

# The sphingosine-1-phosphate receptor agonist FTY720 and its phosphorylated form affect the function of CD4<sup>+</sup>CD25<sup>+</sup> T cells *in vitro*

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**Abstract.** The sphingosine-1-phosphate receptor agonist FTY720 and FTY720-P have a wide variety of fundamental functions. Many studies have demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells engage in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes. Although FTY720 has also recently shown to possess an additional effect that increases the functional activity of Treg cells, the mechanism leading to the enhanced Treg activity after FTY720 treatment is still not clear. We isolated Treg cells, which were co-cultured with FTY720 or FTY720-P. The proliferation of co-cultured Treg cells was detected by the cell counting kit-8. The changes of the phenotype CD25<sup>+</sup> and forkhead box P3 (Foxp3)<sup>+</sup> of co-cultured Treg cells were measured by flow cytometry. The levels of IL-10 and TGF-β1 in the supernatants were detected by ELISA. Cytokine mRNA expressions in co-cultured Treg cells were analyzed by real-time quantitative PCR. Mixed lymphocyte reaction assay examined the suppressive function. We found that neither FTY720 nor FTY720-P affected the proliferation of co-cultured Treg cells. The percentages of CD25<sup>+</sup> and Foxp3<sup>+</sup> were enhanced in the high-dose FTY720-P group. The levels of TGF-β1 in the supernatants were enhanced in the high-dose FTY720 group. Medium and high-dose FTY720-P also enhanced the levels of TGF-β1. TGF-β1 and Foxp3 mRNA expression were upregulated in the high-dose FTY720-P group. The proliferation of effector T (Teff) cells was suppressed significantly in the medium and high-dose FTY720-P group at a Treg/Teff cell ratio of 1:1. At a ratio of 1:1, the proliferation of Teff cells was also suppressed in the

high-dose FTY720 group. It can be concluded that high-dose FTY720-P can enhance the immune function of co-cultured Treg cells, and that medium-dose FTY720-P and high-dose FTY720 could partly enhance the function. The reason may be attributed to enhanced levels of TGF-β1 and Foxp3.

## Introduction

As a synthetic structural analog of myriocin, the sphingosine-1-phosphate (S1P) receptor agonist FTY720 can regulate a wide variety of fundamental functions including cell survival, cytoskeletal rearrangements, and cell motility (1,2). It has been shown to control some autoimmunities and allergic diseases as well as to suppress transplant rejection and graft vs. host disease (3). The drug has shown efficacy in advanced clinical trials for the treatment of multiple sclerosis (4). Once FTY720 is phosphorylated *in vivo* by sphingosine kinase 2 (SphK2), the phosphorylated compound (FTY720-P) acts as an agonist on 4 of the 5 known S1P receptors (S1P1, S1P3, S1P4 and S1P5) (5). In contrast to classical immunosuppressants, such as cyclosporine A or FK506, FTY720 does not impair antigen-driven T-cell activation and does not interfere with T-cell proliferation at therapeutically relevant concentrations, but induces a severe deprivation in lymphocytes in the blood due to modification of S1P signaling (6-8).

Regulatory T (Treg) cells have been demonstrated to engage in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes. Although a specific Treg marker has not been identified as yet, Treg cells display a typical pattern of a constitutively increased expression of several molecules/receptors including CD25, forkhead box P3 (Foxp3), cytotoxic T-lymphocyte antigen-4 (CTLA-4), CD122, and glucocorticoid-induced tumor necrosis factor receptor (GITR) (9). Belkaid *et al* (10) have found that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells play a key role in the maintenance of immunological homeostasis and self-tolerance. There is now considerable evidence that Foxp3 is a key control molecule for Treg cell development and function, and an excellent marker for the study of Treg cells (11). Therefore, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells have been implicated in autoimmune diseases, organ transplantation, and infectious diseases (12-14).

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S1P receptors are expressed in multiple immune cells during different stages of differentiation (15). Both S1P 1 and S1P 4 are expressed in T cells and it appears that at least S1P 1 in T cells is critical (15,16). Liu *et al* (17) have shown a similar inhibitory role for Akt-mTOR, activated by S1P signaling, in both the differentiation and suppressive activity of natural Treg cells derived from the thymus. As an agonist for S1P, FTY720 may interfere with Treg function since it abrogates S1P/S1P1-dependent regression of lymphoid cells from lymphoid organs and due to the fact that Tregs express S1P, which appears to be required for optimal suppression of effector T cell activities (18).

Though FTY720 was also recently shown to possess an additional effect that increases the functional activity of Treg cells, the mechanism leading to the enhanced Treg activity after FTY720 treatment is still not clear (19,20). However, there was a study which had contradictory results about the effects of FTY720 on Treg cells (21). Therefore, this study was undertaken in order to further investigate how FTY720 affected the immune function of Treg cells *in vitro*. We provided a new evidence that FTY720 and FTY720-P enhance the immune function of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells *in vitro*.

## Materials and methods

**Animals.** Male BALB/c mice (4-6-week-old, 18-20 g) were purchased from the Beijing HFK Bio-Technology Co., Ltd. (Beijing, China). All mice were housed in a standard experimental room and exposed to a 12 h:12 h light-dark cycle. The experiment was repeated three times.

**Purification of CD4<sup>+</sup>/CD25<sup>+</sup> T regulatory cells.** Spleen sample were obtained from BALB/C mice and gently dissociated to single cell suspensions. CD4<sup>+</sup>CD25<sup>+</sup> T cells were magnetically separated using a Treg separation kit (Miltenyi Biotec Bergisch Gladbach, Germany). Briefly, CD4<sup>+</sup> T cells were negatively selected from the total spleens using the CD4 isolation kit, yielding a population of CD4<sup>+</sup> T cells. Positive selection on anti-CD25 magnetic microbeads was then used to separate the negative fraction containing CD4<sup>+</sup>CD25<sup>+</sup> T cells from the CD4<sup>+</sup>CD25<sup>+</sup> T cell fraction, using the CD4<sup>+</sup>CD25<sup>+</sup> T Regulatory Cell Isolation kit. Cells were then applied to a second magnetic column, washed, and eluted again. The resulting CD4<sup>+</sup>CD25<sup>+</sup> T cell population was determined by fluorescence-activated cell sorter (FACS) analysis.

**FACS.** Cells were detected by a Mouse Regulatory T cell Staining kit (w/PE Foxp3 FJK-16s, FITC CD4, APC CD25) from eBioscience (San Diego, CA, USA), according to the manufacturer's instructions (eBioscience). Data were acquired using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), and analyzed with Winmid 2.9 software (<http://scripps.edu/software.html>; Scripps Institute, La Jolla, CA, USA). The cells were gated for CD4<sup>+</sup> positivity and the percentages of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> cells were calculated.

**CD4<sup>+</sup>CD25<sup>+</sup>/CD4<sup>+</sup>CD25<sup>-</sup> T cell cultures and activation.** CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells (2x10<sup>6</sup> cells/ml) were activated with plate-bound anti-CD3 (17A2, 5 μg/ml) and soluble anti-CD28 (37.51, 5 μg/ml) mAbs (Biolegend, USA). Cells were cultured in RPMI-1640 with 10% fetal calf serum.

Recombinant murine IL-2 (PeproTech, USA) was added into RPMI-1640 at 200 U/ml. These cells were cultured and activated for 5 days.

**Effect of FTY720 or FTY720-P on CD4<sup>+</sup>CD25<sup>+</sup> T cells.** After 5 days of continuous stimulation, these CD4<sup>+</sup>CD25<sup>+</sup> T cells were divided into one control group, three FTY720 groups with different doses, and three FTY720-P groups with different doses. Three FTY720 groups of CD4<sup>+</sup>CD25<sup>+</sup> T cells were added into respective concentrations (10, 100 and 1000 ng/ml) of FTY720 (provided by the Department of Molecular Drug Design, Institute of Pharmacology and Toxicology Sciences, Beijing, China). The other three FTY720-P groups of CD4<sup>+</sup>CD25<sup>+</sup> T cells were added respectively in the same concentrations of FTY720-P (Echelon Biosciences, Inc., Salt Lake City, UT, USA). All CD4<sup>+</sup>CD25<sup>+</sup> T cells were co-cultured for 48 h under continuous above flow conditions. At 48 h after co-culture, the phenotypic changes in co-cultured Treg cells were detected by the Mouse Regulatory T cell staining kit. The levels of IL-10 and TGF-β1 in the supernatants were also determined using ELISA kits (MultiSciences Biotech Co., Ltd., Hangzhou, China). At 48 h after co-culture, some co-cultured Treg cells (2x10<sup>5</sup> cells/well) were washed and then cultured continuously with the aforementioned stimulating conditions. The proliferations of these co-cultured cells were evaluated using the Cell Counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) on Days 0, 1, 2, 3 and 4 after co-culture.

**RNA isolation and qRT-PCR.** At 48 h after co-culture, the expressions of RNA in all seven groups of CD4<sup>+</sup>CD25<sup>+</sup> T cells were detected. Total-RNA from 2x10<sup>6</sup> cells was isolated using the TRIzol<sup>®</sup> reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcription of mRNA to cDNA was performed in 20 μl reaction volumes with random priming and EasyScript RT using Easy RT-PCR kit (Beijing TransGen Biotech Co., Ltd., Beijing, China). Gene expression was examined in an iCycler IQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using the SYBR-Green real-time PCR Master mix (Toyobo, Osaka, Japan) with respective real-time quantitative PCR (qPCR) primer: IL-10, forward, 5'-TGCCTTCAGCCAGGTGAAGACTTTC-3' and reverse, 5'-CTTGATTTC TGGGCCATGCTTCTCTG-3'; TGF-β1, forward, 5'-ATACC AACTATTGCTTCAGCTCCACAG-3' and reverse, 5'-GTAC TGTTGTCCAGGCTCCAAATAT-3'; Foxp3, forward, 5'-ATG CCCAACCTAGGCCAGCCAAG-3' and reverse, 5'-TGGG CCCCACTTCGCAGGTCCCGAC-3'; and GAPDH, forward, 5'-TGAAGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-GTTGAATTTGCCGTGAGTGGAGTC-3'. The cycle number at which the reporter fluorescence reaches the threshold (CT value) was used for quantitative measurement. The relative expression data was determined by normalizing to the GAPDH expression measured simultaneously from the same sample to calculate a fold-change in value using the 2<sup>-ΔΔCT</sup> method.

**Treg cells suppression assay.** At 48 h after co-culture, CD4<sup>+</sup>CD25<sup>+</sup> T cells were washed and cultured with activated CD4<sup>+</sup>CD25<sup>-</sup> effector T (Teff) cells at various ratios of 1:1, 1:4 and 1:8 (4x10<sup>5</sup> cells/well). All CD4<sup>+</sup>CD25<sup>+</sup>/CD4<sup>+</sup>CD25<sup>-</sup> T cells were exposed to an anti-CD3 (0.5 μg/ml) and anti-CD28 (0.5 μg/ml) monoclonal antibodies and IL-2 (100 U/ml). Five

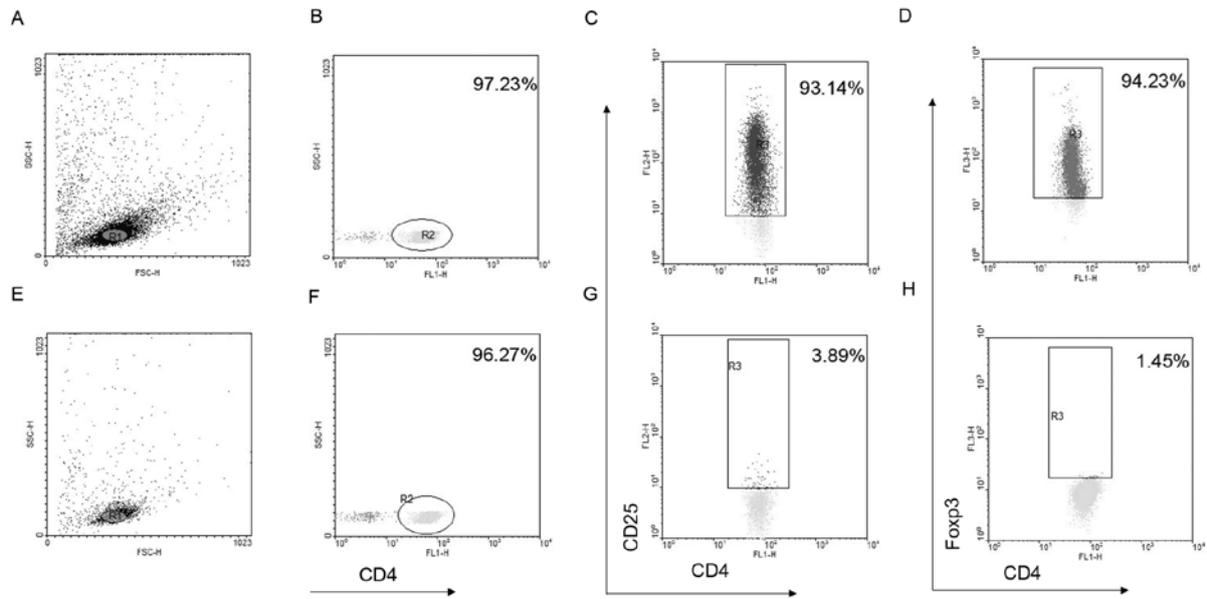


Figure 1. CD4<sup>+</sup>CD25<sup>+</sup>/CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated by a Treg separation kit. (A) Sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells. (B) Percentage of labeled CD4<sup>+</sup> cells among the sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells. (C) Percentage of labeled CD25<sup>+</sup> T cells among the sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells. (D) Percentage of labeled Foxp3<sup>+</sup> T cells among the sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells. (E) All of the sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells. (F) Percentage of labeled CD4<sup>+</sup> among the sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells. (G) Percentage of CD25<sup>+</sup> T cells among the sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells. (H) Percentage of labeled Foxp3<sup>+</sup> T cells among the sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells.

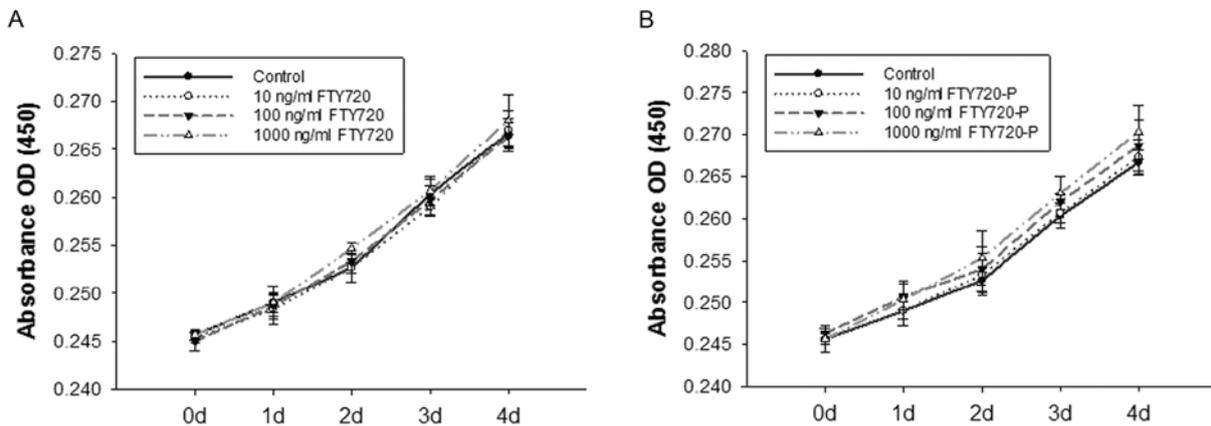


Figure 2. Proliferation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells cultured with or without drugs. (A) Proliferation of Treg cells cultured with or without three doses of FTY720. (B) Proliferation of Treg cells cultured with or without three doses of FTY720-P.

days later, the proliferation of all cells were evaluated using the CCK-8 assay.

**Statistics.** All statistical analyses were performed with SPSS software version 15. Continuous variables were presented as mean ± standard deviation. Differences in the means of various groups were compared using one-way ANOVA. Multiple comparisons of the means of various groups were conducted using the Bonfferoni test. P-values <0.05 were considered to denote statistical significance.

**Results**

**Purification of CD4<sup>+</sup>CD25<sup>+</sup>/CD4<sup>+</sup>CD25<sup>-</sup> T cells.** CD4<sup>+</sup>CD25<sup>+</sup> T cells were isolated by MACS with anti-CD4/anti-CD25 beads. The population of sorted CD4<sup>+</sup>CD25<sup>+</sup> and CD<sup>+</sup>CD25<sup>-</sup> T cells were shown in Fig. 1. The purity of positively selected

CD4<sup>+</sup>CD25<sup>+</sup> T cells was >93% (Fig. 1C) and the percentage of Foxp3<sup>+</sup> cells was >94% (Fig. 1D). The purity of selected CD4<sup>+</sup>CD25<sup>-</sup> cells was nearly 96% (Fig. 1G) and the percentage of Foxp3<sup>+</sup> was 1.45% (Fig. 1H).

**Proliferation of CD4<sup>+</sup>CD25<sup>+</sup> T cells.** CD4<sup>+</sup>CD25<sup>+</sup> T cells were activated by the culture system. Then, the activated cells were co-cultured with or without drugs including three doses of FTY720 and FTY720-P. Slight proliferation in high and medium dose FTY720-P group. But, neither FTY720 nor FTY720-P enhanced the proliferation of Treg cells compared with the control group (both P>0.05) (Fig. 2).

**Phenotypic changes in co-cultured Treg cells.** The percentages of CD25<sup>+</sup> and Foxp3<sup>+</sup> Treg cells after 48 h of co-culture were shown in Fig. 3. There were no statistical differences

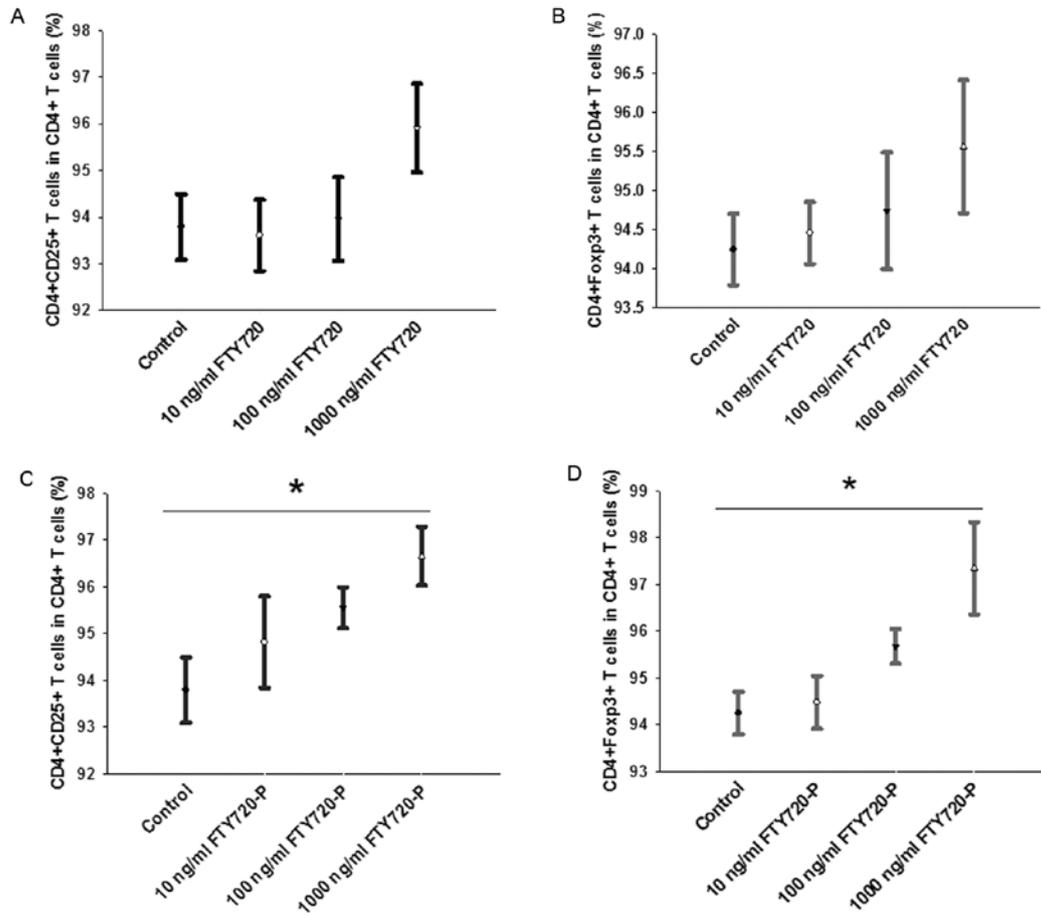


Figure 3. Percentage of phenotypic changes in co-cultured Treg cells. (A) Percentages of CD25<sup>+</sup> in Treg cells with or without three doses of FTY720. (B) Percentages of Foxp3<sup>+</sup> in Treg cells with or without three doses of FTY720. (C) Percentages of CD25<sup>+</sup> in Treg cells with or without three doses of FTY720-P. (D) Percentages of Foxp3<sup>+</sup> in Treg cells with or without three doses of FTY720-P. (\*P<0.05 compared with the control group).

in the percentages of CD25<sup>+</sup> and Foxp3<sup>+</sup> cells between the three FTY720 groups and the control group (both P>0.05) (Fig. 3A and B). The percentages of CD25<sup>+</sup> and Foxp3<sup>+</sup> were enhanced significantly in the high dose FTY720-P group compared with control group (P=0.002 and P=0.007, respectively) (Fig. 3C and D). Representations of the phenotypic changes in co-cultured Treg cells were shown in Figs. 4 and 5.

**Levels of IL-10 and TGF- $\beta$ 1 in the supernatants of co-cultured Treg cells.** The concentrations of IL-10 in the three groups of FTY720 were similar as the control group (both P>0.05) (Fig. 6A). The levels of TGF- $\beta$ 1 in the high dose FTY720 group were increased compared with the control group (P=0.026) (Fig. 6B). No differences in the levels of IL-10 were observed between the FTY720-P groups and the control group (both P>0.05) (Fig. 6C). Compared with the control group, medium and high dose FTY720-P significantly increased the levels of TGF- $\beta$ 1 in the supernatants (P=0.017 and P<0.001, respectively) (Fig. 6D).

**IL-10, TGF- $\beta$ 1 and Foxp3 mRNA expression by co-cultured CD4<sup>+</sup>CD25<sup>+</sup> T cells.** The mRNA expressions of IL-10, TGF- $\beta$ 1 and FoxP3 were calculated as described in Materials and methods. There were no significant differences in the mean ratio of IL-10, TGF- $\beta$ 1 and FoxP3 mRNA to GAPDH mRNA between the three doses of FTY720 and the control group (both

P>0.05) (Fig. 7A, B and C). There was also no significant difference in the expression of IL-10 mRNA between the three dose of FTY720-P and the control group (both P>0.05) (Fig. 7D). However, the expression of TGF- $\beta$ 1 and FoxP3 mRNA in the high dose FTY720-P group was significantly enhanced (P=0.006 and P=0.036, respectively) (Fig. 7E and F).

**Mixed lymphocyte reaction (MLR) co-cultures at various ratios (1:1, 1:4 and 1:8).** To observe the suppressive capacity of these co-cultured Treg cells, MLR was performed. The proliferation of Teff cells was not suppressed in the three FTY720 groups at various ratios of Treg cells to Teff cells ranging from 1:4 to 1:8, when compared with the control group (both P>0.05) (Fig. 8A). At a 1:1 Treg/Teff ratio, the proliferation of Teff cells was suppressed in the a high dose FTY720 group (P=0.031) (Fig. 8A). The proliferation of Teff cells was suppressed significantly in high dose FTY720-P group at Treg:Teff cell ratios ranging from 1:1 to 1:4 (P=0.001 and P=0.003, respectively) (Fig. 8B). The significant inhibition of Teff cells was also achieved in a medium dose FTY720-P group at a Treg/Teff ratio of 1:1 (P=0.016) (Fig. 8B).

## Discussion

It has been proven that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells have significant immune suppressive functions (9,10). Several studies

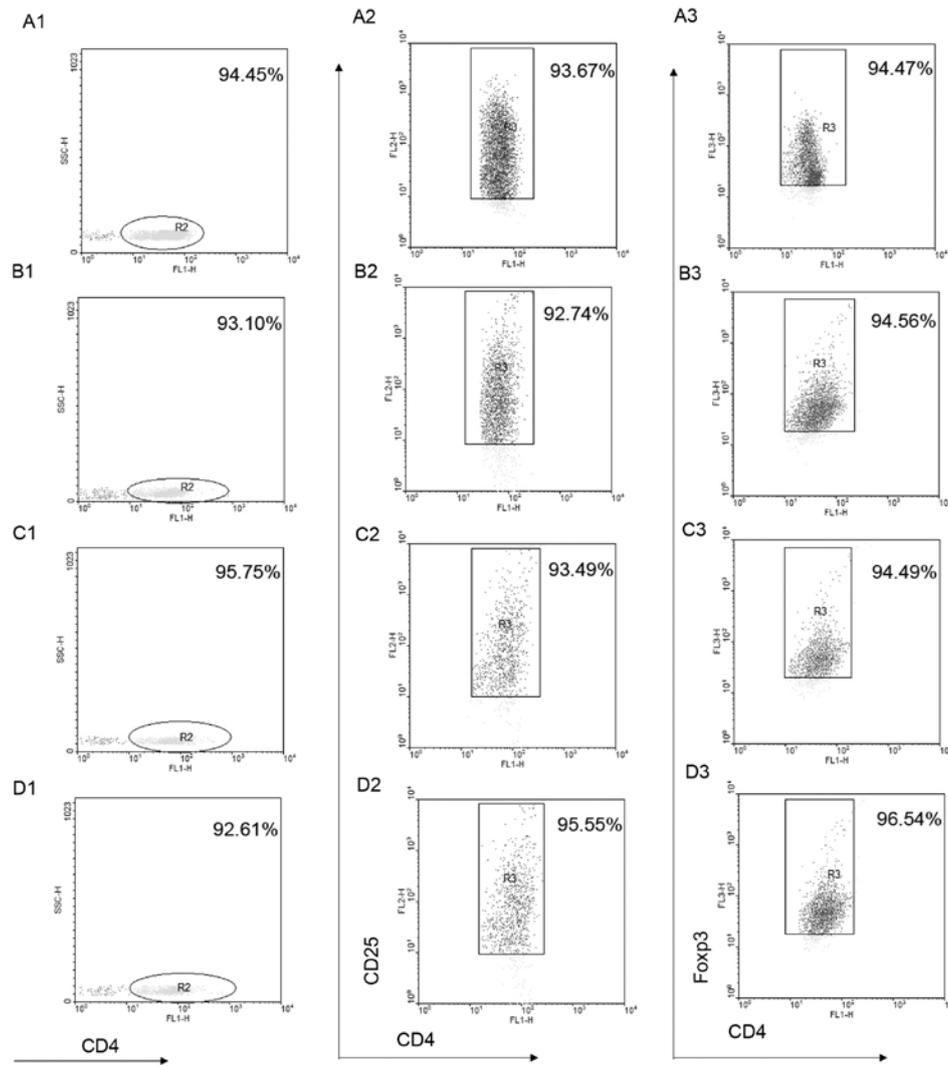


Figure 4. Representation of phenotypic changes in co-cultured Treg cells with or without FTY720. (A1-A3) Phenotypic changes in the control group. (B1-B3) Phenotypic changes in low dose FTY720 group. (C1-C3) Changes in the medium dose FTY720 group. (D1-D3) Changes in the high dose FTY720 group.

found that the S1P receptor agonist FTY720 could partly affect the immune function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (19,20). However, one study found that the proliferation of Treg cells was impaired significantly by FTY720 (21). In this study, we examined whether FTY720 and FTY720-P affect the immune function of Treg cells *in vitro*. In contrast to Wolf *et al* (21), we found that FTY720 did not affect the proliferation of the CD4<sup>+</sup>CD25<sup>+</sup> T cells. Though the proliferation of Treg cells in the high and medium dose FTY720-P group appeared to be enhanced slightly, there was still no significant difference between three FTY720-P groups and the control group.

From the results of the phenotypic changes of CD25 and Foxp3 positivity in co-cultured Treg cells, we observed that the high dose FTY720-P enhanced the percentages of CD25 and Foxp3 positivity in Treg cells. It is known that S1PR is expressed on Treg cells and influences Treg development, maintenance, and suppressor function (22). In addition, it has been reported that S1P1 can block the differentiation of thymic Treg precursors and function of mature Treg cells and affect Treg cell-mediated immune tolerance (17). The function of S1P1 is mediated mainly by Akt-mTOR in Treg cells (17). It is

known that FTY720 can be phosphorylated by SphK2 generating (S)-FTY720-phosphate (FTY720-P) (23). FTY720-P is structurally similar to S1P, and it could enhance the phenotypes of CD25<sup>+</sup> and Foxp3<sup>+</sup> in Treg cells by modulating S1P receptors (S1PR) directly. This indicates that FTY720-P enhances the differentiation and maturity of Treg cells by modulating S1P signaling directly. Though we find that these two phenotypic changes appear to be slightly enhanced in the high dose FTY720 group, there is no significant difference between the three FTY720 groups and the control group. The reason may be that FTY720 modulates the S1P receptor after phosphorylation with SphK2. Its efficiency is less than that of FTY720-P at the same dose level.

From the results of levels of TGF-β1 in the supernatant, we found that higher levels were shown in the high dose FTY720 group than in the control group. Being in accordance with Kim *et al* (24), we also observed that the levels of TGF-β1 in the medium and high dose FTY720-P were higher than in the control group. As we know TGF-β is synthesized as a precursor protein and released in an inactive form as either a small or large latent (25). It can both stimulate and inhibit cell

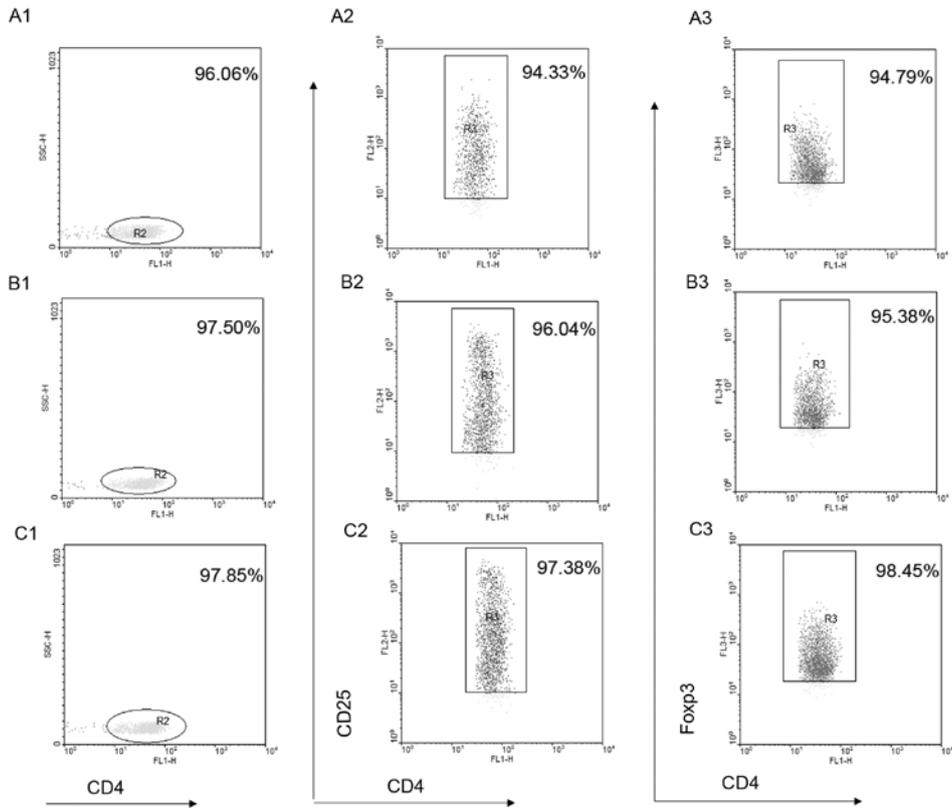


Figure 5. Representation of phenotypic changes in co-cultured Treg cells with FTY720. (A1-A3) Phenotypic changes in the low dose FTY720 group; (B1-B3) changes in the medium dose FTY720 group; and (C1-C3) changes in the high dose FTY720 group.

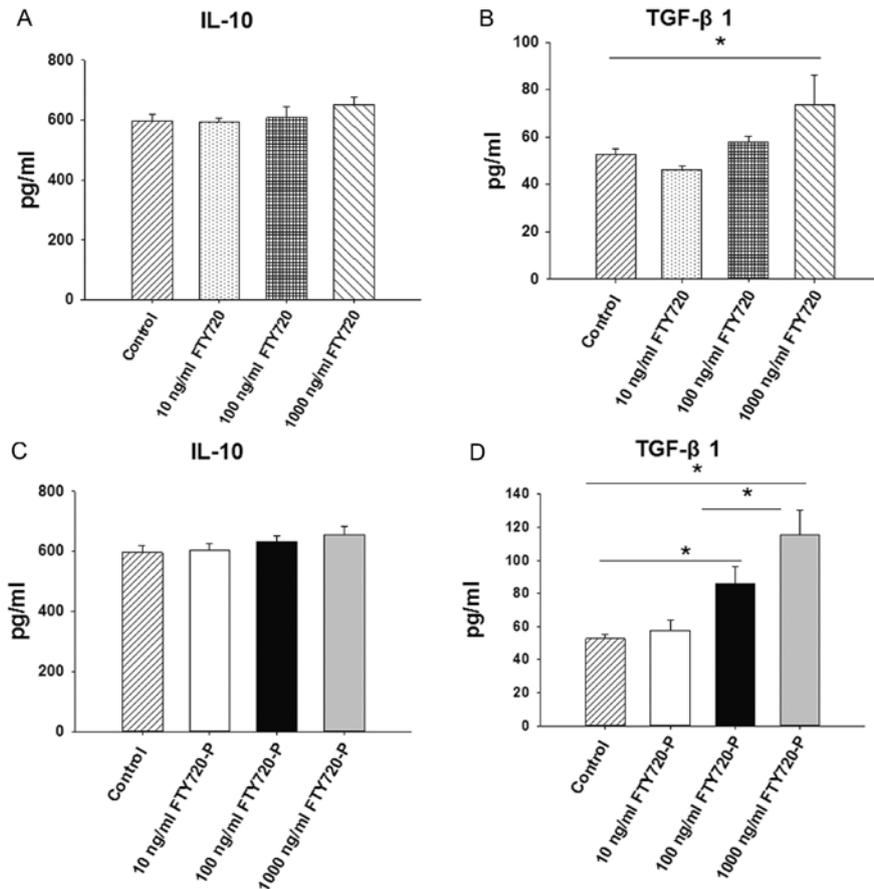


Figure 6. Levels of IL-10 and TGF-β1 in the supernatant of co-cultured Treg cells. (A and B) Levels of IL-10 and TGF-β1 in the supernatant of Treg cells with or without FTY720. (C and D) Levels of IL-10 and TGF-β1 in the supernatant of Treg cells with or without FTY720-P. (\*P<0.05 compared with the control group).

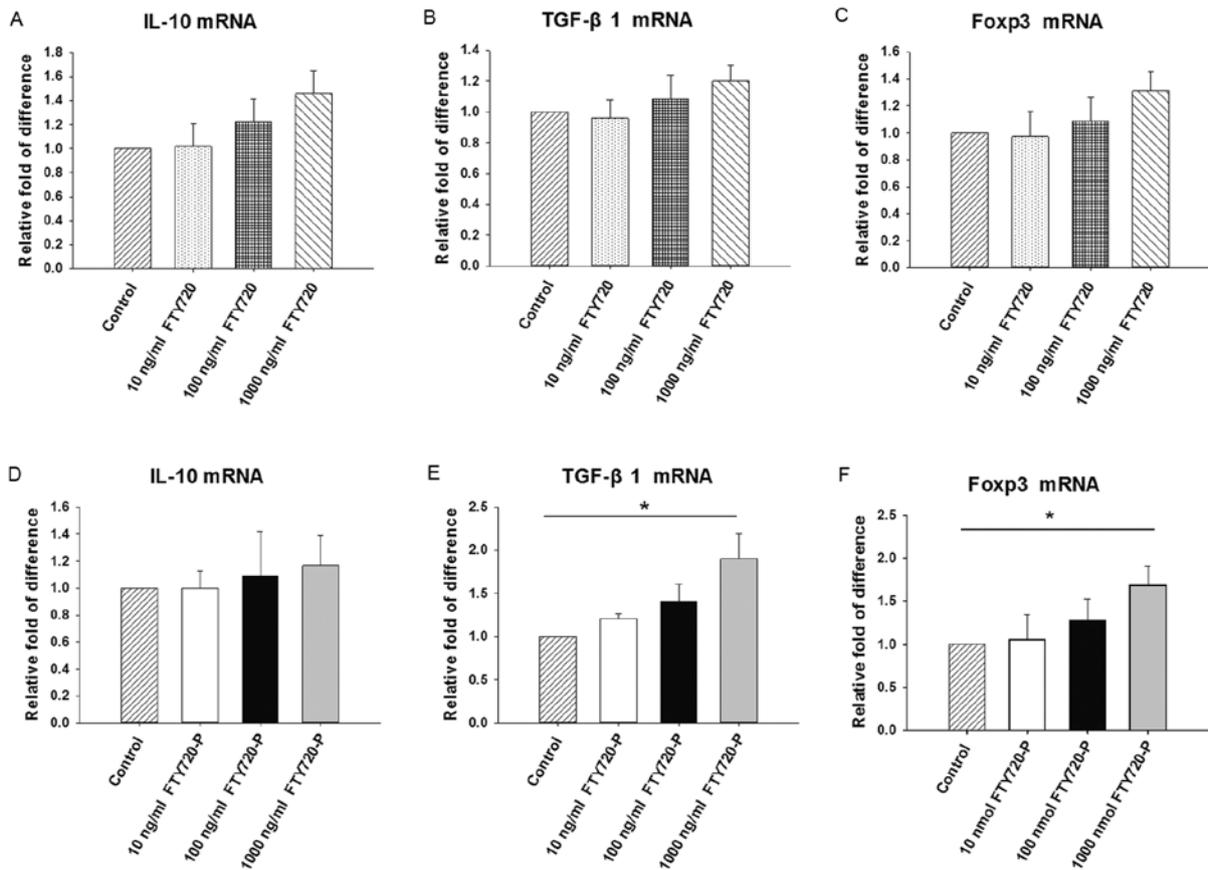


Figure 7. Expression of IL-10, TGF-β1 and Foxp3 mRNA in co-cultured Treg cells. (A, B and C) Levels of IL-10, TGF-β1 and Foxp3 mRNA expression in Treg cells with or without FTY720, respectively. (D, E and F) IL-10, TGF-β1 and Foxp3 mRNA expression in Treg cells with or without FTY720-P, respectively. (\*P<0.05 compared with the control group).

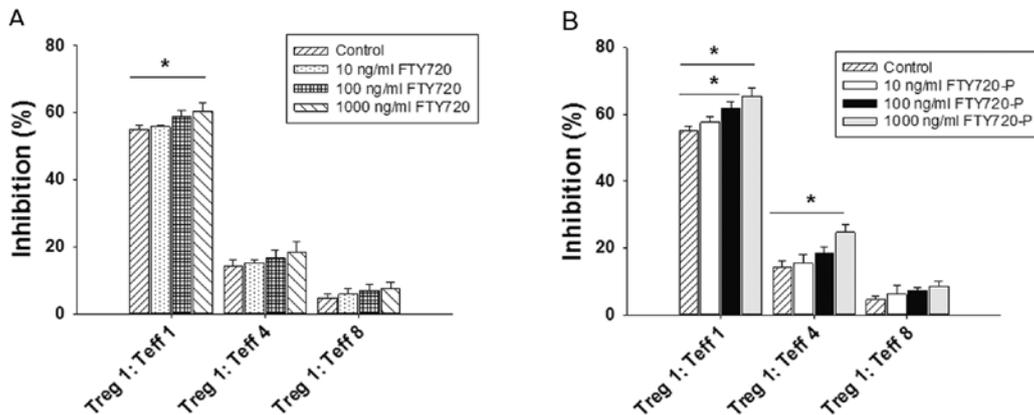


Figure 8. Mixed lymphocyte reaction (MLR) co-cultures at various ratios (1:1, 1:4 and 1:8). (A) Inhibition of effector T (Teff) cells co-cultured with Treg cells which were treated with or without three doses of FTY720. (B) Inhibition of Teff cells co-cultured with Treg cells which were treated with or without three doses of FTY720-P. (\*P<0.05 compared with the control group).

growth and proliferation. TGF-β1 is a regulatory molecule, acting to restore balance after deviations from normal and induce lymphocyte apoptosis (26). Moreover, many studies have reported that TGF-β is produced by Treg cells and controls T cell tolerance, and that TGF-β plays an important role in the induction of Treg and maintenance of immunologic tolerance (27-29). Therefore, we postulate that high dose FTY720 and FTY720-P may enhance the immune function of Treg cells by increasing the secretion of TGF-β1.

The results of the analysis of mRNA levels showed that the expression of TGF-β1 mRNA in the high dose FTY720 group appeared to be higher than in the control group, but there was no statistical difference between the two groups. For the three FTY720-P groups, we demonstrated that the high dose FTY720-P upregulated the TGF-β1 mRNA expression compared with the control group. The result may be attributed to the chemical structure of FTY720-P, which can modulate S1P signaling directly. As for the expression of Foxp3 mRNA, we found similar results.

These results are concordant with several studies which proved that FTY720 enhanced expression of Foxp3 (19,20). We can also explain the aforementioned phenotypic changes according to the upregulated expression of Foxp3 mRNA. Foxp3 is a key control molecule for Treg cell development and function, and is an excellent marker for the study of Treg cells (11,30). The above-mentioned results further indicate that high dose FTY720-P may enhance the suppressive functions of co-cultured Treg cells by upregulating TGF- $\beta$ 1 and Foxp3 mRNA levels.

It is well-known that IL-10 can be produced by many cell types, including B cells, macrophages, dendritic cells, Th2 cells, and distinct populations of Treg cells (31). IL-10 is also an anti-inflammatory cytokine with a crucial role in preventing inflammatory and autoimmune pathologies (32). Moreover, homogeneous populations of IL-10 Treg cells inhibited the *in vitro* proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells with a similar efficiency as that of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (33). However, though the levels of IL-10 in the supernatants both in high dose FTY720 and FTY720-P appeared to be enhanced slightly, there were still no significant differences. There were also no significant differences in IL-10 mRNA expression between the FTY720-P group, the FTY720 group and control group. We do not think the FTY720 and FTY720-P affect the function of Treg cells via the IL-10 mediated pathway according to these results.

In order to confirm that both FTY720 and FTY720-P can affect the immune-suppressive function of co-cultured Treg cells, we designed the MLR. From the results of MLR, we showed that the proliferation of Teff cells was significantly suppressed both in the medium- and the high-dose FTY720-P groups at a ratio of 1:1. High dose FTY720-P can also significantly suppress the proliferation of Teff cells at a ratio of 1:4. We verify that high-dose FTY720-P can significantly enhance the immune-suppressive function of co-cultured Treg cells and medium-dose FTY720-P has a partial effect. For FTY720, we found that only the high dose enhanced the immune-suppressive function of co-cultured Treg cells at ratio of 1:1. There were no significant differences between the three doses of FTY720 and the control group at ratios of 1:4 and 1:8. The results demonstrate that FTY720 affects the immune function of Treg cells slightly, not more than the same dose of FTY720-P. Therefore, we further indicate that FTY720-P enhances the immune function of Treg cells by modulating the S1P receptor directly, while FTY720 should be phosphorylated by SphK2 *in vivo* firstly.

In summary, we find that high dose FTY720-P could significantly enhance the immune function of co-cultured Treg cells, and medium-dose FTY720-P and high-dose FTY720 could partly enhance the function. Our findings suggest that the enhanced immune-suppressive functions of co-cultured Treg cells can be attributed to the high levels of TGF- $\beta$ 1 in the supernatant and upregulated expression TGF- $\beta$ 1 and Foxp3 mRNA in co-cultured Treg cells. High dose FTY720-P could also increase the percentages of CD25<sup>+</sup> and Foxp3<sup>+</sup> in co-cultured Treg cells. For FTY720, only the high dose FTY720 can partly enhance the immune function of co-cultured Treg cells and increase the level of TGF- $\beta$ 1 in the supernatant.

The effects of FTY720 and FTY720-P on Treg cells would be valuable in some clinical situations, such as transplantation, autoimmune diseases and chronic infections. However, the effects of other doses of FTY720-P and FTY720, and the exact mechanism of action need to be further investigated.

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