European Journal of Immunology

Transient T cell accumulation in lymph nodes and sustained lymphopenia in mice treated with FTY720

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Received 18/3/05
Revised 25/8/05
Accepted 12/10/05

[DOI 10.1002/eji.200526218]

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) is an orally available immunomodulatory agent that induces severe peripheral blood lymphopenia. Most studies of these lymphopenic effects have been limited to short-term exposure to FTY720. FTY720 alters the ability of lymphocytes to respond to sphingosine-1-phosphate (S1P) through S1P receptors, particularly $S1P_1$. FTY720 affects different leukocyte populations and their trafficking through major lymphoid organs. We show the dynamics of CD4 T, CD8 T, and B lymphocyte recirculation in all major lymphoid compartments during 21-day FTY720 treatment of normal C57BL/6 mice. Following a transient increase in peripheral lymph nodes and Peyer's patches, lymphocyte recirculation reaches a new steady state. Other lymphoid organs show transient changes in lymphocyte composition with various patterns. At 21 days of FTY720 treatment, total body lymphocyte content is reduced by 20% and blood lymphocytes by 80%. Modeling suggests that the new steady state is due to a combination of reduced naive lymphocyte release from the thymus and a transient reduction of lymphocyte egress from lymph nodes. Our data indicate that the commonly held belief that FTY720 blocks lymphocyte egress from lymph nodes cannot fully explain the lymphocyte dynamics observed with prolonged treatment.

Key words: B cells · Cell trafficking · Lymph nodes · T cells · Thymus

Introduction

Sphingosine-1-phosphate (S1P) is an important regulator of lymphocyte trafficking. This lysophospholipid is derived from ceramide, but unlike ceramide, it promotes cell survival and proliferation. S1P exerts its cellular effects through five G protein-coupled receptors called $S1P_{1–5}$ (formerly EDG receptors) [1]. Signaling through these receptors elicits a potent chemotactic response from lymphocytes [2]. S1P also has potent effects on vascular endothelial cells, which may indirectly alter lymphocyte trafficking [3]. The combined effects of S1P on vascular cells and lymphocytes alter lymphocyte recirculation.

A sphingosine analog, 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride (FTY720), acts

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Abbreviations: **DN:** double negative · **DP:** double positive · **FTY720:** 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride · **MLN:** mesenteric lymph node · **PLN:** peripheral lymph node · **PP:** Peyer's patches · **S1P:** sphingosine-1-phosphate, **$S1P_1$:** sphingosine-1-phosphate receptor 1

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as a strong agonist of S1P receptors following its phosphorylation and inhibits lymphocyte egress from primary lymphoid organs [4], accompanied by an increase in lymphocyte numbers in peripheral lymph nodes (PLN) in the first few days of treatment [5, 6]. FTY720 alters the ability of lymphocytes to respond to S1P, possibly by down-regulating expression of $S1P_1$ on the lymphocyte surface [4, 7].

Although FTY720 significantly alters B cell numbers in the peripheral blood, the effects are far more profound on T cells [4–6]. Myeloid cells do not appear to be significantly affected by FTY720 [8], and NK cells are mildly affected [9]. Most of the lymphopenic effects of FTY720 are due to interactions between $S1P_1$ and FTY720 [10]. The selective $S1P_1$ analog, SEW 2871, induces peripheral blood lymphopenia [10]. This, together with unaltered lymphopenia in $S1P_3^{-/-}$ mice, suggests that the lymphopenic effects are dependent upon interactions of FTY720 with $S1P_1$.

The current explanation for the FTY720 effects on lymphocyte numbers is that mature thymocytes respond to S1P in the blood stream in order to leave the thymus as naive T cells [4]. A similar mechanism has been postulated to be responsible for T cells leaving PLN [11], but this has not been verified experimentally. T cells show increased expression of $S1P_1$ on their surface as they mature. High blood plasma levels of S1P may signal the maturing T cell to leave the thymus and enter the blood stream. Conditional knockout mice lacking $S1P_1$ receptors on T cells show an increase of CD4 and CD8 single-positive lymphocytes in the thymus, but a profound lack of T cells in the periphery [4, 12]. In mice reconstituted with $S1P_1^{-/-}$ hematopoietic cells from fetal livers, no T cells are found in the peripheral lymphoid organs, blood, or lymph [4], consistent with their inability to leave the thymus. B cells are found in peripheral lymphoid organs in near-normal numbers, but their numbers are significantly reduced in peripheral blood and lymph [4]. The B cell defect in the absence of $S1P_1$ may relate to their reduced ability to recirculate from peripheral lymphoid organs back to the bone marrow. Additionally, S1P has been shown to retain B cells in splenic marginal zones. Without $S1P_1$ expression, marginal zone B cells localized to the follicles [13].

Few long-term studies of immunologic function in the presence of FTY720 are available. High-dose FTY720 (1 mg/kg/day) leads to both lymphopenia and immune suppression; however, lymphocyte subsets were not distinguished in this study [14]. During long-term treatment (1 mg/kg/day), emigration of thymocytes was decreased over 21 days [15]. The authors of this work investigated immature ($CD4^-CD8^-$), double-positive ($CD4^+CD8^+$), and mature single-positive thymocytes ($CD4^+CD8^-$, $CD4^-CD8^+$).

Although patients need to be maintained on immunosuppressive therapies for extended periods of time, if not for the remainder of their life, no studies have tried to account for total numbers of lymphocytes from all major lymphoid organs during prolonged FTY720 treatment. As the lymphocyte distribution in one compartment can affect that of other compartments, the present study analyzes the composition of all major lymphoid compartments over an extended period of time. Since the blood compartment accounts for less than 5% of all lymphocytes, blood lymphocytes can easily disperse into the large lymphoid compartments like spleen and bone marrow without significantly increasing the size of these compartments.

Results

Mice treated with FTY720 in their drinking water reached steady state blood concentrations of free FTY720 of 20–30 nM, which did not change during the 21-day treatment course (data not shown). While granulocyte counts were not significantly changed before and after treatment (Fig. 1A), or over the course of 21 days (data not shown), total lymphocyte counts in blood were dramatically diminished after 24 h of treatment (Fig. 1A). Total lymphocyte counts rose slightly on day 7, but dipped again to remain at less than 20% of control at day 14 and 21 (Fig. 1B). B cell counts dropped to 15% of control counts (Fig. 1B). Of the different lymphocyte subsets, all T cell subsets were severely affected, with CD8 cells declining to about 4% and CD4 T cells declining to 5% of normal numbers (Fig. 1C). NK cells were the least affected by FTY720 treatment, dropping to 60% of normal counts within the first 24 h (data not shown). These data confirm the profound lymphopenic effects of FTY720 and the differential effect on T and B cells, and show that a new steady state is reached by day 14 of treatment.

During the course of FTY720 treatment, lymphocytes that normally circulate through the peripheral blood compartment and lymphatics are unable to leave the PLN [11]. Indeed, our data show a transient accumulation of lymphocytes in lymph nodes over the first 2 days of treatment (Fig. 2A). While the increase in the B cell compartment is evident by the increased percentage of B cells in the nodes (Fig. 2B), increases in CD4 and CD8 T cells are not evident by merely studying the FACS plots (Fig. 2C), but the increase becomes obvious when total lymphocyte counts are calculated (shown in Fig. 2A). On days 2.5 and 4, all lymphocyte subsets are increased compared to the day 0 group (Fig. 3A). However, by day 21, the cell counts decreased to levels slightly lower than the day 0 group, with a significant decrease in CD4 T cells by this time. We believe that this is due to a

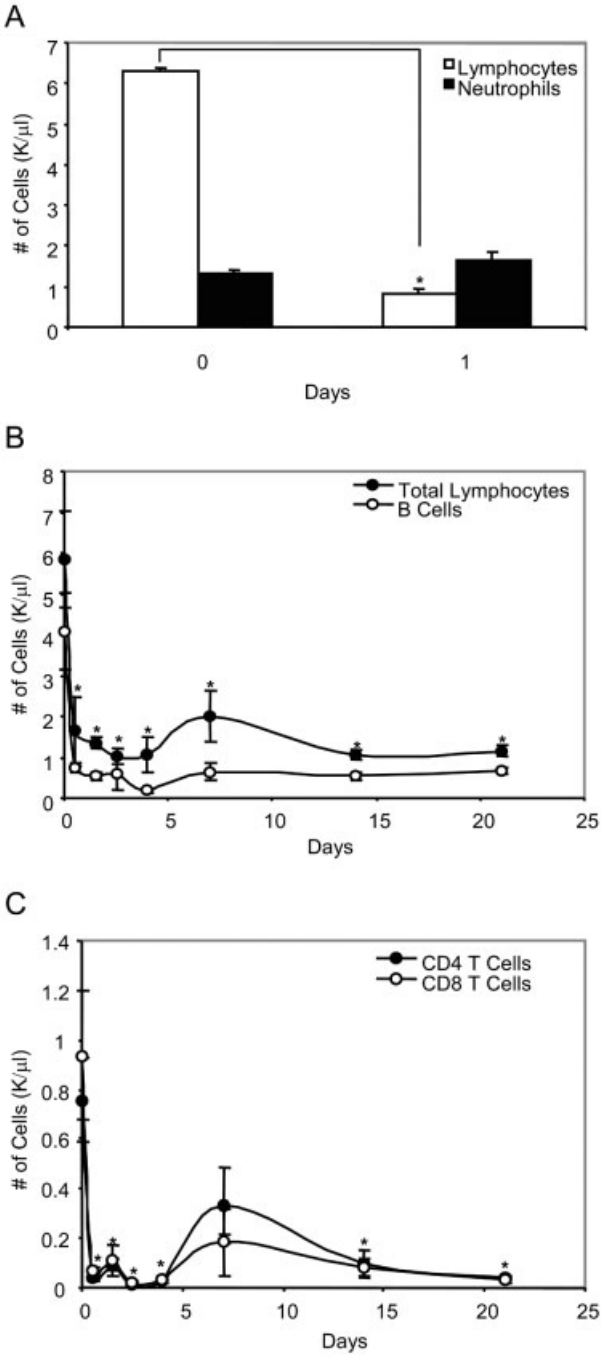

Figure 1. Effect of prolonged FTY720 treatment on lymphocyte subsets in peripheral blood. (A) Comparison of lymphocyte *vs.* neutrophil counts before and 24 h after starting FTY720 treatment. (B) Lymphocyte counts from mice treated with FTY720 over a prolonged 21-day time course. Solid circles indicate total lymphocytes, open circles indicate B cells, and (C) shows CD4 (closed circles) and CD8 (open circles) T cells. All T lymphocyte decreases shown are significantly different when compared to no treatment, with the exception of day 7 CD4 T cells. * $p < 0.05$ in (A) and (C), and $p < 0.01$ in (B). Data are representative of at least five mice per time point, which is a collection of data from at least three different experiments.

reduced release of single-positive naive T cells from the thymus (as suggested by Matloubian *et al.* [4]), which balances a deficit in lymphocyte egress from PLN. This new steady state appears to take effect in the latter part of the treatment, as PLN lymphocyte content starts to plateau by day 14 (Fig. 3A). Trafficking patterns of lymphocytes in the Peyer's patches (PP) follow the same pattern as that seen in the PLN compartment (Fig. 3B).

These data, combined with the data from Fig. 1, suggest that the decrease in lymphocyte numbers seen in PLN is not due to the large-scale release of these cells back into the peripheral blood. It appears that, initially, these cells are unable to leave PLN. Over the course of the treatment, influx of thymocytes into the periphery is decreased, which changes the trafficking equilibrium of affected lymphocyte populations. Since lymphocyte counts in both peripheral blood and lymph nodes never decline to zero, it seems that a small number of thymocytes are still released from the thymus. Thus, the mice are able to maintain a low level of lymphocytes in both the peripheral blood and the lymph nodes.

In the thymus, administration of FTY720 induced a rapid and significant accumulation of total thymocytes, mainly comprising double-positive thymocytes, which reached a peak at day 1.5 (Fig. 4A). A brisk decline in these numbers by days 2 and 4 was followed by a slow recovery that continued through day 14. In particular, there was a significant increase in the number of single-positive (SP) populations above untreated levels. Developing thymocytes ($CD25^+CD44^+$) followed the pattern of the total lymphocyte population of the thymus (Fig. 4B).

We utilized differential equation modeling to characterize the rates of change of cells in each of the three compartments, thymus, blood, and PLN. The rate of change of cells in the thymus compartment [$\delta T(t)/\delta t$] is characterized by two rate constants: $+k\mathrm{T}_{\mathrm{in}}$, the zero-order rate of cells maturing in the thymus compartment; and $-k\mathrm{T}_{\mathrm{B}}$, the first-order rate of cell transfer from the thymus compartment to that of the blood. The number of cells in the thymus compartment, $T(t)$, is calculated based on the model-estimated value T_0 at time zero. The rate of change of cells in the blood compartment [$\delta B(t)/\delta t$] is characterized by four rate constants: $+k\mathrm{T}_{\mathrm{B}}$, representing transfer from the thymus compartment to blood; $-k_{\mathrm{BP}}$, transfer from blood to PLN; $+k_{\mathrm{PB}}$, transfer from PLN to blood; and $-k_{\mathrm{Bout}}$, the first-order rate of cell elimination from the blood compartment. The numbers of cells in the thymus, blood, and PLN are computed based on the model-estimated value T_0 at time zero and these rates. The rate of change of cells in the PLN compartment [$\delta P(t)/\delta t$] depends on three rate constants: $+k_{\mathrm{BP}}$, transfer from blood to PLN; $-k_{\mathrm{PB}}$, transfer from PLN to blood; and $-k_{\mathrm{Pout}}$, cell elimination from the PLN compartment.

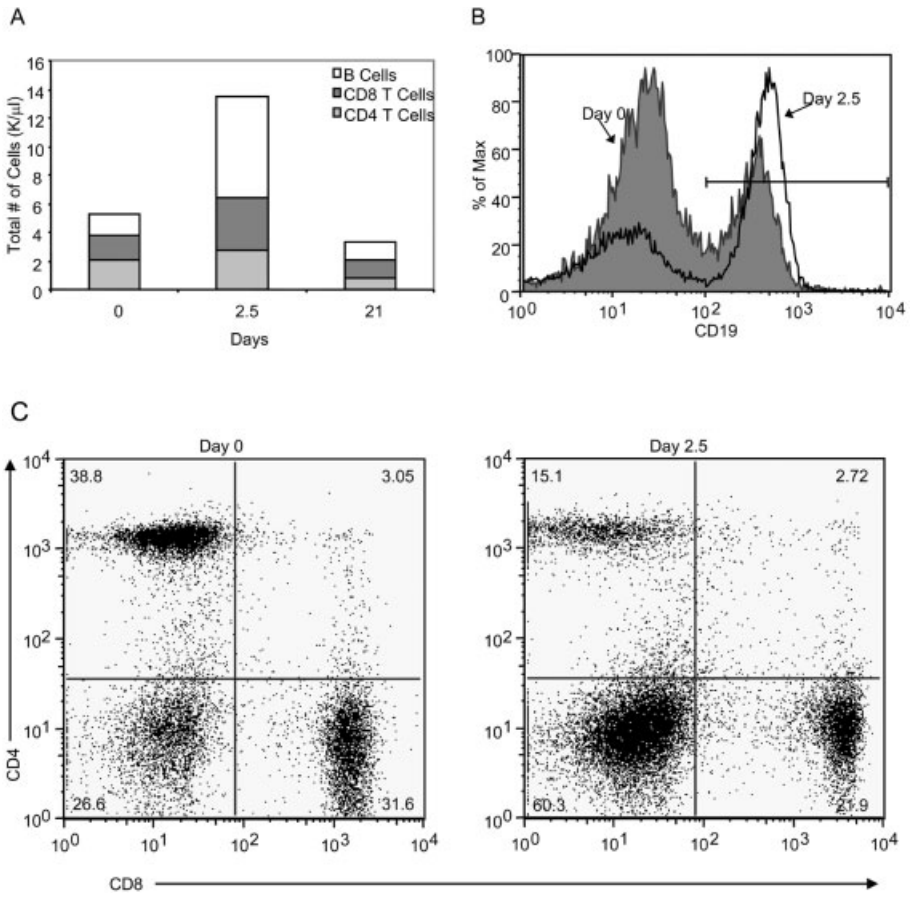

Figure 2. PLN composition on days 0, 2.5, and 21 by lymphocyte counts and flow cytometry. (A) Comparison of lymphocyte subsets on days 0, 2.5, and 21 in the PLN. (B) Histogram of CD19 expression on day 0 (solid) and 2.5 (open) showing an increase in the percentage of B cells in peripheral nodes. (C) Dot plots of day 0 and day 2.5 CD4 and CD8 lymph node T cells. Data shown are representative of at least five mice from three separate experiments.

According to this model, the dynamics of cell transfer into and out of the thymus compartment are independent of the blood and PLN compartments, because peripheral T cells do not enter the thymus. Therefore, parameter values for $+k\mathrm{T}_{\mathrm{in}}$, $-k\mathrm{T}_{\mathrm{B}}$, and T_0 were optimized independently of those of the remaining model parameters. The optimal derived values for $+k\mathrm{T}_{\mathrm{in}}$, $-k\mathrm{T}_{\mathrm{B}}$, and T_0 were subsequently fixed during simultaneous (*i.e.* global) estimation of the remaining model parameters to the data in (C) and (D) of Fig. 5.

Initial modeling efforts with $\mathrm{K}_{\mathrm{p\ out}} = 0$ successfully modeled the increase in mature $\mathrm{CD4}^+$ T cells retained in the thymus, but were unable to realistically match our experimental data in the blood or PLN (data not shown). When we allowed $\mathrm{K}_{\mathrm{p\ out}}$ to take on a finite value, the model consistently converged on a set of curves that were reasonable fits of the measured data (Fig. 5A). Using the measured starting numbers of 2.42×10^6 $\mathrm{CD4}^+$ T cells in the thymus, 9.8×10^6 in the blood, and 27.8×10^6 in the PLN, the model converged on 1.43×10^6 $\mathrm{CD4}^+$ T cells being produced in the thymus per day. The rate of egress from thymus to blood was 10.4% of the number of $\mathrm{CD4}^+$ thymocytes per day, from blood to PLN 2.67 times the number of $\mathrm{CD4}^+$ T cells in blood, and the number of $\mathrm{CD4}^+$ T cells disappearing from the lymphoid compartment without reappearing in blood was 12% of the number of $\mathrm{CD4}^+$ T cells in PLN (see Fig. 5A). The rate of cells leaving PLN and entering blood was approximately zero (1.19×10^{-30}), and almost no $\mathrm{CD4}^+$ T cells were leaving the blood to compartments other than PLN (9.72×10^{-17} times the number of blood $\mathrm{CD4}^+$T cells). The fit of the model to the data is shown in Fig. 5B–D. These data show that $\mathrm{CD4}^+$ T cells must disappear from PLN, most likely by apoptosis. We looked for apoptotic cells, but could not detect any (data not shown), perhaps because they are rapidly cleared by phagocytosis.

To further explore the effects FTY720 has on the total body lymphocyte populations, we continued our analysis by studying spleen, mesenteric lymph nodes (MLN), liver, and lungs. Total lymphocytes in the spleen are significantly decreased over the course of the 21-day treatment, starting at day 2.5 (Fig. 6A). A significant increase in CD8 T cell numbers was seen at 12 h in the

spleen. This was followed by a significant decrease in CD8 T cells by day 21. FTY720 initially did not affect the spleen as much as the PLN, which was suggested in previous studies [11, 16]. This could be due to differences in the lymphocyte composition of the spleen, which typically has many more mature cells, rather than the naive cells in the lymph nodes. However, the data trend seen in the latter portion of the treatment course closely mimics that seen in the PLN. The trafficking patterns seen in the MLN did not follow the same pattern as seen in the PLN (Fig. 6B). The MLN consists of many gut-associated lymphocytes that are not found in other lymphoid organs. In this organ, we saw only minor changes in all lymphocyte subsets studied. Overall lymphocyte numbers and subset distribution were unchanged after the 21-day FTY720 treatment. While there was some initial fluctuation of lymphocytes in the liver, the overall number of lymphocytes was not significantly altered in this organ (data not shown). Similarly, total lung lymphocytes showed small fluctuations over the course of FTY720 treatment, with no overall change in total lymphocyte number (data not shown).

To verify that these changes were due to altered trafficking, we injected C57BL/6*scid* mice ($CD45.2^+$/$CD45.1^-$) with $CD45.1^+$ congenic splenocytes (Fig. 7). We indeed found alterations in lymphocyte trafficking that mimicked results seen in our other studies. $CD45.1^+$ lymphocyte numbers were decreased in blood and spleen, yet increased in PLN and MLN of mice treated with FTY720 when compared to untreated littermate controls.

To analyze the full effect of FTY720 on lymphocytes, we looked at the distribution of total pooled lympho-

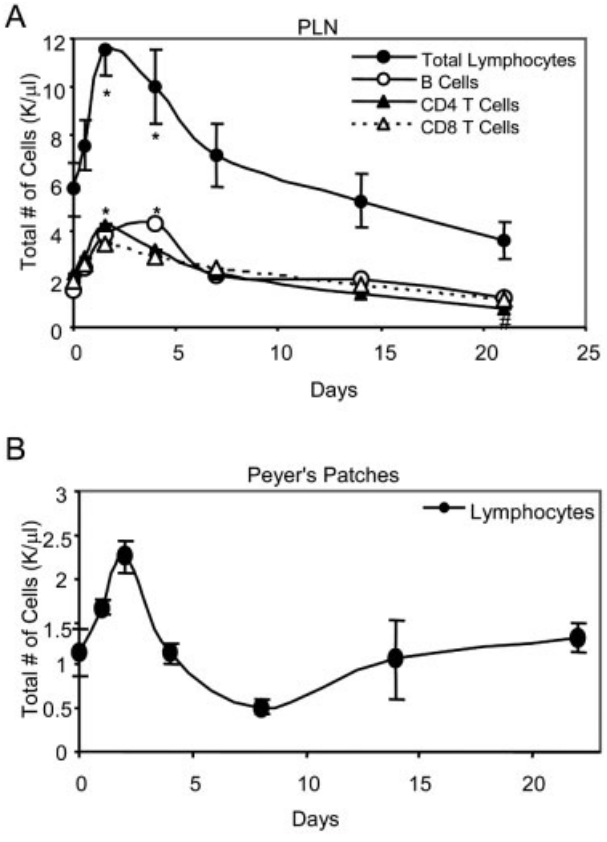

Figure 3. Kinetics of lymphocyte subsets in PLN and PP. (A) A closer look at T and B cell numbers at key time points in PLN. * $p \leq 0.05$, significant increase in all lymphocyte subset compartments as compared to day 0. # $p < 0.05$, significant decrease in CD4 T lymphocytes as compared to day 0. Data shown are the average of at least five mice per time point from at least three separate experiments. (B) Similar trends seen in total lymphocyte kinetics of the PP compartment. Data shown are the average of at least two mice per time point from two separate experiments.

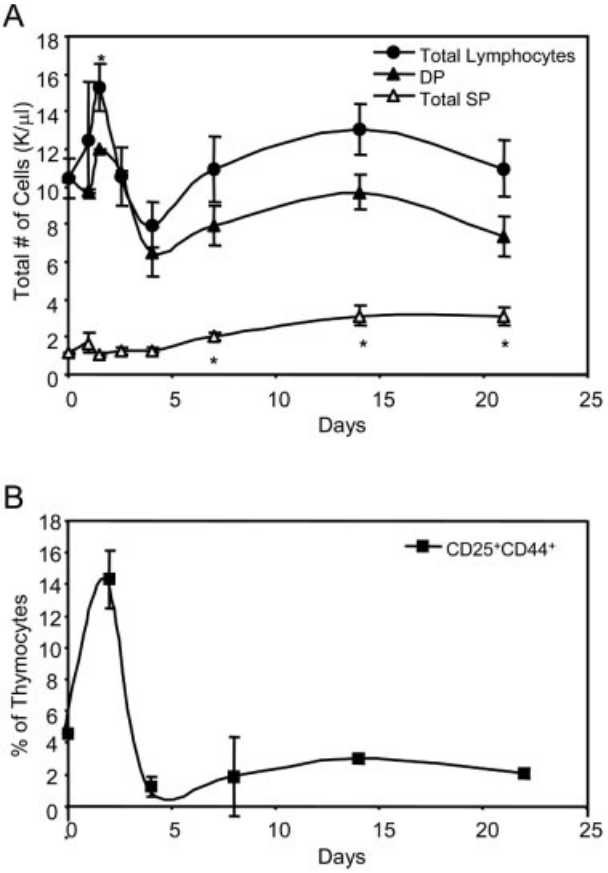

Figure 4. Changes in thymocyte numbers over 21-day FTY720 treatment. (A) Kinetics of thymocyte populations over 21 days of FTY720 treatment, including total lymphocytes (solid circles), as well as double- (solid triangles) and single-positive (open triangles) thymocytes. Data shown are an average of at least five mice from three separate experiments. * $p < 0.05$ indicates a significant increase in either total lymphocytes at day 1.5 or in single-positive lymphocytes at days 7, 14, and 21. (B) Kinetics of developing thymocytes ($CD25^+CD44^+$) upon FTY720 treatment. Data shown in (B) are the average from three mice per time point from two separate experiments.

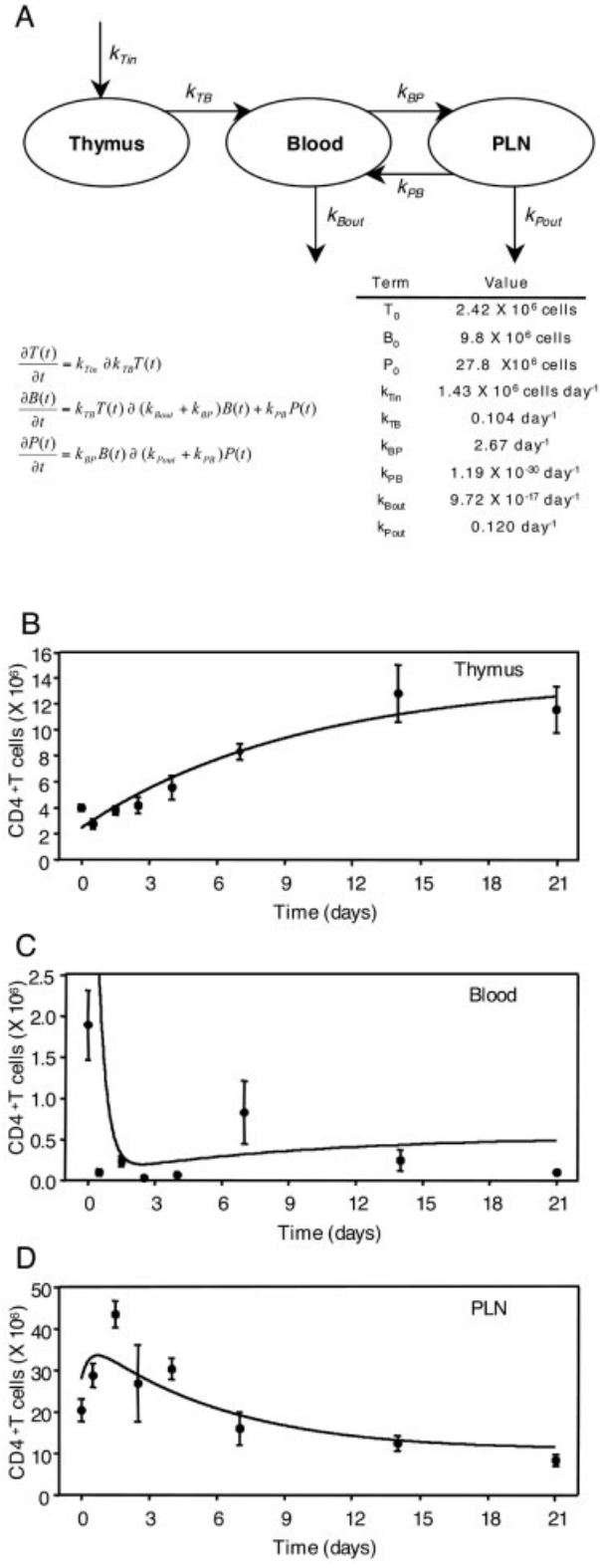

Figure 5. Modeling of T cell trafficking between thymus, blood, and PLN during FTY720 treatment. (A) Schematic of the mathematical model used to determine trafficking patterns of CD4 T cells based upon available data. Also shown are differential equations and constants derived from the model. (B–D) Curve fit of model to actual data for thymus (B), blood (C), and PLN (D). This model was generated using all available data points from all experiments performed.

cytes in lymphoid organs, and organs with large lymphocyte components over time (Fig. 8). Fig. 8A compares total numbers of lymphocytes in each of the organs on days 0, 1.5, and 21. As mentioned before, cell counts in many organs return to normal or reduced levels by day 21, despite a significant increase initially. Total lymphocyte levels in blood and spleen remained low at day 21. The whole body subset composition is shown in Fig. 8B. Fig. 8C shows a comparison of lymphocyte distribution in all organs studied, as well as the different lymphocyte subsets within each organ, on days 0, 1.5, and 21. These graphs illustrate lymphocyte trafficking during the course of FTY720 treatment, and show the profound effects on the T cell subset compared to other subsets. Although T cells are most affected, we also noted initial B cell accumulation in the bone marrow compartment. However, this increase, like that seen in PLN, was transient. It is important to note that blood, the compartment in which FTY720 has the most

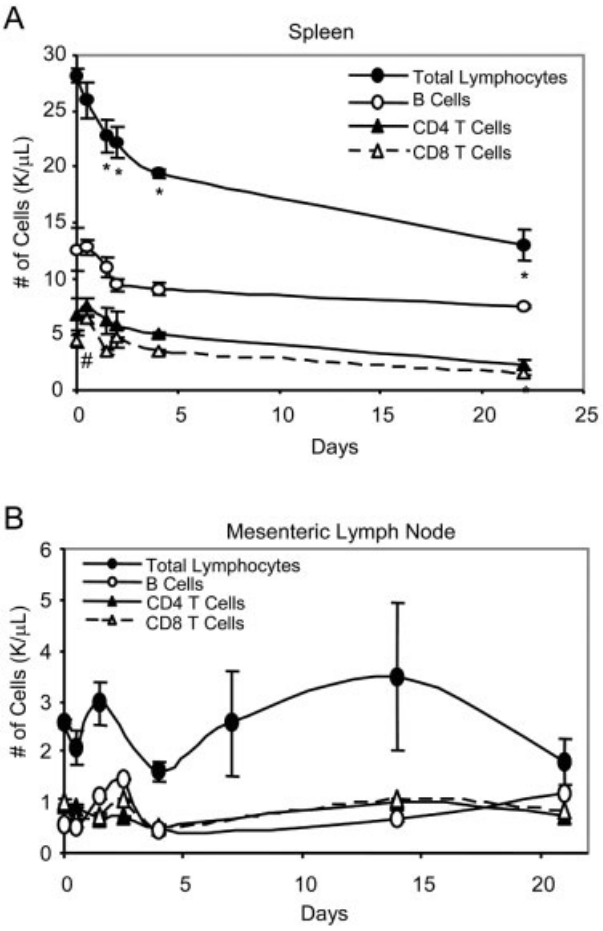

Figure 6. Kinetics of lymphocyte trafficking in spleen (A) and MLN (B). * $p < 0.05$, significant decrease from day 0 group in total lymphocytes and CD8 T cell compartments. # $p < 0.05$, representing a significant increase in CD8 T cells on day 0.5. Data shown are pooled from at least five mice from three separate experiments.

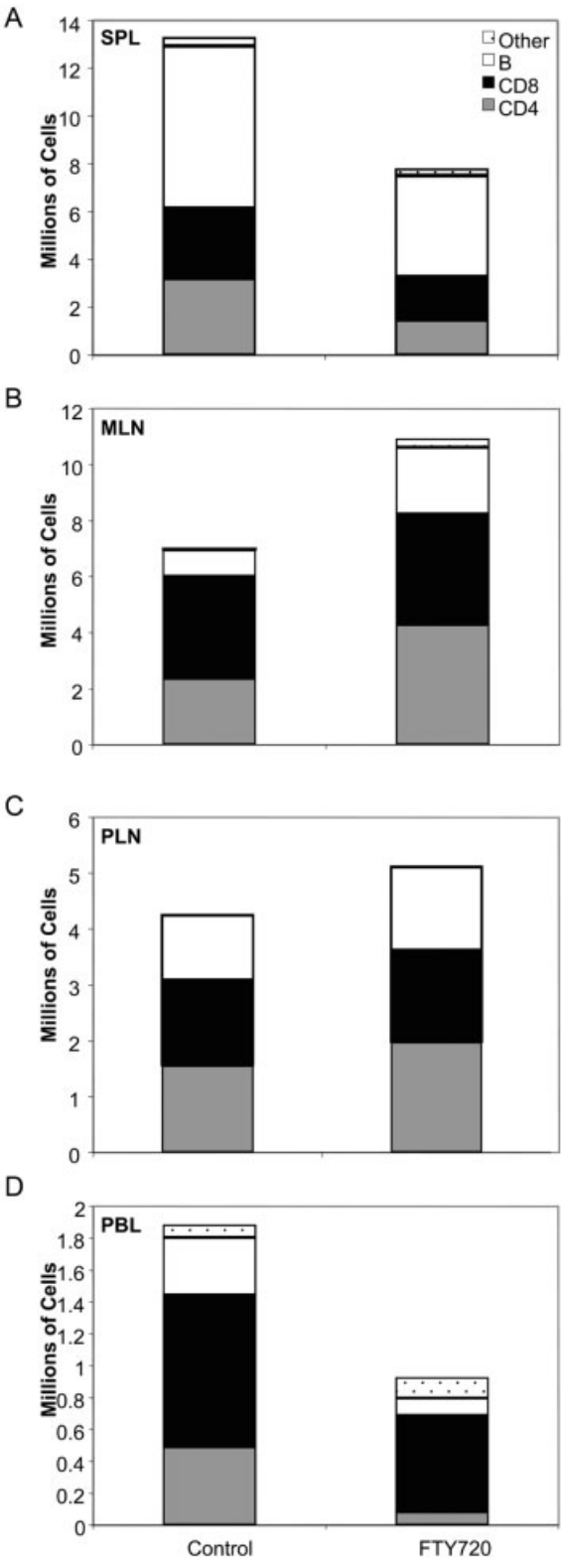

Figure 7. Trafficking of CD45.1$^+$ lymphocytes in C57BL/6*scid* mice. Stacked bar graphs show the lymphocyte distribution in spleen (A, SPL), MLN (B), PLN (C), and blood (D) of host mice that were either untreated (n = 2) or treated with FTY720 for 7 days (n = 3). Gray bars indicate numbers of CD4$^+$ T cells, black bars indicate numbers of CD8$^+$ T cells, white bars indicate numbers of B cells, and dotted bars indicate numbers of other lymphocytes (CD45.1$^+$, CD3$^-$CD19$^-$Gr-1$^-$).

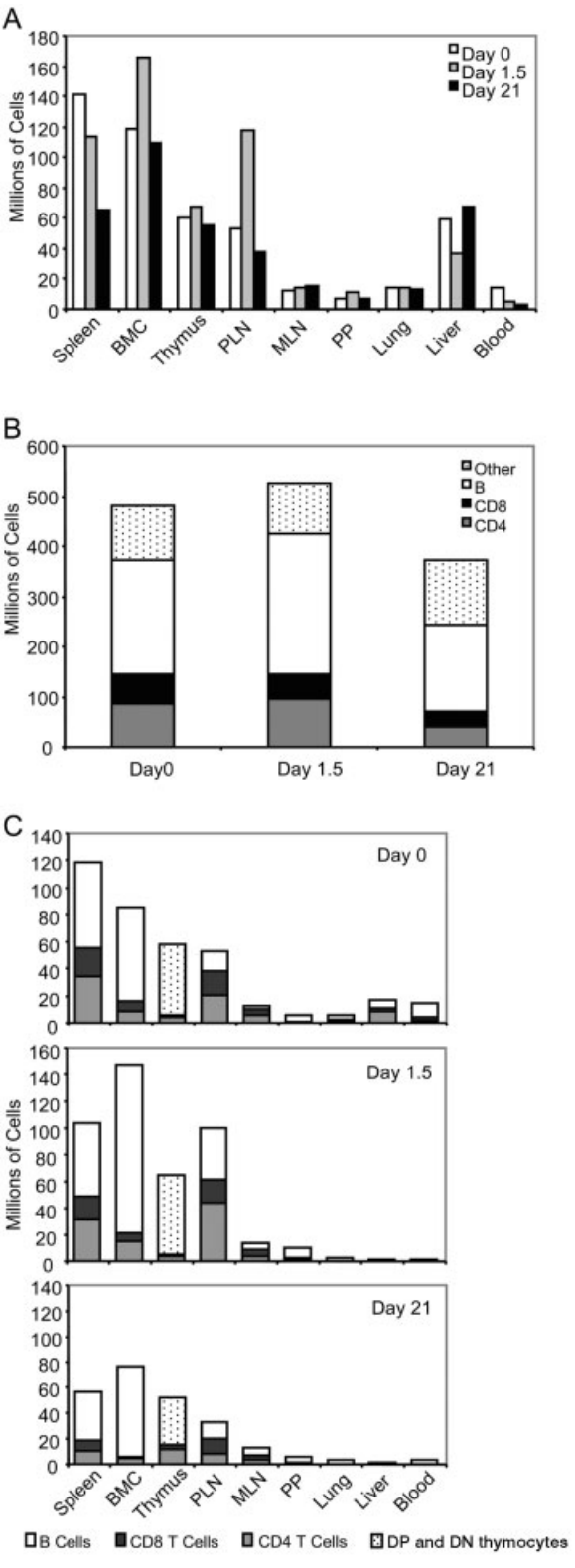

Figure 8. Distribution of lymphocytes in all organs tested on days 0, 1.5, and 21. (A) Total lymphocytes in each organ at each time point. (B) Stacked bars show whole body pooled lymphocyte counts, and lymphocyte subset composition on days 0, 1.5, and 21. (C) Stacked bars show distribution of subsets within each organ on days 0, 1.5, and 21, using at least five mice per time point from a minimum of three experiments performed. BMC: bone marrows *cells*.

Table 1. Total body lymphocytes ($\times 10^6$)

	Day 0				Day 21				Day 21/Day 0, %[a]			
	CD4	CD8	B/DP[b]	Other/DN[b]	CD4	CD8	B/DP[b]	Other/DN[b]	CD4	CD8	B/DP[b]	Other
Blood	1.9 ± 0.4	2.3 ± 0.6	10.2 ± 2.3	3.6 ± 1.6	0.1 ± 0.02	0.08 ± 0.03	1.7 ± 0.2	1.5 ± 0.3	5.3	3.5	16.7	41.7
Liver	8.9 ± 3.5	1.8 ± 1.0	6.1 ± 2.8	42.7 ± 1.1	0.01 ± 0.006	0.2 ± 0.05	0.6 ± 0.2	66.2 ± 8.4	0.1	11.1	9.3	155.0
Lung	1.3 ± 0.2	1.4 ± 0.3	3.7 ± 0.9	7.5 ± 1.0	0.2 ± 0.05	0.2 ± 0.08	3.7 ± 1.7	9.2 ± 3.5	15.4	14.3	100	122.7
PP	0.8 ± 0.1	0.4 ± 0.06	4.5 ± 0.6	1.9 ± 0.4	0.8 ± 0.2	0.6 ± 0.02	4.3 ± 0.6	1.7 ± 0.7	100	150	95.6	89.5
MLN	4.9 ± 0.9	5.1 ± 1.0	2.9 ± 0.5	0.04 ± 0.02	3.5 ± 0.4	4.1 ± 0.08	6 ± 0.06	1.2 ± 0.05	71.4	80.4	206.9	3000
PLN	20.4 ± 2.7	17.7 ± 2.5	14.7 ± 3.0	1.7 ± 1.0	8.3 ± 1.3	12 ± 1.6	12.8 ± 2.1	5 ± 2.1	40.7	67.8	87.1	294.1
Thymus	4 ± 0.3	1.7 ± 0.2	52.5 ± 3.2	1.1 ± 0.1	11.5 ± 1.8	4 ± 0.5	36.7 ± 5.1	2.7 ± 0.8	287.5	235.3	69.9	245.4
BMC[c]	8.2 ± 0.6	6.4 ± 1.1	68.6 ± 0.5	35.1 ± 27	1.2 ± 0.3	0.4 ± 0.3	70.7 ± 20.2	37 ± 6	14.6	6.2	103.1	105.4
Spleen	33.8 ± 7.4	21.8 ± 2.5	62.9 ± 9.7	22.4 ± 3.6	13.5 ± 2.3	8 ± 0.9	37.3 ± 0.7	6.2 ± 5.7	39.9	36.7	59.3	27.7
Body	84.2 ± 2.6	58.6 ± 6.9	226.1 ± 25	116 ± 19	39.1 ± 3.7	29.6 ± 0.3	173.8 ± 9.5	130.7 ± 29	46.4	50.5	76.9	112.6

[a] Last four columns indicate cells at day 21 expressed as percent of day 0.
[b] Indicates either $CD19^+$ B cells or other lymphocytes, except for the thymus compartment, where this population refers to double-positive (DP) and double-negative (DN) thymocytes, respectively.
[c] Bone marrow compartment.

dramatic effects, is the smallest of the lymphoid compartments.

Table 1 summarizes the data presented in this study, showing absolute total body content of lymphocytes at day 0 and day 21, as well as the number of cells at day 21 as a fraction of the initial number. Table 1 emphasizes the retention of developing T cells in the thymus, as well as the reduction in total body lymphocytes. We also saw a sharp increase in other lymphocytes in the MLN by day 21, perhaps due to availability of space in the MLN after the decrease in the T lymphocyte population. A similar but smaller increase was seen in the PLN compartment. It is likely that these increases are due to homeostatic proliferation of other lymphocytes, such as NK and NKT cells, in a setting where the niche is being emptied of other cells [17, 18].

Discussion

Lymphocytes in FTY720-treated mice are thought to be unable to leave the peripheral lymphoid organs and the thymus [11, 15]. This may be due to the inability to respond to S1P in the bloodstream because FTY720 desensitizes $S1P_1$ receptors. Our data show that a new steady state of lymphocyte trafficking is established following initial lymphocyte sequestration. This new steady state involves fewer lymphocytes, but the cells are still able to traffic to PLN, although only minimal numbers of blood lymphocytes populate the peripheral blood.

At the dose used (1.8 mg/L in drinking water, resulting in blood concentrations of 20–30 nM), FTY720 appears to be unable to block $S1P_1$ completely, as indicated by the incomplete lymphopenia compared to the results seen by Matloubian *et al.* in mice with $S1P_1$-deficient leukocytes [4]. $S1P_1$-deficient T cells are not able to migrate into the periphery, even in small numbers [4, 12]. Incomplete blockade has been shown in previous studies of short-term FTY720 exposure [6], but our data demonstrate for the first time that the FTY720 block continues to be incomplete for up to 21 days of treatment, which is probably important in supporting immune system functions in FTY720-treated animals and patients.

It was previously determined that FTY720 does not affect all lymphocytes equally [5, 6, 9]. We found that T cells react much more dramatically to FTY720 treatment than do B cells, and NK cells are only minimally affected. It is surprising that NK cells are not affected by FTY720, although NK cells express $S1P_1$, as determined by RT-PCR [19]. In addition, lymphocyte reaction to chemokines may be altered in the presence of FTY720, as it appears that S1P normally prevents lymphocyte over-reaction to chemokines [6, 20]. Perhaps T and B cells are more dependent on chemokines in their trafficking patterns than NK cells. By eliminating the influence of S1P, T and B cells may respond more vigorously to the available chemokines, leading to extravasation of more of these cells from the blood stream. Evidence supporting this idea resulted from the co-transfer of $S1P_1{}^{+/+}$ and $S1P_1{}^{-/-}$ thymocytes,

in which all $S1P_1^{-/-}$ thymocytes were found in peripheral lymphoid organs, but not in the blood [4].

Unlike the elimination of $S1P_1$ [4], FTY720 treatment does not cause complete blood lymphopenia. It has been suggested that the remaining cells are more mature peripheral effector/memory cells [21]. These cells generally lack homing receptors like CCR7 and CD62L, which are typically responsible for lymphocyte homing to secondary lymphoid organs, such as the lymph nodes and PP (reviewed in [22]).

Our data suggest that in FTY720-treated mice, there is an alteration in B cell trafficking from the bone marrow. It was previously shown that B cell recirculation was altered in the absence of $S1P_1$ [4], but the effect of FTY720 on B cells does not mimic this recirculation defect. Since FTY720 acts not only through $S1P_1$ but also through $S1P_1$[3–5], it is possible that FTY720 also inhibits S1P-mediated suppression of proliferation through $S1P_4$, as has been recently shown for T cells [23].

We have also shown that naive cells cannot leave the thymus effectively. It is clear from our data that the immune system is able to achieve and maintain a new balance of production and circulation of lymphocytes over the course of prolonged FTY720 treatment. Based on other studies, the remaining circulating T cells appear to be sufficient to mount systemic [24] but not localized immune responses [25]. Although these studies did not look at prolonged FTY720 treatment systems, it is possible that the ability of cells to mount an immune response is not altered further after additional days of FTY720 treatment.

In conclusion, the lymphocyte response in lymphoid organs is biphasic, leading to a sustained decrease in total lymphocyte numbers. All lymphatic organs show a decline in lymphocyte numbers as the treatment time is extended. Our data support the idea that FTY720 initially prevents lymphocyte egress from thymus, PLN, PP, and to a lesser degree MLN. The cumulative effect on PLN and PP is transient, because the decreased lymphocyte supply from the thymus prevents further accumulation. A new steady state is achieved in all lymphoid (both secondary and tertiary) organs within 21 days, as the lymphocyte counts reach a plateau.

Materials and methods

Mice

C57BL/6J mice (B6, Jackson Laboratories, Bar Harbor, ME) were maintained in a specific pathogen-free environment under a microisolator containment system. Both adult male and female age-matched mice were used for all experiments, which were reviewed and approved by the Animal Care and Use Committee at the University of Virginia.

Administration of FTY720

FTY720 dissolved in water (1.85 mg/L) was supplied *ad libitum* to C57BL/6 mice. Mice received an estimated dosage of 1.25 mg/kg/day for 0–21 days until tissues were harvested. Water was changed at least once a week. FTY720 is stable in water at room temperature for several months (unpublished observations).

Lymphocyte trafficking

C57BL/6*scid* mice were injected *via* the lateral tail vein with 20×10^6 splenocytes from B6.CD45.1 congenic mice. Host mice were allowed to rest for 7 days, at which point one group of mice was treated with FTY270 in the drinking water (described above) for 7 additional days. Tissues were harvested as described below.

Harvesting tissues

Mice were anesthetized *via* intraperitoneal injections of ketamine hydrochloride (125 mg/kg; Sanofi Winthrop Pharmaceuticals, New York, NY), xylazine (12.5 mg/kg TranquiVed; Phoenix Scientific, St. Joseph, MO), and atropine sulfate (0.025 mg/kg; Fujisawa USA, Deerfield, IL).

Blood, PLN, MLN, thymus, and liver were harvested from at least six mice for each time point following 0, 0.5, 1.5, 2.5, 4, 7, 14, and 21 days of FTY720 treatment. Spleen, bone marrow (data not shown), lungs, and PP were harvested from at least two mice following treatment for 0, 0.5, 1.5, 4, 7, 14, and 21 days. With the exception of blood, liver, and lungs, single-cell suspensions of all tissues were made in 5 mL phosphate-buffered saline (PBS). Cell counts were determined from these suspensions, yielding cell counts in thousands of cells per microliter (K/μL). For total cell counts from organs that were not harvested in full (*i.e.* bone marrow and liver), total numbers were extrapolated based upon the percentage of cells removed. For example, we flushed both femurs and tibias from all mice. We estimated this portion of the bone marrow to be 20% of the total bone marrow based upon previously published data [26]. The bone marrow counts from cells harvested were then multiplied by 5 to yield the total number of bone marrow cells expected in each mouse. The liver cells harvested were approximately half of the total liver cells. The total blood volume was estimated to be 2.5 mL per mouse. We estimated that the cells from PLN harvested (inguinal, axillary, brachial, and cervical nodes) were half of the total PLN cellularity for each mouse based on available data [27]. The total number of lymphocytes harvested from each mouse was determined to be the "total pooled lymphocytes".

Liver and lung fragments were suspended in 5 mL PBS containing collagenase type XI (125 U/mL), deoxyribonuclease (60 U/mL), and hyaluronidase (60 U/mL) (all from Sigma, St. Louis, MO). The suspension was placed in a 37°C water bath for 30 min to allow for enzymatic digestion.

Cell counts

Aliquots (40 μL) were obtained from each thoroughly mixed cell suspension. With the exception of liver samples, cell counts

of the aliquots were recorded *via* a Hemavet (Drew Scientific, Oxford, CT). The Hemavet quantified total white blood cells, neutrophils, monocytes, lymphocytes, eosinophils, and basophils in each sample. The accuracy of the Hemavet was verified by manually counting Kimura-stained cells in a hemocytometer (data not shown).

Aliquots (10 μL) from mixed liver cell suspensions were mixed with 90 μL Kimura stain [11 mL toluidine blue, 0.8 mL 0.03% light green SF yellowish (Sigma), 0.5 mL saturated saponin (Sigma) in 50% ethanol, and 5 mL 1/15 M phosphate buffer, pH 6.4]. Cell counts of lymphocytes, granulocytes, and total leukocytes were obtained by analyzing the solution in a hemocytometer.

Total cell numbers for each compartment were determined either by estimation for larger organs (*i.e.* liver, bone marrow, blood, and PLN, as described above), or by multiplying total suspension volume by cell concentration (*i.e.* spleen, thymus, MLN, and lungs).

Flow cytometry preparation and analysis

To identify and quantify lymphocyte subsets, cell suspensions were analyzed by flow cytometry. Following red blood cell lysis, cells were stained with anti-mouse monoclonal antibodies against CD3, CD4, CD8, CD19, and NK1.1 (BD Biosciences, San Jose, CA). Cells were analyzed *via* four-color flow cytometry on a FACSCalibur (BD Biosciences) in the University of Virginia Cancer Center Core Facility. Lymphocyte subsets, including B cells, total T cells, CD4 T cells, CD8 T cells, double-positive thymocytes, double-negative thymocytes, NK cells, and NK/T cells, were analyzed. The size of each cell population was calculated as the product of the total lymphocyte count recorded by the Hemavet or hemocytometer and the percentage of positive lymphocytes recorded by the flow cytometer. All data were analyzed with BD Biosciences Cell Quest analysis software. This protocol allowed for quantification of the various types of cells in various organs throughout a 21-day period.

Modeling

In this analysis, the numbers listed resulted from an aggressive session of nonlinear least squares (NLLS) optimization [28, 29] of one parameter at a time to only six of the nine parameters. T_0, k_{Tin}, and k_{TB} were constrained to their optima, obtained from analysis of only the *T*-pool of data [*i.e.*, note that the first differential equation exhibits dependence only on *T(t)*, and not on *B(t)* nor on *P(t)*]. The model differential equations were numerically integrated during the NLLS parameter optimization process. All parameter optimizations were performed on a data set comprising the total ensemble of individual data points, not to the averaged data with SEM (as depicted in Fig. 5).

Measurement of FTY720 blood levels

FTY720 blood levels were measured as described [30]. Levels were stable over the course of the 21-day treatment (data not shown).

Statistical analysis

Statistical significance was determined using Student's *t*-test to compare all time points to the day 0 group.

Acknowledgements: The authors would like to acknowledge Dr. Kevin Lynch for his critical reading of the manuscript, and Dr. Yulius Setiady for providing detailed knowledge of the murine lymphatic system. We would also like to thank Dr. Jeffrey Lysiak for his assistance with the apoptosis assays. M.A.M. is supported by a postdoctoral fellowship from the Juvenile Diabetes Research Foundation.

References

1 **Hla, T., Lee, M.-J., Ancellin, N., Thangada, S., Liu, C. H., Kluk, M., Chae, S.-S. and Wu, M.-T.,** Sphingosine-1-phosphate signaling *via* the EDG-1 family of G-protein-coupled receptors. *Ann. N. Y. Acad. Sci.* 2000. **905:** 16–24.

2 **Graeler, M. and Goetzl, E. J.,** Activation-regulated expression and chemotactic function of sphingosine-1-phosphate receptors in mouse splenic T cells. *FASEB J.* 2002. **16:** 1874–1878.

3 **Sanchez, T., Estrada-Hernandez, T., Paik, J.-H., Wu, M.-T., Venkataraman, K., Brinkmann, V., Claffey, K. and Hla, T.,** Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. *J. Biol. Chem.* 2003. **278:** 47281–47290.

4 **Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L. and Cyster, J. G.,** Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 2004. **427:** 355–360.

5 **Chiba, K., Yanagawa, Y., Masubuchi, Y., Kataoka, H., Kawaguchi, T., Ohtsuki, M. and Hoshino, Y.,** FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J. Immunol.* 1998. **160:** 5037–5044.

6 **Henning, G., Ohl, L., Junt, T., Reiterer, P., Brinkmann, V., Nakano, H., Hohenberger, W., Lipp, M. and Forster, R.,** CC Chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: Modulation by FTY720. *J. Exp. Med.* 2001. **194:** 1875–1881.

7 **Graler, M. H. and Goetzl, E. J.,** The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* 2004. **18:** 551–553.

8 **Matsuura, M., Imayoshi, T., Chiba, K. and Okumoto, T.,** Effect of FTY720, a novel immunosuppressant, on adjuvant-induced arthritis in rats. *Inflamm. Res.* 2000. **49:** 404–410.

9 **Mayer, K., Birnbaum, F., Reinhard, T., Reis, A., Braunstein, S., Claas, F. and Sundmacher, R.,** FTY720 prolongs clear corneal allograft survival with a differential effect on different lymphocyte populations. *Br. J. Ophthamol.* 2004. **88:** 915–919.

10 **Sanna, M. G., Liao, J., Jo, E., Alfonso, C., Ahn, M.-Y., Peterson, M. S., Webb, B. *et al.*,** Sphingosine-1-phosphate (S1P) receptor subtypes $S1P_1$ and $S1P_3$ respectively, regulate lymphocyte recirculation and heart rate. *J. Biol. Chem.* 2004. **279:** 13839–13848.

11 **Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R. *et al.*,** Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 2002. **296:** 346–349.

12 **Allende, M. L., Dreier, J. L., Mandala, S. and Proia, R. L.,** Expression of the sphingosine-1-phosphate receptor, $S1P_1$, on T-cells controls thymic emigration. *J. Biol. Chem.* 2004. **279:** 15396–15401.

13 **Cinamon, G., Matloubian, M., Lesneski, M. J., Xu, Y., Low, C., Lu, T., Proia, R. L. and Cyster, J. G.,** Sphingosine-1-phosphate receptor 1

promotes B cell localization in the splenic marginal zone. *Nat. Immunol.* 2004. **5:** 713–720.

14 **Luo, Z.-J., Tanaka, T., Kimura, F. and Miyasaka, M.,** Analysis of the mode of action of a novel immunosuppressant FTY720 in mice. *Immunopharm.* 1999. **41:** 199–207.

15 **Yagi, H., Kamba, R., Chiba, K., Soga, H., Yaguchi, K., Nakamura, M. and Itoh, T.,** Immunosuppressant FTY720 inhibits thymocyte emigration. *Eur. J. Immunol.* 2000. **30:** 1435–1444.

16 **Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C. *et al.*,** The immune modulator FTY720 targets sphingosine-1-phosphate receptors. *J. Biol. Chem.* 2002. **277:** 21453–21457.

17 **Prlic, M., Blazar, B. R., Farrar, M. A. and Jameson, S. C.,** *In vivo* survival and homeostatic proliferation of natural killer cells. *J. Exp. Med.* 2003. **197:** 967–976.

18 **Gudmundsdottir, H. and Turka, L. A.,** A closer look at homeostatic proliferation of $CD4^+$ T cells: Costimulatory requirements and role in memory formation. *J. Immunol.* 2001. **167:** 3699–3707.

19 **Kveberg, L., Bryceson, Y., Inngjerdingen, M., Rolstad, B. and Maghazachi, A. A.,** Sphingosine-1-phosphate induces the chemotaxis of human natural killer cells. Role for heterotrimeric G proteins and phosphoinositide 3 kinases. *Eur. J. Immunol.* 2002. **32:** 1856–1864.

20 **Graeler, M., Shankar, G. and Goetzl, E. J.,** Cutting Edge: Suppression of T cell chemotaxis by sphingosine-1-phosphate. *J. Immunol.* 2002. **169:** 4084–4087.

21 **Brinkmann, V., Cyster, J. G. and Hla, T.,** FTY720: Sphingosine-1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. *Am. J. Transpl.* 2004. **4:** 1019–1025.

22 **Sallusto, F. and Lanzavecchia, A.,** Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev.* 2000. **177:** 134–140.

23 **Wang, W., Graeler, M. H. and Goetzl, E. J.,** Type 4 sphingosine 1-phosphate G protein-coupled receptor ($S1P_4$) transduces S1P effects on T cell proliferation and cytokine secretion without signaling migration. *FASEB J.* 2005. **19:** 1731–1733.

24 **Pinschewer, D. D., Ochsenbein, A. F., Odermatt, B., Brinkmann, V., Hengartner, H. and Zinkernagel, R. M.,** FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory. *J. Immunol.* 2000. **164:** 5761–5770.

25 **Xie, J. H., Nomura, N., Koprak, S. L., Quackenbush, E. J., Forrest, M. J. and Rosen, H.,** Sphingosine-1-phosphate receptor agonism impairs the efficiency of the local immune response by altering trafficking of naive and antigen-activated $CD4^+$ T cells. *J. Immunol.* 2003. **170:** 3662–3670.

26 **Boggs, D. R.,** The total marrow mass of the mouse: A simplified method of measurement. *Am. J. Hematol.* 1984. **16:** 277–286.

27 **Dunn, T. B.,** Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. *J. Nat. Cancer Inst.* 1954. **14:** 1281–1434.

28 **Johnson, M. L. and Frasier, S. G.,** Nonlinear least squares analysis. *Methods Enzymol.* 1985. **117:** 301–342.

29 **Straume, M., Frasier-Cadoret, S. G. and Johnson, M. L.,** Least-squares analysis of fluorescence data in **Lakowicz, J. R.** (Ed.) *Topics in Fluorescence Spectroscopy.* Plenum, New York 1991, p 177–240.

30 **Nikolova, Z., Hof, A., Baumlin, Y. and Hof, R. P.,** Combined FTY720/cyclosporine A treatment promotes graft survival and lowers the peripheral lymphocyte count in DA to Lewis heart and skin transplantation models. *Transp. Immunol.* 2001. **8:** 267–277.

