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The Sphingosine 1-Phosphate Receptor Agonist FTY720 Potently Inhibits Regulatory T Cell Proliferation In Vitro and In Vivo1,2

Anna Maria Wolf,3,8† Kathrin Eller,† Robert Zeiser,§ Christoph Dürr,§¶ Ulrike V. Gerlach,¶ Michael Sixt,§ Lydia Markut,8† Guenther Gastl,8† Alexander R. Rosenkranz,‡ and Dominik Wolf8†

CD4+CD25+ regulatory T cell (Treg) entry into secondary lymphoid organs and local expansion is critical for their immunosuppressive function. Long-term application of the sphingosine-1-phosphate receptor agonist FTY720 exerts pleiotropic anti-inflammatory effects, whereas short-term FTY720 boosts antiviral immunity. In this study, we provide evidence that FTY720 potently inhibits Treg proliferation in vitro and in vivo without affecting their viability, phenotype, or in vitro immunosuppression. In contrast, adoptively transferred Treg exposed ex vivo to FTY720 lost their protective effects in murine models of acute glomerulonephritis and acute graft-vs-host disease. On a cellular level, FTY720 inhibits IL-2-induced STAT-5 phosphorylation, paralleled by a loss of FoxP3 expression during Treg expansion in vitro. Notably, loss of in vivo immunosuppression is not due to impaired migration or to localization within secondary lymphoid organs. We could even show a selective trapping of adoptively transferred Treg in inflammatory lymph nodes by FTY720. Finally, Treg isolated from animals systemically exposed to FTY720 also exhibit a significantly impaired proliferative response upon restimulation when compared with Treg isolated from solvent-treated animals. In summary, our data suggest that sphingosine-1-phosphate receptor-mediated signals induced by FTY720 abrogate their in vivo immunosuppressive potential by blocking IL-2 induced expansion, which is indispensable for their in vivo immunosuppressive activity. The Journal of Immunology, 2009, 183: 3751–3760.

Naturally occurring regulatory T cells (Treg) are emerged as a unique CD4+ T cell subset mainly characterized by coexpression of high CD25 levels together with the Forkhead transcription factor FoxP3 (1–3). Treg play a decisive role in the maintenance of immunological tolerance (2). Various target cells such as CD4+ and CD8+ T cells (4–6) and NK cells (7), as well as NKT cells (8) and dendritic cells (9), and different immunosuppressive mechanisms including TGF-β (10) and CTLA-4 (11) have been described to date. The proliferative potential of Treg is well documented and is assumed to be of critical importance for counter-regulation of an ongoing effector T cell activation, as a rapid expansion of the Treg pool tips the numerical balance in favor of a more immunosuppressive milieu (12). Among various others, IL-2 has been proven to play a critical role for Treg survival and proliferation (13, 14). Accordingly, the expansion of Treg rather than that of effector T cells in cancer trials testing IL-2 as an immunostimulant support this concept (15). Finally, data from murine models further corroborate these clinical observations, as IL-2 is indispensable for peripheral maintenance of naturally occurring Treg (16).

In addition, the immunosuppressive potential of Treg also depends on direct cell-to-cell contact with target cells. Thus, their migration to sites of immune responses has to be tightly regulated. Secondary lymphoid organs (SLO) are considered to be an important environment for Treg-mediated immune regulation (17), as lymph nodes (LN) as well as the spleen are critical for orchestrating T cell-mediated immune responses (18). Obviously, in case of an exaggerated immune activation within target organs, Treg also have to enter end organs to induce local immune regulation. In line with this idea, it has recently been demonstrated in an experimental model of acute graft-vs-host disease (GvHD) that LN-homing, CD62L-expressing Treg are superior to CD62L-negative Treg in terms of disease control (19, 20). In contrast, in a model of chronic GvHD that rather reflects a situation of ongoing T cell activation within damaged end organs, it appeared to be mainly the CD103+ Treg population that was inhibiting GvHD (21). These observations fit perfectly the recent description of various Treg subpopulations characterized by a distinct chemokine and cell surface receptor expression pattern, i.e., CCR7- (22), CCR5- (23), CXCR4- (23), CD62L- (20, 24), and CD103-expressing (21) Treg. In addition to the data derived from the GvHD models, we could recently demonstrate the potent nephroprotective effects of adoptively transferred Treg in an accelerated model of anti-glomerular basement membrane (GBM) glomerulonephritis (GN) (25). In this particular model, Treg do not infiltrate the inflamed end organ but...
primarily migrate to the SLO, which most likely induces inhibition of tissue-destructive effector T cells. Thus, especially in the early phase of inflammatory processes, efficient LN occupancy as well as a high proliferative potential of Treg is required for efficient immunosuppression.

FTY720 (2-amino-2-(4-octylphenyl)ethyl)-1,3-propanediol hydrochloride) is a sphingosine analog and an immunomodulatory agent derived from the Chinese fungus Iscaria sinclairi. FTY720 has been proven to prolong allograft survival in both rodents and men (26, 27) by acting as a sphingosine-1 phosphate (SIP) agonist inducing a prolonged down-regulation of the cell surface expression of the SIP receptor. The latter enables a blockade of SIP-mediated T cell egress from SLO, which is normally induced by a SIP gradient from blood and lymph fluid to tissue (28). Among its various target cell effects, the trapping of lymphocytes in SLO and the subsequent induction of lymphopenia is thought to be the predominant mechanism that prevents effector T cell exit into the blood stream and subsequent entry into target tissues (29). Interestingly, immuno-suppressive function after systemic long-term application of FTY720 has at least in part been attributed to the induction of FoxP3 in naïve T cells, thereby inducing T cells with a regulatory phenotype (30). However, this is just one among the plethora of mechanisms induced by FTY720 in vivo, as it has also been shown to modify lymphatic endothelial (28), dendritic (31), and B cell function (32).

We provide in this report first evidence that the SIP receptor agonist FTY720 potently inhibits Treg proliferation in vitro and in vivo by inhibiting IL-2 mediated mitogenic signals. Despite the fact that FTY720 induced the trapping of Treg in inflamed SLO, the immunosuppressive effects of FTY720-exposed and subsequently adoptively transferred Treg were abrogated in two murine models of inflammation.

Materials and Methods

Animals

C57BL/6 and BALB/c mice (6–12 wk of age) were purchased from Harlan Laboratories. For bone marrow transplant experiments, luciferase-expressing transgenic C57BL/6 L2G85 mice (33) were used. All experiments used age and sex-matched control mice and were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Innsbruck Medical University, Innsbruck, Austria (35/9185.81/G-07/19, Austrian Federal Ministry for Education, Science and Culture (BMBWK)-66.011/0087-Br/GT/2005).

Reagents

CFSE and TAMRA were purchased from Molecular Probes. FTY720 was a gift from Dr. V. Brinkmann (Novartis Pharma) and SEW2871 was purchased from Biomol. Anti-CD3 was purchased from BD Biosciences. The annexin V detection kit was from R&D Systems and IL-2, IL-7, and IL-15 were from Peprotech.

Cell preparation

Spleens and LN were removed and gently dissociated to single cell suspensions. CD4+CD25− T cells (Treg) were magnetically separated using a Treg separation kit (Milteny Biotec) achieving purity of T cells together with equal numbers of either FTY720- or solvent-Treg were

Performed in anti-CD3 precoated (5 μg/ml) wells. As control, the respective T cell population was cultured alone. Proliferation was measured on day 5 by [3H]thymidine incorporation in a beta scintillator.

Treg culture

CFSE-labeled (0.5 μM) Treg at 1 × 10^6/ml were cultured with 100 ng/ml plate-bound anti-CD3 together with 100 U/ml IL-2 for 2–4 days. Stimulation with anti-CD3 plus IL-2 results in cell division with distinct CFSE fluorescence peaks that allows for discrimination between cycled (at least one division) and noncycled cells by FACS analysis.

Flow cytometry

All FACS Abs (except the anti-FoxP3 mAb) were purchased from BD Biosciences. Phosphospecific flow cytometry was performed strictly according to the manufacturer’s manual (protocol 3; BD Biosciences) after stimulation using plate bound anti-CD3 (5 μg/ml; BD Biosciences) together with one of the following common γ-chain cytokines: IL-2 (100 U/ml), IL-7 (10 ng/ml), or IL-15 (25 ng/ml). SIP specificity of FTY720-mediated effects on STAT-5 phosphorylation was tested by using SEW2871 at concentrations ranging from 100 to 1000 nM instead of FTY720. Flow cytometric analyses were performed on a FACS Calibur (BD Biosciences) and data were analyzed with CellQuest software (BD Biosciences). FoxP3 was stained by APC-labeled anti-mouse FoxP3 mAb (eBioscience).

Western blotting

Treg (5 × 10^6) were lysed on ice and lysates were analyzed by electrophoresis on 10% SDS-polyacrylamide gels after being blotted on hydrophobic polyvinylidene difluoride membrane (GE Healthcare). Mouse anti-phosphorylated STAT-5 Ab (clone 8-5-2, dilution 1/1000; Millipore) was added and binding was subsequently detected by HRP-conjugated goat anti-mouse IgG (dilution 1:2000; DakoCytomation). Chemiluminescent reaction was induced using a Lumigen TMA-6 kit (GE Healthcare) and the blots were analyzed by using a ChemiDoc XRS+ molecular imaging system (Bio-Rad). To evaluate expression of total STAT-5 protein, the blots were incubated with 1% mild Ab stripping solution (Chemicon) and with mouse anti-STAT-5 Ab (clone 89/STAT-5, dilution 1/400; BD Biosciences).

Cytokine production

To detect IL-2 and IFN-γ in supernatants, FTY720 or solvent-exposed T cells were restimulated with PMA/ionomycin. Cytokines were detected by ELISA (BD Biosciences).

Adoptive transfers and immunofluorescence

FTY720- and solvent-Treg suspensions were differentially labeled with CFSE (green) or TAMRA (red) dyes for adoptive transfer strictly according to the manufacturer’s recommendations and subsequently injected i.v. (5 × 10^6 of each Treg population) to follow their in vivo migration behavior under steady state or inflammatory conditions. The latter were induced by s.c. injection of 0.02 g of nonviable desiccated Mycobacterium tuberculosis H37a (Difco Laboratories) dissolved in IFA (Sigma-Aldrich) into the footpad. Seven days after the induction of inflammation, differentially labeled FTY720- and solvent-Tregs were adoptively transferred. Peripheral blood, spleen, draining (popliteal), and non-draining (popliteal, contralateral to footpad injection) LN were harvested 4–48 h after Treg transfer and subsequently prepared either as single cell suspension (for FACS) or snap frozen (for preparation of tissue sections). Images were taken with an upright Leica MZ 16 FA stereomicroscope equipped with a spot camera and operated via MetaMorph software (system implemented by Vis- tron Systems). Original magnification was ×10.

Acute GVHD model

Acute GvHD (aGvHD) was induced as described previously (34). In brief, recipients were exposed to lethal irradiation with 850 cGy on day 0 followed by i.v. injection of 5 × 10^6 T cell-depleted bone marrow cells after 1 day. To induce aGVHD, 1 × 10^6 CD4+CD8− T cells in the C57BL6→BALB/c model, respectively, were injected i.v. on day 2. Treg were derived from donors of the same genetic background as those of conventional T cells (Tconv) and injected i.v. on day 0 at the same dosage as the Tconv. Slides of small bowel and large bowel samples collected on day 7 were stained with H&E and scored by a blinded and experienced pathologist (U.V.G.) according to a previously published histopathology scoring system.

In vivo bioluminescence imaging (BLI)

BLI was performed as previously described (33). Briefly, mice were injected i.p. with luciferin (10 μg/kg body weight). Ten minutes later, mice were imaged using an IVIS200 charge-coupled device imaging system...
FIGURE 1. Effects of FTY720 on Treg viability. Isolated Treg were exposed to increasing concentrations of FTY720 as indicated and subsequently taken into culture using anti-CD3 together with 100 U/ml IL-2. Cell death was determined by annexin/propidium iodide and FACS analysis at the indicated time points. Percentages of positive cells are given in the quadrants. A representative experiment is shown ($n = 3$).

Anti-GBM GN

An accelerated model of nephrotic nephritis was induced as previously described (35). In brief, mice were preimmunized s.c. with 2 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories) dissolved in IFA and nonviable desiccated Mycobacterium tuberculosis H37a (Difco Laboratories). Two days later, animals received either $3 \times 10^8$ solv-Treg or equal numbers of FTY-Treg i.v. As control, mice received $3 \times 10^8$ CD4$^+$/CD25$^-$. After 3 days, 5 mg of heat-inactivated rabbit anti-mouse GBM antibodies was administered i.v. For controls, mice received solvent. Twenty-four-hour urine samples were collected in metabolic cages on days 1, 1, 7, and 14 after the injection of GN. Urinary albumin was determined by a double-sandwich ELISA (Abcam), and urinary creatinine was quantified spectrophotometrically using a commercially available kit (Sigma-Aldrich).

**Histology**

Formalin-fixed tissue was embedded in paraffin, cut in 4-$\mu$m sections, and stained with periodic acid Schiff (PAS) for histologic analysis. Sections cut from frozen tissue (4–25 $\mu$m) were used for LN migration studies as well as for immunoperoxidase staining of macrophages and T cells in the kidney (25) using a rat anti-mouse macrophage Ab (Clone F4/80; Serotec), a rat anti-mouse CD4 mAb (clone YTS191;1; Serotec), and an IgG2a isotype Ab as negative control. Biotin-conjugated goat anti-rat IgG Ab (Jackson ImmunoResearch Laboratories) was used as a secondary Ab, followed by incubation with an avidin-biotin complex and subsequent development and HE counterstaining. T cells were quantified by counting the number of cells in six adjacent high-power fields of the renal cortex and medulla. A semiquantitative scoring system was performed for the quantification of macrophages: 0, 0 to 4 cells stained positive; 1+, 5 to 10 cells; 2+, 10 to 50 cells; 3+, 50–200 cells; and 4+, >200 cells stained positive per low-power field.

**Systemic FTY720 administration**

FTY720 was administered i.p. in a dose of 1 mg/kg body weight on two consecutive days, whereas control mice received solvent. Mice were sacrificed 36 h after the first i.p. injection for isolation of Treg and CD4$^+$CD25$^-$ control cells from spleen and LN suspensions. The isolated cells were cultured on plates precoated with 100 ng/ml anti-CD3 together with 100 U/ml IL-2 for 5 days and proliferation was determined by $[^3H]$thymidine incorporation.

**Statistics**

Results represent data from at least three independent biological experiments. After ANOVA, Student’s $t$ test was used. Values of $p < 0.05$ were considered significant. Figures show means ± SEM. Statistical analysis was performed using GraphPad Prism.

**Results**

FTY720 effects on Treg viability, phenotype, and in vitro immunosuppression

Only little information is available regarding the direct effects of FTY720 on isolated Treg. Thus, we analyzed the influence of the drug on characteristic Treg properties. First, we tested the effects of FTY720 on Treg viability. Using increasing doses (0.5–4 µg/ml) of FTY720, we observed an increased rate of cell death only after 72 h at the highest dose level of 4 µg/ml, which is known to be toxic for various cell types (Fig. 1). We therefore used the concentration of 0.5 µg/ml for our subsequent in vitro and in vivo experiments. We next tested whether the characteristic properties of Treg function are modulated by short-term FTY720 exposure. The suppressive potential of FTY-Treg, as measured by standard in vitro suppression assays, remained unaltered as compared with solv-Treg, even when FTY-Treg were mixed with CD4$^+$CD25$^-$ responder T cells in different ratios ranging from 1:1 to 1:10 (Fig. 2A; *p < 0.05 for FTY- and solv-Treg vs responder cells alone). Moreover, FTY-Treg suppressed the production of IFN-$\gamma$ and IL-2 by CD4$^+$CD25$^-$ T cells as effectively as their solvent-treated counterparts (Fig. 2B; *p < 0.05). Finally, FTY-Treg showed a comparable cell surface marker expression (CD62L, CD103, CCR7) as well as intracellular FoxP3 levels after short-term exposure (Fig. 2C; *p < 0.05). Thus, typical functional and phenotypical hallmarks of bona fide Treg are preserved upon short-term exposure to FTY720 in vitro.

FTY720-treated Treg show severe proliferation defects in vitro and in vivo

The critical role of IL-2 for Treg proliferation is well documented (36). Thus, we next focused on in vitro Treg expandability upon stimulation via the TCR together with IL-2. In contrast to solv-Treg, which can be readily expanded, FTY-Treg exhibit a markedly impaired proliferation in vitro (Fig. 3A; *p < 0.05). These data were corroborated in vivo by measuring CFSE dilution upon adoptive
transfer of either CFSE-labeled FTY- or solv-Treg into mice suffering from footpad inflammation. In this model, FTY-Treg also showed a severely reduced expansion in the draining LN when compared with adoptively transferred solv-Treg. As depicted in Fig. 3B, only 24% of transferred FTY720-treated, CFSE-positive Tregs divided 48 h after i.v. injection in vivo (*, \( p < 0.05 \); \( n = 10 \) per group). B, FTY- or solv-Treg were seeded in 24-well plates alone or mixed in a 1:1 ratio with CD4+ CD25− control T cells in a final concentration of 5 × 10^6 cells/ml and stimulated with PMA and ionomycin for 16 h. IFN-γ and IL-2 were determined in triplicate in supernatants by ELISA and compared with the cytokine production of pure populations of CD4+ CD25− control T cells (*, \( p < 0.05 \); \( n = 6 \)). C, CD103, CD62L, and CCR7 surface stainings and intracellular FoxP3 stainings of either FTY- or solv-Treg (a representative example from five independent experiments is shown).

FTY720 blocks IL-2 signaling in Treg

On the molecular level, we found that FTY720 pre-exposure of Treg dose-dependently impaired IL-2-induced phosphorylation of STAT-5, which is a critical component of the IL-2 signal transduction pathway (Fig. 4, A and B; *, \( p < 0.05 \)). In contrast, solvent pre-exposure did not alter IL-2-induced phosphorylation of STAT-5. Reduced sensitivity to IL-2-induced signals is not mediated by down-regulation of the IL-2 receptor α-chain upon FTY720 exposure (Fig. 4C). However, in line with the reduced activation of Treg by IL-2 after FTY720 exposure (which is mirrored by inhibition of proliferation), further up-regulation of the mean fluorescence intensity of CD25 throughout the culture period (at 72 and 96h after culture initiation) is prevented by FTY720 (the percentage of CD25-expressing cells remains unchanged in both fractions; data not shown). It is well documented that the IL-2/STAT-5 pathway plays a decisive role for FoxP3 maintenance in Treg (37). Thus, it is not surprising that the maintenance of FoxP3 expression during in vitro culture of initially FTY720-exposed Treg is affected (Fig. 4D).

Inhibition of STAT-5 phosphorylation is S1P1 and IL-2 specific

SIP receptor 1 specificity of the FTY720-mediated effects was proven by using increasing doses of the S1P receptor 1 specific agonist SEW2871 instead of FTY720. In line with our previous data, SEW2871 also dose dependently inhibited IL-2 induced STAT-5 phosphorylation (Fig. 5A). Of note, phosphorylation of STAT-5 induced by other cytokines of the common γ-chain family (i.e., IL-7 and IL-15) was not prevented by FTY720 (Fig. 5B).

FTY720 pretreatment of Treg abrogates their suppressor function in vivo

We subsequently tested the consequence of impaired Treg proliferation induced by the S1P receptor agonist FTY720 in adoptive Treg transfer models. First, we used an established model for...
FTY720 induces the trapping of adoptively transferred Treg into inflamed LN

To exclude the possibility that additional mechanisms contribute to the observed loss of Treg-mediated immunosuppression in vivo, we next focused on FTY720 effects on Treg migration upon adoptive transfer. Using steady state conditions as well as a well defined model of rapid local inflammation (i.e., footpad injection of mycobacteria together with Freund’s adjuvant), we i.v. coinjected TAMRA-labeled FTY-Treg together with equal numbers of transfer of solv-Treg almost completely prevented the expansion of luciferase transgenic, allogeneic Tconv (Fig. 6, A and B). In contrast, short-term exposure of Treg to FTY720 before injection markedly reduced their suppressive cues, which is reflected by a massive expansion of luciferase-positive effector T cells comparable to that seen in Tconv-transferred animals (Fig. 6, A and B). This reduced suppressor function also resulted in significantly reduced overall survival of the group that received Tconv along with FTY-Treg compared with the group receiving Tconv together with solv-Treg (Fig. 6C; *, p = 0.001). Histopathology scoring revealed a significantly higher GvHD score in the group that received Tconv together with FTY-Treg as compared with the group receiving Tconv plus solv-Treg (Fig. 6D; *, p < 0.05). Overall, these results demonstrate that pre-exposure of Treg to FTY720 impairs Treg function in vivo.

Second, anti-GBM nephritis was selected as an autoimmune model to test FTY-Treg in vivo. According to our previous data (25), transfer of solv-Treg into mice suffering from anti-GBM GN potently inhibited the development of renal inflammation as reflected by significantly reduced proteinuria (Fig. 7A; *, p < 0.05). Histological evaluation revealed only occasional PAS-positive deposits, and the majority of the glomeruli displayed no pathological changes (Fig. 7C). According to the data from the aGvHD model, ex vivo FTY720 treatment of Treg also abrogated their suppressive effect on nphritic inflammation as demonstrated by significantly elevated albumin/creatinine urine levels when compared with animals receiving solv-Treg (Fig. 7A). Kidneys displayed classical histological signs of GN such as hypercellularity and focal deposition of PAS-positive deposits, as well as enhanced infiltration of CD4+ T cells and macrophages (Fig. 7B; *, p < 0.05), which was comparable to mice receiving CD4+CD25+ control T cells (Fig. 7E).

FIGURE 4. FTY720 blocks IL-2 signaling in Treg. A and B, IL-2-induced STAT-5 phosphorylation (p-STAT-5) of Treg exposed to increasing doses of FTY720 was determined at the indicated time points by phosphospecific flow cytometry (n = 8 per group; *, p < 0.05 vs solv-Treg) (A) and Western blotting (B). C, CD25 expression upon either FTY720 or solvent exposure of isolated Treg. Data are presented as mean fluorescence intensity throughout a culture period of 96 h under growth-supporting conditions with anti-CD3 and IL-2 (n = 3 per group; *, p < 0.05 vs solv-Treg). D, IL-2 induced maintenance of FoxP3 expression of in vitro cultured Treg was determined in FTY- vs solv-Treg at the indicated time points (n = 4 per group; *, p < 0.05).

FIGURE 3. FTY-Treg show severe proliferation defects in vitro and in vivo. A, In vitro expansion by TCR stimulation with anti-CD3 together with IL-2 is impaired by FTY720 as shown by CFSE dilution of solv- or FTY-Treg at the indicated time points (n = 7 per group; *, p < 0.05 vs solv-Treg). B, Forty-eight hours after adoptive transfer of either CFSE-labeled FTY-Treg or CFSE-labeled solv-Treg into mice suffering from a footpad inflammation, CFSE dilution of transferred Treg in SLO was determined by flow cytometry (n = 8 per group; *, p < 0.05 vs solv-Treg). aGvHD as an alloimmune model in which Treg are capable of reducing lethality (34). In vivo expansion of aGvHD-inducing T cells was monitored by detecting photons via bioluminescence imaging, as the donor effector T cells carry the luciferase transgene. The extent of the emitted light is known to correlate with T cell aging, as the donor effector T cells carry the luciferase transgene. A FIGURE 4.
CFSE-labeled solv-Treg (Fig. 8A). Under steady state conditions, 6–12 h after transfer increased numbers of FTY-Treg were found in the spleen, but not in peripheral LN (Fig. 8B). Notably, despite their loss of immunosuppression in vivo in the inflammatory models, inflamed LN occupancy of Treg is even increased by ex vivo exposure of Treg to FTY720 (Fig. 8C; *, p < 0.05). No significant increase of FTY-Treg was detectable in the contralateral non-draining LN under inflammatory conditions.

In vivo positioning of Treg within SLO is not altered by FTY720

Next, we analyzed the positioning of FTY720-exposed Treg in T cell areas of SLO upon adoptive transfer, because an altered

FIGURE 5. Inhibition of STAT-5 phosphorylation is specific for S1P1 and IL-2. A, IL-2-induced STAT-5 phosphorylation (pStat5) of Treg exposed to increasing doses of the selective S1P receptor 1 agonist SEW2871 was determined at the indicated time points by phosphospecific flow cytometry (n = 3 per group; *, p < 0.05 vs solv-Treg). B, STAT-5 phosphorylation of FTY720 pre-exposed Treg subsequently stimulated with the common γ-chain cytokines IL-2, IL-7 and IL-15 (n = 3 per group; *, p < 0.05 vs solv-Treg).

FIGURE 6. FTY720 treatment severely impairs Treg suppressor function in a model of acute GvHD. A, Representative BALB/c recipients from the indicated groups (Tconv, CD4/CD8 conventional T cells given on day 2 after bone marrow transplantation (BMT; n = 10); solv-Treg (n = 15); and FTY-Treg (n = 15)) from different time points (days 12, 14, and 16) after BMT are shown. B, Expansion of luciferase transgenic alloreactive T cells as quantified in photons per second per mouse over time. Pooled data from two independent experiments are shown. C, Survival of BALB/c after lethal irradiation with 8.5 Gy and BMT. Data are pooled from three independent experiments (*, p < 0.05 solv-Treg vs FTY-Treg). D, Seven days after transplantation, mice from the indicated groups were sacrificed and sections of small bowel and large bowel were stained with H&E. Slides were analyzed by a pathologist blinded to the treatment groups for evidence of pathologic damage (*, p < 0.05 vs solv-Treg). BM, Bone marrow; ctrl, control; Tc, conventional T cell.
localization might also interfere with proper Treg function in vivo. Therefore, we again coinjected differentially labeled FTY720- and solvent-exposed Treg i.v. into mice under steady state as well as inflammatory conditions. As shown in Fig. 8D, TAMRA labeled FTY-Treg (red fluorescence) perfectly colocalized with CFSE-labeled solv-Treg (green fluorescence) in T cell areas of the spleen and LN after i.v. transfer into mice with local footpad inflammation. This finding was corroborated in healthy mice showing similar positioning patterns of FTY- and solv-Treg in SLO under steady state conditions (data not shown).

**Discussion**

The sphingosine analog FTY720 is a promising immunomodulatory agent with proven therapeutic efficacy in various experimental models of autoimmunity (26, 27). The drug is currently tested as an immunosuppressive agent in various clinical trials and will soon be approved for treatment of relapsing multiple sclerosis (38). Immunosuppressive action of FTY720 is suggested to be primarily mediated via the trapping of lymphocytes in SLO, thereby preventing effector T cell exit into the blood stream and subsequent target tissue entry (29). Interestingly, immunosuppressive functions after continuous systemic application of FTY720 or exposure of the whole PBMC fraction to the drug have at least in part been attributed to the induction of Treg (30, 39, 40). However, the fact that FTY720 induces a plethora of mechanisms in various target cells (i.e., lymphatic endothelial, dendritic, T, and B cells) (31, 32) limits the interpretation of direct FTY720-induced effects on Treg. The latter have been shown to play a decisive role in the maintenance of tolerance and have been considered as cellular immunosuppressants for the treatment of exaggerated autoimmune and alloimmune responses (41–43).
We provide in this report for the first time evidence that the S1P receptor agonist FTY720 is a potent inhibitor of Treg proliferation in vitro and in vivo. Treg expand very efficiently when stimulated appropriately in vitro via the TCR together with IL-2 (14). Accordingly, clinical studies using IL-2 as immunostimulant described a preferential expansion of Treg (15), which can be explained by a low IL-2 receptor signaling threshold that supports Treg development and maintenance (44). Pre-exposure of isolated Treg to FTY720 in vitro almost completely prevented Treg proliferation induced by TCR stimulation and IL-2. Previous reports, which have already demonstrated that S1P inhibits the proliferative response of CD4+ T cells (45–47), support the concept of a T cell inhibitory effect of S1P receptor agonists in addition to their well characterized effects on lymphocyte migration (29). In line with our in vitro observation, we could further show that FTY720 exposure also profoundly impairs Treg expansion in vivo after adoptive transfer under inflammatory conditions (i.e., a model of local LN inflammation). Recent reports have already defined the critical role of IL-2 for Treg maintenance and expansion (44, 48, 49). On the molecular level, FTY720, as well as the S1P receptor 1-specific agonist SEW2871, severely impairs critical components of the IL-2 signaling pathway in Treg (50), as phosphorylation of STAT-5 and the subsequent IL-2-induced maintenance of FoxP3 expression are inhibited. Interestingly, this inhibitory effect appears to be specific for IL-2, as we were not able to detect any inhibitory effect of the compound on STAT-5 phosphorylation induced by other family members of the common γ-chain cytokines, such as IL-7 and IL-15. Inhibition of FoxP3 maintenance during the culture period is most likely due to the fact that STAT-5 binding sites are located in the promoter region of FoxP3 (37, 50), linking IL-2 signaling to the maintenance of FoxP3 expression. It is of particular importance that FTY720 does not simply reduce...
to destabilize LN architecture with the displacement of marginal
fact that a blockade of S1P receptors by FTY720 has been shown
did choose an experimental setup that allowed the evaluation of
recent report demonstrating that systemic application of FTY720
regarding the effects of FTY720 on Treg migration complement a
ally suggests that the increased LN occupancy of Treg (especially
vitreous transfer (25, 33, 51), we could clearly demonstrate that FTY-Treg
immune model), both of which can be inhibited by adoptive Treg
production) and did not affect Treg survival in vitro. However,
concomitantly abrogates their in vivo immunosuppressive poten-
agonist FTY720 induces Treg trapping in inflammatory SLO but
proportion of Treg (17). Thus, it is most likely the FTY720-induced inhibition
Treg LN occupancy is critical for the immunosuppressive function
in vivo. Finally, we could further corroborate our previous findings
by showing that FTY720 also exerts Treg inhibitory effects when applied in a systemic fashion. In line with our in vitro data, Treg
isolated from animals exposed to FTY720 for a short term are
hyporesponsive to IL-2 plus TCR-induced proliferation. Our data
are in line with a very recent report showing that short-term sys-
FTY720 treatment enhanced virus-specific CD4+ and CD8+ T cell responses in a chronic lymphocytic choriomeningitis virus
infection model (54). This observation was primarily explained by
improved T cell priming within SLO induced by transiently in-
creased LN occupancy of T cells by FTY720, but the effect on
Treg function in vivo was not addressed in this report. Our data
suggest that concomitant inhibition of immunosuppressive Treg
might also account for the observed phenomenon. This observation is
supported by a very recent report presented by Liu and col-
leagues (55) demonstrating that S1P delivers via S1P receptor 1 an
intrinsic negative signal activating the Akt/mammalian target of
rapamycin pathway leading to inhibition of the thymic generation,
peripheral maintenance, and suppressive activity of Treg.

In summary, we show for the first time that the S1P receptor
agonist FTY720 induces Treg trapping in inflammatory SLO but
concomitantly abrogates their in vivo immunosuppressive poten-
tial by inhibition of Treg expansion, which is due to the inhibition
of IL-2 induced STAT-5 activation. Our data highlight the need of
proper Treg proliferation after adoptive Treg transfer to induce an
efficient immunosuppressive impact on effector T cells (i.e.,
achievement of an optimal Treg to autoreactive or alloreactive T
cell ratio) and suggest that the S1P receptor agonism induced by
pharmacological agents might represent a rational approach for
Treg inhibition. Finally, our data suggest that the inhibition of Treg
by S1P receptor agonists might represent an innovative adjuvant
for cancer immunotherapy (e.g., in combination with dendritic cell
vaccines).

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Disclosures
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