Modulation of T cell homeostasis and alloreactivity under continuous FTY720 exposure

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Abstract

The immunomodulator FTY720 inhibits lymph node (LN) and thymic egress, thereby constraining T cell circulation and reducing peripheral T cell numbers. Here, we analyzed in mouse models the as yet scarcely characterized impact of long-term (up to 6 months) FTY720 exposure on T cell homeostasis and possible consequences for alloreactivity. In green fluorescent protein (GFP) hemopoietic chimeras, the turnover of (initially GFP⁻) peripheral T cell pools was markedly delayed under FTY720, while normal homeostatic differences between CD4 and CD8 T cell sub-populations were retained or amplified further. Homeostatic proliferation was enhanced, and within shrinking T cell pools, the proportions of effector memory phenotype CD4 T cells (CD4T.addElement) increased in spleens and LNs and of central memory phenotype CD8 T cells (CD8T.Element) in LNs. By contrast, the fractions of CD8T.Element and CD4T.Element remained stably small under FTY720. The enrichment for CD4T.Element and CD8T.Element correlated with larger proportions of IFNγ-producing T cells upon nonspecific but not allospecific stimulation. Splenic CD4 T cells from FTY720-treated mice proliferated more strongly upon transfer to semi-allogeneic hosts. However, heart allograft survival was not compromised in FTY720 pre-treated recipients. It correlated with reduced intra-graft CD8 T cells, and the longest surviving transplants contained the highest numbers of CD4 T cells. Thus, continuous FTY720 exposure reveals differential homeostatic responses by memory phenotype CD4 and CD8 T cell sub-populations, and it may enhance alloreactive CD4 T cell proliferation and tissue infiltration without accelerating allograft rejection.

Introduction

The sphingolipid mimetic FTY720 inhibits transplant rejection and autoimmunity in animal models and was shown to be effective in phase II clinical trials of transplantation (Tx) and multiple sclerosis (1–3). FTY720 is rapidly converted to FTY720 phosphate in vivo and modulates lymphocyte trafficking via sphingosine-1 phosphate receptor-1 (S1P1), expressed to similar degrees by CD4 and CD8 T cells (4, 5). Mature single-positive (SP) thymocytes need S1P1 to leave the thymus (4, 6). Peripheral T cells transiently downregulate S1P1 upon activation and are thus rendered refractory to sphingosine-1 phosphate (S1P)-mediated lymph node (LN) exit signals. FTY720 induces S1P1 downregulation, thereby impairing S1P-dependent thymus and LN egress (4). Changes in lymphocyte trafficking by S1P1 modulators have also been attributed to increased endothelial barrier functions, and the relative significance of T cell autonomous versus endothelial effects has been debated extensively without being fully resolved (7–9). Either mechanism may contribute to the decline in T cell numbers observed in the blood and to a lesser extent also in the spleens of FTY720-treated rodents (10, 11). The effects of FTY720 are reversible, however, thereby requiring continuous therapeutic administration, with as yet scarcely characterized consequences for T cell homeostasis.

In the presence of a functioning thymus, naive peripheral T cells are continuously replaced by new thymic emigrants (12, 13). FTY720 was shown to inhibit rather than completely block thymic egress during a 3-week treatment course (14), thus raising the question as to what extent a turnover of peripheral T cell pools is still possible under long-term continuous FTY720 exposure. While thymic egress inhibition would impair peripheral naïve T cells (T₂) in general, the sequestration of T cells expressing the LN homing receptor CD62L, i.e. mostly T₂, might lead to an additional compartmentalized...
decline in T<sub>N</sub>, as shown for peripheral non-lymphoid tissues in mice and in the blood of FTY720-treated patients (15, 16). Moreover, shrinking peripheral T cell pools might provoke homeostatic proliferation, thereby further promoting a concentration of phenotypic and functional memory T cells, possibly including those with auto- and allo-cross-reactive potential (17, 18).

In this report, we analyzed in mouse models changes in the turnover and in the naive/memory phenotype composition of peripheral CD4 and CD8 T cell pools under continuous FTY720 exposure for up to 6 months. We further investigated the functional consequences of these changes for memory and alloreactivity.

Materials and methods

Mice

Female 6–8-week old C57BL/6 (B6) mice were obtained from Charles River, L’Arbresle, France; congenic B6CD45.1 (B6.SJL-Ptprca/BoAItac) from Taconic, Ejby, Denmark and B6C3F1 (C57BL/6 × C3H) F1 from Charles River, Sulzfeld, Germany. Transgenic mice on a B6 background expressing enhanced green fluorescent protein (GFP) under the β-actin promoter were generated and bred at Novartis, Basel, Switzerland (19). Mice were housed in conventional facilities in filter-top-protected cages with free access to water and a standard laboratory diet, in accordance with Swiss Federal law.

In vivo reagents

FTY720 (2-amino-2-[2-(4-octylphenyl) ethylpropane-1,3-diol) was synthesized at Novartis. FTY720 was supplied in the laboratory diet, in accordance with Swiss Federal law. 5-Bromo-2-deoxyuridine (BrdU) (Sigma, Germany. Transgenic mice on a B6 background expressing GFP under the β-actin promoter were generated and bred at Novartis, Basel, Switzerland) was added to the drinking water at 0.8 mg ml<sup>−1</sup> for an estimated daily dose of 0.5 mg kg<sup>−1</sup>. FTY720 is highly stable in water at room temperature, and FTY720-containing drinking water was changed weekly. 5-Bromo-2-deoxyuridine (BrdU) (Sigma, Buchs, Switzerland) was added to the drinking water at 0.8 mg ml<sup>−1</sup> for 7 days and exchanged daily.

Hemopoietic chimerialism

One day before bone marrow (BM) Tx, B6 recipient mice were injected intraperitoneally with 30 mg kg<sup>−1</sup> busulfan, as described (20). BM was prepared from tibiae, femurs and humeri of B6 GFP transgenic donors; 2.5 × 10<sup>7</sup> viable white BM cells were injected intravenously (i.v.) per recipient. To exclude variability in BM engraftment as a reason for differences in GFP T cell chimerism, the blood of BM recipients was screened, by flow cytometry, for GFP monocyte/granulocyte chimerism, the blood of BM recipients (20). Only BM recipients with similar blood GFP monocyte/granulocyte chimerism were compared for GFP T cell chimerism.

Flow cytometry for GFP T cell chimerism and BrdU

T cells were analyzed by four-color flow cytometry. RBCs were lysed with CAL-Lyse (CALTAG Laboratories, Burlingame, CA, USA) or RBC lysing buffer (Sigma). Antibodies were purchased from BD Pharmingen, Basel, Switzerland. Cells were initially incubated with an Fc receptor-blocking antibody (anti-CD16/CD32, 2.4G2). GFP T cell chimerism was determined by co-staining with anti-CD3–PE (145-2C11), anti-CD4–allophycocyanin (RM4-5) and anti-CD8–Peridinin chlorophyll protein (PerCP) (E13-161.7). T cells in the GFP transfer experiments were analyzed with allophycocyanin-conjugated anti-CD4 or anti-CD8 together with anti-CD62L–PE (Mel-14) and biotinylated anti-CD44 (IM7) followed by streptavidin–PerCP. BrdU detection was modified from previously published methods to retain GFP fluorescence intensity and surface staining (21, 22). Cells were surface stained with allophycocyanin-conjugated anti-CD4 or anti-CD8 and anti-CD44–biotin and incubated in 1% PFA/PBS, 0.05% Tween 20 overnight at 4°C. Samples were incubated in 125 Kunitz units DNase I (from bovine pancreas, Sigma) per 250 μl DNase buffer (4.2 mM MgCl<sub>2</sub>, 0.15 M NaCl, pH 5) for 1 h at 37°C. BrdU staining was performed with anti-BrdU–PE (3D4) for 30 min at room temperature in 10% FCS and 0.5% Tween 20. Cells were finally stained with streptavidin–PerCP for 30 min at 4°C. In studies without GFP chimerism, cells were also surface labeled with anti-CD62L–PE following BrdU staining with anti-BrdU–FITC (3D4). BrdU staining efficiency was confirmed with thymocytes; negative reference cells were prepared from mice without BrdU treatment and stained in parallel with the same reagents. Data were acquired on a FACS Calibur using CellQuest software (BD Pharmingen). Due to the strong T cell depletion, blood samples from FTY720-treated mice were limited to an acquisition of ~400 gated CD4 or CD8 T cells. All data were analyzed by Flowjo software (Tree Star, Ashland, OR, USA).

Intracellular cytokine assay

Spleen and LN (pooled inguinal, axillary and brachial) suspensions were cultured in RPMI containing 10% FCS at 5 × 10<sup>5</sup> cells per well in 96-well round-bottom plates in the presence of brefeldin A and activated with 50 ng ml<sup>−1</sup> Phorbol 12,13-dibutyrate (Sigma) and 300 ng ml<sup>−1</sup> ionomycin (Calbiochem, Merck Chemicals, Nottingham, UK). For allo-specific stimulation, 5 × 10<sup>5</sup> cells were co-cultured with 10<sup>6</sup> irradiated (3000 rad) B6C3F1 splenocytes, following a 1-min spin at 1200 r.p.m. to improve cell–cell contacts. Positive controls were spleen cells from B6 mice that had rejected a B6C3F1 skin graft 2 months before. Cells were harvested after 6 h and surface stained with anti-CD4–allophycocyanin and anti-CD8–PerCP or with anti-H-2K–biotin–streptavidin–PerCP and either anti-CD4–allophycocyanin or anti-CD8–allophycocyanin. Cells were fixed, permeabilized and stained with anti-IFN<sub>γ</sub>–PerCP and either anti-CD4–allophycocyanin or anti-CD8–Peridinin–PerCP or with anti-H-2K–biotin–streptavidin–PerCP and either anti-CD4–allophycocyanin or anti-CD8–allophycocyanin. Cells were fixed, permeabilized and stained with anti-IFN<sub>γ</sub>–PerCP and either anti-CD4–allophycocyanin or anti-CD8–Peridinin–PerCP or with anti-H-2K–biotin–streptavidin–PerCP and either anti-CD4–allophycocyanin or anti-CD8–allophycocyanin.

Systemic graft versus host-driven T cell proliferation

Spleens were taken from B6 45.1 donors treated with FTY720 for 3–4 months or from age-matched controls. RBC-depleted cell suspensions were stained with 10 μM carboxyfluorescein succinimidyl ester (CFSE) for 10 min at 37°C. To compensate for lower T cell proportions by a factor of ~0.75 in spleens from FTY720-treated mice, B6 or B6C3F1 recipients were injected i.v. with either 3 × 10<sup>7</sup> or 4 × 10<sup>7</sup> spleen cells from untreated or FTY720-treated donor mice,
modulation arising from irradiation-induced tissue injury (25–27). FTY720 treatment was started 6 days after BM transfer within a time frame of no detectable thymic and peripheral GFP+ cells. GFP T cell chimerism was monitored during 5–6 months of continuous FTY720 treatment, with efficacy reflected by ~95–97% blood T cell depletion and a 2- to 4-fold increase in SP thymocytes (Fig. 1B and data not shown). While SP thymocytes in FTY720-treated mice reached similar steady state GFP chimerism of ~80–90%, with marginally delayed kinetics, peripheral GFP T cell chimerism differed markedly under FTY720 (Fig. 1Aa and b versus c–h). GFP+ T cells in the blood of FTY720-treated mice appeared with similarly fast kinetics, yet reached lower steady state levels (Fig. 1Ac and d). In the spleens and LNs of FTY720-treated mice, by contrast, GFP T cell chimerism growth was profoundly delayed but appeared to approach steady state levels more comparable to those in control mice (Fig. 1Ae–h). For more instructive quantitative comparisons, the data were fitted to mathematical models (24, 28). They were modeled accurately by the Boltzmann logistic equation for sigmoidal growth. FTY720 treatment approximately doubled the time required to reach inflection points (half maximal GFP chimerism in this model), and maximal growth rates (at the inflection point) were reduced to ~one-half to one-third of controls (Table 1). FTY720 treatment retained or further amplified differences in CD4 and CD8 GFP T cell chimerism that were already apparent in control mice. CD4GFP T cell chimerism increased faster in LNs than in spleens around the inflection point. By contrast, early CD8GFP T cell chimerism growth (at 3 weeks) was higher in spleens than in LNs, and this difference diminished thereafter (Table 1 and Supplementary Table 1, available at International Immunology Online, for data from a second independent experiment). Generally, peripheral T cell numbers declined under FTY720, so that GFP chimerism developed within T cell pools of different sizes in controls and FTY720-treated mice. T cell numbers were most strongly reduced in the blood by ~95–97%, followed by an ~60–70% decline in spleens and a more moderate decrease in LN T cells (Fig. 1B, shown for 12 weeks after GFP+ BM transfer). Similar differences in T cell numbers were measured during 1–6 months of FTY720 treatment in GFP chimerism experiments as well as for FTY720-treated mice that had not undergone any other experimental procedures (data not shown).

Chronic FTY720 increases homeostatic T cell proliferation

Subsequent analyses addressed possible consequences of reduced sizes and slower turnover of peripheral T cell pools under FTY720, in particular altered compositions of T cell compartments and homeostatic proliferation. Indications of homeostatic T cell proliferation under FTY720 initially came from GFP chimerism studies showing that a fraction of splenic CD8 T cells in FTY720-treated mice displayed lower co-receptor expression levels (Fig. 2A). Co-receptor down-regulation was more pronounced among GFP+CD8 T cells than GFP−CD8 T cells and to a lesser extent also noted for CD4 T cells from FTY720-treated mice (Fig. 2Aa–d and data not shown). CD8lo T cells also showed lower surface CD3 expression, they expressed high levels of the activation/
Fig. 1. Delayed turnover of peripheral T cell pools under continuous FTY720 exposure. Busulfan-conditioned C57BL/6 mice received syngeneic BM from GFP transgenic donors. They were left untreated (open circles) or given FTY720 continuously in the drinking water from day 6 after BM transfer (closed circles). At the indicated times, thymi (a and b), blood (c and d), spleens (e and f) and LNs (g and h) from two mice per group were analyzed, by flow cytometry, for GFP+ cells among gated CD3+CD4+ and CD3+CD8+ cells. (A) Each symbol represents an individual data point. The smoothed lines connect the arithmetic means of the two data points for untreated (solid line) and FTY720-treated (dashed line) mice. (B) Total (GFP+ and GFP−) numbers of CD4 or CD8 T cells in the blood (separate scale), spleens and LNs (standardized for both inguinal, axillary and brachial per mouse) are shown for untreated (gray circles) and FTY720-treated (black circles) GFP BM recipients at the 12-week time point. Similar data were obtained in another independent GFP BM chimerism experiment.
memory marker CD44 and they were enriched for recently proliferated cells, as indicated by the incorporation of the nucleotide analog BrdU during 1 week of BrdU exposure (Fig. 2Ae and f and data not shown). Larger proportions of recently proliferated cells were detected among both CD8 and CD4 T cells from FTY720-treated mice (Fig. 2B). We confirmed in another model that lower T cell co-receptor expression indicated a history of homeostatic proliferation rather than an FTY720-related artifact. CD8 and CD3 down-regulations were also observed during homeostatic proliferation of donor BALB/c T cells within SCID recipient mice on a BALB/c background. By contrast, proliferating alloreactive B6 T cells in BALB/c SCID hosts kept high CD8 expression levels (Supplementary Figure 1, available at International Immunology Online).

Differential changes in the composition of CD4 and CD8 naive and memory phenotype T cell pools

The combined effects of thymic egress inhibition and increased homeostatic proliferation would be conducive to smaller T cell pools that are more concentrated in memory phenotype T cells (TPM). In the GFP T cell chimerism studies, CD4 T cells became highly concentrated in effector TPM (TPEM; CD62LhiCD44hi) in the spleens and LN, whereas CD8 T cells contained enlarged proportions of central TPM (TPCM; CD62LhiCD44hi) in LN but not in spleens. These differences were already apparent among GFP-negative (i.e. mostly ‘older’) T cells from control animals, suggesting that FTY720 exposure further enhanced physiological—rather than implementing new—differences in the homeostasis of CD4 and CD8 T cell sub-populations (Supplementary Figure 2, available at International Immunology Online). It was important, however, to exclude the possibility of artifacts related to busulfan treatment and GFP BM transfer. Therefore, the composition of T cell pools was analyzed for age-matched untreated and FTY720-treated but otherwise unmanipulated mice based on CD62L and CD44 expression (Fig. 3, histogram insets). The decline in total T cell numbers after 3 months on FTY720 was comparable to previous experiments (e.g. Fig. 1B), reducing T cell numbers to ~3, 30 and 60% of normal values in the blood, spleen and LN, respectively.

FTY720 exposure preferentially depleted CD4 and CD8 TN from the blood, resulting in an over-representation of CD4 and CD8 TPM and of CD8TPCM among the remaining blood T cells (Fig. 3a and b). Due to the strong overall blood T cell depletion, however, the numbers of TPM also decreased, e.g. CD4 T cells CD4TPEM from 7% of 100% numbers = 7 to 65% of 3% numbers = 2. In spleens, the composition of the CD8 T cell pool did not change with respect to CD62L/CD44 phenotyping (Fig. 3d). By contrast, TN were preferentially lost from the splenic CD4 T cell pool, thereby further enriching CD4TPEM from ~20% in controls to ~50% in FTY720-treated mice (Fig. 3c). Thus, within the degree of data variability, splenic CD4TPEM numbers remained relatively stable or declined moderately (20% of 100% numbers versus 50% of 30% numbers = 20 versus 15). In LN, TN were preferentially lost from the splenic CD4 T cell pool, thereby further enriching CD4TPEM from ~20% in controls to ~50% in FTY720-treated mice (Fig. 3c). Thus, within the degree of data variability, splenic CD4TPEM numbers remained relatively stable or declined moderately (20% of 100% numbers versus 50% of 30% numbers = 20 versus 15). In LN, TPM displayed a strong dichotomy between an exclusive proportional rise in TPEM among CD4 T cells and of TPCM among CD8 T cells (Fig. 3e and f). Given a more variable and smaller drop in LN T cell numbers, these proportional increases, on average, resulted in stable or moderately elevated numbers of LN CD4TPEM and a reproducible increase in LN CD8TPCM (6% of 100% numbers versus 15% of 60% numbers = 6 versus 9; and 6% of 100% numbers versus 20% of 60% numbers = 6 versus 12). It seemed noteworthy that CD8TPCM were not enriched in either spleen or LN and that the small proportions of CD4TPCM did not increase in any compartment tested (including the BM, not depicted).

An analysis of BrdU uptake by individual T cell subsets showed that physiological and FTY720-enhanced homeostatic proliferation was largely confined to TPM, and it was particularly prominent for CD4TFCM from the blood and BM. Homeostatic proliferation was not increased for splenic CD4TPEM and LN CD8TPCM, indicating that their strong local enrichment did not result from enhanced proliferation at those sites (data not shown).

Chronic FTY720 exposure increases the proportions of IFN-producing T cells

We next tested whether the enrichment of CD4TPEM in spleens and LN and of CD8TPCM in LNs of FTY720-treated mice correlated with proportional increases in functional
memory T cells. Splenic and LN cells from untreated and 3-month FTY720-treated mice were analyzed, by flow cytometry, for intracellular IFNγ after stimulation with phorbol ester and ionomycin for 6 h (Fig. 4). During this time, only pre-existing memory T cells are capable of producing IFNγ. CD4 T cells in LNs and spleens of FTY720-treated mice contained 2–3 times larger proportions of IFNγ+ T cells than the same lymphoid organs from age-matched untreated mice. CD8 T cells generally contained larger fractions of IFNγ+ cells than CD4 T cells, and these were increased further in LNs (~doubled) but not consistently in spleens of FTY720-treated mice. In addition to overall functional T cell memory as revealed by nonspecific activation, we also screened for allo-cross-reactive memory T cell to facilitate the interpretation of subsequent Tx experiments. Allo-cross-reactive memory T cells may form upon activation with infectious agents. Although our animals were free from infections, they were housed in conventional facilities, i.e. not germ free or specific pathogen free and rather old at the time of many experiments. A small possibility remained, therefore, that under long-term FTY720 exposure, allo-cross-reactive memory T cells might have arisen through homeostatic proliferation and/or had been enriched under continuous FTY720 treatment. However, no IFNγ+ T cells above background were detected upon stimulation with irradiated B6C3F1 spleen cells (Fig. 4a–d). High IFNγ expression in response to B6C3F1 stimulators was only detected among T cells from B6 mice that had rejected a B6C3F1 skin graft 2 months before and that served as positive controls for this assay (Fig. 4, histogram inset).

Enhanced proliferation by splenic CD4 T cells from FTY720-treated donors within semi-allogeneic recipients

Because FTY720 does not inhibit T cell egress from the spleen, splenic memory T cells might still respond rapidly to allografts other than through immediate effector functions, such as through enhanced alloreactive proliferation. On the other hand, within FTY720-treated hosts themselves, lower T cell numbers might outweigh qualitative changes of T cell sub-populations that could jeopardize allograft integrity. To test for in vivo alloreactive proliferation independently of differences in T cell numbers, equivalent numbers of CFSE-labeled splenic T cells from 3- to 4-month FTY720-treated or untreated B6CD45.1+ donors were transferred to (untreated) congenic B6 or B6C3F1 recipients. Three days later, donor-derived CD45.1+ CD4 and CD8 T cells from recipient spleens were analyzed for their proliferation patterns, as indicated by the 50% loss of CFSE intensity with every cell division (Fig. 5). In congenic B6 hosts, both donor-derived CD4 and CD8 T cells retained maximal CFSE fluorescence intensity, regardless as to whether they originated from untreated or FTY720-treated donors. Upon transfer to B6C3F1 recipients, about 40–45% of CD4 and CD8 T cells from untreated donors as well as CD8 T cells from FTY720-treated donor spleens displayed similar CFSE staining patterns consistent with 1–7 cell divisions. In marked contrast, CD4 T cells from FTY720-treated donors proliferated more vigorously, with >70% cycling cells and with the majority having undergone high numbers (5–7) of divisions.

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Fig. 2. Continuous FTY720 treatment induces homeostatic proliferation. Three months after the transfer of GFP transgenic syngeneic donor BM, as described in Fig. 1, mice received BrdU in their drinking water for 1 week. (A) CD8 expression by splenic T cells from FTY720-treated (a and c) or untreated mice (b and d) gated on GFP+ (a and b) and GFP− cells (c and d); CD8+ and CD8− T cells were analyzed for co-expression of CD44 (e) and BrdU (f); plots (e) and (f) show splenic CD8 T cells from a FTY720-treated mouse. (B) BrdU staining of splenic CD4 and CD8 T cells among GFP+ (a and b) and GFP− (c and d) gated cells. Numbers within histograms indicate percentage of BrdU+ cells.
Continuous FTY720 treatment prolongs heart allograft survival

The enhanced capacity of splenic CD4 T cells from FTY720 pre-treated mice for alloreactive proliferation raised questions regarding their impact on the survival of a solid organ allograft within FTY720 pre-treated recipients themselves. This was tested with FTY720-treated B6 recipients of fully vascularized B6C3F1 heart grafts. When 3- to 4-month pre-treatment with FTY720 was stopped 1 week before heart Tx, allograft rejection was moderately delayed by 4 days compared with recipients that had never received FTY720 (days of rejection 14–17 versus 8–12 after Tx), suggesting that modifications in the composition and function of T cell pools per se did not accelerate allograft rejection, and underpinning the need for continuous FTY720 treatment for effective transplant protection. For recipients on continuous FTY720 after Tx, allograft survival was compared for two groups; one that had been pre-treated with FTY720 for 3–4 months and a second group put on FTY720 only 1 week prior to heart Tx. T cells in the former recipient group had therefore undergone an extended period of homeostatic proliferation and had reached maximal T_{PM} enrichment in all locations. One week of FTY720 treatment, by contrast, only achieved minimal T cell numbers and maximal T_{PM} skewing in the blood but not yet in spleens and LNs (data not shown). Recipients from both pre-treatment groups showed significantly delayed allograft rejection, with a tendency for longer graft survival with the 1-week pre-treatment protocol (days 17–87 and 35–114 versus days 8–12, Table 2). The histopathology of rejected heart grafts was similar for both groups under continuous FTY720 and differed markedly from untreated mice. Untreated recipients lost their heart grafts through severe acute interstitial cellular rejection with endothelialitis obliterating the arterial lumen. By contrast, rejected heart allografts from recipients on continuous FTY720 showed various grades of mild to moderate–severe acute interstitial cellular rejection in combination with fibrinoid necrosis of arteries and mild intimal hyperplasia (Fig. 6). Mild chronic rejection was only observed in the two longest surviving heart allografts. The main overall mechanism of rejection under FTY720 in this model, therefore, was delayed cellular rather than humoral or chronic rejection. Although allospecific IgM and IgG antibodies were not reduced in the blood of FTY720-treated recipients, there were no differences in the intensity or distribution of C3d deposits in untreated or FTY720-treated mice, and infiltrating B cells (B220⁺) were extremely rare in all groups (data not shown). T cell infiltrates in rejected allografts contained more CD8⁺ than CD4⁺ cells, albeit with clear differences between groups (Table 2). With 3- to 4-month FTY720 pre-treatment, both CD8 and CD4 T cell infiltrates tended to be smaller. In marked contrast, 1-week FTY720 pre-treatment resulted in lower numbers of infiltrating CD8 T cells, whereas CD4 T cell numbers were higher, thereby resulting in the lowest CD8:CD4 T cell ratios within the longest surviving allografts.

Discussion

In this report, we analyzed in mouse models the impact of long-term continuous FTY720 exposure on T cell homeostasis and properties pertaining to T cell memory and alloreactivity. In initial studies, syngeneic GFP T cell chimerism indicated the replacement of peripheral T cells by thymic
emigrants and their progeny. It followed sigmoidal growth, characteristic of competitive population dynamics, the rules of which are readily applicable to the competition among lymphocytes for limited space and resources (28, 29). T N, although long lived in the absence of a thymus, were shown to be replaced rapidly in euthymic mice and with kinetics similar to normal GFP T cell chimerism in our model (30, 31). Chronic FTY720 treatment placed challenges on T cell homeostasis, as T N were strongly diminished in the blood, with little replacement by mature thymocytes. This resulted in lower blood steady state levels of GFP+ T cells and their delayed kinetics in spleens and LNs. The kinetics of CD4 and CD8 T cell GFP chimerism differed in spleens and LNs of both untreated and FTY720-treated mice. Because T N and memory T cells are under separate homeostatic control (32, 33), the contribution of thymic emigrants to the turnover of peripheral T cell pools is determined by the proportions of peripheral T N. Thus, the faster replacement of CD4 T cells in LNs by (initially naive) thymic descendants was presumably favored by larger proportions of CD4T N in LNs over spleens. In addition, it may also reflect that CD4T N but not CD8T N absolutely depend on LNs for their survival (34). CD8T N were more similarly distributed in spleens and LNs, although slightly higher proportions of splenic CD8T N might have contributed to initially faster CD8GFP chimerism in spleens. CD8 T cell chimerism within LNs presumably ‘caught up’ through growing contributions from CD8TPCM, i.e., a memory phenotype including the LN homing receptor CD62L. In the light of differential CD4 and CD8 T cell subset compartmentalization and declining total T cell numbers

![Diagram of T cell homeostasis and alloreactivity under FTY720](https://academic.oup.com/intimm/article-abstract/20/5/633/965279)

**Fig. 4.** Continuous FTY720 treatment increases the proportions of IFNγ-producing T cells. LN (a and c) and spleen cells (b and d) from 3-month untreated (gray circles) or FTY720-treated (black circles) B6 mice were activated for 6 h *in vitro* with PdBu and ionomycin (Iono) or with irradiated B6C3F1 ‘allo’ spleen cells in the presence of brefeldin A. Cells were surface stained for CD4 or CD8 and from B6C3F1 co-cultures also for H-2Kk. Fixed and permeabilized cells were stained for intracellular IFNγ and analyzed by flow cytometry. Controls for alloantigen-driven IFNγ production were spleen cells from a B6 mouse that had rejected a B6C3F1 skin graft 2 months previously (histogram inset). Symbols represent data from two individual mice per group. The data are representative of three independent experiments.

**Fig. 5.** Proliferation of T cells from FTY720-treated donor spleens upon transfer to semi-allogeneic hosts. CFSE-stained splenocytes prepared from 3- to 4-month FTY720-treated or untreated B6CD45.1 donors were injected i.v. into B6 or B6C3F1 recipients. For the transfer of equivalent numbers of T cells, recipients were injected with either 3 × 10^7 spleen cells from untreated or 4 × 10^7 spleen cells from FTY720-treated donors. Three days later, recipient spleens were analyzed for proliferation by CFSE fluorescence intensity of donor-derived CD45.1+ CD4 and CD8 T cells. Histograms are representative of data from two syngeneic and three semi-allogeneic recipients per group. Markers indicate the percentage of T cells with loss of CFSE staining intensity. Similar data were obtained in another independent experiment.
infiltrates in FTY720-treated mice

Table 2. Prolonged heart allograft survival and altered T cell infiltrates in FTY720-treated mice

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<th>CD4⁺ cell numbers</th>
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<td>FTY720, from 1-week pre-Tx, n = 4</td>
<td>114</td>
<td>280</td>
<td>160</td>
<td>1.8</td>
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B6 recipients of B6C3F1 heart allografts were untreated or kept on FTY720 following Tx and a 1- or 14-week pre-treatment period with FTY720 before Tx. Data within each row refer to the same recipient and graft tissue. P-values are derived from Wilcoxon-Mann-Whitney rank-sum statistics.

FTY720, from 1-week pre-Tx, n = 4,

Untreated versus FTY720 1-week pre-Tx, P = 0.014; untreated versus FTY720 1-week pre-Tx, P = 0.029; untreated versus FTY720 1-week pre-Tx; P = 0.057, FTY720 1-week pre-Tx versus untreated. 

under FTY720, it seemed possible that altered survival rates as a consequence of changing competition for survival factors also influenced chimerism growth rates. We measured similarly and moderately increased cell sizes (by forward scatter, not depicted) for CD4 and CD8 GFP⁺ and GFP⁻ Tₜ in FTY720-treated mice. In a previous report, an increase in cell size by non-proliferating CD8Tₜ cultured in the presence of IL15 was taken to reflect the pro-survival effect of this cytokine (35). Although CD4Tₜ do not utilize IL15 in addition to IL7 for their survival, increased sizes of non-proliferating Tₜ might generally reflect an improved availability of appropriate survival factors. Furthermore, T cells from FTY720-treated mice had a slightly higher expression of the anti-apoptotic factor Bcl-2, which is up-regulated by IL7 and IL15 (36, 37). Thus, these observations might indicate a moderate generally improved T cell survival capacity under FTY720, yet without favoring any of the CD4 or CD8 GFP⁺ or GFP⁻ T cell subsets, as would be required for a considerable impact on chimerism growth rates.

In addition to delaying the turnover of peripheral T cell pools, continuous FTY720 exposure led to a decline in peripheral T cell numbers, thereby provoking an increase in homeostatic proliferation. This was associated with co-receptor and CD3 down-regulation, which was most pronounced among GFP⁺CD8 T cells. The reasons for stronger proliferation among GFP⁺ T cells remain speculative. A previous study excluded different intrinsic proliferative capacities between wild-type and similar (actin promoter-driven) GFP transgenic T cells (38). Possibly, ‘younger’ T cells, as represented by GFP⁺ T cells, proliferate more readily than ‘old’ peripheral T cells in response to partial lymphopenia. Co-receptor down-regulation was generally observed for a fraction of T cells from FTY720-treated mice in all experiments and was not, therefore, peculiar to GFP chimerism studies. Nor was it directly induced by FTY720, as it was also demonstrated for T cells proliferating in syngeneic SCID hosts, whereas CD8 appeared stably expressed during the proliferation of allo-mismatched T cells in the same SCID transfer model. Regular health reports ruled out infections as a reason for increased proliferation. TCR down-regulation during homeostatic proliferation was described previously (39), and down-regulation of CD8 and the TCR by T cells encountering their cognate antigen in the periphery was identified as a mechanism of peripheral tolerance (40). Given the necessary and sufficient role of self-peptide–MHC in driving homeostatic proliferation (41, 42), co-receptor/CD3 down-regulation may reflect one strategy to preserve peripheral tolerance to self-antigens.

Although homeostatic proliferation was shown to promote transplant rejection and autoimmunity under some conditions (17, 18), continuous FTY720 application resulted in prolonged rather than accelerated allograft survival, even when discontinued before Tx. Furthermore, tissues and organs from mice continuously treated with FTY720 for up to 2 years bore no signs of autoimmunity (our unpublished observations). Several reasons might account for these apparent discrepancies, such as different degrees of T cell depletion and homeostatic proliferation as well as the clonality/specificity of the proliferating T cells. A limited T cell repertoire with strong skewing toward allo-cross-reactive or self-reactive pathogenic T cells might arise through the expansion of small T cell numbers within an initially T-deplete or near-deplete space. Unlike TCR transgenic allospecific T cells that caused enhanced allograft rejection following homeostatic proliferation within T-deplete recipients (18), the degrees of...
T cell depletion and homeostatic proliferation under FTY720 were modest, by comparison, and the T cell repertoire of FTY720-treated mice was not intentionally skewed. Nor is the wild-type B6 strain used in our experiments susceptible to autoimmunity, unlike the non-obese diabetic mouse model where diabetes was promoted by homeostatic proliferation (17). In other studies, strong T cell depletion of 80–90% triggered high degrees of homeostatic proliferation that interfered with the induction of Tx tolerance by co-stimulation blockade (43). It remains to be shown weather tolerance induction would be impaired by a more moderate reduction of T cell numbers and less vigorous homeostatic proliferation.

The highest degrees of homeostatic proliferation were found among CD4 TCM, resulting in ~80 and 95% BrdU+ CD4 TCM in the blood and BM of FTY720-treated mice. Nevertheless, their contribution to the whole CD4 T cell pool never exceeded 4–5% at any site in controls or under long-term FTY720 exposure. The high death rate that must be inferred for CD4 TCM may be supported by their very low levels of Bcl-2 that were lowest among all T cell subsets (our unpublished data). CD8 TCM also retained similarly small proportions in the spleens and LNs of controls and FTY720-treated mice. As total T cell numbers declined under FTY720, the absolute numbers of CD8 TPEM and CD8 TCM also decreased accordingly, possibly indicating that maintaining stable small proportions of CD8 TPEM, and in particular of CD8 TCM, may be critical to the integrity of the immune system.

The strongest FTY720-induced modulation of local T cell pool compositions (other than in the blood) were a further concentration of CD4 TPEM in spleens and LNs and of CD8 TCM within LNs. Homeostatic proliferation of CD4 TPEM and CD8 TCM was also increased under FTY720, albeit not at those sites of their predominant enrichment. Factors that contributed substantially to the concentration of CD4 TPEM presumably included the preferential loss of CD4 TCM from spleens and LNs. The large and selective proportional increase of CD8 TCM within LNs and not spleens translated into reduced total numbers of splenic CD8 TCM and higher numbers of CD8 TCM within LNs, suggesting that their migration to and sequestration within LNs of FTY720-treated mice played a role. Although the proportions of CD4 TPEM and CD8 TCM increased, their overall numbers did not, as implemented by the separate homeostatic control of T N and memory T cells that prevents memory T cells from occupying the void left by contracting T N pools (32, 33). In addition to FTY720-induced T cell redistribution, it remains formally possible that phenotype conversion might have contributed to changes in the compositions of T CM and T PEM. Insight into phenotype conversion in mice largely comes from studies in TCR transgenic models, and prominent proportional changes of effector memory T cells (TEM) and central memory T cells (T CM) through phenotype conversion appeared to be favored by non-physiologically high T responder frequencies in vivo and in vitro (44, 45). Other reports demonstrated various degrees of phenotype flexibility to accommodate T cell migration and location patterns (46, 47). The tendencies towards more prominent CD4 TPEM and CD8 TCM among ‘naturally occurring’ memory T cells in this and previous studies might imply, however, that endogenous T PEM/T PCM patterns are formed during initial rounds of homeostatic proliferation, i.e. during the conversion from a naive to an either central or effector memory phenotype rather than interconversion between memory phenotypes (47–49). Furthermore, as indicated by the GFP-negative T cell pool in the chimerism studies, these CD4 TPEM/CD8 TCM biases become more prominent as the proportions of T N decline with the age of the T cell pool. Thus, given the preferential loss of T N and increased homeostatic proliferation under FTY720, the resulting proportional increases in CD4 TPEM and CD8 TCM presumably further accentuate physiological tendencies rather than reflect memory T cell conversion. Enlarged proportions of CD4 TPEM and CD8 TCM in our model correlated with higher percentages of rapidly IFN-γ-producing CD4 T cell from spleens and LNs and CD8 T cells from LNs of FTY720-treated mice. Although ‘central’ and ‘effector’ memory phenotypes define functionally distinct human memory T cells, with the former lacking immediate effector functions (50), this does not apply to murine CD8 TCM and CD8 TPEM that were shown capable of mediating effector functions (51, 52). IFN-γ-producing T cells were only obtained upon nonspecific activation and not in the presence of semi-allogeneic (B6C3F1) cells. This was expected since allogene-reactive IFN-γ-producing memory T cells induced by infectious agents, often viruses, are rare in ‘clean’ mice but may be a serious obstacle in clinical Tx (53). Surprisingly, however, CD4 T cells from FTY720-treated donor spleens proliferated more vigorously within B6C3F1 hosts than CD4 T cells from untreated controls. This might be accounted for by the larger content of CD4 TPEM among donor spleen cells from FTY720-treated mice and/or the strong proliferative capacity of CD4 TCM. In either case, these data might suggest that all or certain subsets of CD4 TCM, i.e. cells with a history of self-MHC-driven homeostatic proliferation, can gain functional memory for allo-cross-reactive proliferation. Nevertheless, despite an enhanced alloreactive proliferation potential of splenic T cells, i.e. those not ‘trappable’ by FTY720, heart allograft rejection was strongly delayed. Cellular graft infiltrates were dominated by CD8 T cells, and prolonged allograft survival correlated with lower numbers of intra-graft CD8 T cells rather than CD4 T cells. Presumably, FTY720-induced LN sequestration of CD8 TCM contributed to allograft protection, consistent with previous studies demonstrating that murine allospecific CD8 TCM were far more potent mediators of allograft rejection than CD8 TCM (54). The tendency of the longest surviving allografts to contain higher numbers of CD4 T cells might reflect an intrinsically weaker productive alloreactive potential at the level of individual intra-graft CD4 T cells, thereby requiring larger numbers of them to accumulate for effective allograft rejection. Because FTY720 does not inhibit T cell effector functions directly (55), this would imply that the short FTY720 pre-treatment protocol promoted CD4 T cell infiltrates with inherently weaker alloreactivity at the single cell (rather than the population) level. There is no precedence, however, to support this possibility. Alternatively, the enlarged CD4 T cell infiltrates, even though not capable of mediating indefinite allograft survival, included an active protective component, as might be achieved with contributions from regulator T cells (Tregs). Because alloantibodies were not reduced in FTY720-treated mice, putative Tregs more likely acted within the allograft rather than...
lymphoid tissue. Transplant protection in the presence of Tregs and alloantibodies was reported previously, as was, within allografts, the accumulation of Tregs that modulate the effector rather than the induction phase of cellular immunity (56–58). The properties of graft-infiltrating T cells in this model are currently under investigation. It also remains to be clarified why short and not long pre-treatment with FTY720 favored larger allograft CD4 T cell infiltrates. This could result from reduced total numbers of a potential Treg type or Treg ‘precursor’, as would apply to CD4+CD25+ or CD4+CD25− T cell subsets but did not promote allograft rejection. Ongoing studies investigate whether homeostatic tuning under FTY720 may be harnessed rationally to promote Treg tissue infiltrates for therapeutic purposes.

Supplementary data
Supplementary Figures 1 and 2 and Table 1 are available at International Immunology Online.

Disclosures
All authors are employees of Novartis, Basel, Switzerland.

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Abbreviations
BM bone marrow
BrdU 5-bromo-2-deoxyuridine
CD4+CD25+ central memory phenotype CD8 T cells
CD4−CD25− effector memory phenotype CD4 T cells
CFSE carboxyfluorescein succinimidyl ester
GFP green fluorescent protein
i.v. intravenously
LN lymph node
PDPr phosphor 12,13-dibutyrate
PerCP peridinin chlorophyll protein
S1P sphingosine-1 phosphate
SP single positive
S1P1 sphingosine-1 phosphate receptor-1
Tcm central memory T cells
TEM effector memory T cells
Tn naive T cells
Tpm memory phenotype T cells
Treg regulator T cell
Tx transplantation

T cell homeostasis and alloreactivity under FTY720

References
15 Hofmann, M., Brinkmann, V. and Zerwes, H. G. 2006. FTY720 preferentially depletes naive T cells from peripheral and lymphoid organs. Int. Immunopharmacol. 6, 1902.