Evaluation of Experimental Autoimmune Uveitis in Mice Treated with FTY720

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PURPOSE. FTY720 (fingolimod) is an immunomodulatory drug capable of preventing T-cell migration to inflammatory sites by binding to and subsequently downregulating the expression of sphingosine-1 phosphate receptor 1 (S1P1) leading to T-cell retention in lymphoid organs. Additional effects of FTY720 by increasing functional activity of regulatory T cells have recently been demonstrated, raising the conversion of conventional T cells into regulatory T cells and affecting the sequestration of regulatory T cells in normal mice. In this study, the action of FTY720 in the ocular autoimmune model in mice was investigated.

METHODS. Mice were immunized with 161-180 peptide and pertussis toxin and were treated with 1 mg/kg/d FTY720 by gavage (7–21 days postimmunization [dpi]) or left untreated. Spleen cells, harvested 21 dpi, were cultured and assayed for cytokine production. Draining lymph node, spleen, and eye cells 21 dpi were assayed for quantification of T-cell populations. Disease severity was evaluated by histologic examination of the enucleated eyes at 21 and 49 dpi. In addition, anti-IRBP antibodies were analyzed by ELISA.

RESULTS. FTY720 was effective in suppressing the experimental autoimmune uveitis score. Although there was a reduction in the number of eye-infiltrating cells, FTY did not prevent Treg accumulation at this site. FTY720 leads to a significant increase in CD4+IFN-γ+ and CD4+Foxp3+ cell percentages in lymph nodes, suggesting that this site could be the source of Treg cells found in the eye.

CONCLUSIONS. The data showed that treatment in vivo with FTY720 was able to suppress EAU in mice. These results are indicative of the possible therapeutic use of FTY720 in ocular autoimmune processes. (Invest Ophthalmol Vis Sci. 2010;51: 2568–2574) DOI:10.1167/iovs.09-4769

Experimental autoimmune uveitis (EAU) is an organ-specific, T cell–mediated disease that targets the posterior pole of the eye. It is also a valuable, well-characterized model for the study of human idiopathic uveitis. This disease can be induced in susceptible primates and rodents after immunization with retinal self-antigens, such as interphotoreceptor retinoid-binding protein (IRBP) or S-antigen (arrestin), or by the adoptive transfer of uveitis antigen-specific T cells.1–3

It has been shown that autoreactive Th1 cells mediate EAU4,5; thus, its induction is correlated with the production of IFN-γ by T cells. Moreover, inhibition of Th1 responses by anti-IL-12 treatment suppresses the disease.6 Recently, Th17 cells have also been suggested to be involved in EAU.7,8

The EAU mouse model has contributed significantly to the establishment of parameters for the evaluation of possible therapies for posterior uveitis in humans.9 Studies of genetic susceptibility and resistance to EAU,9 characterization of uveitogenic epitopes,10 and studies of tolerance in EAU by anterior chamber-associated immune deviation11,12 or by systems of oral tolerance13,14 have obtained success according to this model.

In 2005, our group demonstrated that treatment with an α4 active peptide inhibitor, which targets the α4 integrin of the VLA-4 adhesion molecule, had a significant ameliorating effect on EAU.15 Clinical ocular pathology can also be prevented by the administration of recombinant Galectin-1 [rGal-1] either early or late during the course of EAU.16 Associated mechanisms were recently described because Gal-1 induces dendritic cells to secrete IL-27, which induce T cells to become Tr1 cells and decreasing allogeneic T-cell proliferation.17

Recently, various reports clearly showed that some molecules, such as LX211 (voclosporin),21 anti–IL-17,22 and α-melanocyte-stimulating hormone,23 were effective in suppressing EAU. In this context, fingolimod (FTY720; Novartis, Basel, Switzerland) is an immunomodulatory drug capable of preventing T-cell infiltration by binding to and subsequently downregulating the expression of sphingosine-1 phosphate receptor 1 (S1P1).24 S1P1 signaling is required for T-cell egress from secondary lymphoid tissue.25 Therefore, blocking this pathway leads to T-cell retention in lymphoid organs and prevents their migration to inflammatory sites.

In transplantation, FTY720 prolonged mouse (C3H to BALB/c) corneal allograft survival by reducing the infiltration of CD4+ and F4/80+ cells and decreasing allogeneic T-cell proliferation.26 In rats, penetrating keratoplasty was performed (Fisher to Lewis), and FTY720 treatment caused significant rejection-free graft survival and reduction of CD4+ and CD8+ and NK infiltrating cells.27 In addition, FTY720 treatment suppressed the incidence and intensity of EAU in Lewis rats immunized with S-antigen. Suppression of antibody serum levels and antigen-specific lymphocyte proliferation was observed.28 In B10.RIII mice induced to autoimmune uveoretinitis, the retinal infiltration was prevented by FTY720 treatment 2 days

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before disease onset. This finding was associated with the suppression of histologic disease. A single dose of fingolimod administered after disease onset (14 days after induction) not only abolished retinal infiltrates but prevented disease relapse for at least 3 weeks.29

Additional effects of FTY720 have recently been demonstrated by increasing the functional activity of regulatory T cells, enabling the conversion of conventional T cells into regulatory T cells in normal mice.31

Daniel et al.32 used a model of experimental colitis to evaluate the role of FTY720 in disease development. It was observed that FTY720 substantially reduced all clinical, histopathologic, macroscopic, and microscopic parameters of colitis analyzed. FTY720 treatment caused a downregulation of IL-12p70 and TNF-alpha expression in colon protein extracts. LP CD4+ cells from Peyer’s patches were evaluated by ELISA and presented a dose-dependent increase of IL-10 and TGF-beta expression in FTY720-treated mice. In addition, induction of FoxP3 and CTLA4 protein expression was observed in colon. This finding provides evidence that, in addition to its well-established effects on migration, FTY720 leads to specific downregulation of proinflammatory signals while it simultaneously induces the functional activity of CD4+CD25+ Treg cells.32

Zhang et al.33 evaluated experimental autoimmune neuritis (EAN) in rats and observed by flow cytometry that treatment with FTY720 at disease onset (day 10 until day 18) caused an increase in Foxp3 cells in blood but a decrease in lymph node. In sciatic nerves of EAN rats, FTY720 increased the percentages of Foxp3+ cells as observed by immunohistochemistry.33

Considering that different T-cell subsets may present uveitogenic capability and that FTY720 not only increases Treg cells generation but also affects their homing properties,27 it was our aim to investigate the effects of FTY720 administration in the EAU model and possible associated mechanisms.

MATERIALS AND METHODS

Animals

Six- to 8-week-old B10.RIII mice were obtained from the animal facilities of the University of Sao Paulo in Brazil. All animals were housed under specific pathogen-free conditions and treated in accordance with institutional guidelines and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal care and use committee at the Institute of Biomedical Sciences of the University of Sao Paulo approved all the procedures used in this study.

Induction of EAU and In Vivo Treatment with FTY720

Mice were immunized subcutaneously at the base of the tail with 30 μg 161-180 peptide emulsified in 0.2 mL CFA (vol/vol). At the same time, mice were injected intraperitoneally with 0.5 μg PTX in 0.1 mL as an additional adjuvant. Animals were treated with 1 mg/kg/d FTY720 administered in 200 μL PBS by gavage (7–21 days postimmunization [dpi]) or were left untreated.

Histopathology EAU

Eyes were collected and prepared for histopathologic evaluation at the end of each experiment (21 and 49 dpi). The eyes were immersed for 1 hour in phosphate-buffered glutaraldehyde 4%, transferred to phosphate-buffered formaldehyde 10% for 24 hours, and replaced with ethanol 70% until processing. Fixed and dehydrated tissue was embedded in paraffin wax, and 4–6 μm sections were cut through the papillary-optic nerve plane. Sections were stained by hematoxylin and eosin. Presence or absence of disease was evaluated in a double-blinded fashion by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale from 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semi-quantitative system described previously35 and according to lesion type, size, and number. In brief, the minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroids, or retina (EAU grade 0.5). Progressively higher grades were assigned for the presence of discrete lesions in the tissue such as vasculitis, granuloma formation, retinal folding or detachment, and photoreceptor damage.

Determination of Cytokine Production

Spleen cells harvested 21 dpi were cultured in 24-well plates (106 cells/well) and stimulated with 30 μg/mL IRBP. Supernatants were collected for cytokine analysis after 48 or 72 hours and were stored at −80°C until assayed. The level of IFN-γ was assessed by ELISA using a kit from BD PharMingen (La Jolla, CA), and the level of IL-17 was assessed by ELISA using a kit from eBioscience (San Diego, CA). All kits were used according to the manufacturers’ instructions.

Flow Cytometry Analysis

Eyes. Eyes were nucleated and gently crushed with the flat surface of a sterile syringe plunger in a plastic Petri dish containing PBS. The cell suspension was then filtered through a 400-μm stainless iron mesh and a 70-μm nylon cell strainer (BD Biosciences-Labware, Bedford, MA) and adjusted for 5 × 106 cells/tube for staining with rat anti–mouse CD25FITC and rat anti–mouse CD4PerCP-Cy5.5 (eBioscience). After incubation for 30 minutes, cells were washed with PBS, fixed for 15 minutes with 1% paraformaldehyde, permeabilized for 6 minutes with 0.2% Triton X-100, and stained with rat anti–mouse Foxp3 FITC for 30 minutes (eBioscience). Stained cells were acquired (FACSCalibur Flow Cytometer; BD Biosciences), and data were analyzed (Cell Quest Pro software; BD Biosciences). At least 30,000 events were acquired for each analysis.

Spleen and Lymph Nodes. The frequency of CD4+ and CD8+ in spleen cells and CD4+CD25+Foxp3+ T cells in lymph nodes was evaluated in cell suspensions first incubated with anti–CD3/CD16 (Fc block) antibody for 20 minutes at 4°C. Cell suspensions were further incubated with combinations of fluorescent antibody for 50 minutes at 4°C (all purchased from eBioscience). All samples were acquired (FACSCalibur; BD Biosciences), and data were analyzed with appropriate software (FlowJo; TreeStar, Ashland, OR).

Intracellular Cytokine Staining and FACS Analysis

For detection of intracellular expression of IL-17 and IFN-γ by FACS analysis, cells were collected and were left unstimulated or were stimulated for 5 hours with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) and 750 ng/mL ionomycin (Calbiochem, La Jolla, CA) in the presence of either protein transport inhibitor containing Brefeldin A (Golgiplug; BD PharMingen) or protein transport inhibitor containing monensin (Golgistop; BD PharMingen) at the recommended concentration. Standard intracellular cytokine staining was performed. Briefly, cells were stained extracellularly with fluorescent-conjugated anti–CD4+ and were fixed. Then they were permeabilized with solution (Cytofix/Cytoperp; BD PharMingen) and stained intracellularly with phycoerythrin-conjugated anti–IL-17 and anti–IFN-γ. All samples were acquired (FACSCalibur; BD Biosciences), and data were analyzed (FlowJo; TreeStar).

Analysis of Anti–IRBP Antibodies

Levels of anti–IRBP IgG1 and IgG2a isotypes were determined by ELISA in untreated and in FTY-treated mice sera. Briefly, 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 1 μg/ well IRBP diluted in 0.1 M NaHCO3 , pH 9.6, overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween 20. Serum was serially diluted starting at 1:16. Peroxidase-labeled anti-mouse
IgG1 (1:1000) or IgG2a (1:1000) was obtained from BD PharMingen. Antibody titers were expressed as log₂ of the reciprocal serum dilution giving an absorbance value of 20% of the saturation level.

### Statistical Analysis

Data are expressed as mean ± SD. Statistical analyses were performed (Prism version 5.00 for Windows; GraphPad Software, San Diego CA). Parametric Student’s *t*-test was used, and *P* < 0.05 was considered significant.

### RESULTS

**FTY720 Was Associated with a Decreased EAU Score**

Mice were immunized and treated with oral FTY during the migration of cells to the eye. We observed that treatment with FTY was able to inhibit the development of the disease (Fig. 1A). Histopathologic examination showed retinal disorganization and the presence of inflammatory cells in the vitreous in mice with EAU on day 21 (Fig. 1B). In treated animals (EAU × FTY) it is possible to observe a normal organization of the retina.

**FTY720 Prevents Cell Migration to the Eye**

It has been shown that FTY720 sequesters T lymphocytes in secondary lymphoid organs and inhibits their migration to the inflammatory site. In agreement with this finding, our results show that 21 dpi nontreated mice had a significantly higher number of infiltrating cells in the eye than did FTY-treated mice (Fig. 2).

**FTY720 Reduces the Percentage of CD4⁺CD25⁺ T-Cell Population in the Eye**

It is known that in animals with EAU, CD4 T cells migrate to the eye and are responsible for the induction of the inflammatory response, leading to the lesions observed in disease progression. In addition, it is known that FTY prevents T-cell migration to the inflammatory site with a more important effect in CD4⁺ T cells. Therefore, we analyzed the percentages of cells in the eyes of mice treated with FTY and of mice that did not receive treatment. Lower percentages and absolute cell numbers of CD4⁺CD25⁺ T cells were observed in the eyes of treated mice than of nontreated mice (Fig. 3A). Also noted was a decrease in the frequency of CD4⁺CD25⁺Foxp3⁺ regulatory T cells inside the eye of treated mice, though the absolute cell numbers were similar to those of nontreated mice (Fig. 3B). On the other hand, although there was no difference in the percentage of the CD4⁺IL-17⁺ T-cell population when EAU and EAU + FTY mice were compared, we observed a decrease in absolute cell numbers of this cell population in FTY-treated mice (Fig. 3C).

**Treatment with FTY720 Causes a Change in the Percentage of CD4⁺Foxp3⁺ and CD4⁺IFN-γ⁺ T Cells in Lymph Nodes**

We analyzed the percentages and absolute cell numbers of CD4⁺CD25⁺ and CD4⁺Foxp3⁺ T cells in the lymph nodes of animals treated with FTY and those left untreated. Our study showed a significant increase in the percentage of CD4⁺Foxp3⁺ cells in treated animals when compared with control animals (Fig. 4A), but no difference was found in absolute cell numbers.

Our study also showed a significant increase in the frequency of CD4⁺IFN-γ⁺ T cells in the lymph nodes of treated animals (Fig. 4B), whereas CD4⁺IFN-γ⁻ absolute cell numbers were similar when EAU mice were compared to EAU + FTY mice. On the other hand, percentages and absolute numbers of CD4⁺IL-17⁺ T cells were similar in control and treated animals (Fig. 4D).

**FTY720 Is Capable of Decreasing the Absolute Cell Numbers of CD4⁺ T Cells in the Spleen**

It has been shown that FTY720 has a more prominent effect in CD4⁺ than in CD8⁺ T-cell populations. In agreement with this, our data from spleen cells showed a significant decrease in absolute cell numbers of CD4⁺ compared with CD8⁺ T cells (Fig. 5A). Considering that autoreactive Th1 cells mediate EAU, this decrease corroborates with the lower level of disease observed in FTY720-treated mice. We also found in this group a reduced absolute cell (lymphocyte) number in spleen...
and lymph node that did not reach statistical significance (Fig. 5B).

**Analysis of IFN-γ and IL-17 Levels in Spleen**

Spleen cells of treated animals produced the same amount of IFN-γ that the cells of control animals produced (Fig. 6A).

Similarly, with regard to the production of IL-17, there was no difference in cytokine production between treated animals and control animals (Fig. 6B).

**Anti–IRBP IgG2a and IgG1 Levels Presented No Change after FTY Treatment**

As shown in Figure 7A, anti–IRBP IgG2a levels were not altered in the sera of treated animals compared with control. In addition, no significant difference was observed in the levels of anti–IRBP IgG1 antibodies in both groups (Fig. 7B).

**FTY720 Inhibits the Severity of EAU after Drug Withdrawal**

FTY-treated (7–21 days) animals were killed at 49 dpi of EAU induction. Histopathologic analysis showed that FTY was able to maintain the animals without disease recurrence (Fig. 8). Figure 8A shows that treated mice were compared with untreated mice but had no evidence of disease. Figure 8B shows that treated animals continued without intraocular lesions after the drug was withdrawn.

**DISCUSSION**

The model of EAU in mice contributes significantly to the establishment of parameters for possible therapies for poste-
rior human uveitis. Studies of genetic susceptibility and resistance to EAU, characterization of uveitogenic epitopes, and studies of tolerance in EAU by anterior chamber-associated immune deviation or systems of oral tolerance have been successful according to this model. Our study showed that the treatment with FTY (fingolimod) was able to inhibit the development of disease in animals immunized with the IRBP peptide. This can be seen in histopathologic analysis in which treated animals did not have retinal lesions, in contrast to untreated animals with disorganized retinal layers, intense retinal folds, and inflammatory infiltrating cells in the vitreous.

We also observed, by harvesting and counting eye-infiltrating lymphocytes, that FTY significantly impaired the migration of these cells to the eye. In agreement with our data, Raveney et al. showed that treatment with fingolimod rapidly reduces ocular infiltrates in EAU in mice.

EAU development in mice is provided by CD4 T cells with a Th1-like phenotype, high levels of IFN-γ, and low levels of IL-4 production. We evaluated by flow cytometry the phenotype of eye-infiltrating cells and observed that there was a decrease in the percentage of CD4+CD25+ and CD4+CD25+Foxp3+ cells, whereas the percentage of CD4+IL-17+ cells was similar when compared with nontreated animals. Moreover, the absolute numbers of CD4+CD25+ and CD4+IL-17+ infiltrating cells was lower in FTY-treated mice. CD4+CD25+Foxp3+ absolute cell numbers infiltrating the eye were similar in EAU and FTY+EAU groups.

Our findings in the eye confirm the already known role played by FTY, which is to prevent cell migration to the inflammatory site. The results also suggest an additional role for FTY: the improvement of CD4+CD25+Foxp3+ function. Similar absolute numbers of eye-infiltrating cells with Treg phenotype were observed in both groups, but disease was prevented only in FTY-treated mice (Figs. 1, 3). Therefore, we can argue that FTY200 promoted the improvement of Treg function. This cell population was capable of inhibiting CD4+IL-17+ -producing cells. In accordance with our data, Zhang et al. showed that FTY200 administered at the onset of EAN caused the accumulation of Foxp3+ cell in sciatic nerve during the period of recovery from neurologic disease, suggesting a contribution of Foxp3+ cells to the resolution of EAN. To evaluate a possible effect of FTY200 treatment in the periphery, we counted spleen and lymph nodes cells of mice induced with EAU and performed flow cytometry.

FTY-treated mice showed significantly higher expression of CD4+Foxp3+ and CD4+IFN-γ+ cells in lymph nodes. We believe that in our model, the increase in IFN-γ production in
lymph node was associated with the enhancement of CD4\(^+\)Foxp3\(^+\) cells in this site (Figs. 4A, B). Consistent with our hypothesis, Kelchtermanns et al.\(^{37}\) showed that in the experimental model of collagen-induced arthritis, the absence of IFN-\(\gamma\) was associated with disease susceptibility and impaired function of CD4\(^+\)CD25\(^+\) Treg cells. In transplantation, mice tolerized to alloantigen experience enhanced IFN-\(\gamma\)-production by CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells. The binding of IFN-\(\gamma\) to its receptor on Tregs upregulates STAT1 activation, causing in turn the maintenance of these cells in the graft and an enhancement of their regulatory role.\(^{38}\) Altogether these findings suggest that, in our model, FTY treatment led to the accumulation of a regulatory T-cell population and to improved function in lymph nodes that was supported by the production of IFN-\(\gamma\). Evaluating the profile of the migration of these cells is needed to clarify their role in EAU. Yopp et al.\(^{39}\) showed that T cells migrate from peripheral lymph nodes in response to both chemokine (CCL12) and S1PR activation. It is possible that in our model, FTY caused a preferential migration of Treg cells from lymph nodes to the eye and, thus, contributed to the inhibition of EAU development.

Absolute cell numbers in lymph nodes from FTY-treated mice decreased but did not reach statistical significance. Treatment caused a significant decrease in splenic CD4\(^+\) T cells, but the decrease in CD8\(^+\) T cells did not reach statistical significance. The major effect of FTY on CD4\(^+\) T cells has been reported by others.\(^{34}\) At early periods (as early as 3 hours) after FTY720 administration, an important increase of cells in lymph node has been shown.\(^{40}\) However, in C57BL/6 mice treated for 21 days with FTY720, Morris et al.\(^{41}\) found that after a transient increase in peripheral lymph nodes and Peyer’s patches, lymphocyte recirculation reaches a new steady state. All lymphatic organs showed a decline in lymphocyte numbers as the treatment time was extended to 21 days.

Antibodies were investigated in mice sera, and we confirmed the previously described\(^{16}\) higher levels of IgG2a than IgG1, suggesting that the inhibition of EAU development by FTY was not the result of immune deviation. Moreover, FTY treatment did not cause any statistical difference in the levels of evaluated antibodies compared with those of nontreated mice.

The inflammatory process in the eye occurs between 6 and 9 dpi.\(^{42}\) After 9 days it is possible to observe an infiltrate of small cells in the retina and choroid in the eyes of B10.RIII mice. Among these cells are macrophages, CD4\(^+\) T lymphocytes, and dendritic cells that increase in number between 12 and 15 dpi, causing the onset of structural damage in retina. In 3 weeks (21–26 dpi), there was a decrease in the infiltration of inflammatory cells because of lower numbers of cells migrating to the eye. However, the intense intraocular inflammatory response already set causes retinal degeneration.\(^{43}\) We started treatment with FTY early (7 days after EAU induction) in cell migration to the eye. Even when the drug was withdrawn (21 dpi), EAU development was inhibited. This result suggests that FTY, which prevents cell migration to the eye during the inflammatory period, is a drug with an early mechanism of action capable of inhibiting EAU development. Treatment extended to 21 days also enabled the development of Treg cells capable of regulating EAU antigen-specific effector cells.

It is important to emphasize the need to study the effect of FTY during relapse, which, according to Chan et al.,\(^{44}\) occurs 5 and 10 weeks after immunization. Studies of long-term treatment are important; the effect on the production of specific antibody has been previously reported.\(^{44}\) Our group has observed that treatment with IVLA-4 in the efflent phase is effective at reducing ocular damage in animals killed in 49 days.\(^{45}\)

In conclusion, FTY was able to prevent EAU development when administered between 7 and 21 dpi. In addition, the drug withdrawn did not lead to disease, suggesting the development of a regulatory state. We believe that FTY caused a decrease of eye-infiltrating cells without inhibiting Treg accumulation in this site, that Treg cells in the eye were enhanced by FTY treatment and thus impaired the effects of CD4\(^+\)IL-17\(^+\) cells, and that, in lymph nodes, the increased percentage of CD4\(^+\)Foxp3\(^+\) cells, which could have been the source of Treg cells found in the eye. Our results suggest a possible therapeutic use of this immunomodulatory drug for the treatment of ocular autoimmune disease of autoimmune etiology.

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**References**


**Figure 8.** Treatment with FTY prevents ocular pathology until 49 dpi even after the drug is withdrawn. B10.RIII mice were immunized with 25 μg 161-180 IRBP peptide on day 0 and were treated (gavage) with FTY or saline solution (vehicle control) until 21 dpi. Eyes were collected for histopathology on 49 dpi. (A) EAU score was assigned by histopathologic examination of the eyes. (B) Histopathologic features representative of the EAU scores: mice immunized with IRBP and treated with vehicle control (EAU) show ocular lesions characterized by cells infiltrating the vitreous and retinal folds; mice treated with FTY (EAU + FTY) exhibit a normal retinal architecture corresponding to nonimmunized naïve mice. Representative photographs (hematoxylin-eosin staining). Original magnifications: 100× (left); 400× (right).


