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FTY720 Ameliorates Th1-Mediated Colitis in Mice by Directly Affecting the Functional Activity of CD4$^+$CD25$^+$ Regulatory T Cells[1]

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Following the present concepts, the synthetic sphingosine analog of myriocin FTY720 alters migration and homing of lymphocytes via sphingosine 1-phosphate receptors. However, several studies indicate that the immunosuppressive properties of FTY720 may alternatively be due to tolerogenic activities via modulation of dendritic cell differentiation or based on direct effects on CD4$^+$ CD25$^+$ regulatory T cells (Treg). As Treg play an important role for the cure of inflammatory colitis, we used the Th1-mediated 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis model to address the therapeutic potential of FTY720 in vivo. A rectal enema of TNBS was given to BALB/c mice. FTY720 was administered i.p. from days 0 to 3 or 3 to 5. FTY720 substantially reduced all clinical, histopathologic, macroscopic, and microscopic parameters of colitis analyzed. The therapeutic effects of FTY720 were associated with a down-regulation of IL-12p70 and subsequent Th1 cytokines. Importantly, FTY720 treatment resulted in a prominent up-regulation of FoxP3, IL-10, TGFβ, and CTLA4. Supporting the hypothesis that FTY720 directly affects functional activity of CD4$^+$CD25$^+$ Treg, we measured a significant increase of CD25 and FoxP3 expression in isolated lamina propria CD4$^+$ T cells of FTY720-treated mice. The impact of FTY720 on Treg induction was further confirmed by concomitant in vivo blockade of CTLA4 or IL-10R which significantly abrogated its therapeutic activity. In conclusion, our data provide clear evidence that in addition to its well-established effects on migration FTY720 leads to a specific down-regulation of proinflammatory signals while simultaneously inducing functional activity of CD4$^+$CD25$^+$ Treg. Thus, FTY720 may offer a promising new therapeutic strategy for the treatment of IBD. ***The Journal of Immunology,*** **2007, 178: 2458–2468.**

The FTY720 is a synthetic sphingosine analog (2-amino-(2-(2-(4-octophenyl) ethyl)-1,3-propanediol hydrochloride) of myriocin (1–4). Currently, the discussion about its mode of action is mainly focused on its property to cause lymph node homing or sequestration of T cells. In this scenario, FTY720 activates sphingosine-1-phosphate (S1P)[4] receptors and modulates migration after being effectively phosphorylated in vivo by sphingosine kinase 2 (SphK2) (5, 6). FTY720-phosphate (FTY720-P) exhibits a potency comparable to S1P itself as an agonist at four of the five known G-protein-coupled S1PRs ($S1P_{1,3,4,5}$). Interference of FTY720 with S1P signaling hampers entry of lymphocytes into efferent lymphatics within lymph nodes, thereby delaying their subsequent return into circulation (7, 8). $S1P_1$ and $S1P_4$ are the main S1PRs on T and B cells. FTY720 treatment does not result in an impairment of T cell activation, expansion, and generation of memory to systemic viral infections. Also it is not inducing T cell apoptosis at clinically relevant concentrations (7, 9–12), FTY720 is highly potent in animal models for the treatment of allograft rejection and autoimmunity. Despite ongoing discussions about adverse effects, FTY720 has entered clinical trials and was shown to be beneficial in human kidney transplantation (1, 13–17).

Inflammatory bowel disease (IBD) representing a family of chronic, relapsing, and tissue-destructive diseases is characterized by a dysfunction of mucosal T cells, imbalanced cytokine production and cellular inflammation leading to damage of the intestinal mucosa (18). Clinically, IBD is subdivided into Crohn's disease and ulcerative colitis. Although the detailed etiology of IBD remains to be determined, there is circumstantial evidence to link IBD to the mucosal immune system's failure to attenuate immunity to luminal Ags (19, 20).

CD4$^+$CD25$^+$ Treg play a critical role in the maintenance of self tolerance, control of autoimmune diseases, transplant rejection and have also been documented to offer a therapeutic option in severe inflammatory colitis (21–23). These regulatory T cells (Treg) are typically anergic and unresponsive to TCR stimulation. They prevent proliferation and activation of inflammatory CD4$^+$ or CD8$^+$ T cells via cell-cell contact-dependent mechanisms and also prominently by the production of suppressor cytokines such as IL-10 and/or TGFβ (24). Recently, S1P was demonstrated to be required for optimal suppression of effector T cell activities by Treg (25). Additionally another group showed that the S1P mimetic FTY720 differentially affected the recirculation of Treg vs memory effector T cells (26). However, in the latter investigation it was also

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[4] Abbreviations used in this paper: S1P, sphingosine-1-phosphate; IBD, inflammatory bowel disease; Treg, regulatory T cell; DC, dendritic cell; GITR, glucocorticoid-induced TNFR-related gene; TNBS, 2,4,6-trinitrobenzene sulfonic acid; BW, body weight; MPO, myeloperoxidase; LP, lamina propria; LPMC, LP mononuclear cell.

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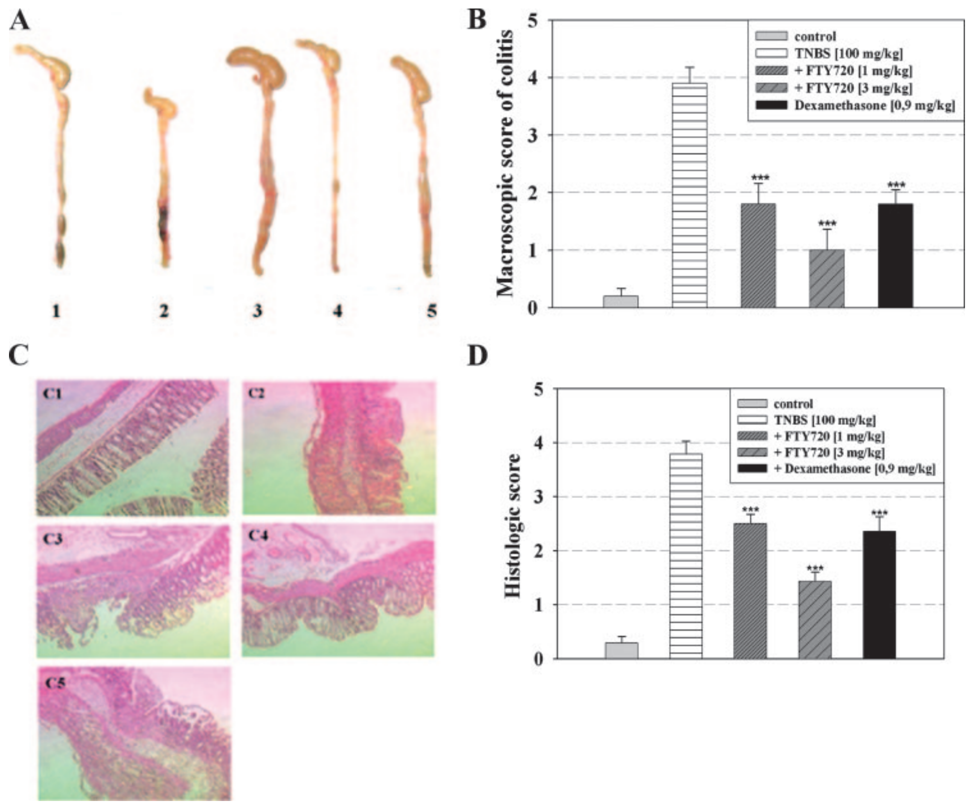

FIGURE 1. Macroscopic and microscopic analysis of colons from mice with acute TNBS colitis. Mice were treated with 1, 3 mg/kg FTY720, or 0.9 mg/kg dexamethasone, respectively. *A*, A representative photograph of colons from day 3 after the induction of TNBS-colitis. 1, Ethanol-treated control; 2, TNBS-treated; 3, TNBS plus 1 mg/kg FTY720; 4, TNBS plus 3 mg/kg FTY720; 5, TNBS plus 0.9 mg/kg dexamethasone. *B*, Macroscopic score of colitis. Results are the mean ± SEM from eight mice per group. *C*, Photomicrographs of colon sections after treatment with 45% ethanol (*C1*), TNBS in 45% ethanol (*C2*); TNBS plus 1 mg/kg FTY720 (*C3*); TNBS plus 3 mg/kg FTY720 (*C4*), and TNBS plus 0.9 mg/kg dexamethasone (*C5*). Histopathological scoring (*D*); original magnification, ×250. Results are the mean ± SEM from eight mice per group. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs TNBS-treated mice.

considered that modulation of migration might not be the end of the story regarding its impact on Treg. Indeed, FTY720 may additionally alter the functional activity of Treg directly or via "tolerogenic" DC (25–30). Thus, a more complex mode of action may be supported by the fact that the affinity profile of FTY720-P on S1PRs is not identical to S1P. Furthermore, the definition of T lymphocytes as the prime and major target of FTY720/S1P has been challenged by several publications showing significant effects on other immune competent cells comprising monocytes and dendritic cells (DC) (26–28, 31–33). These investigations raise the possibility that not only migratory but rather differentiating effects are involved early during an immune response, which include e.g., a direct intracellular blockade of cPLA2 that is independent of FTY720 phosphorylation and significantly contributes to its therapeutic activity in experimental autoimmune encephalomyelitis (33).

Our data clearly indicate that FTY720 effectively treats Th1-mediated colitis in mice. Importantly these therapeutic effects of FTY720 are closely related to changes of functional Treg activity. Although our results do not exclude that a modulation of Treg sequestration contributes to its immunosuppressive capacities, they are highly supportive for the view that FTY720 exerts its prominent therapeutic effects through a differential down-regulation of proinflammatory signals of DC, which subsequently favor education of Treg, as assessed by analysis of IL-10, TGFβ, FoxP3, CTLA4, glucocorticoid-induced TNFR-related gene (GITR), as well as of FoxP3 and CD25 expression in isolated lamina propria (LP) $CD4^+$ positive T cells. The hypothesis put forward here that FTY720 modulates the function of Treg is further supported by the fact that in vivo administration of anti-CTLA4 Ab or anti-IL-10R Ab significantly abrogated the FTY720-mediated immune suppression in 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis. Thus, focusing on this alternate mode of action FTY720 may offer a promising new approach for the treatment of chronic, lymphocyte dependent immune disorders including IBD.

Materials and Methods

Mice

Male, 8-wk-old, BALB/c mice weighing ~20 g were obtained from Charles River Laboratories. All studies were performed under approval of the Ethics Committee of Darmstadt/Hessen (Germany, F134/03) and are in agreement with the guidelines for the proper use of animals in biomedical research. At the end of the experiments mice were sacrificed by cervical dislocation under isoflurane anesthesia (Forene; Abbott).

Table I. *Effect of FTY720 on clinical parameters of acute TNBS colitis*[a]

	Δ BW (%)	CAS	Colon Length (cm)	Colon Weight (mg)
Control	0.75 ± 0.73	0.36 ± 0.15	12.75 ± 0.23	147.44 ± 7.53
TNBS	−17.61 ± 1.55	9.40 ± 0.57	10.12 ± 0.11	319.94 ± 6.12
FTY720 (1 mg/kg)	−9.98 ± 1.71[c]	5.12 ± 0.73[d]	11.02 ± 0.17[d]	251.65 ± 12.13[d]
FTY720 (3 mg/kg)	−6.91 ± 1.10[d]	2.87 ± 0.40[d]	11.76 ± 0.14[d]	210.14 ± 9.13[d]
Dex (0.9 mg/kg)	−10.10 ± 2.88[b]	4.11 ± 0.48[d]	11.10 ± 0.39[c]	228.44 ± 12.08[d]

[a] TNBS colitis mice were treated i.p. with FTY720 or dexamethasone. Body weight (BW) change on day 3 in percent of day 0, clinical activity score (CAS), colon length, and colon weight (distal 6 cm) were determined on day 3. Data represent mean ± SEM from three separate experiments (eight mice per group per experiment). [b] $p < 0.05$; [c] $p < 0.01$; [d] $p < 0.001$ vs TNBS-treated mice.

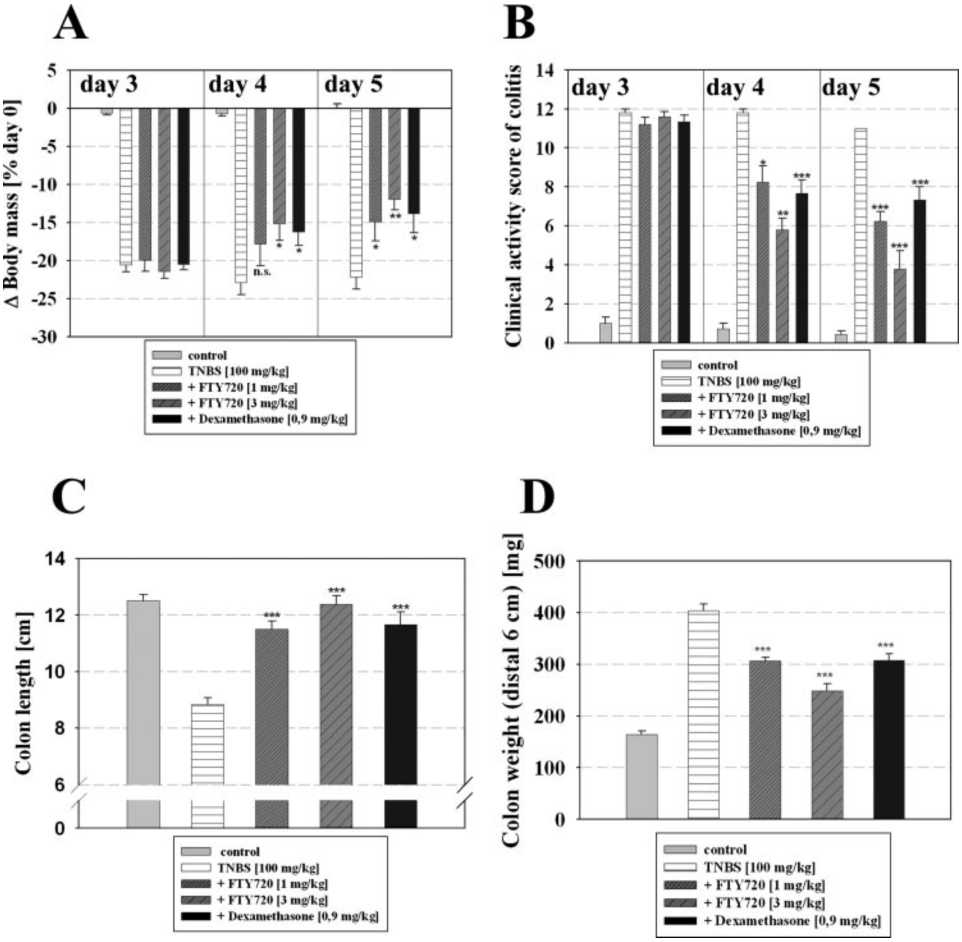

FIGURE 2. FTY720 ameliorates established TNBS colitis. FTY720 was first administered i.p. on day 3 following the TNBS enema and on the next 2 days. The colitis severity was assessed by analysis of BW change on days 3–5 in percent of day 0 (*A*), clinical activity score of colitis from days 3 to 5 (*B*), colon length (*C*), and colon weight of 6-cm colon portions (*D*) on day 5. Results represent the mean ± SEM from eight mice per group. FTY720 treatment blocked development of disease and led to a significant improvement of colitis in all analyzed parameters. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs TNBS-treated mice.

Induction of Th1-mediated colitis by the haptenating agent TNBS

The haptenating agent TNBS (Sigma-Aldrich) was used at a concentration of 2% in 45% ethanol. TNBS was administered (100 mg/kg (body weight (BW)) to slightly anesthetized mice through a 3.5 F catheter carefully inserted into the rectum. The catheter tip was inserted 4 cm proximal to the anal verge. To ensure proper distribution of TNBS within the entire colon and cecum, mice were kept in a vertical position for 1 min after the instillation of the TNBS enema. Control animals received 45% ethanol alone using the same technique.

Administration of FTY720 and study design

FTY720 (2-amino-2-[2-(octyl-phenyl) ethyl]-1,3-propanediol hydrochloride) was synthesized by Witega. Identity and purity were checked by mass spectrometry and ^{1H}NMR and was >99% as assessed by HPLC analysis. FTY720 was dissolved in sterile distilled water with the solutions prepared fresh daily. The drug was administered i.p. at a dose of 1 or 3 mg/kg BW, respectively. To test the therapeutic efficacy of FTY720, two protocols have been used: 1) prevention of colitis: FTY720 was administered 2 h before the instillation of the TNBS enema and for the subsequent 3 days. On day 3, the colon was removed. 2) Treatment of established colitis: FTY720 was administered i.p. from day 3 to 5 following the TNBS enema. The colon was removed at day 5. In all experiments performed dexamethasone (Dex; D-2915, water-soluble; Sigma-Aldrich) was used as a reference compound. For in vivo CTLA4-blockade an ultra-purified functional grade anti-mouse CTLA4 Ab (Natutec; eBioscience) was used at a concentration of 7.5 mg/kg i.p. The dose for the in vivo use of anti-CTLA4 Ab was optimized according to a previously documented study (21). To block IL-10R function an ultra-purified IL-10R Ab (1B1.3a; BD Pharmingen) was used at 10 mg/kg i.p. following previously documented usage (21, 34). Control mice received normal IgG (anti-mouse IgG; Sigma-Aldrich) at the same doses.

Assessment of inflammation and colitis severity

Clinical activity score of colitis. For the assessment of the clinical severity of colitis the BW, as well as the stool consistency and rectal bleeding were recorded daily with a scoring system described in detail previously (35). In brief, the loss of BW was scored as follows: 0, no weight loss; 1, weight loss of 1–5%; 2, weight loss of 5–10%; 3, loss of 10–20%; and 4, weight loss >20%. Assessment of diarrhea: 0, normally formed pellets; 2, pasty and semiformed pellets; 4, liquid stools. Bleeding: 0, no blood in hemoccult; 2, positive

Table II. *Effect of FTY720 on the inflammatory response in established TNBS colitis*[a]

	MPO Activity (U/g Colon Tissue)	TNF-α (pg/mg Protein)	IL-12p70 (pg/mg Protein)
Control	0.23 ± 0.18	3.17 ± 0.47	3.83 ± 0.20
TNBS	17.33 ± 1.20	16.78 ± 0.90	18.94 ± 0.97
FTY720 (1 mg/kg)	8.84 ± 0.49[c]	8.24 ± 0.48[c]	11.60 ± 1.05[c]
FTY720 (3 mg/kg)	3.66 ± 0.89[c]	4.74 ± 0.60[c]	6.32 ± 0.76[c]
Dex (0.9 mg/kg)	9.02 ± 1.23[b]	9.12 ± 0.97[b]	10.55 ± 0.82[c]

[a] TNBS colitis mice were treated i.p. with FTY720 or dexamethasone from days 3 to 5. MPO activity, TNF-α, and IL-12p70 were determined in colon protein extracts on day 5. The results show the mean ± SEM from eight mice per group. [b] $p < 0.01$; [c] $p < 0.001$ vs TNBS-treated mice.

Table III. *Effect of FTY720 on the inflammatory response in acute TNBS colitis*[a]

	MPO Activity (U/g colon tissue)	TNF-α (pg/mg protein)	IL-12p70 (pg/mg protein)
Control	0.40 ± 0.24	1.86 ± 0.25	2.78 ± 0.29
TNBS	11.60 ± 1.48	8.95 ± 0.45	10.83 ± 1.17
FTY720 (1 mg/kg)	3.87 ± 1.51^c	4.13 ± 0.44^c	5.70 ± 0.33^b
FTY720 (3 mg/kg)	1.81 ± 0.99^c	2.91 ± 0.47^c	4.15 ± 0.60^c
Dex (0.9 mg/kg)	2.78 ± 1.19^c	3.46 ± 0.40^c	5.55 ± 0.46^b

[a] TNBS colitis mice were treated i.p. with FTY720 or dexamethasone. MPO activity, TNF-α, and IL-12p70 were determined in colon protein extracts on day 3. The results are the mean $\pm$ SEM from three separate experiments (eight mice per group per experiment). $^bp < 0.01$; $^cp < 0.001$ vs TNBS-treated mice.

hemoccult; 4, gross bleeding from the rectum. The resulting scoring parameters were added resulting in a total clinical score ranging from 0 (healthy) to 12 (maximal ill/activity of colitis).

Colon weight and colon length. The length of the colon was determined as well as the wet weight of the distal 6-cm colon to be used as indirect markers of disease-associated intestinal wall thickening correlating with the intensity of inflammation.

Macroscopic scoring system. The assessment of the macroscopic colon damage was performed using the scoring system of Wallace and Keenan (36). The criteria for the evaluation of macroscopic damage were based on a semiquantitative scoring system. Features were graded as follows: 0, no ulcer, no inflammation; 1, no ulcer, local hyperemia; 2, ulceration without hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending >2 cm.

Histological analysis of the colon. For histological examination, a sample of colon tissue located precisely 3 cm above the anal canal was obtained from the mice of all treatment groups. Tissues were graded semiquantitatively from 0 to 5 (0: no changes to 5: marked transmural inflammation with severe ulceration and loss of intestinal glands) in a blinded fashion according to previously described criteria (37, 38).

Measurement of myeloperoxidase (MPO) activity

MPO activity was assessed as a marker for neutrophil leukocyte infiltration and accumulation into the inflamed colon tissue. The MPO-activity assay was performed using a modification of the method described by Bradley et al. (39). The enzyme activity was determined photometrically as the MPO catalyzed change in absorbance occurring in the redox reaction of 3,3,5,5-tetramethylbenzidine dihydrochloride (Sigma-Aldrich) at 650 nm. MPO (Sigma-Aldrich) was used as an internal standard. Values are expressed as MPO units per gram of wet tissue.

Isolation of LP CD4$^+$ T cells

LP cells were isolated using a modification of the technique described by van der Heijden and Stok (40). Briefly, after removal of all visible Peyer's patches colonic samples of the different treatment regimens were washed thoroughly in cold Ca/Mg-free HBSS to remove debris and were cut into 0.5-cm pieces. The epithelium was removed from the LP by incubation with 2 mM DTT and 1 mM EDTA in HBSS at 37°C for 2×20 min under gentle shaking. Tissues were subsequently minced into 2×2 mm pieces and digested using collagenase D (4000 Mandl units/ml) and DNase (1 mg/ml; both from Boehringer Mannheim) in complete RPMI1640 medium. Incubation was performed in a 37°C waterbath for 90–120 min, manually shaking the tube every 5 min. LP mononuclear cells (LPMCs) were harvested by discontinuous Percoll (44–67%; Amersham Bioscience) and lymphocyte-enriched populations were isolated from the cells at the 44–67% interface. LP CD4$^+$ T cells were purified from LPMCs by using the anti-CD4 (L3T4; Miltenyi Biotec) MACS system.

LP CD4$^+$ T lymphocyte cytokine production

Cell cultures of LP CD4$^+$ T cells were performed in complete RPMI 1640 medium supplemented with 3 mM L-glutamine, 10 mM HEPES buffer, 10 mg/ml gentamicin, 100 U/ml of each penicillin and streptomycin, 0.05 mM 2-ME (Sigma-Aldrich), and 10% FCS. Anti-CD3ε precoated T cell activation plates (BD Biosciences) were used for analysis of cytokine production. LP CD4$^+$ T cells (5×10^5/ml) were incubated in the presence of soluble anti-CD28 (clone 3751; 1 μg/ml; BD Pharmingen) at 37°C in 5% CO_2 humidified air. Samples were performed in quadruplicates in a total volume of 200 μl/well, respectively. After 48 h of culture, supernatants were harvested and assayed for cytokine contents by specific ELISAs (IL-10, TGFβ) according to the manufacturer's instructions (R&D Systems).

Protein extraction

Protein extraction of colon tissue or LP T cells was performed using the Active Motif Nuclear cell extraction kit according to the manufacturer's instructions for extraction procedures starting from tissue (Active Motif Nuclear extract kit; Rixensart). Aliquots of the resulting extracts were analyzed for their protein content using the BioRad colorimetric assay according to the method of Bradford (Bio-Rad) and stored at -80°C until use.

Cytokine assays

The amount of murine TNF-α, IL-12p70, IL-10, and TGFβ in the protein lysates were quantified by commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions and adapted to the protein content of the colon tissue probe.

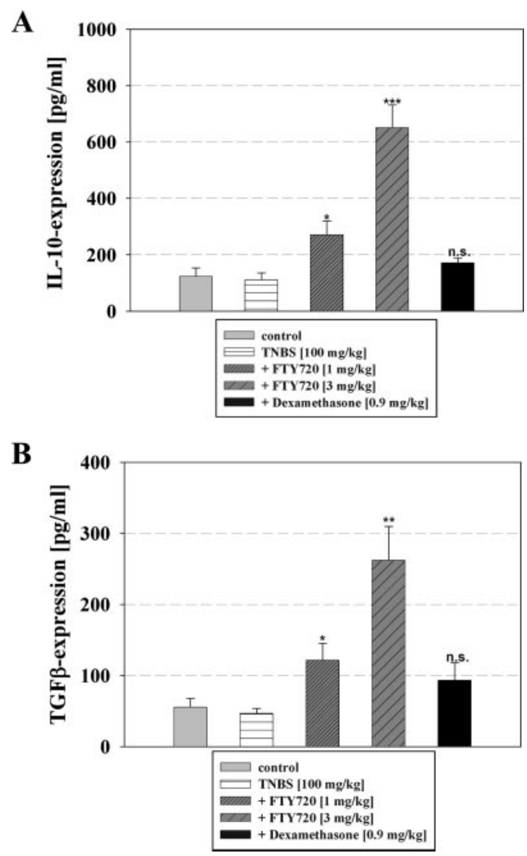

FIGURE 3. IL-10 (*A*) and TGFβ expression (*B*) of CD4$^+$ T cells isolated from the LP of either untreated TNBS colitic mice ($n = 5$) or colitic TNBS mice treated with FTY720 (1 or 3 mg/kg, respectively) or with dexamethasone (0.9 mg/kg) from days 0 to 3. CD4$^+$ T cells (5×10^5 cells/ml) were cultured in 96-well plates with coated anti-CD3ε (5 μg/ml) and costimulated with anti-CD28 (1 μg/ml). Supernatants were harvested after 48 h of culture. IL-10 and TGFβ levels were assayed by ELISA. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs control group.

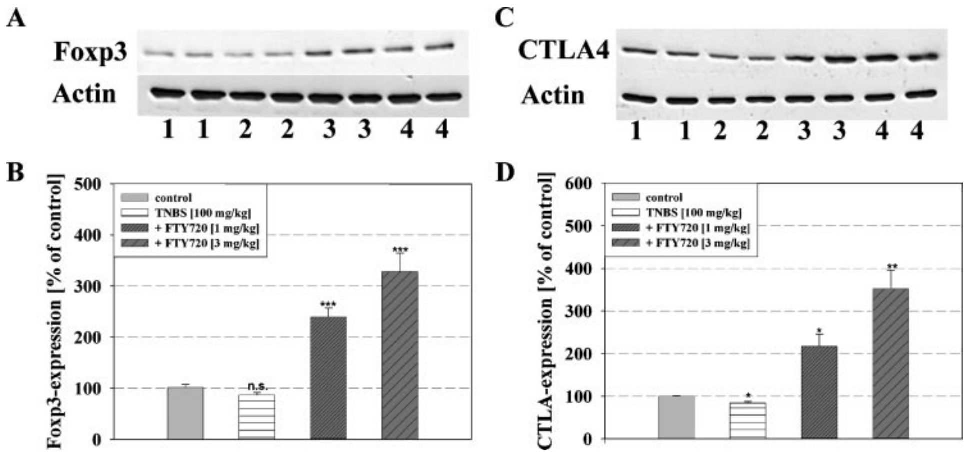

FIGURE 4. FTY720 treatment leads to an induction of FoxP3 and CTLA4 protein expression. *A*, A representative immunoblot of FoxP3 expression is shown. 1, Ethanol-treated control group; 2, TNBS in 45% ethanol; 3, TNBS plus FTY720 (1 mg/kg); 4, TNBS plus FTY720 (3 mg/kg). Two different mouse colon probes per treatment regimen have been blotted. *B*, Densitometric evaluation of FoxP3 immunoblot analysis. The bars represent the mean ± SEM of three separate experiments ($n = 6$) per group. $*$, $p < 0.05$; $**$, $p < 0.01$; and $***$, $p < 0.001$ vs control group. *C*, CTLA4-protein expression. 1, Ethanol-treated control group; 2, TNBS in 45% ethanol; 3, TNBS plus FTY720 (1 mg/kg); 4, TNBS plus FTY720 (3 mg/kg). Two different mouse colon probes per treatment regimen have been blotted. *D*, Densitometric evaluation of the CTLA4 immunoblot analysis. The bars represent the mean ± SEM of three separate experiments ($n = 6$) per group. $*$, $p < 0.05$; $**$, $p < 0.01$; and $***$, $p < 0.001$ vs control group.

Western blot analysis

After addition of sample buffer to the colon protein extracts or T cell extracts and boiling samples at 95°C for 5 min 150 μg of total protein lysates were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell) and the membrane was blocked for 1 h at room temperature with 4% skim milk in TBST. The level of proteins was assayed using the primary mouse FoxP3, CD25, CTLA4, or GITR Abs (1:1000, 1:200, or 1:500, respectively; eBioscience) overnight at 4°C. Immunoreactivity was visualized by an ECL system (Amersham) using an appropriate HRP-conjugated secondary Abs (NA931; Amersham Biosciences; 1:2000; anti-Armenian hamster IgG, 1:1000; Rockland; NA935; Amersham Biosciences; 1:2000). Bands were detected after exposure to Hyperfilm-MP (Amersham International). Blots were reprobed with actin Ab (Santa Cruz Biotechnology). For quantitative analysis, the bands were detected with scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProViDoc software (Desaga).

Statistical analysis

All data are expressed as mean ± SEM. Statistical significance of differences between TNBS- and FTY720-treated groups of mice was determined by the unpaired two-tailed Student's t test (SigmaStat). Differences were considered statistically significant with $p < 0.05$.

Results

FTY720 prevents acute and established Th1-mediated TNBS colitis

TNBS-treated mice developed severe diarrhea accompanied by an extensive wasting disease. Treatment with FTY720 resulted in a striking improvement of the wasting disease, as assessed by animal weight loss, as well as clinical, macroscopic, microscopic and immunological parameters of colitis (Fig. 1 and Table I). Macroscopic analysis of

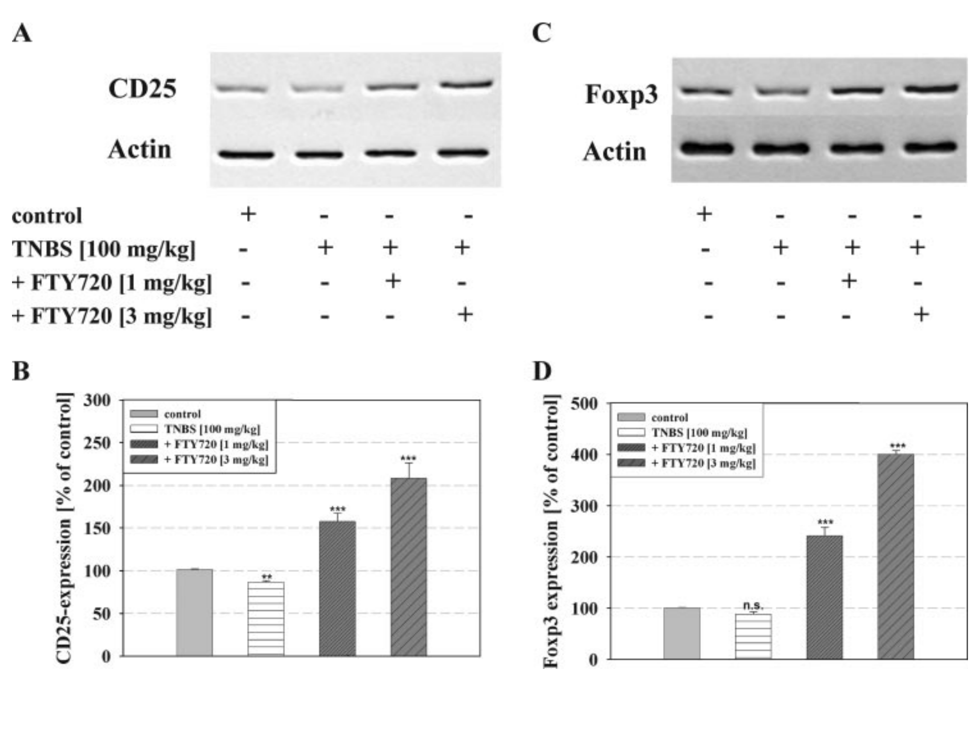

FIGURE 5. TNBS-treated mice were treated i.p. with FTY720 from days 0 to 3. CD25 (*A* and *B*) or FoxP3 protein expression (*C* and *D*) of $CD4^+$ T cells isolated from the LP of either untreated TNBS colitic mice ($n = 5$) or colitic TNBS mice treated with FTY720 (1 or 3 mg/kg, respectively) is depicted. Densitometric evaluations of the immunoblot analysis are demonstrated in *B* and *D*. One representative experiment of three is shown. $*$, $p < 0.05$; $**$, $p < 0.01$; and $***$, $p < 0.001$ vs control group.

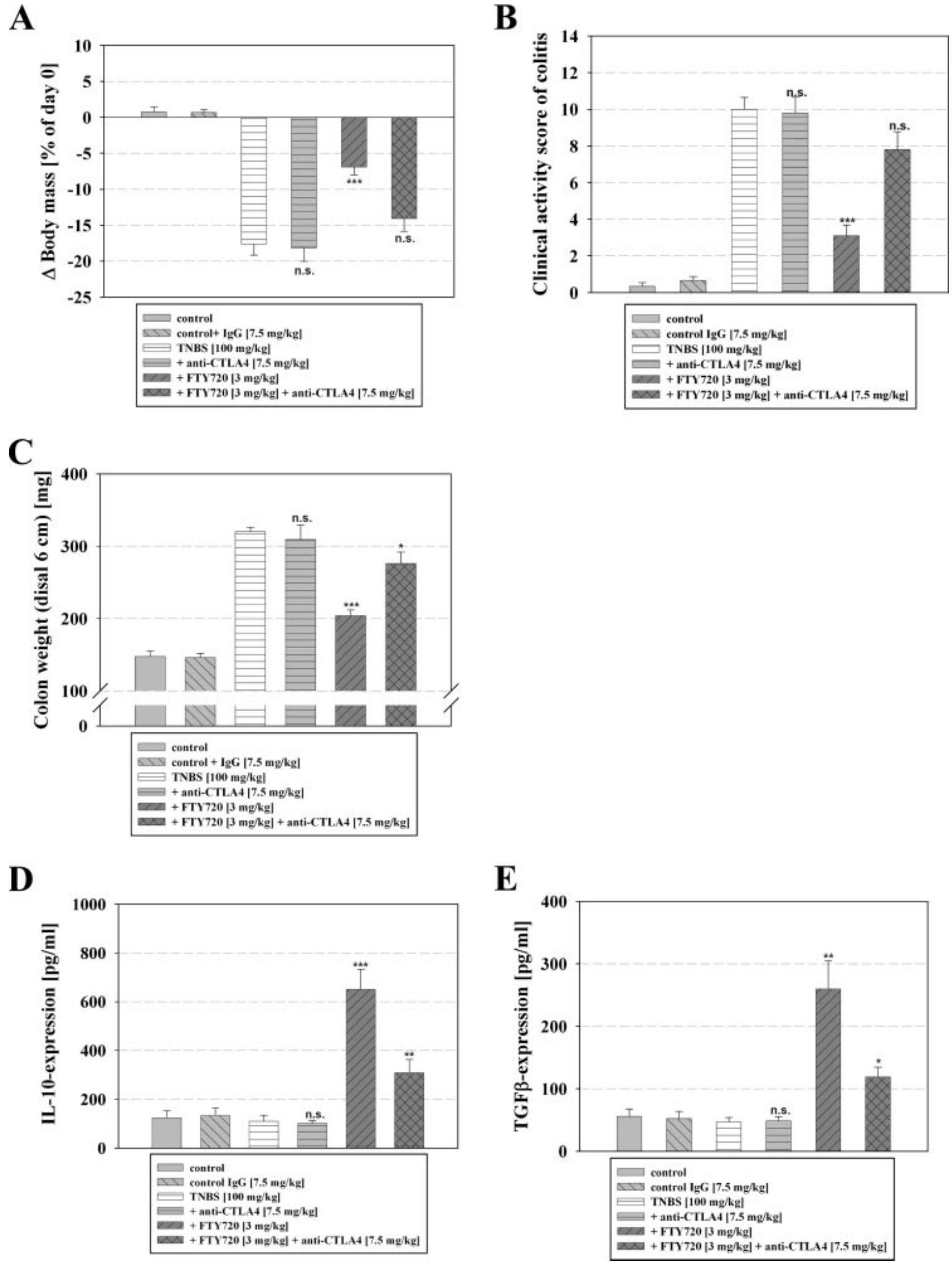

FIGURE 6. Clinical parameters of acute TNBS colitis following in vivo blockade of CTLA4. Mice were treated i.p. with the anti-CTLA4 Ab (7.5 mg/kg) alone, FTY720 (3 mg/kg), or the combination of FTY720 (3 mg/kg) and the anti-CTLA4 Ab (7.5 mg/kg). Control mice received normal IgG at the same dose. BW change on day 3 in % of day 0 (*A*), CAS (*B*), and colon weight (distal 6 cm; *C*) were determined on day 3. Data represent mean ± SEM from three separate experiments (eight mice per group per experiment). $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$ vs TNBS-treated mice. *D* and *E*, IL-10 (*D*) and TGFβ (*E*) expression was determined following ex vivo T cell culture of $CD4^+$ T cells isolated from the LP of either untreated TNBS colitic mice ($n = 5$) or colitic TNBS mice treated with FTY720 (3 mg/kg), the anti-CTLA4 Ab (7.5 mg/kg) alone, or the combination of FTY720 and the anti-CTLA4 Ab ($n = 5$ each). $CD4^+$ T cells (5×10^5 cells/ml) were cultured in 96-well plates with coated anti-CD3ε (5 μg/ml) and costimulated with anti-CD28 (1 μg/ml). Supernatants were harvested after 48 h of culture; IL-10 and TGFβ levels were assayed by ELISA. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs control group.

colons obtained 3 days after TNBS administration showed a striking hyperemia, necrosis, and inflammation compared with ethanol-treated control groups, which almost showed no signs of inflammation. FTY720 administration significantly improved macroscopic scores three days after TNBS instillation (Fig. 1, *A* and *B*). The severity of colon inflammation and ulceration was evaluated further by histological examinations (Fig. 1*C*). By day 3, transmural inflammation characterized by infiltration of inflammatory cells, predominantly neutrophils and lymphocytes, was associated with ulcerations, loss of globule cells, and fibrosis throughout the colon. Administration of FTY720 dose-dependently improved these signs restoring the histological appearance of the mucosa and submucosa when compared with the TNBS and the ethanol-treated control group (Fig. 1*D* and Table I).

In a subsequent set of experiments we were able to demonstrate that FTY720 treatment beginning on day 3 significantly alleviated the development of the disease and led to an improvement as assessed by analysis of clinical, macroscopic, microscopic and immunological signs of colitis (Fig. 2 and Table II). These data indicate that FTY720 is effective not only as an experimental preventive drug but also as a true therapeutic for established colitis.

FTY720 down-regulates the inflammatory response in acute Th1 TNBS-induced colitis

In the acute phase of TNBS colitis (day 3), colonic MPO activity values were significantly increased in comparison with the ethanol-treated control group. In contrast, in FTY720-treated animals MPO levels were significantly and dose-dependently down-regulated. Additionally, administration of FTY720 significantly reduced the TNBS-induced production of TNF-α and the DC-derived IL-12p70 (Table III).

FTY720 directly affects function and activity of Treg in acute Th1-mediated colitis

The mechanism of pathogenesis of colitis is currently unknown, but it can be controlled by the suppressor cytokines IL-10 and

TGFβ. We isolated LPMCs from either untreated or FTY720-treated colitis mice. LP CD4$^+$ T cells were further purified from LPMCs by using an anti-CD4 magnetic bead cell sorting system. Of note FTY720 significantly and dose-dependently increased IL-10 and TGFβ production in LP CD4$^+$ T cells, while dexamethasone-treated animals revealed no significant change of IL-10 and TGFβ (Fig. 3). Several lines of evidence have demonstrated a distinct role for FoxP3 in the development and function of CD4$^+$CD25$^+$ Treg. In parallel to the observed increase of IL-10 and TGFβ the treatment with FTY720 resulted in a concomitant dose-dependent and significant induction of FoxP3 expression in protein extracts of colon tissue (Fig. 4, *A* and *B*). To further address the role of FTY720 in modulating the activity of Treg we next investigated the expression of cytotoxic T lymphocyte-associated Ag 4 (CTLA4), which was exclusively expressed by CD4$^+$CD25$^+$ Treg in naive mice. In accordance with the results of FoxP3 expression CTLA4 expression was significantly induced following application of FTY720 (Fig. 4, *C* and *D*). Recently, the member of the TNFR superfamily glucocorticoid-induced TNFR-related gene (GITR), which is constitutively expressed on CD4$^+$CD25$^+$ Treg, was also considered to function as a Treg marker. The GITR system seems crucial in regulating immunity, but its involvement seems to be complex and warrants further studies. In vivo, GITR activation was shown to cause the development of autoimmune disease. To address a possible impact of GITR mediating the FTY720-induced modulation of CD4$^+$CD25$^+$ Treg function, we analyzed the GITR protein expression in mice following i.p. application of FTY720. GITR expression in TNBS colitis in mice remained unchanged following FTY720 treatment (data not shown).

To further support our contention that the therapeutic potential of FTY720 might be mediated via induction of CD4$^+$CD25$^+$ FoxP3$^+$ T cells we performed cellular analysis of the lymphocytes present in the colon tissue. Therefore, we isolated LP CD4$^+$ T cells from either untreated or FTY720-treated colitis mice. As demonstrated in Fig. 5, *A* and *B*, FTY720 treatment dose-dependently led to a significant increase of CD25 expression in LP CD4$^+$ T cells. Along these lines we then analyzed FoxP3 expression in LP CD4$^+$ T cells. FTY720 resulted in a considerable up-regulation of FoxP3 expression in a dose-dependent fashion (Fig. 5, *C* and *D*). These results therefore support our hypothesis that the therapeutic effect of FTY720 in this model is indeed mediated via a direct induction of CD4$^+$CD25$^+$FoxP3$^+$ T cells.

As it has been shown that anti-CTLA4 Ab abrogated CD4$^+$CD25$^+$ Treg activity in vitro and in vivo, we next analyzed, whether the immunosuppressive capacities of FTY720 might be abolished by in vivo blockade of CTLA4. To this end we injected FTY720 i.p. in combination with anti-CTLA4 Ab. The dose for the anti-CTLA4 Ab was optimized according to a previously documented work underlining the role of CTLA4 for CD4$^+$CD25$^+$ Treg in experimental colitis (21). To evaluate the impact of the CTLA4 Ab on TNBS colitis in the absence of FTY720 the Ab was also administered alone. As demonstrated in the previous study focusing on the in vivo impact of CTLA4 on Treg induction using SCID mice the disease outcome was not significantly influenced by the anti-CTLA4 Ab alone. Control mice were either untreated or received normal IgG. As already demonstrated above, FTY720 led to a prominent amelioration of TNBS colitis. However, the curative potential of FTY720 was significantly abolished using the combination of FTY720 and the anti-CTLA4 Ab (Fig. 6, *A*–*C*). Like TNBS-treated mice the mice receiving the combination of FTY720 and the anti-CTLA4 Ab showed a distinct wasting disease with an increase in body weight loss as well as in the clinical activity score of colitis. These results therefore clearly demonstrate

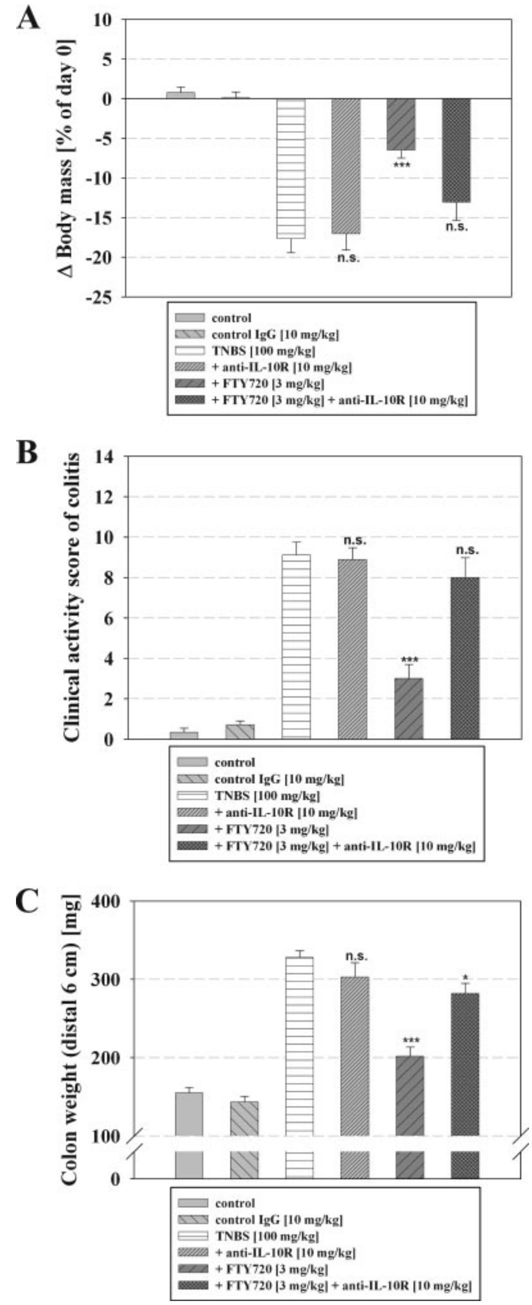

FIGURE 7. Clinical parameters of acute TNBS colitis following in vivo blockade of IL-10. Mice were treated i.p. with the anti-IL-10R Ab (10 mg/kg) alone, FTY720 (3 mg/kg), or the combination of FTY720 (3 mg/kg) and the anti-IL-10R Ab (10 mg/kg). Control mice received normal IgG at the same dose. BW change on day 3 in % of day 0 (*A*), CAS (*B*), and colon weight (distal 6 cm; *C*) were determined on day 3. Data represent mean ± SEM from three separate experiments (eight mice per group per experiment). $*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$ vs TNBS-treated mice.

that the therapeutic effect of FTY720 in this model is critically dependent on CTLA4.

As we have shown that in our colitis model FTY720 led to a distinct increase of CTLA4 expression and as CTLA4 engagement and up-regulation have recently been reported to up-regulate IL-10 and TGFβ finally leading to the generation and maintenance of CD4$^+$CD25$^+$ Treg (21, 41, 42), we analyzed IL-10 and TGFβ expression in LP CD4$^+$ T cells of TNBS-treated mice receiving either anti-CTLA4 Ab and/or FTY720 (3 mg/kg). The anti-CTLA4

Ab alone and the control Ab did not result in any significant change of IL-10 and TGFβ expression. FTY720, as indicated before, caused a substantial up-regulation of IL-10 and TGFβ expression. However, using the combination of FTY720 and the anti-CTLA4 Ab the inductive capacity of FTY720 on IL-10 and TGFβ production of LP $CD4^+$ T cells was significantly blunted (Fig. 6, *D* and *E*).

Recently it was shown that the curative activity of $CD4^+CD25^+$ Treg is also critically dependent on IL-10. So, to further underline the suggested induction of $CD4^+CD25^+$ Treg activity by FTY720 we used an IL-10R Ab to block IL-10 functions in vivo. The dose for the anti-IL-10R was optimized according to recently published studies focusing on the role of IL-10 for $CD4^+CD25^+$ Treg in experimental colitis and experimental allergic encephalomyelitis (21, 34). Treatment with control IgG or the IL-10R Ab alone did not result in a significant change of disease severity and intensity of inflammation. As already indicated above, FTY720 treatment markedly reversed disease progression. However, the anti-IL-10R Ab evidently abolished the therapeutic potential of FTY720 in TNBS colitis (Fig. 7). These data further support our hypothesis that FTY720 might increase function and activity of DC or $CD4^+$ $CD25^+$ T cells which is critically dependent on IL-10.

Discussion

The data in this study clearly indicate that the significant immunosuppressive capacities of FTY720 in Th1-mediated colitis resulted from a prominent increase of the functional activity of $CD4^+$ $CD25^+$ Treg. The observed enhancement of the potency of $CD4^+$ $CD25^+$ Treg might additionally be the result of a differential down-regulation of proinflammatory signals of DC subsequently favoring the education of Treg. It is important to emphasize that the clear reduction of the inflammatory process could be observed in acute as well as in established ongoing Th1-mediated colitis.

Although currently changing, up to now the molecular basis for the mode of action of FTY720 was regarded to be mainly based on its interference with cellular traffic between lymphoid organs and blood following S1PR interactions. Whereas several studies support its effects on lymphocyte trafficking, more recent investigations indicate that the FTY720 action distinct from S1P might not only be due to its interaction with other S1PR subtypes but may be based on direct intracellular effects on e.g., the S1P lyase or the cytosolic PlA_2 (33, 43). For S1P it has recently been documented that the S1P-$S1P_1$ axis mediates many of the T cell-associated activities. Still, these data define its major impact on immunity just through the control of T cell trafficking and migration (25, 44). Extending these results of S1P on T cells Wang et al. (44) recently reported that S1P action through $S1P_4$ does not affect migration of T cells. Rather, $S1P_4$ transduced stimulatory and inhibitory signals on T cell secretion of several important cytokines. In T cells exclusively expressing functional $S1P_4$ S1P revealed inhibitory effects on IFN-γ, IL-4, and IL-2, while IL-10 production was enhanced. Additionally, S1P modulates the DC-mediated T cell response in favor of Th2 lymphocyte-dominated immunity, i.e., the suppression of IL-12 and enhancement of IL-10 production by S1P-treated DC was associated with a Th1 to Th2 switch of an in vitro primed T cell response (31). To extend these findings regarding S1P toward FTY720 Muller et al. (27) clearly showed that in vitro treatment of monocyte-derived DC with therapeutic concentrations of FTY720 and FTY720-P led to a significant change of the cytokine production profile of DC from high IL-12/low IL-10 to low IL-12/high IL-10 production, thus also biasing T cell responses toward Th2 or Treg. This shift from high IL-12/low IL-10 to low IL-12/high IL-10 following treatment with FTY720 indicative for DC targets could also be observed in our in vivo system of TNBS colitis. Thus, these results underscore that in addition to the well-established effects on the regulation of cellular homing and migration through $S1P_1$, S1P and its mimetic FTY720 also prominently reduces inflammatory T cell cytokine profiles. Although some of these effects may be mediated through interactions with $S1P_4$ (8), we expect that further studies with endogenous S1P or the therapeutic analog FTY720 will be more successful when molecular targets in DC are included.

In a previous work FTY720 has been studied in IL-$10^{-/-}$-mice (45). Although at first sight this approach seems comparable to ours, it is important to emphasize that in this study knock-out mice were used, which developed a chronic enterocolitis very late at 2–3 mo of age, and different from our study, FTY720 was administered during a long-term treatment regimen over a period of four weeks. Therefore it seems reasonable to assume that a direct comparison regarding the therapeutic efficiency and mode of action of FTY720 in the IL-$10^{-/-}$ and the acute Th1-mediated TNBS-colitis model used in our study is difficult at best. Additionally, the disruption of the IL-10 suppressive and regulatory pathway does not allow to relate FTY720 action to differentiation-inducing capacities. Only if this activity of FTY720 could be exerted, an enhanced generation of tolerogenic DC cells might lead to an induction of Treg, which–as mentioned above–has already been suggested in several in vitro studies before (27, 28).

As an important new aspect of the immunosuppressive mechanism of FTY720, our results indicate that FTY720 strongly affects the activity of Treg in vivo. In a first set of experiments, we could demonstrate that FTY720 led to a significant induction of FoxP3 expression. Recent work has confirmed that FoxP3 can be regarded as the most reliable Treg marker (46). It is predominantly expressed in $CD4^+CD25^+$ Treg and it is sufficient for their development and function. The induction of FoxP3 was paralleled by a dose dependent increase of IL-10 and TGFβ. Different subpopulations of Treg are responsible for immunological tolerance in the gut thereby preventing mucosal inflammation (47, 48). Their regulatory capacity has been linked to the expression of IL-10 and TGFβ. TGFβ has been clearly implicated in the conversion of naive $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells via the induction of FoxP3 (49, 50). Recently, it was also shown using mice with a reporter for FoxP3 mRNA that TGFβ may directly influence FoxP3 (51). Although IL-10 has been characterized to function directly as an important and essential regulatory cytokine, it also seems to contribute via indirect pathways to the regulatory role of TGFβ (49, 50, 52). Signaling events that are initiated by TGFβ are comprised of the phosphorylation and homotrimerization of Smad proteins (53). Mechanistic insights to understand the observed increase of Treg function following treatment with the S1P-mimetic FTY720 may be introduced by the fact that S1P cross-activates the TGFβ signaling cascade, thus leading to an activation of at least three Smad proteins (Smad1, 2, and 3) with subsequent gene transcription (29). Recently, such a direct interference with TGFβ signaling was also proven for FTY720, since FTY720 led to a significant phosphorylation and thereby activation of Smad proteins. Thus, in different cell types these data underline the possibility of a cross-talk between FTY720 and TGFβ signaling, which may also contribute to the observed induction of Treg capacities seen in our study (30). Further evidence to support possible overlapping signaling pathways of S1P and TGFβ has recently been provided by our own study demonstrating a clear interaction between S1PRs and TGFβ signaling on the level of Smad protein activation (28). In this in vitro study, we proposed a role of S1P as an endogenous immunosuppressive mediator affecting migration, but moreover the development of tolerogenic DC. Already in 2000, insight regarding the importance of the TGFβ signaling to mount a regulatory response in acute TNBS colitis has been provided by a study of Kitani et al. (54). In that investigation intranasal administration of a TGFβ1 plasmid led to the expression of TGFβ1 mRNA in the intestinal LP as well as to the appearance of TGFβ1-producing T cells. Subsequently, a substantial amelioration of TNBS-colitis severity resulted on day 3. Like in our study such a counterregulatory response may proceed from a rise of TGFβ to IL-10, then followed by increased numbers and activity of Treg to distinctly increase FoxP3 and CTLA4, and finally resulting in the suppression of acute colitis (54). Focusing on our data, i.e., the significant initial increase of TGFβ and IL-10, the studies discussed may support our hypothesis that such an immune modulatory sequence could also be induced by FTY720.

Shimizu et al. (55) demonstrated that the member of the TNFR superfamily glucocorticoid-induced TNFR-related gene (GITR) is predominantly and constitutively expressed on $CD4^+CD25^+$ Treg. GITR was shown to abrogate the suppressive capacity of $CD4^+CD25^+$ Treg as determined by anti-GITR Ab application that triggers rather than inhibits GITR function (55, 56). In vivo GITR activation caused the development of autoimmune diseases (56, 57) indicating that stimulation of GITR can break immunological self tolerance. Ronchetti et al. (58) further demonstrated that GITR triggering resulted in an inhibition of Treg activity, opening the view that blockade of the GITR signaling pathway might be applied for treatment of inflammatory diseases and additionally useful for the cure of autoimmune diseases. As shown here, FTY720 treatment does not significantly alter the GITR expression in TNBS colitis. The fact that FTY720 did not change the expression of GITR as well as the point that GITR seems to be constitutively expressed on $CD4^+CD25^+$ Treg might again underline our hypothesis that FTY720 directly alters the functional activity of $CD4^+CD25^+$ Treg. Still, we consider that this direct impact on $CD4^+CD25^+$ Treg function might additionally be facilitated by a differently regulated sequestration of $CD4^+CD25^+$ Treg vs inflammatory effector T cells resulting in a higher ratio of Treg/effector cells in blood and inflammatory sites (25, 26).

To support the concept regarding the increase of functional activity of $CD4^+CD25^+$ Treg following treatment with FTY720 we performed analysis of the lymphocytes in the colon tissue. Isolation of LP $CD4^+$ T cells documented a significant increase of CD25 and FoxP3 expression in FTY720-treated mice. This increase in CD25 and FoxP3 expression may also be seen as a relative increase due to selective reduction of effector T cells by the selective sequestrating activity of FTY720. However, the fact that the CD25 and FoxP3 expression is significantly higher in FTY720-treated $CD4^+$ T cells than in the control group, in which no effector T cells should be present, may possibly support the concept that FTY720 directly affects Treg induction. By others the importance of $CD4^+CD25^+$ Treg in mediating the inhibition of colitis was also documented using SCID mice. In that study $CD45RB^{low}CD4^+$ T cells were sorted into $CD25^+$ and $CD25^-$ fractions and tested for their ability to inhibit colitis induced by transfer of $CD45RB^{high}$ cells (22). Control of intestinal inflammation resided dominantly in the $CD25^+$ fraction, as these cells significantly inhibited wasting disease and development of colitis. In contrast, transfer of $CD25^-CD45RB^{low}$ transferred mediated no significant regulatory function. These results support the concept that the $CD4^+CD25^+FoxP3^+$ T cells function as the major players in mediating the regulatory potential in experimental colitis instead of $CD25^-CD4^+FoxP3^+$ T cells. In accordance with the observations in our present study these findings underline the potential of FTY720 to cure Th1-mediated experimental colitis in mice by increasing $CD4^+CD25^+FoxP3^+$ T cells. $CD4^+CD25^+$ T cells are the only $CD4^+$ T cells that significantly express the CTLA4 Ag. To put our hypothesis one step further we could also demonstrate that FTY720 caused a significant induction of CTLA4 protein expression. Signaling via CTLA4 is essential for the function of Treg (22), also illustrated by the fact that CTLA4 mediates the release of suppressive cytokines such as TGFβ (41). Anti-CTLA4 Ab abrogated $CD4^+CD25^+$ T cell mediated suppression in vitro and in vivo, also the protective effect of $CD4^+CD45RB^{low}$ T cells in colitis induced by cell transfer with $CD4^+CD45RB^{high}$ T cells was blunted by treatment with anti-CTLA4 Ab in SCID mice, both of which observations being consistent with earlier results for a pivotal role of CTLA4 Ag in autoimmune diseases in vivo (21, 22).

The prominent role of $CD4^+CD25^+$ Treg and especially CTLA4 was further emphasized in our study by in vivo blockade of CTLA4 using anti-CTLA4 Ab, which significantly abrogated the therapeutic potential of FTY720 measured in TNBS colitis. The fact that application of the anti-CTLA4 Ab did not result in a significant modulation of disease severity supports the concept that the Ab suppresses $CD4^+CD25^+$ Treg function and does not lead to the induction of Th1 effector T cells. Similar results have been observed in the SCID transfer colitis model, where anti-CTLA4 Ab treatment of mice transferred with $CD4^+CD25^-$ T cells alone did not affect the pathogenicity of these cells (21). Using this model the kinetics of wasting disease as well as of the incidence and severity of colitis induced by transfer of $CD45RB^{high}CD4^+$ T cells was indistinguishable in anti-CTLA4-treated or untreated mice with colitis (22), which is exactly what we observed in our Th1-mediated TNBS colitis model. In the study cited above CTLA4 blockade did not lead to the development of a pathogenic T cell population after transfer of $CD45RB^{low}CD4^+$ T cells. Thus, the anti-CTLA4 treatment inhibiting the function of Treg as opposed to enhancing the pathogenicity of $CD45RB^{high}$ cells or uncovering pathogenic T cells among the $CD45RB^{low}$ population are in accordance with our data presented here. Also in another autoimmune situation, i.e., islet transplantation, a similar mode of action of CTLA4 blockade was described (59, 60). CTLA4 Ab completely prevented tolerance induction, obviously through an effect that was directed at the cellular targets of Treg action (59, 60). Of note, and extending findings in total intestinal extracts, we were able to demonstrate that in vivo blockade of CTLA4 resulted in significant down-regulation of IL-10 and TGFβ in LP $CD4^+$ T cells. Our findings are in agreement with recent studies indicating that CTLA4 engagement up-regulates IL-10 and TGFβ (21, 41, 42). Additionally supporting the connectivity, FoxP3 was demonstrated to induce CTLA4 expression (41, 61, 62). Then, in a final set of experiments we blocked the IL-10 axis using an ultra-purified IL-10R Ab. To an extent already shown for the CTLA Ab the curative potential of FTY720 was significantly inhibited in mice receiving the anti-IL-10R Ab. In conclusion, and to our understanding the data shown in this study clearly support a functional tolerogenic in vivo activity of FTY720 via a TGFβ/IL-10/FoxP3/CTLA4-positive loop that generates and maintains the function of $CD4^+CD25^+$ Treg.

We expect that these data provide a new focus for further studies, which will help to define the complex role and interaction of the S1P-analog FTY720, especially regarding its possible impact on tolerogenic DC and Treg functions. Although warranting further investigations, these findings strongly support the potential of FTY720 as a new auspicious therapeutic option for the treatment of IBD in the clinic.

Disclosures

The authors have no financial conflict of interest.

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