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Circulating Monocytes Are Reduced by Sphingosine-1-Phosphate Receptor Modulators Independently of S1P₃


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Circulating Monocytes Are Reduced by Sphingosine-1-Phosphate Receptor Modulators Independently of S1P3


Sphingosine-1-phosphate (S1P) receptors are critical for lymphocyte egress from secondary lymphoid organs, and S1P receptor modulators suppress lymphocyte circulation. However, the role of S1P receptors on monocytes is less clear. To elucidate this, we systematically evaluated monocytes in rats and mice, both in naive and inflammatory conditions, with S1P receptor modulators FTY720 and BAF312. We demonstrate that S1P receptor modulators reduce circulating monocytes in a similar time course as lymphocytes. Furthermore, total monocyte numbers were increased in the spleen and bone marrow, suggesting that S1P receptor modulation restricts egress from hematopoietic organs. Monocytes treated ex vivo with FTY720 had reduced CD40 expression and TNF-α production, suggesting a direct effect on monocyte activation. Similar reductions in protein expression and cytokine production were also found in vivo. Suppression of experimental autoimmune encephalomyelitis in mice and rats by FTY720 correlated with reduced numbers of lymphocytes and monocytes. These effects on monocytes were independent of S1P3, as treatment with BAF312, a S1P1,4,5 modulator, led to similar results. These data reveal a novel role for S1P receptors on monocytes and offer additional insights on the mechanism of action of S1P receptor modulators in disease. The Journal of Immunology, 2013, 190: 3533–3540.

Sphingosine-1-phosphate (S1P) is a bioactive lipid mediator that regulates multiple cellular processes, including immune cell trafficking (1). A gradient of S1P is recognized by S1P receptors on immune cells, leading to their entry into circulation (2). The biological relevance of this pathway was highlighted by the development of FTY720, a potent S1P1,3,4,5 modulator, as a therapy for multiple sclerosis (3). FTY720 leads to internalization and degradation of S1P receptors on lymphocytes; consequently, lymphocyte egress from secondary lymphoid organs is inhibited, resulting in a significant decrease in circulating lymphocyte levels (4) and reduced lymphocyte infiltration into the CNS (5).

S1P receptors may also be involved in innate immune cell trafficking (2). In vitro, mast cells (6), dendritic cells (7), eosinophils (8), NK cells (9), macrophages (10), and monocytes (11) have been shown to migrate toward S1P, albeit weakly. In vivo, NK cell trafficking is dependent upon S1P (12); and similar to lymphocytes, FTY720 treatment restricts NK cell egress from lymph nodes and bone marrow (13). Additionally, migration of in vitro generated, mature S1P1−/− or S1P3−/− dendritic cells is defective following injection into mice (14).

The role of S1P receptors on monocytes in vivo is less clear. Several reports suggest that monocyte trafficking may be restricted by modulation of S1P receptors based on reduced numbers of macrophages in tissue, such as the following: the peritoneal cavity following thioglycolate-induced peritonitis (10), peripheral nerves in experimental autoimmune neuritis (15), the CNS in experimental autoimmune encephalomyelitis (EAE) (16), renal glomeruli in experimental mesangioproliferative glomerulonephritis (17), atherosclerotic plaques (18), and heart allografts (19). However, an effect on circulating monocytes has not been reported. Moreover, FTY720 has been shown to increase the number of macrophages in mesenteric lymph nodes, suggesting that S1P receptors may be important for monocyte egress from lymph nodes in a manner similar to lymphocytes (20). However, it is not clear whether this is a direct or indirect effect on either the numbers or trafficking of circulating monocytes.

In this work, we examined the effect of S1P receptor modulators on monocytes in vivo and observed reduced circulating monocyte numbers in mice and rats, in both normal and inflammatory conditions. We demonstrate that this effect is independent of S1P3 by using S1P receptor modulators with different selectivities. Furthermore, we provide evidence that FTY720 restricts monocyte egress from the spleen and bone marrow, as monocyte cell numbers are significantly increased at these sites. We also show that monocytes express S1P receptors and that FTY720 can act directly upon monocytes to alter their activation, as demonstrated by reduced surface protein expression and cytokine production. These findings demonstrate an important role for S1P receptors on monocytes in rodents and suggest that additional studies on the functions of S1P receptor modulators on humans may be merited.

Materials and Methods

Animals

Female C57BL/6 mice (6–10 wk of age) were purchased from The Jackson Laboratory. Female Lewis rats (150–200 g) were purchased from Charles...
FTY720 reduces monocyte numbers in the circulation

River Laboratories. Animals were fed and watered ad libitum under 12-h light/dark cycles. All studies were performed in accordance with Institutional Animal Care and Use Committee guidelines.

Chemical synthesis and dosing

FTY720 was purchased from Cayman Chemical. BAFl2 (21) was prepared as the zwitterion, according to the synthetic procedures described (22), and crystallized, as described (23). Compounds were dosed in a vehicle containing 0.5% methylcellulose (Spectrum Chemical) and 0.2% Tween 80 (EMD Chemicals). Mice and rats were dosed once daily by oral gavage. Prophylactic dosing of mice began 1 d before immunization, and therapeutic dosing of mice began once mice had a clinical score $\geq 1$ or a score of 1 for at least 2 d. For in vitro studies, cell culture medium was used as the vehicle.

Whole-blood differential analysis

Whole blood was collected from animals by retro-orbital bleeding and analyzed immediately using the Hemavet 1700FS (Drew Scientific).

Tissue preparations and flow cytometry

Whole blood was isolated retro-orbitally, and 100 µl was used for staining. Spleens were smashed in 2 ml Cell Staining Buffer (BioLegend) over a 40-µm strainer, and 30 µl was used for staining. Cell Staining Buffer was added to make a final volume of 100 µl. The right tibia was used to isolate bone marrow. Bone marrow was resuspended in 800 µl Cell Staining Buffer, and 100 µl was used for staining. TruStain FixCyt (10 µg/ml; BioLegend) was added directly to the samples and incubated on ice for 10–15 min to block FcRs. The following Abs in a total volume of 50 µl were then added at the concentration suggested by the manufacturer. Abs were purchased from BioLegend (Ly6C, CD11b, CD115, CD86, CD40, CD69, H1.2F3; CD80, 16-10A1; CD44, IM7; CD62L, MEL-14; B220, RA3-6B2; CD4, RM4-5; CD8, 14-5C11; CD19, 53-67; Gr-1, RB6-8C5; NK1.1, PK136), Becton Dickinson Biosciences (CD45, 30-F11; CD11b, M1/70), and eBioscience (CD335 [Nkp46], 29A1.4). Isotype control Abs were used at the same protein concentrations as their corresponding markers. Cells were stained in the dark on ice for 20 min, and then 1 ml One-Step Fix/Lyse Solution (eBioscience) was added, mixed, and incubated with the cells for 30 min at room temperature. The cell solution was then centrifuged, and the supernatant was discarded. The cells were then washed twice with Cell Staining Buffer, re suspended in 200 µl Cell Staining Buffer, run on the FACS Canto II (Becton Dickinson Biosciences), and analyzed using FlowJo 7.6.3.

The total cell numbers shown in Fig. 1B were obtained by multiplying the percentage positive of each cell type (as determined by flow cytometry) by the whole blood cell count obtained from the Hemavet, then dividing that number by 100. For Fig. 2B, the total cell counts were obtained by multiplying the percentage of monocytes by the total cell concentrations. The cell concentrations were derived by taking the total number of cells run through the flow cytometer for each sample, divided by the run time of the sample to get cells/time. This number was then divided by the speed of the cytometer to get the cell concentration. The cell counts were then multiplied by their respective dilutions and the total volume of the cell suspension. Mice were assumed to have a total blood volume of 58.5 ml/kg body weight.

SIP receptor expression analysis

SIP receptor expression data were obtained from publicly available microarray datasets using NexusBio [www.nexusbio.com (24)]. The data were processed and normalized by NexusBio to compare between different datasets, as described. Expression levels of SIP receptor mRNA for C57BL/6 cells (GSE1446), B220+ B cells (GSE6095), NK.1.1+ NK cells (GSE6506), granulocytes (Mac-1+ Gr-1+; GSE10246), Ly6C high and low monocytes (GSE17256), and corticortical neurons (GSE17784). All cell types were obtained from C57Bl/6 mice. Data are presented as the mean ± SD.

Monocyte enrichment and stimulation

Blood was isolated from C57BL/6 mice and lysed using RBC lysis buffer (BioLegend), according to the manufacturer’s instructions. The EasySep Mouse Monocyte Enrichment kit (Stemcell Technologies) was used to enrich for monocytes. This was performed according to the manufacturer’s instructions. Cell purity was assessed by CD11b+ Ly6G− according to the manufacturer’s instructions and was on average 90.2% pure.

Monocytes were cultured at 37°C in RPMI 1640 medium containing 10% FBS, 10% L929-conditioned medium, 1× penicillin/streptomycin, 2 mM t-glutamine, and 10 mM HEPES. FTY720 was dissolved in this medium and was used at 10 nM. LPS was used at 10 ng/ml. After 2 h, cells were centrifuged and supernatants were collected and frozen until needed. Cytokine levels of TNF-α were assessed using a sandwich immunoassay (Meso Scale Discovery). Cells were also stained for CD40 and analyzed by flow cytometry.

In vivo LPS challenge and cytokine measurement

C57BL/6 mice were dosed with either 0.3 mg/kg FTY720, BAFl2, or vehicle (0.5% methylcellulose and 0.2% Tween 80). After 22 h, mice received an i.p. injection of 200 µg LPS or vehicle (saline). Two hours later, whole blood was collected. The blood was then centrifuged, and the serum was stored at −20°C until assessed for cytokine levels. Serum levels of CCL2 (MCP-1), TNF-α, IL-6, and IL-10 were measured using a multiplex sandwich immunoassay (Meso Scale Discovery).

EAE induction

Mice were s.c. injected with 100 µg myelin oligodendrocyte glycoprotein35-55 (AnaSpec) in IFA (Difco) containing 250 µg Mycobacterium tuberculosis (Difco). On the same day and again 48 h later, mice received an i.p. injection of 200 ng pertussis toxin (List Biological Laboratories). Mice were scored as follows: 0, no symptoms; 1, limp tail; 2, limp tail and inability to right itself when placed on its back; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, complete hind limb paralysis with forelimb involvement or moribund. EAE was induced in rats by s.c. injection of 100 µg guinea pig myelin basic protein68-86 (AnaSpec) in IFA containing 800 µg M. tuberculosis. Rats were scored as follows: 0, no symptoms; 1, limp tail; 2, ataxia and/or altered gait; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, complete hind limb paralysis with forelimb involvement.

Statistics

For the EAE clinical score data, area under the curve (AUC) was calculated and the Kruskal-Wallis test was performed, followed by a Dunn’s posttest to determine significance between multiple groups. For other data, either an unpaired t test was used when comparing two groups or a one-way ANOVA followed by the Newman-Keuls posttest was used for comparison between multiple groups. These calculations were performed using GraphPad Prism 5.04.

Results

FTY720 reduces circulating levels of monocytes and NK cells without affecting neutrophil levels

To determine the effects of FTY720 on innate immune cells, we dosed mice with FTY720 at 0.03, 0.3, and 3 mg/kg and performed a differential analysis of peripheral blood collected at 4 and 24 h after dosing. In agreement with previous reports, using size-based analysis (Hemavet), we found a significant, dose-dependent decrease in circulating lymphocytes at both time points when compared with vehicle alone (up to an 87% reduction). We also observed a significant reduction in circulating monocytes. Monocyte levels were decreased up to 78% in a dose-dependent manner with FTY720 treatment (Fig. 1A). We also monitored circulating neutrophil levels and found that there was no significant effect in response to FTY720 treatment (Fig. 1A).

Using flow cytometry, we confirmed the lymphocyte and monocyte lowering in mice and extended our analysis to additional cell types. Mice were dosed with 0.03, 0.1, 0.3, and 3 mg/kg FTY720, and whole blood immune cell counts were assessed by flow cytometry. We confirmed that CD4+ (97% reduction) and CD8+ T cells (91% reduction) along with B220+ B cells (60% reduction) were decreased in peripheral blood 24 h after dosing with all tested doses of FTY720 (Fig. 1B). Significant decreases in monocytes (CD11b+CD115+; 56% reduction) and NK cells (NK1.1+ NKP46+; 43% reduction) were also detected; however, no effect on neutrophils (CD11b+Gr-1+) was observed (Fig. 1B). Thus, the number of circulating monocytes and NK cells is reduced following FTY720 with a similar time course as lymphocytes.

We dosed rats with FTY720 at 0.03, 0.1, and 0.3 mg/kg and performed a differential analysis of peripheral blood collected at 4
and 24 h after dosing. Similar to our results in mice, we found a significant, dose-dependent decrease in circulating lymphocytes at both time points when compared with vehicle alone (up to an 88% reduction; Fig. 1C). Monocyte levels were also decreased in a dose-dependent manner with FTY720 treatment up to an 80% reduction (Fig. 1C). We also monitored circulating neutrophil levels by Hemavet, and, although a decrease in response to FTY720 treatment was observed, it was not a significant effect (Fig. 1C).

**FIGURE 1.** FTY720 reduces circulating lymphocyte, monocyte, and NK cell levels, but not neutrophils. (A) C57BL/6 mice were dosed with vehicle, 0.03 mg/kg, 0.3 mg/kg, or 3 mg/kg FTY720 (n = 7 mice per group). Whole blood was taken at 0, 4, and 24 h after the administration of the dose. Lymphocyte, neutrophil, and monocyte cell counts were assessed using the Hemavet. (B) C57BL/6 mice were dosed with vehicle, 0.03, 0.1, 0.3, or 3 mg/kg FTY720. After 24 h, whole blood was collected and CD4⁺ T cells, CD8⁺ T cells, B220⁺ B cells, CD11b⁺ CD115⁺ monocytes, NK1.1⁺ NKp46⁺ NK cells, and CD11b⁺ Gr-1⁺ neutrophil cell counts were assessed by flow cytometry (n = 29–30 mice in the untreated and vehicle groups; n = 10 in the 0.03, 0.1, and 0.3 mg/kg groups; and n = 20 in the 3 mg/kg group). (C) Lewis rats were dosed with vehicle, 0.03 mg/kg, 0.1 mg/kg, or 0.3 mg/kg FTY720. Whole blood was taken at 0, 4, and 24 h after the administration of the dose. Lymphocyte, neutrophil, and monocyte cell counts were assessed using the Hemavet (n = 12 rats for the 0-h time point, and n = 5 rats per group for all other time points). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle. Mean ± SEM is shown.

FTY720 restricts monocyte egress from the spleen and bone marrow

Due to the decreased levels of circulating monocytes observed upon administration of FTY720, we wanted to determine whether monocyte levels were also altered in lymphoid tissues. We dosed mice with FTY720 at 0.3 mg/kg and 2 mg 5-ethynyl-2'-deoxyuridine (EdU) (a 5-bromo-2'-deoxyuridine alternative) and isolated the blood, spleen, and bone marrow 4 h later and performed flow cytometry to assess monocyte levels in these tissues. In addition to our observation that FTY720 reduced circulating monocyte levels, we found that FTY720 causes a significant accumulation of monocytes in both the spleen and bone marrow (Fig. 2B). This effect was observed for both Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes. Additionally, we calculated the sum of the total monocytes in these tissues and found that there were significantly more Ly6C<sup>hi</sup> monocytes, but not Ly6C<sup>lo</sup> monocytes (Fig. 2C). These data suggest that for the Ly6C<sup>lo</sup> monocytes, the changes
we observed were due to cell relocation. However, the observed changes for Ly6C<sup>hi</sup> monocytes could be due to increased proliferation or reduced cell turnover. We next analyzed the number of EdU<sup>+</sup> bone marrow monocytes and found that FTY720 increased their numbers, suggesting an increase in proliferation (Fig. 2D). This effect, however, was restricted to the Ly6C<sup>hi</sup> subset, thus explaining our results found in Fig. 2C. These results demonstrate that FTY720 causes monocytes to accumulate in the spleen and bone marrow while also causing increased proliferation in the bone marrow.

**FTY720 directly restricts monocyte activation**

Our previous results demonstrate that FTY720 changes monocyte levels by reducing their numbers in the circulation and increasing their numbers in the spleen and bone marrow. These data, however, do not address whether this effect is direct or indirect. The first step we took to address this was to analyze S1P receptor expression on monocyte subsets and other immune cells by accessing and analyzing publicly available microarray datasets using NextBio (Fig. 3A). These data demonstrate that monocytes express S1P receptors with the highest levels observed for S1pr2 and S1pr5. These data also confirm previous publications that lymphocytes express high levels of S1pr1 and NK cells have high levels of S1pr5. Expression of S1pr4 was not included in these datasets; nevertheless, other studies have shown low-level expression of S1pr4 on monocytes as compared with other immune cells (25).

To further determine whether FTY720 acts directly upon monocytes, we enriched monocytes from the blood (∼90.2% pure) and cultured them in the presence of FTY720 (10 nM) and LPS (10 ng/ml). After 2 h, we found FTY720 reduced CD40 protein levels on monocytes (Fig. 3B). In the presence of LPS, there was an induction of CD40 levels that was significantly reduced with FTY720. Furthermore, we analyzed cytokine production and found LPS induced the production of TNF-α, which was significantly reduced in the presence of FTY720 (Fig. 3C). These data demonstrate that FTY720 acts directly on monocytes to alter surface protein expression and cytokine production.

**S1P receptor modulation in vivo alters monocyte surface protein expression and cytokine production**

To assess whether S1P receptor modulation alters monocyte activation in vivo, mice were dosed with 0.3 mg/kg FTY720. Twenty-four hours later, we collected peripheral blood and assessed the cell surface expression of multiple proteins on monocyte subsets. We found that on Ly6C<sup>hi</sup> monocytes, PD-L1, CD40, and CD69 were decreased by FTY720, whereas CD80 was increased (Fig. 4A). On Ly6C<sup>lo</sup> monocytes, CD40 and CD69 were also decreased by FTY720 compared with control. We also monitored cell apoptosis and necrosis using annexin V and propidium iodide and found no significant difference between the groups (data not shown). These results suggest that FTY720 alters monocyte surface protein expression in vivo and may affect monocyte function.

Because we demonstrated that FTY720 reduced circulating levels of monocytes and altered their surface protein expression and cytokine production, we wanted to determine whether S1P receptor modulation would have an impact on serum cytokine levels after an inflammatory stimulus in vivo. To test this, we dosed mice with 0.3 mg/kg FTY720 and BAF312, a S1P<sub>1,4,5</sub> modulator, for 22 h and challenged them with 200 ng LPS. After 2 h, we collected whole blood and assessed the levels of proinflammatory cytokines. We found that induction of IL-6, TNF-α, CCL2, and IL-10 by LPS was significantly decreased by FTY720 and BAF312 (Fig. 4B). These results demonstrate that S1P receptor modulation decreases cytokine production after an LPS challenge.

**FTY720 reduces monocytes during EAE**

To determine whether the impact of FTY720 on monocytes is transient or sustained in a long-term model of inflammation, we induced EAE in both mice and rats and measured the effects of 0.03 and 0.3 mg/kg FTY720 on clinical disease and circulating levels...

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**FIGURE 2.** FTY720 restricts monocyte egress from the spleen and bone marrow while enhancing proliferation. C57BL/6 mice were orally dosed with vehicle or 0.3 mg/kg FTY720 and simultaneously given an i.p. injection of 2 mg EdU. After 4 h, the blood, spleen, and bone marrow were isolated. Single-cell suspensions were made and analyzed by flow cytometry. (A) Monocyte gating is shown for each tissue. (B) Total cell counts are shown for total monocytes, Ly6C<sup>hi</sup> monocytes, and Ly6C<sup>lo</sup> monocytes in the blood, spleen, and bone marrow. (C) The sum of the cell counts is shown for Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes. (D) Gating of bone marrow EdU<sup>+</sup> monocytes and total cell counts are shown. n = 8–10 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.
of lymphocytes, monocytes, and neutrophils. When treated prophylactically, FTY720 suppressed clinical disease in mice at 0.3 mg/kg (Fig. 5A). Peripheral blood was analyzed at day 14 post-immunization, and the levels of lymphocytes, monocytes, and neutrophils were significantly reduced in mice at both tested doses, as follows: 0.03 mg/kg (reductions of 56, 61, and 56%, respectively) and 0.3 mg/kg (reductions of 86, 82, 74%, respectively; Fig. 5A).

We also treated EAE mice with a therapeutic regimen of FTY720 (i.e., daily dosing began after mice developed disease; Fig. 5B). Although disease was not completely abolished at 0.3 mg/kg, the clinical score was significantly decreased, which correlated with decreased levels of circulating monocytes and lymphocytes. These results indicate that FTY720 also restricts circulating levels of monocytes under sustained inflammatory conditions.

In rats, prophylactic treatment with 0.03 and 0.3 mg/kg FTY720 significantly reduced clinical disease. Peripheral blood was analyzed at the end of the study, and lymphocytes, monocytes, and neutrophils were also significantly reduced at doses of 0.03 mg/kg (reductions of 89, 66, and 47%, respectively) and 0.3 mg/kg (88, 70, and 64%, respectively; Fig. 5C).

Monocyte trafficking is not dependent on S1P₃
S1P₃ has been implicated in controlling monocyte trafficking (10); therefore, we wanted to assess whether decreased circulating monocytes could be achieved with a S1P receptor modulator that does not agonize S1P₃. We synthesized BAF312, a S1P₁,₄,₅ agonist, confirmed its potency (EC₅₀) in vitro with GTPₗₛ assays for S1P receptors, and demonstrated potencies of 0.2 nM for S1P₁, >10 μM for S1P₃, and 0.5 nM for S1P₅. FTY720 was assayed in parallel and demonstrated potencies of 2 nM for S1P₁, 175 nM for S1P₃, and 2.5 nM for S1P₅ (L. Patnaude, S. Lukas, S. Haxhinasto, and L. Modis, manuscript in preparation). We tested BAF312 for potency in naive (0.03, 0.3, and 3 mg/kg) and EAE (0.03 and 0.3 mg/kg) conditions. In naive mice, similar to FTY720, we observed significant decreases in lymphocytes and monocytes at 4 h that continued through 24 h (reductions of 83 and 86%, respectively; Fig. 6A), and we did not observe any effects on neutrophils. In FIGURE 3. FTY720 acts directly on monocytes to alter cell surface expression and cytokine production. (A) S1P receptor mRNA expression is shown for the indicated cell types. Data were obtained from publicly available microarray datasets using NextBio, as described in Materials and Methods. (B and C) Monocytes were isolated from peripheral blood and cultured for 2 h in the presence of 10 nM FTY720 and 10 ng/ml LPS. (B) CD40 levels measured by flow cytometry (n = 8). (C) TNF-α cytokine levels measured by sandwich immunoassay (n = 14). *p < 0.05 versus vehicle and **p < 0.001 versus LPS.

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FIGURE 4. S1P receptor modulation altered monocyte protein expression and reduced serum cytokine levels after a LPS challenge. (A) C57BL/6 mice were dosed with 0.3 mg/kg FTY720 or vehicle, and, after 24 h, peripheral blood was isolated. Monocyte subsets (Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup>) were assessed by flow cytometry for cell surface expression of several proteins. Mean fluorescence intensity data are shown (n = 5–6 mice per group). *p < 0.05, **p < 0.1 versus vehicle. (B) C57BL/6 mice were dosed with 0.3 mg/ml FTY720, BAF312, or vehicle. After 22 h, mice received an i.p. injection of 200 ng LPS or vehicle. Two hours later, whole blood was collected, and IL-6, TNF-α, IL-10, and CCL2 were assessed by a sandwich immunoassay. *p < 0.05, **p < 0.01, ***p < 0.001.
EAE, we demonstrated that BAF312 significantly reduced clinical scores when dosed prophylactically or therapeutically in mice at 0.3 mg/kg (Fig. 6B, 6C). As expected, lymphocyte levels were significantly reduced when assessed near the peak of disease (i.e., days 14 and 16 postimmunization for the prophylactic [73% reduction] and therapeutic [37% reduction] regimens, respectively). We also observed significant decreases in monocyte levels, up to a 61% reduction with the prophylactic regimen, and up to a 57% reduction with the therapeutic regimen. BAF312 also inhibited clinical disease in rats in a dose-dependent manner, which correlated with reduced levels of circulating lymphocytes and monocytes (92 and 66% reductions, respectively; Fig. 6D). These results indicate that direct S1P3 agonism is not necessary for an impact on monocyte numbers in circulation.

Discussion

In this study, we demonstrate in mice and rats using two different S1P receptor modulators (FTY720 and BAF312) that S1P receptors are involved in maintaining monocyte numbers in the circulation under normal and inflammatory conditions. We further demonstrate that FTY720 causes monocytes to accumulate in the spleen and bone marrow. These observed effects on monocytes are similar to the effects observed on lymphocytes, that S1P receptor modulators restrict egress from hematopoietic organs. We also demonstrate that FTY720 acts directly upon isolated monocytes to alter their activation, as demonstrated by reduced surface protein expression and cytokine production. Similar results were also observed in vivo. Thus, our findings reveal that S1P receptors play a significant role in monocyte trafficking and may also alter monocyte function.

A reduction in the number of macrophages has been observed in several tissues of FTY720-treated mice, including the following: atherosclerotic plaques in ApoE-deficient mice (18), peritoneal cavity with thioglycolate-induced peritonitis (10), peripheral nerves in experimental autoimmune neuritis (15), CNS in EAE (16), renal glomeruli in experimental mesangioproliferative glomerulonephritis (17), and heart allografts (19). However, no mechanism has been demonstrated. Given that tissue macrophages typically derive from circulating monocytes, and under conditions of tissue injury there is increased monocyte trafficking into diseased tissues (26, 27), our results demonstrating decreased circulating monocyte levels upon FTY720 administration provide an explanation for these findings.

Our observation that FTY720 altered several cell surface markers on monocytes suggests that FTY720 may alter monocyte function. In both the Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocyte subset, we observed significant decreases in CD69 expression. CD69 has been demonstrated to interact with S1P<sub>1</sub> in T cells and inhibit T cell responsiveness to S1P (28). Because FTY720 causes internalization and degradation of S1P<sub>1</sub>, CD69 may be internalized also due to their interaction. Additionally, ligation of CD69 on monocytes triggers monocyte activation, which may be reduced by FTY720 (29). Similarly, CD40 was also reduced on both monocyte subsets, further suggesting that FTY720 may decrease monocyte activation. These data are also substantiated by our observation that FTY720 reduced monocyte cytokine production and serum cytokine levels after an in vivo challenge with LPS. We hypothesize that this reduced serum response can be mediated by both reduced monocyte numbers and activation state.

Pathogenesis in EAE models can be blocked by either reducing T cells (30) or monocytes, as illustrated by treating mice and rats with clodronate liposomes (31). Similarly, GM-CSF<sup>−/−</sup> mice, which have a monocyte defect, are protected from EAE (30–32).

FIGURE 5. FTY720 suppresses clinical signs of disease in mouse and rat EAE while decreasing lymphocytes, monocytes, and neutrophils. EAE was induced in both C57BL/6 mice and Lewis rats, as described in Materials and Methods. Animals with EAE were either untreated, or treated daily with a vehicle, 0.03 mg/kg, or 0.3 mg/kg FTY720. (A) Prophylactic dosing of mice began 1 d before immunization, and clinical scores were evaluated daily beginning on day 7 postimmunization. Whole blood was assessed for circulating lymphocytes, monocytes, and neutrophils at day 14 postimmunization. For clinical score data, n = 9–10 mice per group. For cell counts, n = 4–5 mice per group. (B) Therapeutic dosing of mice began once mice had a clinical score >1 or a score of 1 for at least 2 d. Whole-blood assessment of circulating lymphocytes, monocytes, and neutrophils was performed at day 16 postimmunization. For clinical score data, n = 16–21 mice per group. For cell counts, n = 4–5 mice per group. (C) Prophylactic dosing of rats began 1 d before immunization, and clinical scores were evaluated daily beginning on day 7 postimmunization. Whole-blood assessment of circulating lymphocytes, monocytes, and neutrophils was performed at day 20 postimmunization. For clinical score data, n = 10 rats per group. For cell counts, n = 5 rats per group. Mean ± SEM is shown. Significant differences in the clinical score data were assessed using the AUC data from individual mice. *p < 0.05 for 0.03 mg/kg versus vehicle, **p < 0.01, and ***p < 0.001 for 0.3 mg/kg versus vehicle.
It has also been shown that inhibition of the M-CSF receptor (CD115) reduces clinical disease during EAE (33). Additionally, monocytes from CCR2−/− mice are reduced in number and unable to traffic into the CNS (34); consequently, these mice are also protected from EAE (35). Our data are consistent with these observations, as we see reductions in the proinflammatory Ly6Chigh monocyte subset, which would be expected to directly affect the levels of macrophages at sites of inflammation. Furthermore, the reduction in monocyte numbers parallels that of lymphocytes, both in timing and with respect to the PK/PD effect (both are maximally reduced at 0.3 mg/kg in healthy mice). Therefore, the reduction in the number of circulating monocytes may be partially responsible for the observed efficacy of S1P receptor modulators in EAE.

We demonstrated that BAF312, which does not modulate S1P3, reduced circulating monocyte levels in a dose-dependent manner similar to FTY720, suggesting that monocytes traffic independently of S1P3. This is in contrast to a report that monocyte trafficking is dependent upon S1P3 (10). However, when the authors compared wild-type mice containing S1P3−/− bone marrow with S1P3−/− mice containing wild-type bone marrow, no difference was observed in macrophage infiltration into the peritoneum. This observation, along with our data utilizing BAF312, suggests that monocyte expression of S1P3 is not solely responsible for monocyte trafficking.

Although other groups have mentioned that they did not observe reductions in circulating monocytes with FTY720 treatment (15, 18, 20, 36, 37), monocytes were not the focus of these studies, monocyte levels were assessed in a disease model in which monocytes are already affected (18), or surface markers were used that are not specific for monocytes (36). Our studies, however, provide an in-depth analysis of the effects of FTY720 and BAF312 on monocytes at several doses under multiple conditions and were consistent in both mice and rats. To confirm that the role of FTY720 on monocytes trafficking is direct, a future step using...
mice in which S1P receptors are specifically deleted from myeloid cells, it is important to note the limitations and potential implications in future studies.

Disclosures

The authors are employed by Boehringer Ingelheim Pharmaceuticals.

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