of phosphorylated FTY720, the biologically active S-enantiomer AML629 and the R-enantiomer AML627 (at least 100-fold less active as agonist on S1P receptors) were obtained from Novartis Pharmaceuticals. Stock solutions were prepared at a 10-mM concentration. Both compounds were dissolved in acidified DMSO (DMSO/50 nM HCl) and used at a final concentration of 1 μM.

Cells were incubated in RPMI 1640 supplemented with L-glutamine, 10% heat-inactivated FBS, 1% nonessential amino acids mixture, 5×10^{-5} M 2-ME, 1×10^{-3} M sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Invitrogen Life Technologies).

Purification of $CD4^+/CD25^+$ T-regulatory cells

$CD4^+$, $CD4^+/CD25^+$, or $CD4^+/CD25^-$ T cells were isolated from the spleens or lymph nodes by magnetic cell sorting (MACS; Miltenyi Biotec) according to the manufacturer's instruction. The resulting cell population was >95% pure as determined by FACS analysis. To obtain highly purified populations of $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells for RT-PCR analysis, $CD4^+$ cells were first isolated from spleens of C57BL/6 mice using MACS system. The $CD4^+$ fraction was then stained with rat anti-mouse CD25-PE mAb (7D4) (BD Pharmingen) and sorted by flow cytometry (MoFlo; Cytomation) into $CD25^+$ and $CD25^-$ fractions.

Proliferation and migration assay

$CD4^+/CD25^-$ and $CD4^+/CD25^+$ were isolated from spleens of OVA-immunized mice. $CD4^+/CD25^-$ or $CD4^+/CD25^+$ cells were stimulated with OVA (100 μg/ml) at 1×10^4 cells per well in the presence of T cell-depleted, mitomycin C (25 μg/ml for 20 min at 37°C)-treated APCs at 1:1 ratio in 96-well round-bottom plates. Where indicated, $CD4^+/CD25^+$ cells were titrated with $CD4^+/CD25^-$ cells at 1:2, 1:4, or 1:8 ratio and 1 μM of FTY-P or vehicle was added. In some experiments, blocking anti CTLA-4 (clone no. 63828) (R&D Systems) or ICOS (Insight Biotechnology) Abs were added at 25 μg/ml for the duration of the assay. Proliferation was assessed after 5 days by addition of 1 μCi of [^{3}H]thymidine for a further 6 h before harvesting and liquid scintillation counting (Topcount NXT; Packard Bioscience).

Cytokine levels were assessed in duplicate culture after 48 h using commercially available ELISA kits (R&D Systems).

The response of $CD4^+/CD25^+$ and $CD4^+/CD25^-$ toward S1P was studied using 24 Transwell plates with a 3-μm pore size (BD Bioscience). The assay was performed using RPMI 1640 with 1% fatty acid-free BSA (Sigma-Aldrich). The lower chamber was loaded with 600 μl of medium and S1P (Sigma-Aldrich) or medium alone as a control. Upper chambers were loaded with 0.5 ml of $CD4^+/CD25^+$ or $CD4^+/CD25^-$ cells at a concentration of 1×10^6/ml. The assay was performed at 37°C for 3 h. Cells that migrated to the lower chamber were enumerated by collecting events for a fixed time (30 s) on a FACSCalibur (BD Bioscience) flow cytometer.

RNA isolation, cDNA preparation, and real-time quantitative PCR

To obtain highly purified populations of $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells for RT-PCR analysis, $CD4^+$ cells were first isolated from spleens of C57BL/6 mice using MACS system. The $CD4^+$ fraction was then sorted by flow cytometry (MoFlo; Cytomation) into $CD25^+$ and $CD25^-$ fractions. These T cell subsets were lysed in TRIzol (Invitrogen Life Technologies) at 1–5 $\times 10^6$ cells/ml and total RNA was isolated according to the recommendation of the manufacturer. Total RNA was resuspended in RNase-free water and further purified using RNeasy Mini columns (Qiagen) including a DNase I-digestion on the column to remove any genomic contamination. RNA was eluted in water and stored at −80°C. Concentration and quality of total RNA was assessed using the RNA 6,000 Nano Assay (Agilent) on an Agilent 2100 bioanalyzer, and all samples were tested by PCR for the complete removal of genomic DNA. To generate a template for real-time PCR, first-strand cDNA was synthesized using the TaqMan reverse transcription reagents and random hexamer primers from Applied Biosystems in a two-step RT-PCR at 20 ng/μl total RNA. Synthesis was performed in a Biometra T3 Thermocycler at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Control PCR were then performed using GAPDH to confirm cDNA synthesis. Gene-specific primers and probes were obtained as Assay-on-Demand reagents from Applied Biosystems for FoxP3 (Mm00475156_m1), $S1P_1$ (Mm00514644_m1), $S1P_3$ (Mm00515669_m1), $S1P_4$ (Mm00468695_m1), and $S1P_5$ (Mm00474763_m1). The $S1P_2$ primers and probes were designed using the Primer Express software (Applied Biosystems). The sequence of the $S1P_2$ primers and probe were as follows: forward 5′-TTGCAGTGGCCAG GAACA, reverse 5′-CAGGTTGCCAAGGAACAGGTA, probe 5′-CAAGTTCCACTCAGCAAT.

Quantitative RT-PCR were performed in triplicate in 25-μl final volumes and contained final concentrations of 1× TaqMan Universal PCR master mix containing AmpliTaq Gold DNA Polymerase (Applied Biosystems) with 20 ng of target cDNA. The primer concentrations were 900 nM for the forward and reverse primers and 250 nM for the labeled probe. All probes for the target genes were labeled with the reporter dye FAM and the internal control gene GAPDH with the reporter dye VIC (Applied Biosystems). Experiments were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) and analyzed using ABI Prism 7700 Sequence Detection System software. Amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves were generated for each target and the internal control gene using serial dilutions of cDNA prepared from mouse reference RNA (Clontech) and data for the experimental samples expressed as the percentage of the internal control gene.

Flow cytometry

To determine the percentage of $CD25^+$ or $CD62L^+$ lymphocytes in spleens and lymph nodes of FTY720-treated and untreated mice, leukocytes were stained with fluorochrome-conjugated rat anti-mouse CD25 mAb (7D4) or rat anti-mouse CD62L (MEL-14) (BD Pharmingen). Isotype-matched mAb were used as negative controls. Flow cytometry was conducted with a FACSCalibur (BD Bioscience) and the data were analyzed with CellQuest software (BD Bioscience).

OVA-sensitization and challenge model

C57BL/6 mice were immunized i.p. on days 0 and 14 with 10 μg of chicken OVA (Grade V; Sigma-Aldrich) in 0.2 ml of alum (Serva). On day 21, animals were exposed for 20 min to an aerosol of OVA in sterile PBS (50 mg/ml) or PBS alone. At the specified time point, BAL was collected by injection of 0.4 ml of PBS three times into the lung. Total cell counts were determined and cytospin preparation (Shandon Scientific) was performed. Cells were stained with DiffQuick (Baxter Dade) and a differential count of 200 cells performed using standard morphological criteria.

CFSE cell labeling and tracking

$CD4^+/CD25^+$ and $CD4^+/CD25^-$ cells were resuspended at 5×10^7/ml in PBS. CFSE (Molecular Probes) was added to a final concentration of 0.5 μM, and the suspension was incubated at 37°C for 10 min. At the end of the incubation period, the cells were immediately washed three times in cold RPMI 1640/10% FCS. CFSE-labeled cells were transferred i.v. into recipients at 5×10^6 cells per mouse. One hour later, animals were treated

with FTY720 (0.1 mg/kg) or vehicle alone. The percentage of fluorescent cells in spleens and lymph nodes were analyzed by flow cytometry.

Adoptive transfer of T-regulatory cells

Donor C57BL/6 mice were treated with 0.1 mg/kg of FTY720 or vehicle on 3 consecutive days. Spleens were collected 24 h after the last treatment for isolation of $CD4^+/CD25^+$ T-regulatory cells, which were then transferred into immunized recipients 24 h before Ag challenge. In some experiments, T-regulatory cells were isolated from naive untreated mice and preincubated in vitro with 1 μM of active (AML629) or inactive (AML627) enantiomer of FTY-P for 16 h before adoptive transfer. BAL fluid eosinophils and lymphocytes were determined 48 h after the Ag challenge.

Statistical analysis

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

Results

Increased numbers of $CD4^+/CD25^+$ lymphocytes in blood and spleens after treatment of naive mice with FTY720

FTY720 acts by selectively and reversibly sequestering lymphocytes from blood and spleen into lymph nodes and Peyer's patches (8, 10, 21). However, multiple studies have demonstrated that the depletion of lymphocytes from the circulation is not complete, suggesting the presence of a T cell subpopulation that is resistant to FTY720 (23, 24). Because little is known about the effect of FTY720 on the sequestration of $CD4^+/CD25^+$ T-regulatory cells, the distribution of this lymphocyte subpopulation in blood and spleen after treatment with FTY720 was investigated. Various doses of FTY720 were orally administered to C57BL/6 mice on three consecutive days. Blood, spleens, and lymph nodes were collected 24 h after the last treatment. As expected, treatment with FTY720 results in a dose-dependent reduction of blood lymphocytes, reaching maximal inhibition at a concentration of 0.1 mg/kg (Fig. 1*a*). The remaining blood lymphocytes were then analyzed by flow cytometry for the presence of $CD4^+/CD25^+$ T cells. As shown in Fig. 1*b*, a significantly higher relative proportion of $CD4^+/CD25^+$ T cells was found after treatment with FTY720 as compared with vehicle-treated mice, again reaching maximal levels at 0.1 mg/kg. Similarly increased numbers of $CD4^+/CD25^+$ T cells were also found in spleens (Fig. 1*c*) but not lymph nodes (data not shown) in the FTY720-treated group of mice. These data suggest that the compound may differentially affect the sequestration of at least a subpopulation of $CD4^+/CD25^+$ T-regulatory cells. Indeed, it recently has been shown that FTY720 differentially affects the lymph node sequestration of T-regulatory cells lacking the lymph node homing receptor CD62L (25). Therefore, the residual T-regulatory cells obtained from spleens of FTY720-treated mice were analyzed for their expression of CD62L. As shown in Fig. 2, a significantly lower proportion of CD62L expressing T-regulatory cells were found in spleens of FTY720-treated mice,

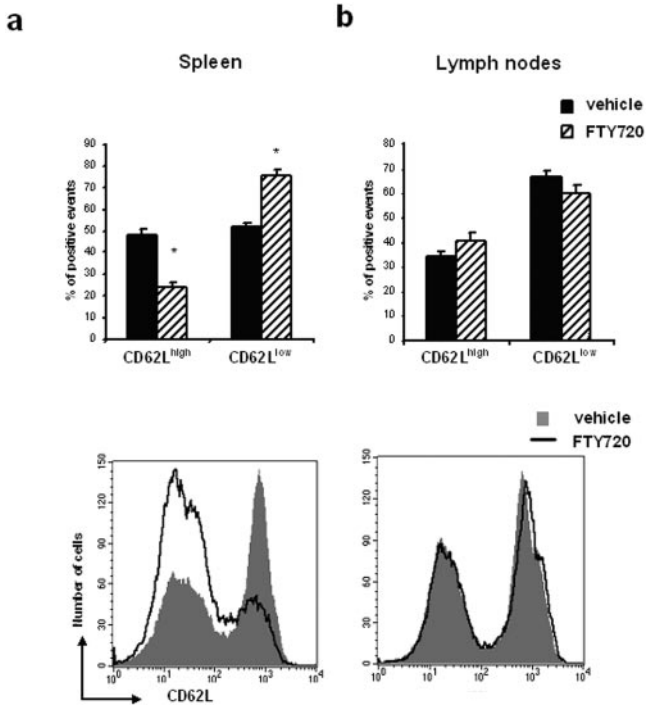

FIGURE 2. FTY720 differentially affects homing of $CD4^+CD25^+/CD62L^{low}$ T cells to spleens and lymph nodes. C57BL/6 mice were treated with FTY720 or vehicle as described in Fig. 1. $CD4^+$ cells were isolated from the spleens (*a*) and lymph nodes (*b*) and analyzed for the expression of CD25 and CD62L by flow cytometry. *c*, Representative histograms of cells isolated from spleens and lymph nodes of vehicle or FTY720-treated mice showing the change in ratio of CD62L low/high cells after FTY720 treatment. Data are mean ± SEM, $n = 8$ mice in each group; *, $p < 0.05$.

clearly indicating that S1P receptor agonists differentially affect the homing of a subset of T-regulatory cells into lymphoid tissues.

CFSE-labeled $CD4^+/CD25^+$ T cells preferentially accumulate in spleens but not lymph nodes after FTY720 treatment

To answer the question of whether the increase in $CD4^+/CD25^+$ T cells in the spleens of FTY720-treated animals was indeed due to the difference in the response of these cells to FTY720 and not a result of T cell activation, a cell tracking experiment with labeled cells was performed. For that purpose, $CD4^+/CD25^+$ and $CD4^+/CD25^-$ cells were isolated from naive, untreated C57BL/6 mice, labeled with CFSE, and transferred i.v. (5×10^6 per mouse) into another group of naive recipient mice. Mice were then treated with a single dose of FTY720 (0.1 mg/kg) or vehicle. Twenty-four hours later, spleens and lymph nodes were removed and $CD4^+$ T cells were isolated and analyzed for the presence of CFSE-positive cells by flow cytometry. A significant increase of CFSE-positive

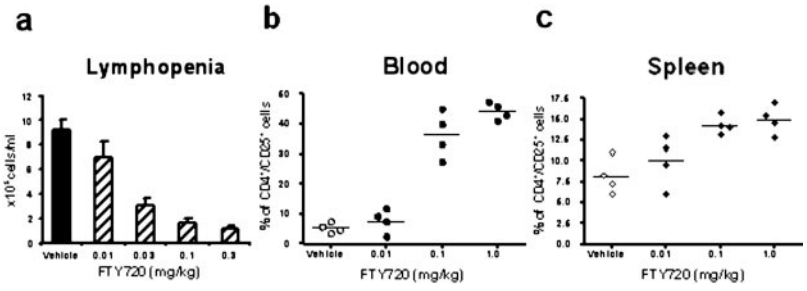

FIGURE 1. Increase of $CD4^+/CD25^+$ T cells in blood and spleens of FTY720-treated mice. C57BL/6 mice were treated with increasing doses of FTY720 (0.01–1 mg/kg) or vehicle on 3 consecutive days. *a*, Total blood lymphocyte count (mean ± SEM, $n = 8$ mice/group). Blood (*b*) and spleen (*c*) $CD4^+/CD25^+$ cells 24 h after the last treatment analyzed by flow cytometry. Data are shown as the percentage of $CD4^+/CD25^+$ cells from four individual animals.

$CD4^+/CD25^+$ T cells was found in spleens after FTY720 treatment as compared with vehicle-treated mice (Fig. 3). In contrast, a significantly lower number of CFSE-labeled $CD4^+/CD25^+$ T cells was found in lymph nodes of FTY720-treated mice. These data are in clear contrast to the expected and well-established observation that naive $CD4^+$ T lymphocytes accumulate within lymph nodes after FTY720 treatment and clearly indicate a different responsiveness of $CD4^+/CD25^+$ T cells to this drug. Indeed, significantly increased numbers of CFSE-labeled $CD4^+/CD25^-$ T cells were found in lymph nodes, whereas the numbers of $CD4^+/CD25^+$ T cells were significantly reduced (Fig. 3).

Differential expression of S1P receptors and functional response to S1P between $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells

To analyze whether the divergent effects of FTY720 on $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells could be a consequence of different expression pattern of S1P receptors and the functional response of these two lymphocyte subsets to S1P, highly purified, FACS-sorted $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells obtained from spleens of naive C57BL/6 mice were analyzed for their distribution of S1P receptors by quantitative RT-PCR. Both cell populations express mRNA for all five S1P receptors (Fig. 4*a*). Moreover, the levels of $S1P_1$ and $S1P_4$ were significantly higher compared with the other three receptors. Interestingly, $CD4^+/CD25^+$ T cells have only ~50% of the expression of $S1P_1$ and $S1P_4$ mRNA compared with $CD4^+/CD25^-$ T cells. In contrast, $CD4^+/CD25^+$ T cells contain higher levels of $S1P_3$ mRNA. No quantitative differences were detectable in the expression of $S1P_2$ and $S1P_5$ mRNA. Treatment of mice with FTY720 before isolation of $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells resulted in an ~50% reduction in the expression of $S1P_1$ and $S1P_4$ in both T cell subsets (Fig. 4*b*). In contrast, the mRNA levels of $S1P_2$, $S1P_3$, and $S1P_5$ were not significantly affected by the treatment.

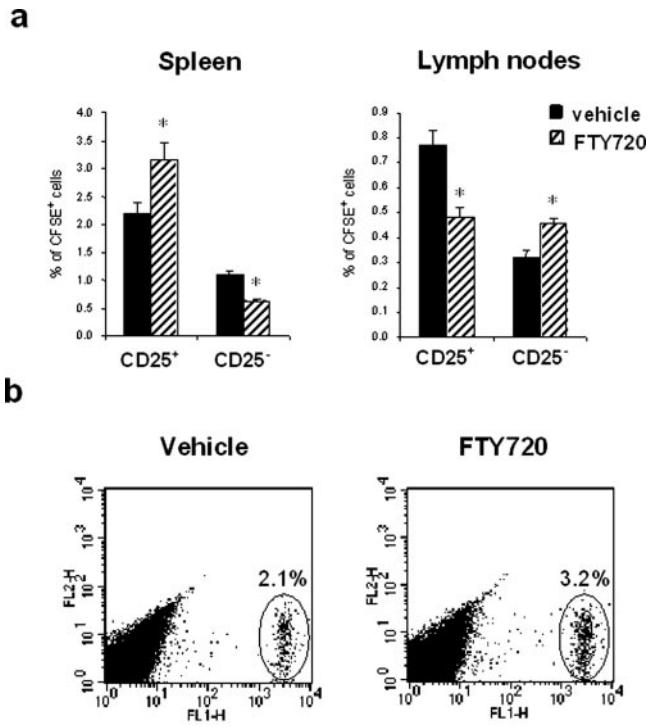

FIGURE 3. FTY720 differently affects the distribution of $CD4^+/CD25^+$ T-regulatory cells. $CD4^+/CD25^+$ and $CD4^+/CD25^-$ cells were isolated from spleens of naive C57BL/6 mice, labeled with CFSE, and injected i.v. at 5×10^6 into recipient mice, which then received a single dose of FTY720 (0.1 mg/kg; □) or vehicle alone (■). $CD4^+$ T cells were isolated from spleens and lymph nodes 24 h posttreatment and analyzed by FACS. *a*, Quantitative analysis of CFSE-positive cells in spleens and lymph nodes of the recipient mice. *b*, Representative FACS dot plot from the spleen sample of $CD4^+/CD25^+$ recipient mouse. Data are mean ± SEM, $n = 6$ mice in each group; ∗, $p < 0.05$.

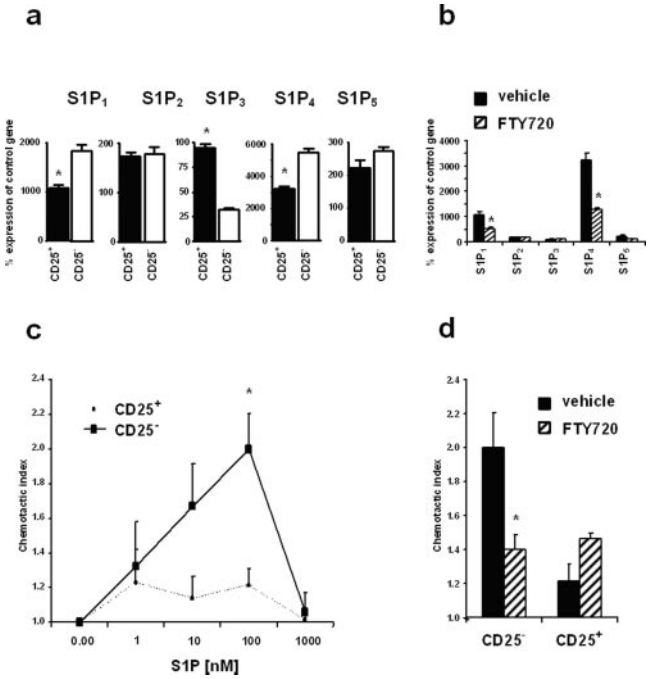

FIGURE 4. $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells express different levels of mRNA for S1P receptors and respond differently to S1P. Quantitative RT-PCR for $S1P_{1-5}$ genes from freshly isolated $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells from spleens of naive mice (*a*), $CD4^+/CD25^+$ cells isolated from spleens of FTY720 (0.1 mg/kg) of vehicle-treated C57BL/6 mice (*b*). Data are presented as average percentage of expression of control gene GAPDH. *c*, Chemotactic response of freshly isolated $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells to S1P. *d*, Chemotaxis of $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells from vehicle or FTY720-treated mice at the optimum concentration of S1P (100 nM). Data are representative of three independent experiments; ∗, $p < 0.05$.

These data indicate that $CD4^+/CD25^+$ T cells may express lower levels of $S1P_1$ and $S1P_4$ receptors, which may alter their functional response to S1P. Therefore, the chemotactic response of $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells to S1P was analyzed. As shown in Fig. 4*c*, both T cell subsets migrate toward S1P gradients. Moreover, consistent with the lower mRNA expression of $S1P_1$ and $S1P_4$ receptors, a significantly lower response was found within the $CD4^+/CD25^+$ T cell subset. In contrast, no difference in the chemotactic response to stromal cell-derived factor 1 (3 ng/ml) was observed between the two subsets (data not shown).

To further investigate this observation, cells were also analyzed for their chemotactic response to S1P after FTY720 treatment. Again, consistent with the reduced mRNA expression of $S1P_1$ and $S1P_4$ receptors after FTY720 treatment, $CD4^+/CD25^-$ T cells showed a significantly lower chemotactic response to S1P (Fig. 4*d*). In contrast, no significant differences were found in the $CD4^+/CD25^+$ T cell subset, despite the fact that a further reduction of mRNA for $S1P_1$ and $S1P_4$ receptors were found in these cells after FTY720 treatment. Thus, these data indicate that $CD4^+/CD25^+$ T cells are less responsive to S1P and that this altered response may explain the differences seen in sequestration of this T cell subset after FTY720 treatment in vivo.

$CD4^+/CD25^+$ T-regulatory cells from FTY720-treated mice inhibit allergic airway inflammation

Next, we sought to analyze whether these $CD4^+/CD25^+$ T cells indeed exert T-regulatory cell activity. To address this matter, $CD4^+/CD25^+$ T cells were obtained from spleens of naive mice that were pretreated either with vehicle or 0.1 mg/kg FTY720 on three consecutive days. These cells were then injected i.v. at 5.0×10^5 per mouse (optimal cell number, as determined by preliminary experiments) into actively OVA-sensitized C57BL/6 mice 24 h before the aerosolized allergen exposure. As presented in our previous studies, the allergen challenge induces a characteristic eosinophilic airway inflammation with significantly increased numbers of BAL fluid eosinophils, neutrophils, and lymphocytes (26). Although transfer of $CD4^+/CD25^+$ cells from vehicle-treated mice had no effect on OVA-induced airway inflammation, $CD4^+/CD25^+$ cells from FTY720-treated animals inhibited the infiltration of both eosinophils and lymphocytes into the airway in a dose-dependent manner (Fig. 5). Thus, $CD4^+/CD25^+$ T cells obtained from spleens of FTY720 pretreated donor mice contain either more T-regulatory cells, or the T-regulatory cells are more activated and, therefore, capable of inhibiting an allergen-induced airway inflammation.

FTY720 increased the T-regulatory cell activity in vitro

To further investigate the observation that FTY720 not only plays a role in enrichment of T-regulatory cells in the spleen but also alters their functional activity, a classical in vitro proliferation assay was performed. $CD4^+/CD25^+$ and $CD4^+/CD25^-$T cells isolated from spleens of OVA-sensitized C57BL/6 mice were stimulated in vitro with OVA for 5 days in the presence or absence of FTY720. $CD4^+/CD25^+$ T cells did not proliferate in response to the specific antigenic stimuli, whereas a high proliferation rate was observed with $CD4^+/CD25^-$ T cells (Fig. 6*a*). The addition of FTY720 (up to 1 μM) to $CD4^+/CD25^-$ T cells had no significant effect on the proliferative capacity of these cells (data not shown). As expected, addition of increasing numbers of $CD4^+/CD25^+$ T cells from OVA-sensitized mice to the $CD4^+/CD25^-$ T cell population from the same mice lead to a dose-dependent inhibition of the proliferative response (Fig. 6*a*). More importantly, the presence of FTY720 in the cultures resulted in a significantly more pronounced inhibition as compared with vehicle-treated cells. These results were further supported by findings that the T-regulatory cell-specific transcription factor FoxP3 (27, 28) was considerably up-regulated in $CD4^+/CD25^+$ T cells obtained from FTY720-treated mice as compared with the vehicle-treated control group (Fig. 6*b*). In contrast, no significant expression of FoxP3 was found in $CD4^+/CD25^-$ cells.

Similarly, the production of cytokines such as IL-4 and IFN-γ were significantly more inhibited in the presence of FTY720 (Fig. 6*c*). In contrast, no significant effect of FTY720 on the production of IL-10 or TGF-β could be measured, suggesting that the inhibition of the proliferative response was not mediated by these two cytokines. Indeed, the T-regulatory cell-mediated inhibition of proliferation was completely lost by using a Transwell culture system, suggesting that the suppressive mechanisms is cell-cell contact dependent (data not shown). To further investigate the potential mechanisms responsible for the suppressive effect, cell cultures were performed in the presence of blocking Abs against CTLA4 or ICOS, two surface molecules associated with suppressive activity of T-regulatory cells (29–30). As shown in Table I, the addition of blocking CTLA4 Abs to the cultures almost completely reversed the FTY720-induced increase in T-regulatory cell activity. In contrast, no significant effect could be demonstrated using blocking Abs against ICOS (data not shown). Taken together, these results clearly indicate that FTY720 alters the functional activity of T-regulatory cells by a cell-cell contact-dependent mechanism.

Inhibition of allergen-induced airway inflammation by in vitro FTY720 pretreatment of $CD4^+/CD25^+$ T cells

To further investigate the significance of the above in vitro finding in relation to controlling an inflammatory response, FTY720 pretreated $CD4^+/CD25^+$ splenocytes from naive mice were adoptively transferred into OVA-sensitized recipients before airway exposure to the allergen. $CD4^+/CD25^+$ T cells were preincubated for 16 h with 1 μM of two chiral analogs of phosphorylated FTY720, the active S-enantiomer AML629, or the inactive R-enantiomer AML627. At the end of this incubation period, cells were washed and injected i.v. into OVA-sensitized recipient animals before aerosol OVA challenge. The airway inflammation was assessed 48 h postchallenge. There was no difference in the magnitude of the eosinophilic airway inflammation between the positive control group and the group of mice receiving $CD4^+/CD25^+$ cells pretreated with the inactive enantiomer AML627 (Fig. 7). In contrast, the OVA-induced inflammatory response was significantly inhibited in the group of animals treated with $CD4^+/CD25^+$ cells preincubated with the active enantiomer AML629, confirming the increased suppressive capacity of FTY720-treated T-regulatory cells as shown in the in vitro experiments (Fig. 6). Taken together,

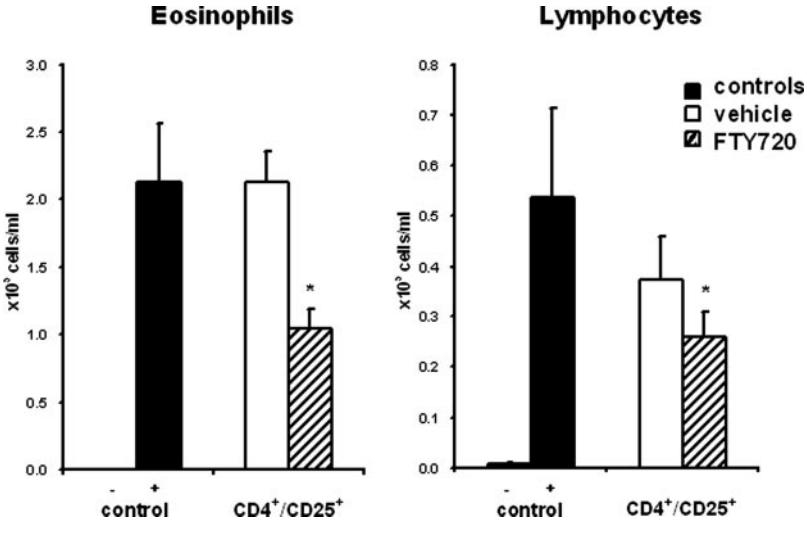

FIGURE 5. $CD4^+/CD25^+$ T-regulatory cells from FTY720-treated mice inhibit allergic airway inflammation. C57BL/6 mice were treated with FTY720 or vehicle as described in Fig. 1. $CD4^+/CD25^+$ T-regulatory cells were isolated from the spleens and transferred into immunized recipients 24 h before Ag challenge at optimum numbers (5×10^5 cells per mouse). BAL fluid eosinophils and lymphocytes were determined 48 h after the Ag challenge. Data are mean ± SEM, $n = 8$ mice in each group; *, $p < 0.05$.

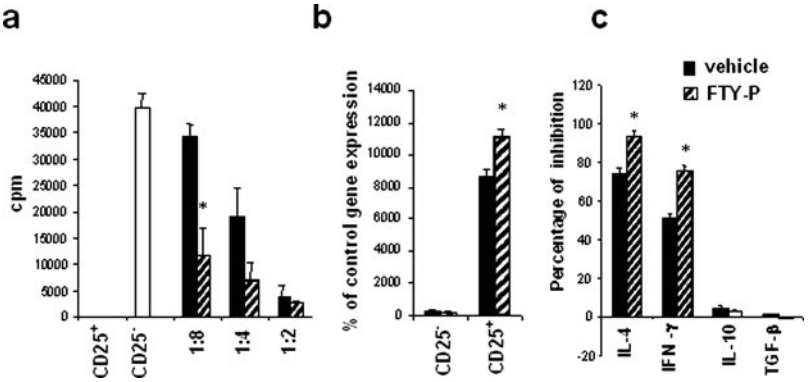

FIGURE 6. $CD4^+/CD25^+$ T cells inhibit proliferation and cytokine production more potently and express increased levels of FoxP3 in the presence of FTY720. *a*, Proliferation assay of $CD4^+/CD25^-$ and $CD4^+/CD25^+$ T cells, isolated from spleens of OVA-immunized recipient mice. Both cell populations were cultured either alone or in 1:2, 1:4, and 1:8 ratio of $CD4^+/CD25^+$ to $CD4^+/CD25^-$ cells in the presence of OVA (100 μg/ml) and APCs. Proliferation was assessed after 5 days by addition of 1 μCi of [^{3}H]thymidine for a further 6 h. *b*, Quantitative RT-PCR for FoxP3 gene. $CD4^+/CD25^+$ T-regulatory cells were isolated from spleens of C57BL/6 mice that were treated either with 0.1 mg/kg of FTY720 or vehicle on three consecutive days. Data are presented as average percentage of expression of control gene GAPDH. *c*, Cytokine production measured at 48 h from duplicate cultures of optimal inhibitory conditions, as assessed earlier in proliferation assay (1:2 ratio). Results are expressed as a percentage of inhibition of positive control ($CD4^+/CD25^-$ cells). Data are representative of three independent experiments; *, $p < 0.05$.

these data clearly demonstrate that FTY720 alters both the sequestration of $CD4^+/CD25^+$ T-regulatory cells as well as their regulatory activity and thus may have long-term disease modifying potential in inflammatory diseases such as asthma.

Discussion

The S1P receptor agonist FTY720 has striking effects on lymphocyte migration and homing without apparent alteration in host defense (6, 10). These observations may have diverted the attention from the possibility that this compound may also modulate certain lymphocyte functions and only few analyses of cellular mechanisms of action of FTY720 have been conducted with lymphocytes to date. The data presented in this study demonstrate that FTY720 not only differentially affects the sequestration of $CD4^+/CD25^+$ T-regulatory cells but also alters their functional activity. More specifically, we demonstrated that 1) $CD4^+/CD25^+$ T-regulatory cells are more resistant to the FTY720-induced sequestration of lymphocytes from blood and spleen to secondary lymphoid organs; 2) $CD4^+/CD25^+$ T-regulatory cells express lower levels of mRNA for $S1P_1$ and $S1P_4$ receptors and demonstrate a reduced chemotactic response to S1P; 3) $CD4^+/CD25^+$ T cells obtained from spleens of FTY720 pretreated donor mice contain regulatory T cells capable of inhibiting an allergen-induced airway inflammation; and 4) FTY720 induces an increased suppressive activity of T-regulatory cells.

Mechanistically, phosphorylated FTY720 acts in vitro as an agonist at four of the five S1P receptors, namely $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ and is a substrate for sphingosine kinases (3, 4, 31). A more recent study has demonstrated that FTY720 acts as a high affinity agonist at the $S1P_1$ receptor on thymocytes and lymphocytes, thereby inducing aberrant internalization of the receptor, which renders these cells unresponsive to S1P, and depriving them from an obligatory signal to egress from lymphoid organs (8, 32). As a consequence, lymphocytes are unable to recirculate to peripheral inflammatory tissues but remain functional in the lymphoid compartment. Although depletion of blood lymphocytes reaches as much as 80% under most experimental conditions, including our own, it is noteworthy that there are up to 20% of lymphocytes in the circulation that do not respond to the drug (23). Similarly, in nonhuman primates treated with FTY720 for >100 days, ~5% of peripheral blood $CD4^+$ T cells and 30% of CD8 T cells were refractory to depletion by the drug (24). The current view is that these cells represent long-lived effector memory T cells because in contrast to naive and central memory T cells they lack the lymph node homing receptors CCR7 and CD62L and reside in nonlymphoid tissues (33–35). However, our data demonstrate that at least a subpopulation of $CD4^+/CD25^+$ T cells appears to be resistant to the effect of FTY720 as an increased number of these cells were found in blood and spleens of treated mice. Moreover, the experiments using transfer of CFSE-labeled $CD4^+/CD25^+$ and $CD4^+/CD25^-$ T cells suggest that FTY720 differently affects the sequestration of this T-regulatory cell population, because treatment with the compound resulted in an increase of these cells in spleens but not lymph nodes. Recent studies have demonstrated that FTY720 differentially affects homing of $CD4^+/CD25^+$ $CD62L^-$ T-regulatory cells to lymph nodes (25), suggesting that at least some T-regulatory cell subsets express an effector memory cell phenotype with no expression of CD62L and thus follow the recirculation pattern of the latter cell type. Indeed, our results confirm this hypothesis, as a significantly higher proportion of T-regulatory cells were found in spleens of FTY720-treated mice, which were predominantly CD62L negative.

The data discussed in the preceding paragraph suggest that the recirculation of a subset of T-regulatory cells is less dependent on $S1P_1$ compared with naive T cells. This hypothesis is further supported by our findings that the $CD4^+/CD25^+$ T-regulatory population expressed significantly lower levels of $S1P_1$ as well as $S1P_4$ receptor mRNA than $CD4^+/CD25^-$ T cells. These cells also showed a significantly lower chemotactic response to S1P, suggesting that the differences seen in mRNA expression translate into differences in receptor expression. Indeed, a recent study demonstrated that T cell mRNA levels of S1P receptors always accurately reflect protein levels as measured by Western blots (22). However, and in contrast to our study, these investigators could not detect any differences in S1P receptor expression between $CD4^+/CD25^+$ and $CD4^+/CD25^-$ cell populations (22). Moreover, no expression of $S1P_2$, $S1P_3$, and $S1P_5$ was found, although the same authors demonstrated low $S1P_5$ expression on $CD4^+$ T lymphocytes in their earlier study (36). This discrepancy is currently difficult to explain and might be due to different cell purification and analysis methods. One possibility to explain the discrepancies in the levels of S1P receptor levels that have been reported is that the ex vivo

Table I. *CTLA-4 dependency of FTY-P enhanced CD4$^+$/CD25$^+$ T regulatory cell activity*

FTY-P[a]	CTLA-4[b]	Inhibition of [^{3}H]Thymidine Incorporation (%)
−	−	21.4 ± 6.1[c]
−	+	19.9 ± 4.2
+	−	50.6 ± 3.7
+	+	26.2 ± 1.7

[a] FTY-P was used at 1 μM.
[b] Anti-CTLA-4 neutralizing Abs were used at 25 μg/ml.
[c] Mean ± SEM; baseline [^{3}H]thymidine incorporation (100% value) 61 × 10^{-5} cpm.

process of isolation of lymphocytes might result in activation and subsequent down-regulation of their S1P receptors (7). However, because both populations of cells compared in this study were exposed to the same purification and isolation process, it is very unlikely that artificial and selective activation of T-regulatory cells in our models account for the differential expression of S1P receptor mRNA. However, similar to their studies, in which T cell activation induced a dramatic decrease in S1P receptor expression, we found that FTY720 treatment reduces the mRNA expression of $S1P_1$ and $S1P_4$ in both populations of cells to a similar degree. Again, and consistent with the reduced mRNA expression, CD4$^+$/CD25$^-$ T cells showed a significantly lower response to S1P. In contrast, no significant differences were found in the CD4$^+$/CD25$^+$ T cell subset, indicating that CD4$^+$/CD25$^+$ T cells might be less responsive to S1P-induced chemotaxis. In contrast, in some lymphoid organs, B cells were shown to express higher levels of $S1P_3$ than T cells, and this receptor seems to be required for the migration of B cells in the spleen (37, 38). A similar role for $S1P_3$ might exist in T-regulatory cells because the $S1P_3$ mRNA levels were significantly higher in T-regulatory cells without changes after FTY720 treatment. Additional studies, using genetically modified mice lacking one or more S1P receptors or using S1P receptor subtype-specific pharmacological tools, are necessary to identify which receptor or combination of receptors are responsible for the observed effects.

In addition to the difference in the sequestration of T-regulatory cells after FTY720 treatment, we could also demonstrate that this compound directly affects the regulatory activity of these cells. FTY720 added to in vitro cultures significantly enhanced the anti-proliferative function of CD4$^+$/CD25$^+$ T-regulatory cells on CD4/CD25$^-$T cells. Moreover, and more importantly, both CD4$^+$/CD25$^+$ T-regulatory cells obtained from in vivo FTY720-treated mice as well as from in vitro-treated cells suppressed an OVA-induced eosinophilic airway inflammation, clearly demonstrating the immunomodulatory potential of FTY720 in a relevant inflammatory disease model.

The mechanisms leading to the enhanced T-regulatory cell activity after FTY720 treatment is currently not well understood. Previous studies have already demonstrated that S1P has the ability to alter the activity of CD4$^+$/CD25$^+$ T cells (22, 36). By enhancing IL-10 production from this cell population as well as the anti-proliferative activity on naive T cells, S1P was found to be required for the optimal activity of CD4$^+$/CD25$^+$ T-regulatory cells. Moreover, besides a potential role for IL-10, CTLA-4 was also suggested as a mechanism for the enhanced T-regulatory cell activity (29). In contrast to this study, we could not demonstrate any changes in IL-10 levels after FTY720 treatment. Moreover, the mechanisms of enhanced T-regulatory cell activity appears to be exclusively cell-cell contact dependent because the effect was completely abolished by using a Transwell culture system and the addition of blocking CTLA-4 Abs to the cultures almost completely reversed the FTY720-induced increase in T-regulatory cell activity.

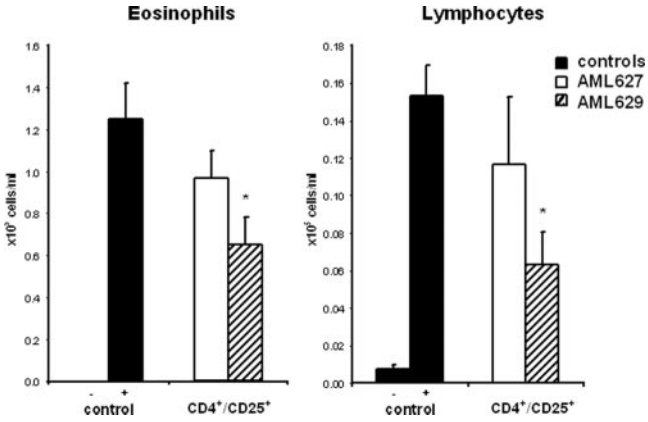

FIGURE 7. The active enantiomer of FTY-P activates CD4$^+$/CD25$^+$ T-regulatory cells. CD4$^+$/CD25+ T-regulatory cells isolated from spleens of naive C57BL/6 mice were incubated for 16h at 37°C with active (AML629) or inactive (AML627) enantiomer of FTY-P. After the incubation period, cells were transferred into immunized recipients at optimum numbers (1×10^6 cells per mouse) 24 h before Ag challenge. BAL fluid eosinophils and lymphocytes were determined 48 h after the Ag challenge. Data are mean ± SEM, $n = 8$ mice in each group; *, $p < 0.05$.

In contrast, we cannot completely rule out the possibility that FTY720 does not directly alter the functional activity of T-regulatory cells but increases the number of this cell type present in the CD4$^+$/CD25$^+$ T cell population. One of the main drawbacks when working with T-regulatory cells is that there is no clear marker available that would allow the detection of the entire T-regulatory cell population or the relative proportion of this cell type within the CD4$^+$/CD25$^+$ T cell subset. The best molecular marker known today is FoxP3, which is selectively expressed in T-regulatory cells and essential for their regulatory activity (27, 28). Indeed, the expression of FoxP3 was found to be increased in CD4$^+$/CD25$^+$ T cells obtained from FTY720-treated mice, but it is currently not clear whether this is due to an increased number of FoxP3 expressing cells or the induction of this transcription factor within the same cell population. However, our data also demonstrate that FTY720 treatment of CD4$^+$/CD25$^+$ T cells in vitro enhances their anti-proliferative activity on CD4$^+$/CD25$^-$T cells suggesting that this compound directly affects the regulatory function rather than the numbers of this cell population.

In conclusion, the present study demonstrates that the S1P receptor agonist FTY720 differently affects the sequestration of CD4$^+$/CD25$^+$ T-regulatory cells resulting in a higher ratio of T-regulatory/T-effector cells in blood, spleen, and potentially inflammatory sites. Moreover, our data clearly indicate that FTY720 is also capable of enhancing the activity of CD4$^+$/CD25$^+$ T-regulatory cells. Indeed, the manipulation of the ratio and potency of T-regulatory cells by FTY720 could have added benefits in transplant rejection or inflammatory or autoimmune diseases because the induction of T-regulatory cell activity in addition to blocking the infiltration of effector cells to inflammatory sites may lead to long-term disease modification.

Disclosures

E. Sawicka, G. Dubois, G. Jarai, M. Edwards, M. Thomas, A. Nicholls, R. Albert, C. Newson, V. Brinkmann, and C. Walker are full-time employees of the Novartis Institutes for BioMedical Research and FTY720 is a development product within the company.

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