The effect of immunosuppressive drug rapamycin on regulatory CD4$^+$CD25$^+$Foxp3$^+$T cells in mice

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Abstract

CD4$^+$CD25$^+$Regulatory T (Treg) cells are crucial for negatively regulating immune responses. Rapamycin (rapa) is an immunosuppressive agent which is widely used for preventing acute graft rejection in patients and has been used to induce operational tolerance in mouse models. The aim of the present study was to determine the effect of rapa on CD4$^+$CD25$^+$Foxp3$^+$Treg cells in a mouse model. After C57BL/6 mice were intraperitoneally given 1.5 mg/kg/day of rapa for 14 days, the percentages, cell numbers, phenotype and function of CD4$^+$CD25$^+$Treg cells were determined by flow cytometry as well as the in vitro and in vivo functional assays. The cell numbers of CD4$^+$ and CD4$^+$CD25$^+$Treg cell subsets were markedly decreased in rapa-treated mice as reported. However, rapa significantly enhanced the ratios of CD4$^+$CD25$^+$Treg cells or CD4$^+$CD25$^+$Foxp3$^+$Treg cells to CD4$^+$T cells in spleens and thymi of mice ($P<0.01$) respectively. Furthermore, splenic CD4$^+$CD25$^+$Treg cells in rapa-treated mice showed immunosuppressive ability on the immune response of T effector cells to alloantigens or mitogen as efficiently as the control CD4$^+$CD25$^+$Treg cells in vitro and in vivo. Thus, rapa could significantly enhance the percentages of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in the thymus and the periphery while keeping these cells functional, indicating that CD4$^+$CD25$^+$Treg cells are more resistant to rapa than other CD4$^+$T cells. The different effects of rapa on CD4$^+$CD25$^+$Treg and T effector cells make rapa to be a favorable choice for inducing immune tolerance to self-, allo-, or xeno-antigens.
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1. Introduction

T cell tolerance is achieved at least by two fundamental pathways. One is through elimination of self-reactive T cells in the thymus during positive and negative selections, the other one is by generation of a variety of regulatory T cells (Treg cells) in the thymus or in the periphery [1–3]. It is becoming apparent that CD4$^+$CD25$^+$Treg cells, which comprise approximately 5%–10% of the peripheral CD4$^+$T cells in humans and mice, play an important role in immune tolerance to self antigens and allografts [4,5]. Enhancement of CD4$^+$CD25$^+$Treg cell activity may protect individuals from autoimmune diseases and graft rejection, while suppression of CD4$^+$CD25$^+$Treg cell activity may increase the potential occurrence of graft rejection, autoimmune diseases and tumors [6,7]. Furthermore, recent studies have demonstrated that the forkhead family transcription factor Foxp3 predominantly expressed in CD4$^+$CD25$^+$Treg cells and play a critical role for their development and function. Foxp3 is recognized as one of the specific Treg cell markers [8,9].

Rapamycin (rapa), a novel macrolide immunosuppressive drug, has been widely used in preventing clinical allograft rejection and some autoimmune diseases [10,11]. Recently, rapa was used to induce transplant tolerance in some experimental models. The molecular mechanism related to rapa-mediated immunosuppression is that it binds to FKBP12 and the formed complex inhibits the function of mammalian target of rapa

(mTOR), which in turn reduces protein phosphorylation and cell cycle progression [12].

Recent studies have shown that FK506 and cyclosporin A (CsA) could significantly decrease the cell numbers and function of CD4$^+$CD25$^+$Treg cells in animal models [13,14]. Compared with CsA and FK506, rapa blocks different cellular signaling pathways. In a mixed chimeric mouse model, CsA or FK506 could inhibit the development of long-term chimerism and abrogate tolerance, whereas rapa had no negative effect on chimerism or tolerance development [15]. It has been reported that rapa could selectively expand the murine naturally occurring CD4$^+$CD25$^+$Foxp3$^+$Treg cells in vitro [16]. However, the in vivo effect of rapa on the level and function of the CD4$^+$CD25$^+$Foxp3$^+$ Treg cell population need to be elucidated. In the present study, significantly higher percentages of CD4$^+$CD25$^+$Foxp3$^+$Treg cells with unaltered immunosuppressive function were observed in the spleens and thymi of rapa-treated mice. These data might have significant impacts on the application and selection of immunosuppressive medicines for preventing graft rejection and transplant tolerance induction in clinics.

2. Materials and methods

2.1. Mice

C57BL/6 (H-2^b, weighing 18 to 22 g, 6–8 week-old, female), and Balb/c (H-2^d) mice were purchased from Institute of Genetics and Development, Chinese Academy of Sciences (Beijing, China). All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. C57BL/6 mice were divided into two groups: control group and rapa-treated group. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

2.2. Monoclonal antibodies (mAbs) and reagents

The following mAbs were purchased from BD Biosciences PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 mAb (RM4-5; rat IgG2a), FITC-labeled anti-mouse CD8 mAb (53–6.7; rat IgG2a), FITC-labeled rat anti-mouse CD25 mAb (7D4; IgM), FITC-conjugated anti-mouse GITR mAb (DTA-1, rat IgG2b), phycoerythrin (PE)-labeled rat anti-mouse CD4 mAb, PE-labeled anti-mouse CD8α mAb (53–6.7; rat IgG2a), and Cy5-labeled anti-mouse CD25 mAb. Cy-chrome-labeled anti-mouse CD4 mAb. PE-labeled anti-mouse CD44 mAb (IM7; rat IgG2b), PE-labeled anti-mouse CD45RB mAb (16A; rat IgG2a), PE-labeled anti-mouse CD69 mAb (FN50; mIgG), PE-labeled anti-mouse CD62L mAb (SK11; mIgG1), PE-labeled anti-mouse GITR mAb (DTA-1; rat IgG2b), PE-labeled anti-mouse CD152 mAb (BNI3; mIgG2a). In addition, PE-labeled anti-mouse Foxp3 mAb (FJK-16s) and its staining kit were obtained from eBioscience (San Diego, CA). Rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, Maryland) in our laboratory.

Mitomycin C (C$_{15}$H$_{18}$N$_4$O$_5$) was obtained from Kyowa Hakko Co, Ltd. (Tokyo, Japan). [^{3}H] thymidine was purchased from China institute of atomic energy (Beijing, China). The culture medium used in the present study was RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate (all from) and 50 μM 2-ME (Sigma, St. Louis, MO).

2.3. Rapa treatment

Rapa (World NCB Co., China) was dissolved in the vehicle containing sodium CMC (C-5013 high viscosity, Sigma-Aldrich) and polysorbate 80. Rapa stock solution was stored at 4 °C in dark according to the manufacture's instruction. Rapa was administered intraperitoneally (IP) at a dose of 1.5 mg/kg/

day for 14 days. Solution including 0.2% CMC and 0.25% polysorbate 80 was administered to the control mice in some experiments. At least three independent experiments were performed for each assay.

2.4. Cell preparation

Mouse peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient (Sigma, St. Louis, MO) as reported before [17]. After mice received rapa treatment for 14 days, thymus, spleen and lymph nodes (LNs, including cervical, inguinal and axillary LNs) were harvested. Single cell suspensions were prepared by grinding the tissues with the plunger of a 5-ml disposable syringe and were then suspended in RPMI1640 medium. Splenocytes were treated with a hemolysis buffer (17 mM Tris–HCl and 140 mM NH$_4$Cl, pH7.2) to remove RBCs as described before [18].

CD4$^+$CD25$^+$Treg cells populations were isolated from mouse splenocytes using a CD4$^+$CD25$^+$Treg Cells Isolation Kit with MidiMACS™ Separator according to the manufacturer's protocols (Miltenyi, Bergisch Gladbach, Germany). Briefly, splenocytes were incubated with a Biotin-antibody cocktail against: CD8α (Ly2), CD11b (Mac-1), CD45R (B220), CD49B (DX5) and Ter-119, for 20 min at 4°C, and then with microbead-conjugated anti-biotin mAb (Bio318E7.2) and PE-labeled anti-CD25 mAb. The cell suspension was loaded on a LD column, which is placed in magnetic field of a MACS Separator. The remaining fraction in the column is the enriched CD4$^+$ cells. For the isolation of CD4$^+$CD25$^+$ cells, the PE-labeled CD25$^+$ cells in the enriched CD4$^+$ cells fraction were magnetically labeled with anti-PE MicroBeads. The magnetically labeled CD4$^+$CD25$^+$ cells were enriched from the CD4$^+$ cells fraction by MACS sorting. The purities of the sorted CD4$^+$CD25$^+$ populations were always >95% as confirmed by flow cytometry (FCM).

2.5. Immunofluorescence staining and FCM

PBMCs, splenocytes or thymocytes were incubated with 2.4G2 to block FcRs and then incubated with and optimal concentration of fluorochrome-labeled mAbs for 30 min at 4 °C in the dark. Cells were washed three times and resuspended by FCM buffer (PBS with 0.1% BSA and 0.1% NaN$_3$). At least ten thousand cells were assayed using a FASCalibur flow cytometry (Becton Dickinson, CA), and data were analyzed with CellQuest software. In some experiments, non-viable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAb from staining in the same dot-plot region with the anti-mouse mAbs.

For the intracellular staining, cells were incubated with Cy-chrome-labeled anti-CD4 and FITC-labeled anti-CD25 mAbs first. After washing, these cells were then fixed and stained with anti-mouse Foxp3 mAb, according to the instruction offered by the manufacturer (eBioscience, San Diego, CA).

2.6. Mixed lymphocyte reaction (MLR)

C57BL/6 splenic CD4$^+$CD25$^+$Treg cells were isolated from either control or rapa-treated mice as described above. Naïve C57BL/6 CD4$^+$CD25$^-$T cells were used as responder T cells. Balb/c splenocytes, which were pre-treated with mitomycin C at the concentration of 30 μg/ml at 37 °C for 30 min, were used as allogeneic stimulator cells. In general, 8×10^4 responder cells (naïve C57BL/6 CD4$^+$CD25$^-$T cells) and 8×10^4 stimulator cells (Balb/c splenocytes) per well in RPMI1640 medium supplemented with 10% FCS were added in 96-well round-bottomed plates. CD4$^+$CD25$^+$Treg cells were subsequently added to each well in different ratios to CD4$^+$CD25$^-$T cells. Cells were cultured at 37 °C and 5% CO$_2$ for 5 days. 0.5 μCi [^{3}H] thymidine (185 GBq/mmol; Atomic Energy Research Establishment, China) was added for the last 16 h. Cells were harvested with an automatic cell harvester (Tomtec, Toku, Finland). The radioactivity of each sample was assayed in a Liquid Scintillation Analyzer (Beckmon Instruments, America). Values are expressed as counts per minute (cpm) of triplicate wells.

2.7. The proliferation of T cells to Con A

C57BL/6 CD4$^+$CD25$^-$T cells (4×10^4 cells/well) were cultured in U-bottom, 96-well plates with syngeneic accessory cells (4×10^4 splenocytes/well, pre-treated with 30 μg/ml mitomycin C at 37 °C for 30 min), 3 μg/ml Con A and the indicated numbers of C57BL/6 CD4$^+$CD25$^+$Treg cells isolated from either

control or rapa-treated mice for 72 h at 37 °C, 5% CO_2. 0.5 µCi [^{3}H] thymidine was added to each well for the last 12 h. Cells were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a Liquid Scintillation Analyzer (Beckmon Instruments, America). Values are presented as counts per minute (cpm) of triplicate wells.

2.8. Delayed type hypersensitivity (DTH)

Sensitized C57BL/6 effector T cells were generated by immunization with allogeneic Balb/c splenocytes. Ten days after immunization, C57BL/6 CD4$^+$T cells were enriched using the negative selecting MACS kit for CD4$^+$T lymphocytes (BD Biosciences PharMingen). Balb/c splenic macrophages (SPMs) were used as stimulator cells. Sensitized C57BL/6 effector CD4$^+$T cells and allogeneic or syngeneic macrophage stimulators (5×10^5 cells/each) were co-injected intradermally into the pinnate of naïve C57BL/6 mice. The changes in ear thickness were measured using an engineer's micrometer at 24 or 48 h after challenge. The ear thickness change was calculated by subtracting the thickness of the ear before injection from the thickness of the same ear after injection.

2.9. Statistical analysis

All data are presented as the mean±SD. Student's unpaired t test for comparison of means was used to compare groups. A P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. The effect of rapa on the levels of CD4$^+$CD25^{high}Treg cells in the periphery of C57BL/6 mice

Early studies have shown that CD4$^+$CD25$^{+/high}$T cells had significantly higher immunosuppressive activity than CD4$^+$CD25$^{+/low}$T cells [19,20]. CD4$^+$CD25^{high}Treg cells may be more likely to be typical Treg cells in vivo. Thus, in order to evaluate the effects of rapa on Treg cells in mice, we compared the percentages and cell numbers of CD4$^+$CD25^{high}Treg cells in rapa-treated mice and control mice.

The percentages of both CD4$^+$ and CD8$^+$T cells in the PBMCs were significantly reduced after the treatment with rapa for 2 weeks (data not shown), as reported before. However, significantly decreased percentages of CD4$^+$CD25^{high}Treg cells in CD4$^+$ PBMCs were observed by the injection of rapa for 2 weeks as determined by a two-color FCM ($P<0.001$, Fig. 1).

After injection of rapa for 2 weeks, lymph nodes (LNs; axillary, inguinal and cervical regions) of mice were dramatically withered (data not shown) and the total cell number was reduced to almost one half than that of normal mice (Fig. 2, $P<0.05$). However, the percentages of CD4$^+$T cells, CD8$^+$T cells and CD4$^+$CD25^{high}Treg cells as well as the ratio of CD4$^+$CD25^{high}Treg cells to CD4$^+$T cells did not show detectable changes ($P>0.05$). As it is recently determined that Foxp3 is specifically expressed in CD4$^+$CD25$^+$Treg cells and plays a critical role in the development and function of CD4$^+$CD25$^+$Treg cells [21,22], we evaluated the expression of Foxp3 in CD4$^+$CD25^{high}Treg cells in mice treated with or without rapa. The ratio of CD4$^+$CD25^{high}Foxp3$^+$Treg cells in CD4$^+$CD25^{high}Treg cells in LNs had no significant change after rapa treatment (data not shown). Due to the significant decrease of the total cell numbers in LNs, the cell numbers of CD4$^+$T cells, CD4$^+$CD25^{high}Treg cells and CD4$^+$CD25^{high}Foxp3$^+$Treg cells markedly decreased after administration of rapa for 2 weeks (Fig. 2E, F and G).

The size and the weight of the spleen were reduced in mice injected with rapa for 2 weeks (data not shown). Consistently, the total cell number of splenocytes decreased 47.96% in these mice, too (Fig. 3). The percentages of CD4$^+$T cells in the spleens of rapa-treated C57BL/6 mice were significantly lower than that of control mice ($P<0.001$, Fig. 3). The percentages of CD4$^+$CD25^{high}Treg cells among all splenocytes were not significantly altered after the treatment with rapa, but the percentage of
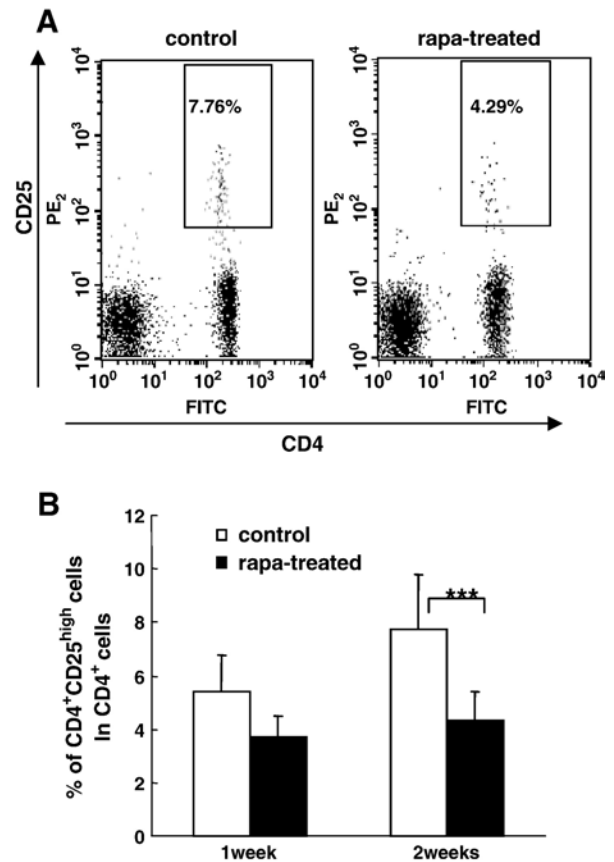

Fig. 1. Rapa treatment reduced the proportion of CD4$^+$CD25^{high}T cells in the peripheral blood. C57BL/6 mice were treated with rapa as described in the Section 2. One week and 2 weeks after treatment, PBMCs were stained with FITC-labeled anti-CD4 mAb and PE-labeled anti-CD25 mAb and assayed by FCM. (A) One representative of the PBMCs stained with anti-CD4 and CD25 mAbs. Numbers in the dot plot indicate the percentage of CD4$^+$CD25$^+$Treg cells. (B) The proportion of CD4$^+$CD25^{high}T cells in CD4$^+$ PBMCs after injection of rapa for 1 and 2 weeks. ***$P<0.001$ compared with the indicated groups. The data are one representative of 3 separated experiments with similar results.

CD4$^+$CD25^{high}Treg cells in CD4$^+$ splenocytes in these mice was markedly increased ($P<0.01$, Fig. 3C). However, the total cell numbers of splenic CD4$^+$T cells, CD4$^+$CD25^{high}T cells and CD4$^+$CD25^{high}Foxp3$^+$Treg cells were remarkably reduced in rapa-treated mice (Fig. 3).

3.2. The phenotype of CD4$^+$CD25^{high}Treg cells in rapa-treated mice

Before evaluating the effects of rapa on CD4$^+$CD25$^+$Treg cells function, we assessed the expression of CD4$^+$CD25$^+$Foxp3$^+$Treg cell-associated proteins, including CTLA-4, GITR, CD44, CD45RB, CD62 L and CD69 [23–25]. Surprisingly, no significant changes for the expressions of these molecules on CD4$^+$CD25^{high}Treg cells or CD4$^+$CD25$^-$T cells in rapa-treated mice were detected by a three-color FCM in three independent experiments (Fig. 4, and data not shown). Thus, rapa treatment did not change the phenotypes of CD4$^+$CD25$^+$Treg cells and CD4$^+$CD25$^-$T cells in mice.

3.3. The effect of rapa on the immunosuppressive ability of CD4$^+$CD25$^+$Treg cells in vitro and in vivo

CD4$^+$CD25$^+$Treg cells have been demonstrated to suppress the proliferation of CD4$^+$CD25$^-$T cells induced by antigens in vitro [4]. We
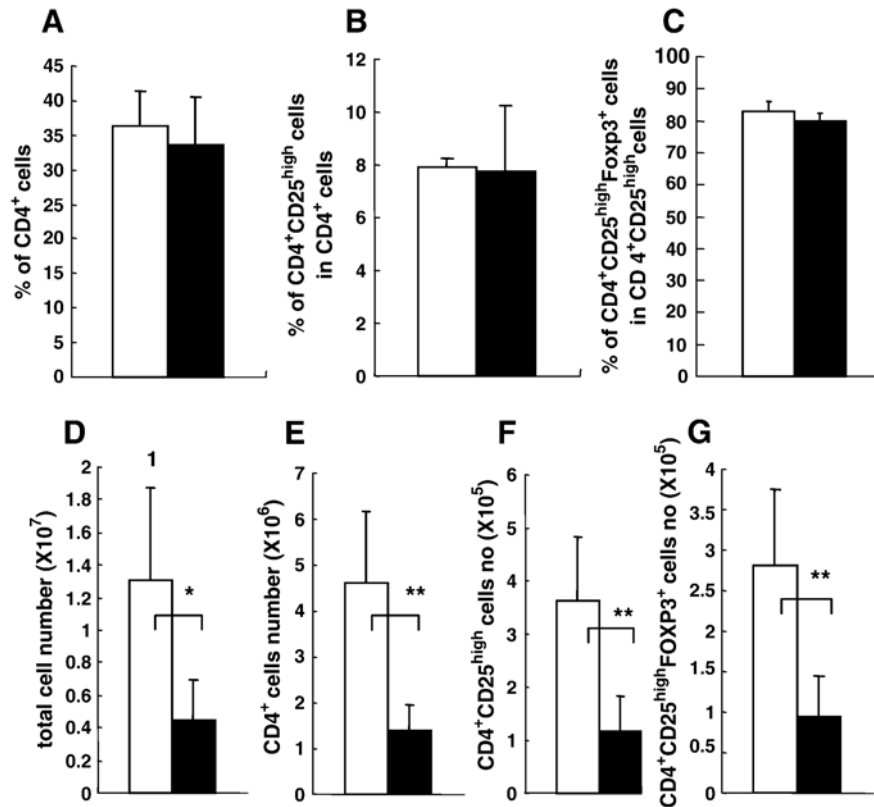
Fig. 2. The percentages and total cell numbers of CD4$^+$CD25^{high}T cells in LNs of mice treated with or without rapa. LNs were isolated from C57BL/6 mice after injections of rapa for 2 weeks. The cells were analyzed for the expression of CD4 and CD25 by FCM. The proportion of CD4$^+$T cells (A) the ratio of CD4$^+$CD25^{high}T cells to CD4$^+$T cells (B) and the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells in CD4$^+$CD25^{high}T cells (C) in LNs cells of control and rapa-treated mice. The total cell numbers of all cells (D), CD4$^+$T cells (E), CD4$^+$CD25^{high}T cells (F) and CD4$^+$CD25^{high}Foxp3$^+$T cells (G) in LNs of control and rapa-treated mice. Results were shown as mean±SD, which was one representative of three independent experiments (n=6 in each group). *P<0.05, **P<0.01, compared with the indicated groups.

then compared the immunosuppressive effects of CD4$^+$CD25$^+$Treg cells sorted by bead separation from rapa-treated and control mice in an in vitro culture system. As is shown in Fig. 5A, regardless CD4$^+$CD25$^+$Treg cells from rapa-treated mice or control mice, they showed excellent suppressive function on the proliferation of CD4$^+$CD25$^-$T cells induced by allogeneic antigens. Importantly, no detectable significant difference for the inhibiting ability of CD4$^+$CD25$^+$Treg cells from rapa-treated or control mice was observed in three independent assays (Fig. 5A), whereas the proliferation of splenic CD4$^+$CD25$^-$T cells in rapa-treated mice was markedly lower than that of splenic CD4$^+$CD25$^-$T cells in the control mice (P<0.01, data not shown). Identical results were observed when the inhibiting effects of CD4$^+$CD25$^+$Treg cells on the proliferation of CD4$^+$CD25$^-$T cells induced by mitogen Con A were detected (Fig. 5B). These data indicated that rapa did not impact the immunosuppressive function of CD4$^+$CD25$^+$T cells on the immune responses of syngeneic CD4$^+$CD25$^-$T cells to alloantigens or mitogen in vitro.

To further determine whether or not rapa has the ability to alter the function of CD4$^+$CD25$^+$Treg cells in vivo, we have performed adoptive transfer DTH assays as described in Section 2. As is shown in Fig. 5C and D, allogeneic Balb/c macrophages induced strong DTH reaction of pre-sensitized C57BL/6 CD4$^+$T cells in naïve C57BL/6 recipients. Consistently with the in vitro assays, CD4$^+$CD25$^+$Treg cells separated from rapa-treated C57BL/6 mice showed similar inhibiting activity on DTH reaction of pre-sensitized C57BL/6 CD4$^+$T cells to alloantigens as that of CD4$^+$CD25$^+$Treg cells separated from rapa-treated C57BL/6 mice (P>0.05, Fig. 5C and D). These results supported the conclusion that rapa treatment did not significantly impact the immunosuppressive function of CD4$^+$CD25$^+$Treg cells in mice.

3.4. The percentages of CD4$^+$ CD8$^-$CD25$^+$Treg cells in the thymus were significantly enhanced by the treatment with rapa

The thymus produces the majority of natural CD4$^+$CD25$^+$Treg cells as a functionally mature T cell subpopulation [26]. As reported before, the size and the weight of the mouse thymus were remarkably reduced after the treatment with rapa for 2 weeks (data not shown). The total cell numbers of thymocytes, CD4$^+$CD8$^-$, CD8$^+$CD4$^-$, CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ thymocytes decreased by about 70% (Fig. 6). Unexpectedly, the percentage of CD4$^+$CD8$^-$CD25$^+$Treg cells in CD4$^+$CD8$^-$T cells in rapa-treated mice was almost two fold higher than those of the control mice (Fig. 6C), but the total cell numbers of CD4$^+$CD8$^-$CD25$^+$Treg cells and CD4$^+$CD25$^+$Foxp3$^+$Treg cells in the thymus did not show any detectable changes compared with the control mice. These data indicated that rapa did not impact the development of CD4$^+$CD25$^+$Foxp3$^+$Treg cells but remarkably inhibited other thymocyte subsets in mice.

4. Discussion

In the present study, we observed that rapa significantly enhanced the relative levels of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in the spleens and thymi of mice while most of T cell subsets were decreased. Although rapa remarkably inhibited the immune response of CD4$^+$T cells to allogeneic antigens or mitogens as reported by others as well as confirmed by our studies using CD4$^+$CD25$^-$T cells, the immunosuppressive function of the
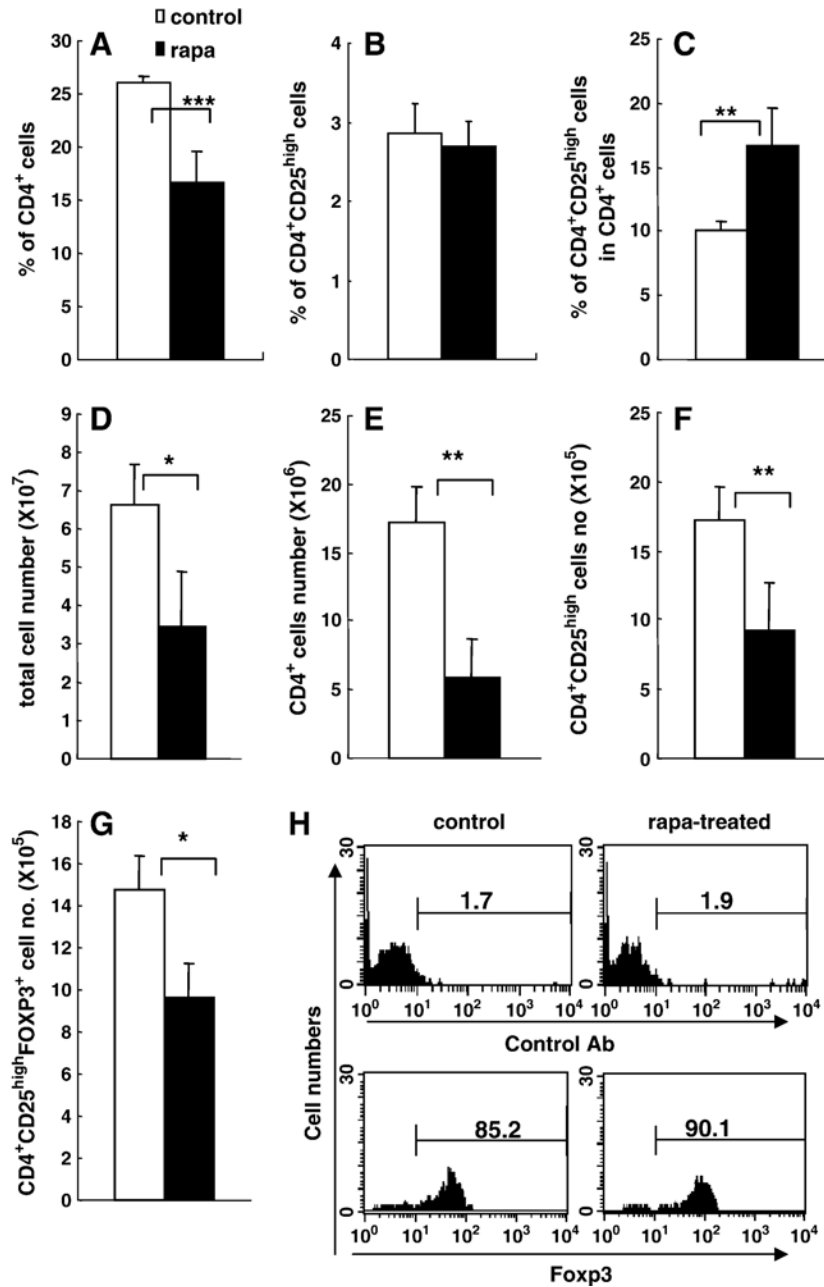
Fig. 3. The enhanced ratios of CD4$^+$CD25^{high}T cells to CD4$^+$T cells in spleens of mice treated with rapa. Splenocytes were isolated from C57BL/6 mice after rapa treatment for 2 weeks. The cells were analyzed for the expressions of CD4, CD25 and Foxp3 by FCM. The proportion of CD4$^+$T cells (A), the levels of CD4$^+$CD25^{high}Treg cells (B) as well as the ratios of CD4$^+$CD25^{high}T cells in CD4$^+$T cells (C) in spleen cells in control and rapa-treated mice. The total cell numbers of splenocytes (D), CD4$^+$T cells (E), CD4$^+$CD25^{high}T cells (F) and CD4$^+$CD25^{high}Foxp3$^+$T cells (G) in spleens of control and rapa-treated mice. Results were shown as mean±SD, which was one representative of three independent experiments ($n=6$ in each group). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the indicated groups. (H) One representative of Foxp3 expression on CD4$^+$CD25^{high} cells assayed by FCM.

CD4$^+$CD25$^+$Treg cells was not impacted by the treatment of rapa as determined by the in vitro and in vivo functional assays. Our present results were indirectly supported by the observation showing that rapa did not block transplant tolerance induction by some protocols while CsA did so in vivo [15]. These data supports that the treatment with rapa may selectively inhibit the host immune rejection against grafts while still keep the host with the potential ability to induce immune tolerance. The separated effect of rapa on Treg and non-Treg cells will be greatly beneficial to patients who have transplant grafts or autoimmune diseases, as they have the tendency to achieve immune tolerance. Very strikingly, FK506 and CsA could significantly decrease the cell numbers and function of CD4^{+-}CD25$^+$Treg cells in vivo [13,14]. Thus, when immune tolerance in attempt to be induced by different approaches, if immuno-suppressive drugs were needed, rapa should be considered as a favor one.

Recent studies have demonstrated that signaling through the interleukin-2 (IL-2) receptor is crucial for the functional activity of CD4$^+$CD25$^+$Treg cells [13,27,28]. On the other hand, It is
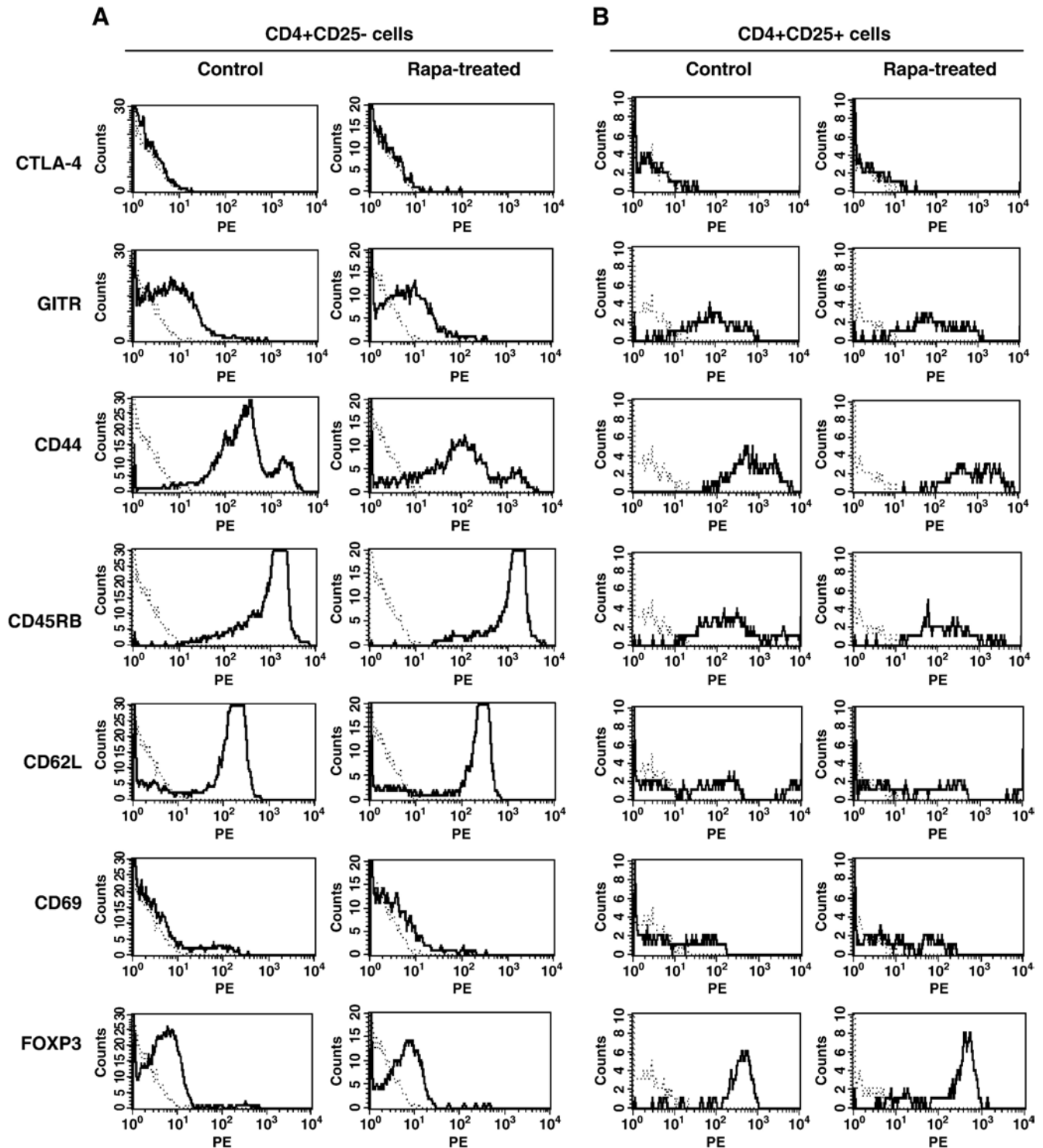
Fig. 4. The expression of some surface molecules and transcript factor Foxp3 on $CD4^+CD25^{high}$ and $CD4^+CD25^-$ splenocytes in mice treated with or without rapa. The surface markers including CD69, CD62 L, CD45RB, CD44 and CTLA-4 as well as the Treg-specific transcript factor Foxp3 on $CD4^+CD25^{high}$T cells and $CD4^+CD25^-$ splenocytes were assayed by multi-color FCM as described in Section 2. One representative for the staining of $CD4^+CD25^-$T cells (A) and $CD4^+CD25^{high}$T cells (B) in control and rapa-treated mice was shown. The thin dotted curve was the isotype control staining, the thick curve indicates specific staining. The data are one representative of at least four separate experiments with similar results.

well known that CsA inhibits TCR-mediated activation and IL-2 production, whereas rapa blocks intracellular signal in response to T cell growth factors like IL-2 [29]. Why do CsA and rapa have different effects on the function of $CD4^+CD25^+$

$Foxp3^+$Treg cells? The mechanism is not addressed yet. The major molecular target for CsA is calcineurin, a serine/threonine phosphatase [30], whereas the one for rapa is the mammalian target of rapa (mTOR) [31,32]. It is possible that calcineurin and
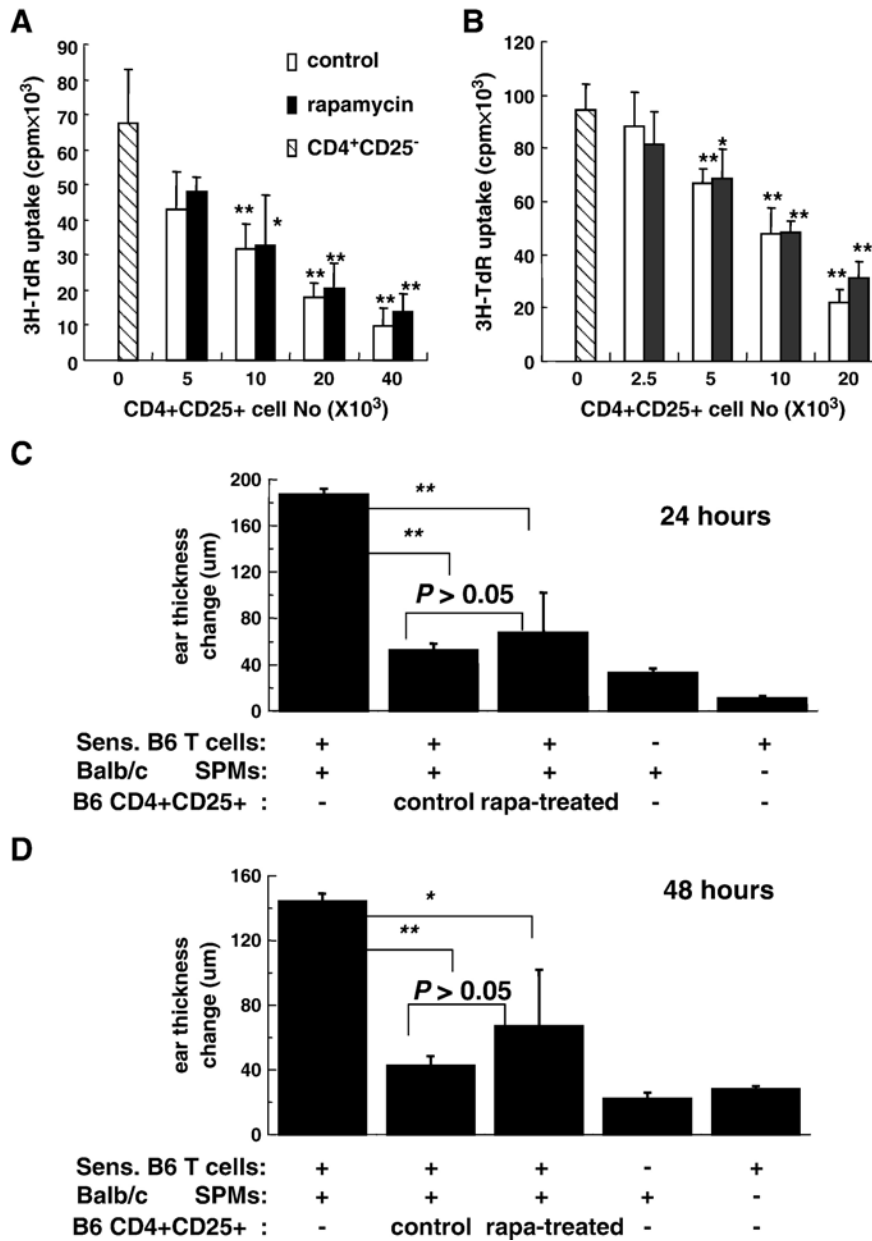
Fig. 5. The unimpacted function of CD4+CD25+Treg cells in rapa-treated mice. CD4+CD25+Treg cells in control and rapa-treated mice significantly inhibited the proliferation of CD4+CD25−T cells to alloantigen (A) or mitogen Con A (B). No significance was observed between samples cultured with CD4+CD25+Treg cells from control and rapa-treated mice at the same dose. CD4+CD25+Treg cells from control or rapa-treated mice significantly inhibited the DTH reaction of syngeneic T cells to alloantigens observed by 24 h (C) or by 48 h (D). *$P<0.05$, **$P<0.01$, compared between the indicated groups.

mTOR signal pathways play different roles in CD4+CD25+Treg cells.

Foxp3 is predominantly expressed in the CD4+CD25+Treg cells and is recognized to be a specific marker for Treg cells. Expression of Foxp3 in CD4+CD25−T cells can convert these cells to Treg cells that displayed similar phenotype and function as naturally occurring CD4+CD25+Treg cells [9,22], so we have detected the expression of Foxp3 in CD4+CD25+T cells in spleens, LNs and thymi of rapa-treated mice. No detectable changes on the expression of Foxp3 in CD4+CD25+Treg cells were observed after the treatment with rapa, as determined by FCM. Together with the unchanged levels of co-molecules expressed on CD4+CD25+Treg cells, these results may explain

why rapa treatment does not alter the immunosuppressive ability of CD4+CD25+Treg cells.

Interestingly, although the significantly higher percentages of CD4+CD25+Treg cells in both thymus and spleen were observed after the treatment with rapa, the total cell number of CD4+CD25+Treg cells in the thymi was unchanged, whereas the total cell number of CD4+CD25+Treg cells in spleens was significantly decreased in rapa-treated mice. The seemingly disparate effects of rapa on CD4+CD25+Foxp3+Treg cells in the thymus and periphery is nicely consistent with the recent report showing that IL-2 signaling does not have a nonredundant function in the development of CD4+CD25+Foxp3+Treg cells but seems to be critically required for maintaining the
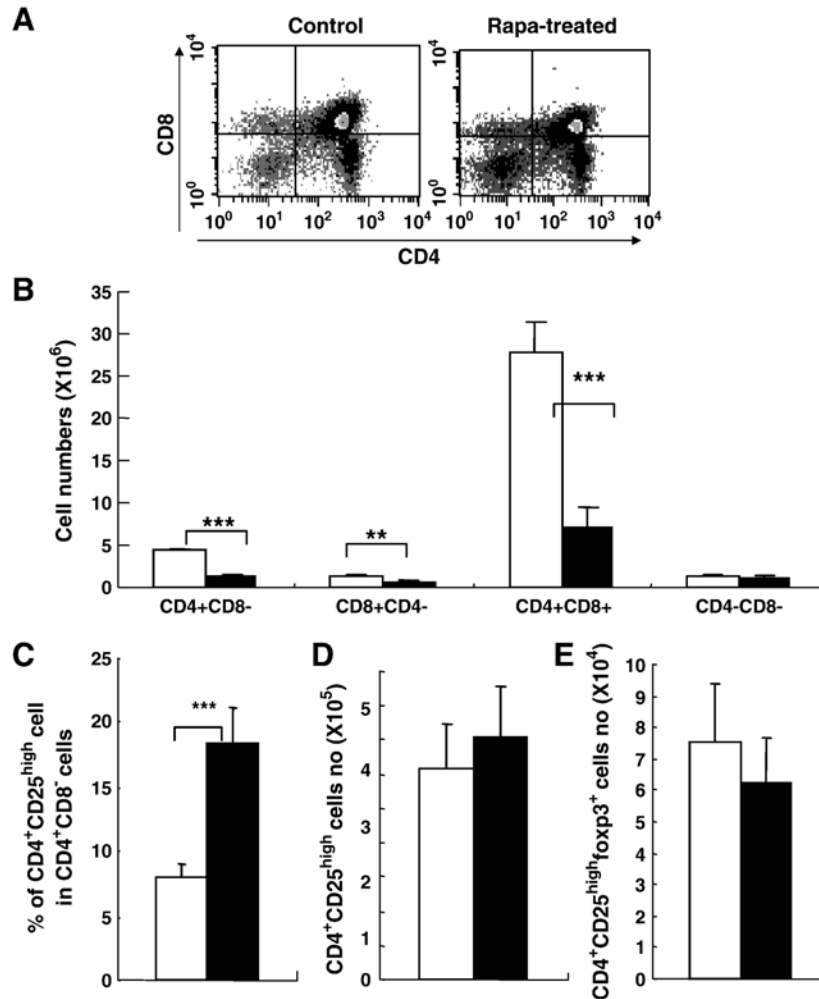
Fig. 6. Significantly enhanced percentages of CD4$^+$CD25^{high}T cells in the thymi of rapa-treated mice. After injection of rapa for 2 weeks, thymocytes were stained with Cy-chrome-labeled anti-CD4 mAb, FITC-labeled anti-CD8 mAb and PE-labeled anti-CD25 mAb and analyzed by FCM. (A) One representative of thymocytes of control and rapa-treated mice staining with anti-CD4 and anti-CD8 mAbs assayed by FCM. (B) The total cell numbers of CD4$^+$CD8$^-$, CD8$^+$CD4$^-$, CD4$^+$CD8$^+$, or CD4$^-$CD8$^-$ thymocytes in the control and rapa-treated mice. (C) The mean percentages of CD4$^+$CD25^{high}T cells in CD4$^+$CD8$^-$T cells in the thymi of the control and rapa-treated mice. (D) The total cell numbers of CD4$^+$CD25^{high}T cells in the thymi of the control and rapa-treated mice. (E) The total cell numbers of CD4$^+$CD25^{high}Foxp3$^+$T cells in the thymi of the control and rapa-treated mice. **$P<0.01$, ***$P<0.001$ compared with the indicated groups. Results were shown as mean±SD which were one representative of three independent experiments ($n=6$ in each group and in each experiment).

homeostasis and competitive fitness of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in vivo [28]. Furthermore, the different effects of rapa on CD4$^+$CD25$^+$Treg cells and CD4$^+$CD25$^-$T cells may be due to the different signal pathways activated by IL-2 or others. Indeed, IL-2R signaling through mTOR is required for the differentiation of T effector cells, whereas CD4$^+$CD25$^+$Foxp3$^+$ Treg cells may use a different signaling pathway, namely the Janus kinase/signal transducer and activator of transcription 5 (JAK/STAT5) pathway [33].

Our in vivo results showed that rapa relatively enhanced the percentages of CD4$^+$CD25$^+$Foxp3$^+$Treg but their absolute cell number was significantly decreased without signs for the expansion of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in this model indicating that rapa does not induce the expansion of CD4$^+$ CD25$^+$Treg cells in the periphery of mice. Rapa seems to induce less cell death of CD4$^+$CD25$^+$Treg cells than that of T effector cells (CD4$^+$CD25$^-$T cells) in the periphery. Battaglia et al. recently provide evidence showing that rapa selectively expanded

the murine naturally occurring CD4$^+$CD25$^+$Foxp3$^+$Treg cells in vitro, and these expanded Treg cells had the ability to suppress proliferation of syngeneic T cells in vitro [16]. The different results may be explained to be due to the different systems, the in vitro vs the in vivo system. Anyway, all the data support that rapa seems at least not to inhibit the development and function of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in all conditions.

In summary, rapa significantly enhanced the relative levels of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in the spleens of mice without altering their immunosuppressive function, while it remarkably decreased the cellularity of all lymphocyte subsets, indicating that the CD4$^+$CD25$^+$Foxp3$^+$Treg cells may be more resistant to the inhibiting effects of rapa than CD4$^+$CD25$^-$T cells. In addition, rapa markedly enhanced the percentages of CD4$^+$CD25$^+$Treg cells while keeping the cell number unchanged, but it significantly reduced the total cell numbers of all thymocyte subsets including CD4$^+$CD8$^-$, CD8$^+$CD4$^-$, CD4$^+$CD8$^+$ or CD4$^-$CD8$^-$ thymocytes. These data suggest that rapa has distinct effects on Treg and

non-Treg cells in the thymus and periphery. The insensitivity of CD4$^+$CD25$^+$Treg cells to rapa makes rapa to be a favorable choice for inducing immune tolerance to allo-, xeno- or self antigens.

Acknowledgements

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