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ORIGINAL ARTICLE

The thymus is required for the ability of FTY720 to prolong skin allograft survival across different histocompatibility MHC barriers

María L. del Rio,[1] Oliver Pabst,[1] Pablo Ramirez,[2] Giovanna Penuelas-Rivas,[2] Reinhold Förster[1] and Jose-Ignacio Rodriguez-Barbosa[1,3]

1 Institute of Immunology, Hannover Medical School, Hannover, Germany
2 Department of Liver Transplantation, Arrixaca University Hospital, Murcia, Spain
3 Institute of Biomedicine, University of León, León, Spain

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Correspondence
Dr Jose-Ignacio Rodriguez-Barbosa PhD, DVM, Institute of Biomedicine, University of León, 24071 León, Spain. Tel.: +34 629 990605; fax: +34 987 291304; e-mail: ignacio.barbosa@unileon.es

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Abstract

The immunosuppressive effect of FTY720 is associated with the reversible sequestration of lymphocytes from the blood and the spleen into secondary lymphoid organs and reduced egress of mature thymocytes from the thymus. This work was designed to dissect the differential effect of FTY720 on CD4 and CD8 T cell-mediated mechanisms of skin graft rejection in the presence (euthymic) or absence (thymectomized) of thymic output. To that end, untreated and FTY720-treated euthymic (Euthy) and thymectomized (ATX) mice received skin allografts across a full, class II or class I major histocompatibility complex (MHC) mismatched (MM) barriers and graft survival was monitored. We demonstrate that a short course of FTY720 treatment significantly augments the survival of full, class I and class II MHC MM skin grafts compared to the nontreated controls. Interestingly, FTY720-treated Euthy recipients showed a significantly prolonged skin allograft survival compared to FTY720-treated ATX mice. These results together show that FTY720 impairs both CD4 and CD8 T cell-mediated mechanisms of rejection and, more importantly, the presence of the thymus is necessary for the ability of FTY720 to modulate skin allograft rejection across different histocompatibility MHC barriers.

Introduction

FTY720 (2-amino-2[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) is a potent immunosuppressant derived from ISP-I (myriocin and thermozymocidin) molecule isolated from *Isaria sinclairii* [1,2]. FTY720 has been shown to prolong the survival of the heart [3,4], liver [5], small bowel [6,7], and skin allografts in animal models at doses ranging from 0.1 to 1 mg/kg [8–11]. FTY720 also prevents the development of graft-versus-host disease and autoimmune diseases [12–14]. Of remarkable interest is to mention that FTY720 has advanced to the clinical arena in multiple sclerosis [15]. Phase III clinical trials are currently in progress to prove definitely the therapeutic efficacy of this immunosuppressant to treat patients suffering from multiple sclerosis [16,17]. FTY720 is rapidly phosphorylated *in vivo* by sphingosine kinase 2, and the active form FTY720-phosphate (FTY720-P) acts as an agonist on four of the five shingosine-1-phosphate receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$) [18,19]. FTY720-P induces the internalization of $S1P_1$ rendering lymphocytes unable to respond to S1P. The absence of $S1P_1$ signaling impedes the entry of B and T cells into the efferent lymphatics of lymph node, and prevents their return to the periphery [20–22].

It was initially postulated that FTY720-induced immunosuppression was, in part, caused by the pro-apoptotic

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effect on lymphocytes [23–25]. However, this hypothesis has been neglected particularly for the range of dosage of clinical relevance (0.25–3.5 mg). Thus, increasing evidence points out in the direction that the immunosuppressive effect of FTY720 in humans is associated with migration and sequestration of lymphocytes from the periphery and not with the induction of lymphocyte apoptosis [26–28]. Additionally, the drug also affects the homing of T cells to secondary lymphoid organs (SLO) across high endothelial venules in an integrin-dependent [29], G-alpha(i)-dependent (Pertussis toxin sensitive), CCR7-CCL19/CCL21-independent manner [30]. In consequence, FTY720-mediated immunosuppression is associated with reduced numbers of mature $CD4^+$ and $CD8^+$ single positive cells leaving the thymus as well as sequestration of B and T cells in the lymph nodes.

Several studies have demonstrated that FTY720 modulates the kinetics of the skin and vascularized solid organ allograft rejection across a full MHC MM barrier [10,14,31,32]. Herein, we provide evidence that FTY720 impairs both CD4 and CD8 T cell-dependent alloimmune responses. Strikingly, we observed that the presence of the thymus significantly prolonged skin allograft survival across a full and class II MHC MM barrier in FTY720-treated mice compared to similarly treated ATX mice. This indicates that the thymus contributes to the FTY720-mediated modulatory effects on the course of skin allograft rejection.

Material and methods

Strains of mice

Six-to-eight week old female Euthy and ATX C57BL/6 (K^b, IA^b) mice were treated with FTY720 or left untreated as controls, and used as recipients of female BALB/c, B6.C-H-2^{bm12}/KhEg (bm12) (K^b, IA^{bm12}) or B6.C-H-2^{bm1}/By (bm1) (K^{bm1}, IA^b) tail skin allografts. bm1 and bm12 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred in our animal facility, whereas C57BL/6 (H-2^b, CD45.2) and BALB/c (H-2^d) were acquired from Charles River Laboratories (Sulzfeld, Germany). Ly5.1 (C57BL/6.SJL-Ptprca, CD45.1) mice were bred in the animal facility of Hannover Medical School. All experiments with rodents were handled and cared in accordance with institutional guidelines for Animal Care and Use of Laboratory Animals that have been approved by the local committees.

Thymectomy and transplantation surgery

Allogeneic skin graft transplantation and transplantation surgery were carried out under anesthesia as previously described [33]. Thymectomies were performed 15 days before skin graft transplantation according to the protocol described previously [34].

FTY720 administration

FTY720 (Novartis Pharma AG, Basel, Switzerland) was dissolved in sterile water and added to the drinking water at 2.5 μg/ml (considering that a mouse of 25 g of body weight drinks 3 ml daily, the dose received would be approximately 0.3 mg/kg body weight). Euthy and ATX C57BL/6 recipients that received full, class I or class II MHC MM skin grafts were treated with a 15-day course of FTY720. Congeneic bone marrow chimeric mice were treated chronically with FTY720, starting at the day of bone marrow transplantation. Drinking water containing FTY720 was replaced every 3–4 days.

FTY720 chronically treated T cell-depleted congeneic bone marrow chimeric mice

Given that FTY720 partially impairs the egress of T cells from the thymus, we designed a long-term experimental strategy to monitor the accumulation of T cells in the periphery released from the thymus under the continuous effect of the drug.

To that end, chimeric mice were prepared using bone marrow cells collected from tibia and femur of Ly5.1 C57BL/6 mice. Bone marrow cells were T cells depleted by negative selection using LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) and biotinylated rat anti-mouse Thy1.2 mAb (CD90.2, clone 30-H12) followed by streptavidin magnetic beads. A total of 15×10^6 T-cell depleted bone marrow cells were injected i.v. to sublethally (5.5 Gy) irradiated C57BL/6 (CD45.2) recipients that were continuously treated with FTY720 in the drinking water (0.3 mg/kg). T-cell reconstitution was monitored 6 weeks after bone marrow transplantation.

The spleen and pooled pLNs (axilar, brachial, inguinal, cervical, and mesenteric) were collected and stained with anti-CD4 and -CD25 monoclonal antibodies (mAb) and the ratio of the absolute number Treg ($CD4^+CD25^+$)/non-Treg ($CD4^+CD25^-$) was calculated. Thus, we could evaluate to what extent donor-derived Ly5.1 Treg and non-Treg were exported to the periphery and whether the distribution of Treg ($CD4^+CD25^+$)/non-Treg ($CD4^+CD25^-$) was affected in the SLO under the continuous influence of FTY720.

Flow cytometry

To ensure complete thymectomy, residual thymopoietic activity was checked at the time of euthanasia by using two-color flow cytometry of cell suspensions prepared

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from the tissue collected around the area where the thymus was originally located. Six weeks after congeneic Ly5.1 to Ly5.2 bone marrow transplantation, donor-derived T-cell reconstitution was analyzed and the following mAbs were used: TcR$\alpha\beta$ (H57-597), CD4 (RM4-5), CD8β (53–6.7), and CD45.1 (A20). Regulatory T-cell recovery was also examined using rat anti-mouse CD25 (PC61 5.3) and rat anti-mouse CD4 mAbs. All the antibodies were purchased from BD Biosciences (San Jose, CA, USA), except Alexa 405 and Cy5-labeled anti-mouse CD4 (RM4-5) and Alexa 405, which were prepared in our laboratory. The extent of the T-cell depletion achieved before bone marrow transplantation was checked by flow cytometry with anti-CD4 and anti-CD8 mAbs.

Nonspecific FcγR binding was routinely blocked with rat anti-mouse mAb 2.4 G2 (CD16/CD32) [35]. Dead cells were excluded by propidium iodide or 4,6-diamido-2-phenylindole hydrochloride staining and gated living cells were analyzed from each sample. Flow cytometric analysis was carried out using LSR II cytometer (Becton Dickinson, Mountain View, CA, USA) and the data analysis was performed using a WINLIST 5.0 (Verity Software House, Inc., Topsham, ME, USA).

Statistical analysis

Student's *t*-test was used to compare the means of the groups that follow normal distribution. The nonparametric Mann–Whitney *U*-test was used to compare the medians of groups that did not follow a normal distribution according to the Shapiro-Witt test. A P-value < 0.05 was considered significant. EXCEL (Microsoft, Redmond, WA, USA) and GRAPHPAD PRISM version 4 software (GraphPad Software Inc., San Diego, CA, USA) were used to perform the statistical analyses. Skin graft survival curves were calculated by using the Kaplan–Meier life table method and statistical analysis for the comparison of the survival curves was performed by the log rank test.

Results

FTY720 impairs CD4 and CD8 T cell-mediated mechanisms of rejection

It has been widely reported in the literature that FTY720 significantly delays skin graft rejection across a full MHC MM barrier [8,10,31]. In line with this observation, here, we demonstrate that FTY720 significantly delays skin graft rejection across full MHC MM barrier not only in Euthy, but also in ATX recipients compared to nontreated controls ($P < 0.005$ and $P < 0.05$ respectively, Fig. 1a). However, little is known about the effect of FTY720 in modulating CD4 or CD8 T cell-mediated mechanisms of rejection. To that end, we were interested in determining whether FTY720 treatment affected differently CD4 or CD8 T cell-mediated mechanisms of rejection across class II and class I MHC MM barriers.

It is generally accepted that rejection of class II MHC disparate skin grafts is a process mainly mediated by CD4 T cells [36]. We first determined whether a short course of FTY720 affected CD4 T cell-mediated mechanisms of graft rejection. Thus, class II MHC MM bm12 tail skin was grafted into C57BL/6 recipient mice. We observed that both FTY720-treated Euthy and ATX mice rejected significantly later than their untreated counterparts ($P < 0.001$, Fig. 1b).

Next, we went on to evaluate the modulatory effect of FTY720 in the rejection process of class I MHC MM skin allografts. The major contributors to rejection of class I MHC MM grafts are CD8 T cells [36,37]. Class I MHC MM skin allografts from bm1 donors were placed in C57BL/6 mice and graft survival was monitored. Similar to class II MHC MM skin allografts, FTY720-treated Euthy mice showed a significant prolonged graft survival compared to nontreated Euthy and ATX controls ($P < 0.01$, Fig. 1c).

Together, these data clearly reveal that a short course of FTY720 administration downmodulates both CD4 and CD8 T cell-mediated allogeneic responses.

The thymus is required for the ability of FTY720 to prolong graft survival

We unexpectedly observed that FTY720-treated Euthy C57BL/6 (H-2^b) recipients of a full MHC MM skin graft from BALB/c (H-2^d) showed a statistically significant prolonged graft survival compared to similarly treated ATX recipients ($P < 0.05$, Fig. 1a). Similarly, a significant prolonged graft survival was also observed in FTY720-treated Euthy C57BL/6 mice that received a class II MHC MM skin allograft compared to equally treated ATX mice ($P < 0.05$, Fig. 1b). However, a nonsignificant trend was observed when graft survival in FTY720-treated Euthy was compared to that of similarly treated ATX recipients receiving a class I MHC MM skin allograft (Fig. 1c). These observations together favor the idea that the presence of the thymus is critical for the ability of FTY720 to mediate its modulatory effect across a full and class II MHC MM barriers.

Partial T-cell reconstitution is achieved under FTY720 treatment

It has been shown that FTY720 induces a partial blockade of T-cell export from the thymus, leading to a 75% reduction of mature single positive T cells being released into the periphery of mice that received daily 1 mg/kg body weight of FTY720 [22,38].

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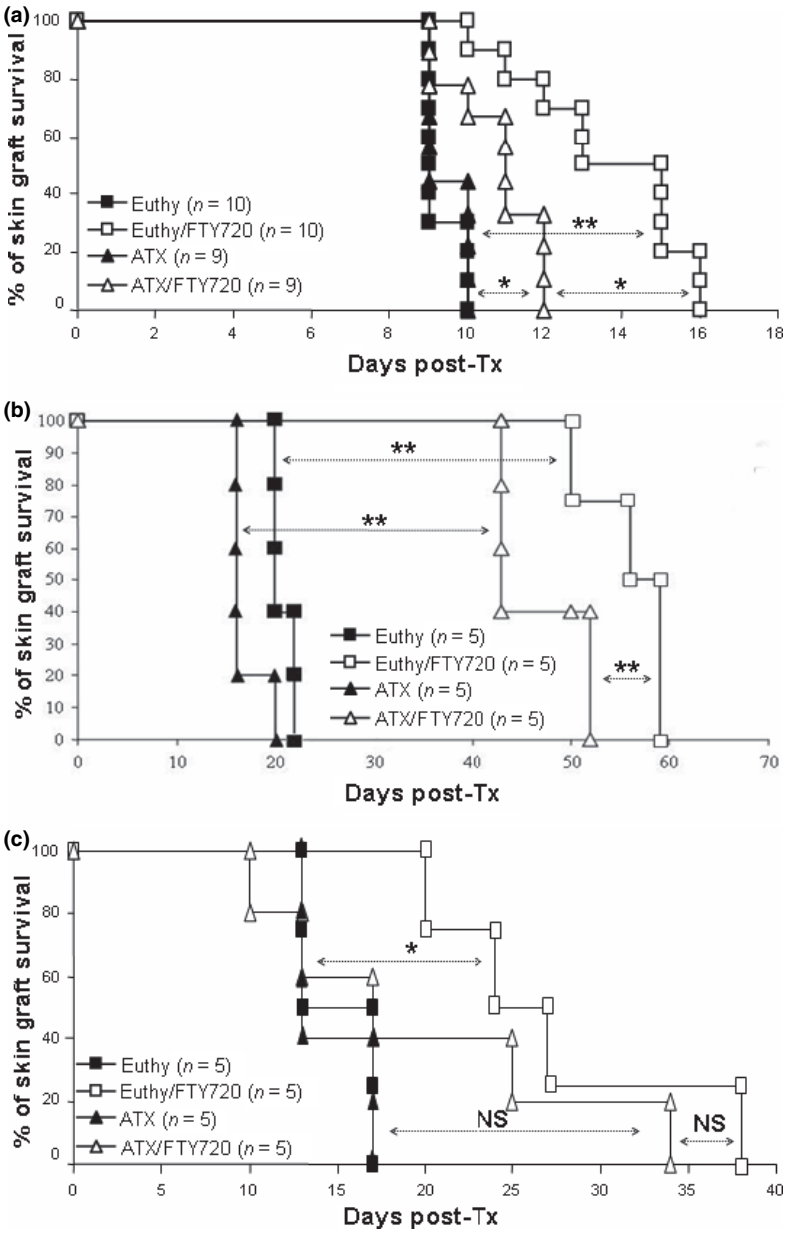

Figure 1 FTY720 enhances graft survival across fully, class II, and class I MHC MM barriers. (a) FTY720 significantly enhances fully MHC MM skin graft survival in both Euthy and ATX mice. Euthy and ATX recipient mice received in the drinking water a dose equivalent to 0.3 mg/kg body weight of FTY720 for 15 days. FTY720-treated Euthy (□) and ATX (Δ) mice exhibited a significantly prolonged graft survival compared to non-treated Euthy (■) and ATX (▲) controls (**$P < 0.005$ and *$P < 0.05$, respectively). Interestingly, FTY720-treated Euthy mice displayed a statistically significant prolonged skin graft survival compared to similarly treated ATX mice (*$P < 0.05$), suggesting a role of the thymus in prolonging skin graft survival. Data from two experiments are shown. (b) FTY720 significantly diminished CD4 T cell-mediated rejection of class II MHC MM skin grafts and the presence of the thymus contributed to the enhanced graft survival across this MHC barrier. Recipient mice received a 15-day course of FTY720 in the drinking water (0.3 mg/kg body weight). FTY720-treated Euthy (□) and ATX (Δ) mice exhibited a significantly longer graft survival than the respective untreated Euthy (■) and ATX control mice (▲) (**$P < 0.005$ for both). Remarkably, the presence of the thymus significantly enhanced skin graft survival of FTY720-treated Euthy (□) compared to similarly treated ATX mice (Δ,) (**$P < 0.005$). (c) FTY720 delays CD8 T cell-mediated graft rejection of class I MHC MM allografts. FTY720-treated Euthy mice (□) showed enhanced graft survival compared to nontreated Euthy (■) and ATX mice (▲) (*$P < 0.05$). However, unlike full and class II MHC MM grafts, the presence of the thymus did not significantly improve graft survival of FTY720-treated Euthy mice (□) compared to similarly treated ATX mice (Δ). Skin graft survival curves were calculated by using the Kaplan–Meier life table method and statistical analysis for the comparison of the survival curves was performed by the log rank test.

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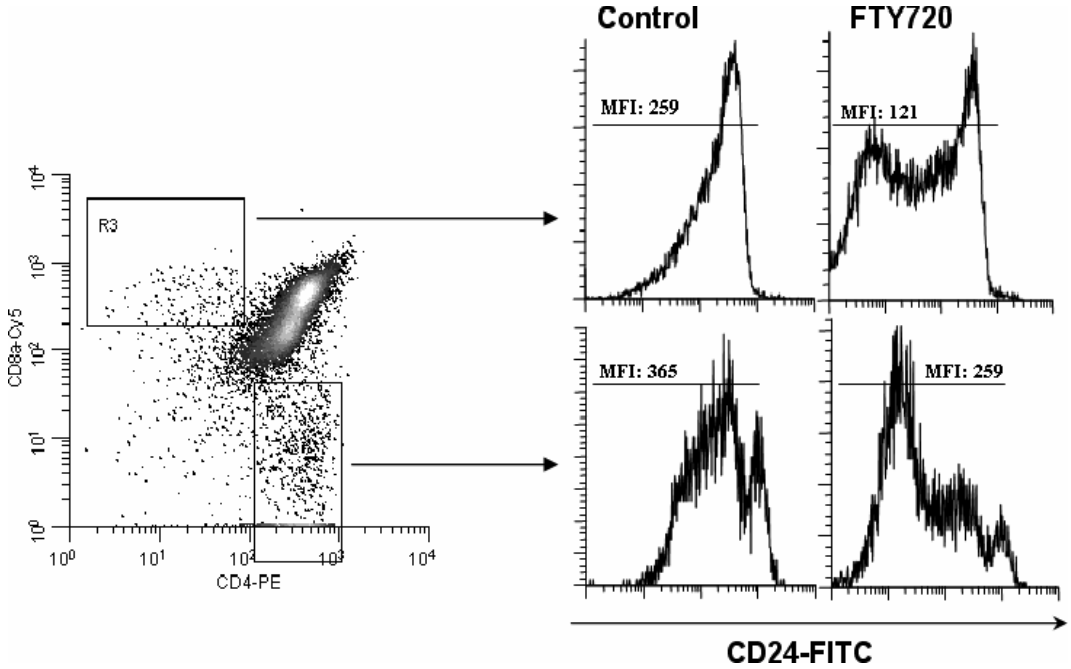

Figure 2 T cells differentiated in the thymus under the effect of FTY720 display an immature phenotype based on CD24 expression. Thymopoiesis of untreated control and FTY720-treated Ly5.1 to Ly5.2 chimeric mice was assessed. T-cell differentiation was monitored in the thymus and CD4 and CD8 single positive T cells were gated and the expression of CD24 was represented as histograms. One mouse representative of five, all with similar results.

The observation that the thymus influenced the course of graft rejection of FTY720-treated mice led us to design an experimental approach to evaluate the effect of long-term administration of the drug on thymic export and peripheral T-cell reconstitution. Thus, FTY720 chronically treated congeneic Ly5.1 to Ly5.2 bone marrow chimeras were prepared and donor-derived thymopoiesis and peripheral T-cell reconstitution was monitored at 6 weeks after bone marrow transplantation. These chimeric mice displayed a peripheral T-cell repertoire composed of T cells that have been recently exported from the thymus under the constant influence of the drug. We could observe that donor-derived thymopoiesis progressed normally in FTY720-treated as in nontreated chimeric controls. This suggests that FTY720 affected neither the migration of donor-derived lymphoid precursors from the bone marrow to the thymus nor the process of T-cell differentiation (data not shown) [22]. However, CD4 and CD8 single positive thymocytes accumulating in the thymus exhibited a phenotype corresponding to more immature cells compared to untreated chimeric control mice based on the CD24 expression as shown in Fig. 2.

We then went on to determine to what extent FTY720 affected host- and donor-derived TcR$\alpha\beta^+$CD4$^+$ and TcR$\alpha\beta^+$CD8$^+$ T-cell reconstitution in Ly5.1 to Ly5.2 bone marrow chimeric mice. In agreement with the concept that FTY720 administration reduces thymic T-cell egress, we observed that T-cell reconstitution was significantly less efficiently achieved in FTY720-treated than in untreated controls. Thus, untreated mice showed a significant higher absolute number of host-derived CD4 and CD8 T cells in the spleen compared to FTY720-treated mice ($P < 0.0005$). Statistically significant differences were also found in pLNs of untreated versus FTY720-treated mice in host-derived CD4 T cells ($P < 0.005$) and in host-derived CD8 T cells ($P < 0.05$) (Fig. 3a and b).

Similarly, the absolute number of donor-derived TcR$\alpha\beta^+$CD4$^+$ and TcR$\alpha\beta^+$CD8$^+$ T-cell reconstitution was also significantly decreased in FTY720-treated than in untreated controls not only in the spleen (FTY720 versus control, $P < 0.0005$), but also in pLNs (FTY720 versus control $P < 0.005$) (Fig. 3c and d).

These data indicate that despite the blocking effect of FTY720 in limiting thymic T-cell egress, T cells still managed to exit and contribute to a certain degree to peripheral T-cell reconstitution.

FTY720 administration augments the ratio Treg/non-Treg in secondary lymphoid organs

To gain further insight into the role of the thymus in facilitating graft survival under FTY720 treatment, we

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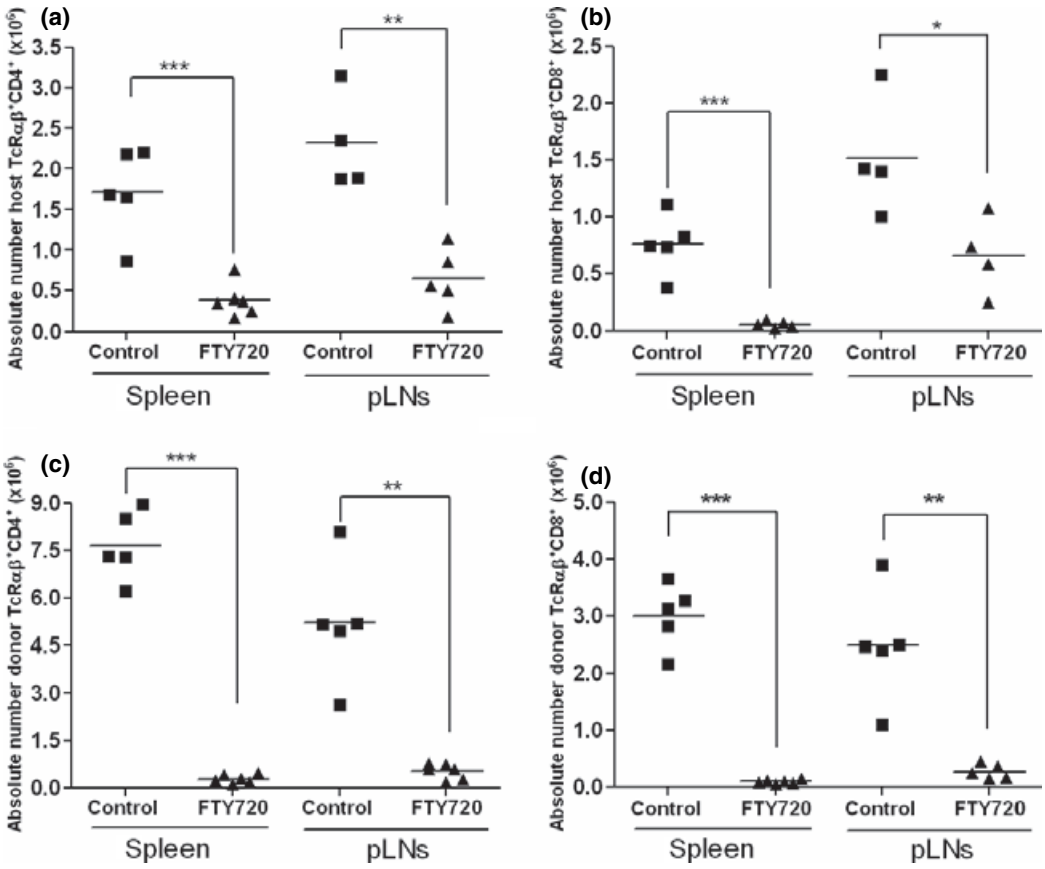

Figure 3 T-cell recovery is achieved to certain extent despite FTY720 blockade of thymic output. Congeneic Ly5.1 bone marrow cells were transplanted to sublethally irradiated Ly5.2 recipients that were treated chronically with FTY720 (0.3 mg/kg in the drinking water) starting at the day of bone marrow transplantation. T-cell reconstitution was assessed after 6 weeks. The absolute number of host-derived TcRαβ$^+$CD4$^+$ (a) and TcRαβ$^+$CD8$^+$ T cells (b) was significantly reduced in FTY720-treated chimeric mice (▲) compared to untreated controls (■) in both spleen and pLNs (***$P < 0.0005$ and **$P < 0.005$, respectively). The absolute number of donor-derived TcRαβ$^+$CD4$^+$ (c) and TcRαβ$^+$CD8$^+$ T cells (d) was also significantly lower in FTY720-treated chimeric mice (▲) compared to untreated controls (■) in both spleen (***$P < 0.0005$) and pLNs (**$P < 0.005$). Each symbol represents an individual animal. Student's *t*-test was used for the comparison of the groups.

assessed the effect of the drug in the distribution of Treg (CD4$^+$CD25$^+$) and non-Treg (CD4$^+$CD25$^-$) in SLO of congeneic bone marrow chimeric mice that were chronically treated with FTY720.

The rational for the use of bone marrow chimeric mice was based on the hypothesis that these mice would only display a T-cell repertoire of Treg and non-Tregs that managed to egress the thymus under the continuous effect of the drug.

We observed that FTY720 affected the distribution of Treg/non-Treg in SLO of chimeric mice. Thus, the ratio absolute number of donor-derived Treg/non-Treg was significantly augmented in FTY720-treated chimeric mice compared to the nontreated chimeric controls in the spleen and pLNs ($P < 0.05$, Fig. 4).

The information gathered from this experiment indicates that the administration of FTY720 affects the distribution of peripheral Treg/non-Treg cells that managed to leave the thymus under the effect of the drug.

Discussion

Our report provides new knowledge to understand the immunosuppressive mechanism of action of FTY720. We demonstrate that (i) FTY720 modulates CD4 and CD8 T cell-mediated mechanisms of rejection; (ii) the presence of the thymus modulates the ability of FTY720 to exert its immunosuppressive effect.

Numerous reports have clearly demonstrated that FTY720 prolongs graft survival in the skin and vascularized murine models of transplantation [4,6,7,9]. The increased graft survival observed in mice receiving FTY720 has been attributed to impaired cellular and humoral-mediated immune responses [31,39–42].

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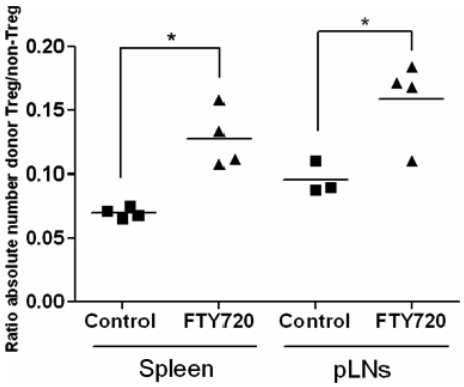

Figure 4 FTY720 increases the ratio absolute number Treg/non-Treg in SLO of chimeric mice. Continuous administration of FTY720 to congeneic bone marrow chimeras significantly augmented the ratio absolute number donor-derived Treg ($CD4^+CD25^+$) non-Treg ($CD4^+CD25^-$) in spleen of FTY720-treated mice (▲) compared to untreated controls (■) (*$P < 0.05$). Similar significant increase of the ratio absolute number donor-derived Treg/non-Treg was observed in pLNs of FTY720-treated chimeric mice (▲) compared to nontreated controls (■) (*$P < 0.05$).

A short-course administration of FTY720 extends fully MM skin graft survival in rats in a dose-dependent manner and the effect is already evidenced at doses as low as 0.1 mg/kg body weight [9,31,43]. Our results are in agreement with these studies as we observed a significant increase in graft survival in FTY720-treated Euthy and ATX recipients compared to the respective untreated controls. Besides, FTY720 also prolonged graft survival across class II and class I MHC MM barriers indicating that the drug was capable interfering with both CD4 and CD8 T cell-dependent mechanisms of graft rejection [44].

Interestingly, the presence of the thymus appeared to account for the significant graft survival observed in FTY720-treated Euthy compared to similarly treated ATX mice in skin allografts transplanted across full and class II MHC MM barriers. This suggests that the thymus, or the cells released to the periphery under the influence of the drug, may contribute to the immunosuppressive effect of FTY720. Although speculative, several possible scenarios could be contemplated. One possibility would be that FTY720 affects thymic stromal cells stimulating them to release substances with immunosuppressive activity, which seems very unlikely. The other possibility may derive from the fact that FTY720 leads to the accumulation of immature CD4 and CD8 single positive T cells in the thymic medulla. These immature T cells that manage to leave the thymus may display a diminished efficiency in rejection or may interfere with the ability of pre-existing peripheral T cells to organize the immune response that leads to graft rejection.

FTY720 also modulated the distribution of Treg/non-Treg in SLO. Along this line, Sawicka *et al.* have demonstrated that FTY720 treatment leads to sequestration of Treg in the spleen. Moreover, untreated Treg expressed lower levels of $S1P_1$ and $S1P_4$ than non-Treg and FTY720 treatment induced a significant down-modulation of these two receptors and, as a consequence, Treg cells exhibited a diminished chemotaxis toward peripheral S1P [45]. One prediction of these observations would be that Treg would be more predisposed to be retained in the lymphoid compartments than non-Treg.

Although the kinetics of T-cell infiltration on skin allografts has not been characterized in this preliminary work, our group has recently initiated ongoing experiments to explore thoroughly the impact of FTY720 administration on regulatory and effector T-cell infiltration on skin grafts transplanted across class I and class II MHC-mismatched barriers, not only in naïve mice receiving a short course of FTY720, but also in lethally irradiated mice that are reconstituted with syngeneic bone marrow and chronically treated with FTY720. This approach would allow us to dissect specifically how this immunosuppressant interferes with the migration of effector CD4 and CD8 T cells and its influence in the recruitment of regulatory T cells to the graft site. Besides, it would provide some insight into the role of the thymus and the ability of the drug to interfere with the competence of recently emigrated T cells to respond and reject skin allografts.

The thymus represents a single anatomical site where control of lymphocyte egress could have a significant long-term impact on peripheral immune diversity. The present research emphasizes a potential clinical application of FTY720-mediated inhibition of thymic egress and modulation of the distribution of Treg/non-Treg in SLO. This drug may have a potential application particularly in young patients with a high rate of thymic output subjected to tolerization protocols, in which the peripheral T cells are controlled during the induction phase of the protocol. FTY720 administration would shrink the diversity of the repertoire of T cells by reducing thymic output, and thus may reinforce the maintenance of the operational tolerance [46] induced by the tolerization protocol [47].

Multiple sclerosis is a cellular-mediated autoimmune pathology, in which the encephalitogenic autoreactive CD4 T cells and macrophages attack autoantigens expressed on oligodendrocytes that synthesize the axonal myelin sheaths in the central nervous system (CNS). This culminates in gradual focal demyelination and neurologic disorders [48]. The migration of encephalitogenic effector T cells from the draining lymph node to the CNS is a critical step in the process of the disease. Therefore, any intervention aiming at preventing or delaying the migration of autoreactive T cells would influence the course of the disease. For these reasons, FTY720 has incited tremendous enthusiasm for its obvious potential in the treat-

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ment of MS. In line with these concepts, and the observed effect of short course administration of FTY720 in delaying CD4-mediated skin graft rejection, it would not be venturous to claim that this compound will certainly contribute to a better control of disease progression and relapsing episodes in multiple sclerosis.

To conclude, FTY720 prolongs graft survival by acting on both CD4 and CD8 T cell-mediated mechanisms of rejection and the thymus seems to be essential for the ability of FTY720 to modulate allograft rejection.

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Authorship

M-LdR performed experiments, collected and analyzed the data. OP, PR, RF contributed with suggestions, advice, reagents and to the critical reading of the manuscript. GP-R performed the skin graft experiments, plotted the results and compared the survival curves. J-IR-B designed the experiments, supervised the study and wrote the manuscript.

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