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The effects of leflunomide on $CD4^+CD25^+Foxp3^+$ T regulatory cells in mice receiving allogeneic bone marrow transplantation

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Abstract
Objective and design Leflunomide (LEF) is effective not only in different animal models of autoimmune diseases and the therapy of patients with rheumatoid arthritisbut also in graft rejection. The effect of LEF on $CD4^+CD25^+$T regulatory cells (Treg) was determined in a mouse model of allogeneic bone marrow transplantation.
Material or subjects BALB/c and C57BL/6 mice were used as donors and recipients, respectively.
Treatment C57BL/6 mice were given 2 Gy total-body irradiation, followed by an intravenous injection of 2×10^7 BALB/c bone marrow cells (BMCs). Mice were treated with LEF daily at a dose of 30 mg/kg/day for 2 weeks.
Results In naïve mice, LEF significantly decreased the percentage of $CD4^+CD25^+$ Treg cells in spleens ($P < 0.05$), but not in lymph nodes, though LEF enhanced the percentages of $CD4^+CD25^+$ Treg cells in CD4 single positive cells in the thymocytes and blood ($P < 0.05$). Furthermore, LEF significantly decreased the percentage of $CD4^+CD25^+$ Treg cells in the spleens of mice that received allogeneic BMCs.
Conclusions LEF decreases peripheral $CD4^+CD25^+$ Treg cells in un-immunizing and immunizing recipients, indicating that LEF might not be an ideal candidate for the treatment of autoimmune diseases or graft rejection with respect to induction of immune tolerance.

Keywords $CD4^+CD25^+Foxp3^+$ Treg cells · Foxp3 · Leflunomide · Autoimmune diseases · Bone marrow transplantation

Abbreviations

Treg	Regulatory T cells
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
LNs	Lymph nodes
MFI	Median fluorescence intensity
PBMCs	Peripheral blood mononuclear cells
PE	Phycoerythrin
PI	Propidium iodide

Introduction

$CD4^+CD25^+$ T regulatory (Treg) cells are crucial for the maintenance of immunological tolerance to self and transplant antigens. It has been shown that $CD4^+CD25^+$ Treg cells regulate effector T cells, natural killer (NK) cells, B cells, macrophages and dendritic cells directly or indirectly via cell-to-cell contact or in a cytokine-dependent manner [1, 2]. Decreased ratios of $CD4^+CD25^+$ Treg cells to T effector cells (Teff) or impairment of their suppressive capacity are closely related to the occurrence of autoimmune diseases, such as insulin-dependent diabetes

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mellitus, autoimmune hemolytic anemia, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and experimental allergic encephalomyelitis [3–6]. Increasing Treg cell numbers or enhancing their suppressive activity may lead to the inhibition of autoimmunity and the induction of tolerance. Foxp3 is a recently identified transcription factor specifically expressed in $CD4^+CD25^+$ Treg cells [7–9]. Accumulating evidence has clearly demonstrated that the expression of Foxp3 is essential and sufficient for the development and immunosuppressive function of murine $CD4^+CD25^+$ Treg cells [10]. Allogeneic bone marrow transplantation (BMT) has been widely used as a potentially curative therapy for patients with a variety of diseases, including hematological disorders, congenital immunodeficiencies, metabolic disorders, autoimmune diseases and solid tumors, as well as for induction of transplant tolerance [11, 12]. However, the critical challenge to a successful allogeneic BMT is how to control graft-versus-host disease (GVHD), a major cause of post-transplantation morbidity and mortality. It has been reported that $CD4^+CD25^+$ Treg cells are able to suppress the progression and severity of GVHD by infiltrating GVHD target organs and inhibiting the function of Teff cells and other immune cells in the recipient, which is closely associated with the development of GVHD in allogeneic stem cell transplantation [13].

Immunosuppressive drugs such as cyclosporine A (CsA), tacrolimus (FK506) and rapamycin have been widely used for many years to prevent organ rejection after transplantation. Obviously, these immunosuppressive drugs function through distinct pathways to impact T-cell-related immunity. For example, rapamycin favors Treg expansion and survival by differentially regulating signaling, proliferation and sensitivity to apoptosis of human Teff cells and $CD4^+CD25^+$ Treg cells after TCR/IL-2 activation [14]. Leflunomide (LEF), a new disease-modifying antirheumatic drug of the isoxazol family, is clinically used in the treatment of rheumatoid arthritis, sarcoidosis, solid organ transplantation, lupus nephritis and the course of several autoimmune diseases [15–17]. Interestingly, LEF also exerts its activity against ganciclovir-resistant cytomegalovirus (CMV) infection, which is an emerging problem in transplant recipients [18–20]. LEF combined with sirolimus may be an effective therapy for BK virus reactivation, whose nephropathy is now the leading cause of early renal graft loss [21]. It also reported that LEF could be useful in prostate cancer chemoprevention and effective in reducing immune activation in the setting of chronic HIV-1 infection [22]. Molecular studies have revealed that LEF inhibits the enzymatic activity of both dihydro-orotate dehydrogenase [23, 24], an enzyme involved in pyrimidine biosynthesis, and protein tyrosine kinases, thus affecting multiple biological processes in different cell types, including T and B lymphocytes as well as non-lymphoid cells. LEF is also a potent inhibitor of NF-κB activation and is considered to be a potential agent for the treatment of acute pancreatitis [25]. In the present study, we evaluated the effect of LEF on $CD4^+CD25^+Foxp3^+$ Treg cells in mice with or without allogeneic bone marrow transplantation, and found that LEF treatment resulted in changes of $CD4^+CD25^+Foxp3^+$ Treg cells in $CD4^+$ T cells, and significantly decreased the ratios of $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells in spleens but not in lymph nodes (LNs), regardless of whether the recipients received BMT or not. Because of the poor induction of $CD4^+CD25^+$ Treg cells, LEF may not be ideal for the induction of immune tolerance.

Materials and methods

Mice

Six- to eight-week-old female BALB/c and C57BL/6 mice were obtained from the Institute of Genetics and Development, Chinese Academy of Sciences (Beijing, China). All mice were maintained in a specific pathogen-free facility and housed in microisolator cages containing sterilized food, autoclaved bedding and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals. For the allogeneic bone marrow transplantation, C57BL/6 mice were given 2 Gy total-body irradiation, followed by intravenous (i.v.) injection of 2×10^7 BALB/c bone marrow cells (BMCs).

Reagents

Leflunomide was obtained from Chang Zheng Pharmaceutical Inc. A 2 mg/ml solution of LEF was prepared in the vehicle containing 0.2% sodium CMC (C-5013 high viscosity, Sigma-Aldrich) and 0.25% polysorbate 80 dimethyl sulfoxide, and stored as small aliquots at 4°C. LEF was administered by gastric gavage each day at a dose of 30 mg/kg/day for 2 weeks. The solution containing 0.2% CMC and 0.25% polysorbate 80 was used for the control mice. At least three independent experiments were performed for each assay.

Monoclonal antibodies and reagents

The following monoclonal antibodies (mAbs) were purchased from BD Biosciences PharMingen (San Diego, CA, USA): 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

antimouse CD25 mAb (7D4; IgM), phycoerythrin (PE)-labeled rat anti-mouse CD4 mAb, PE-labeled anti-mouse CD8α mAb (53-6.7; rat IgG2a), Cy5-labeled anti-mouse CD25 mAb, and Cy5-labeled anti-mouse CD4 mAb. In addition, PE-labeled anti-mouse Foxp3 mAb (FJK-16 s) and its staining kit, FITC-labeled antimouse H2Dd mAb (mouse IgG2a), and PE-labeled anti-mouse H2Db mAb (mouse IgG2a), were obtained from eBiosciences (San Diego, CA, USA). Rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, MD, USA) in our laboratory.

Cell preparation

Mouse peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Sigma, St. Louis, MO, USA) gradient centrifugation as described previously [39]. After LEF treatment for 2 weeks, the 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 in RPMI1640 medium. Splenocytes were treated with a hemolysis buffer (17 mM Tris–HCl and 140 mM NH_4Cl, pH 7.2) to remove red blood cells before staining [26].

Immunofluorescence staining and flow cytometry

PBMCs, lymphocytes from LNs, splenocytes and thymocytes were incubated with the 2.4G2 mAb to block FcR-mediated non-specific staining and then incubated with an optimal concentration of fluorochrome-labeled mAbs for 30 min at 4°C in the dark. Cells were washed once and resuspended in the FCM buffer (PBS with 0.1% BSA and 0.1% NaN_3). At least 10,000 cells were assayed using a FASCalibur flow cytometer (Becton–Dickinson, Mountain View, CA, USA), and data were analyzed with CellQuest software (Becton–Dickinson). 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 was determined by subtracting the percentage of cells stained nonspecifically with the control mAb from those stained in the same dot-plot region with the anti-mouse mAbs. For intracellular Foxp3 staining, cells were first incubated with Cy5-labeled anti-CD4 and FITC-labeled anti-CD25 mAbs. After washing, these cells were then fixed and stained with anti-mouse Foxp3 mAb, according to the manufacturer's instruction (eBioscience).

Statistical analysis

All data are presented as mean ± SD. Student's unpaired *t* test for comparison of means was used to compare groups. $P < 0.05$ was considered to be statistically significant.

Results

Impact of LEF on $CD4^+$ T cell subsets in the peripheral blood of mice receiving BMT or not

C57BL/6 recipient mice were given 2 Gy irradiation, followed by i.v. injection of BALB/c bone marrow cells. The mice were then treated with LEF or vehicle solution by gastric gavage for 2 weeks, and the population and percentage of $CD4^+$ Teff and $CD4^+CD25^+Foxp3^+$ Treg cells in the peripheral blood were determined. There were clearly no donor lymphoid cells detected in the recipient's peripheral blood, peripheral lymphoid tissues and thymus by 2 weeks after BMT (data not shown). LEF treatment significantly enhanced the percentage of $CD4^+$ T cells and $CD8^+$ T cells in the peripheral blood of mice receiving BMT or not ($P < 0.01$ and $P < 0.001$, respectively, Fig. 1a). However, the ratio of $CD4^+CD25^+$ T cells and $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells was not significantly changed in the presence or absence of LEF, although it was slightly lower in the mice with BMT (Fig. 1b–d). Thus, LEF has no significant effect on the ratios of $CD4^+CD25^+$ T cells and $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells in the peripheral blood, suggesting that LEF has similar effects on the $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ Teff cells in the peripheral blood during the immune response.

Decreased percentages of $CD4^+CD25^+$ Treg cells in the spleens but not LNs of mice treated with LEF

There was no significant change in the size and mass of the spleens from both non-BMT and BMT mice after treatment with LEF (Fig. 2a and data not shown). The population of $CD4^+$ T cells and $CD8^+$ T cells in the LEF-treated spleens was significantly higher than controls ($P < 0.05$ or $P < 0.001$, Fig. 2b). However, the ratio of $CD4^+CD25^+Foxp3^+$ Treg cells or $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells was slightly but significantly lower in the spleens of both non-BMT and BMT mice after treatment with LEF ($P < 0.05$, Fig. 2b–e). Similar to the effect of LEF on the Teff cells and $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen, there was a minor change in the population of $CD4^+$ T cells in the LNs from BMT mice treated with LEF ($P < 0.05$, Fig. 3a), but no significant alteration in the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in LNs of mice treated with LEF, regardless of whether these mice received BMT or not (Fig. 3b, c).

On the other hand, the phenotype of $CD4^+CD25^+$ Treg cells, including GITR, CTLA-4 and Foxp3 expression, in these mice was determined using multi-color FCM. As shown in Fig. 4, the majorty of the gated $CD4^+CD25^+$ Treg cells express GITR, intracellular CTLA-4 and Foxp3 molecules, regardless of whether these cells were from

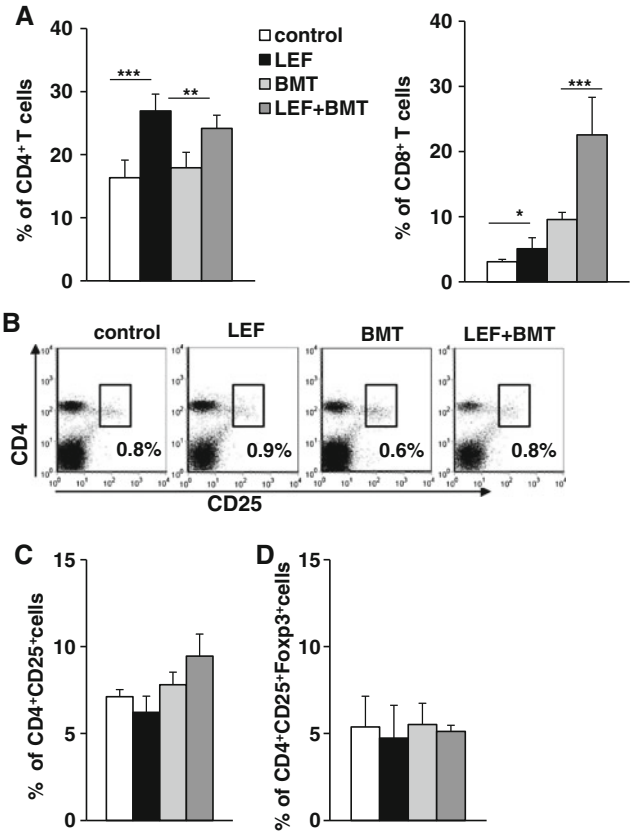

Fig. 1 Effect of LEF treatment on peripheral T cells. C57BL/6 mice were treated with LEF as described in "Materials and methods". After treatment with LEF for 2 weeks, PBMCs were stained with PE-labeled anti-CD4 mAb and FITC-labeled anti-CD25 mAb and assayed by FCM. **a** The percentages of T cell subsets including $CD4^+$ and $CD8^+$T cells in the control and LEF-treated mice. The percentages of $CD4^+CD25^+$ T cells (**b** and **c**) and $CD4^+CD25^+Foxp3^+$ T cells (**d**) in $CD4^+$ T cells in control and LEF-treated mice. Data are shown as mean ± SD ($N = 5$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the indicated groups. The data are one representative of three separate experiments

control, LEF, BMT or LEF + BMT treated mice, indicating that $CD4^+CD25^+$ Treg cells in LEF and/or BMT-treated mice display a normal Treg cell phenotype.

LEF treatment significantly enhanced the percentage of $CD4^+CD8^-CD25^+$ Treg cells in the thymus

We next evaluated the effect of LEF on $CD4^+CD25^+$ Treg cells in the thymus. Unlike the spleen and lymph nodes, LEF treatment resulted in shrinking of the thymus masses in both non-BMT and BMT mice. This observation was further confirmed by the decreased total number of thymocytes isolated from these mice (data not shown). Interestingly, the percentage of $CD4^-CD8^+$ T cells was significantly increased from 2.6 to 5.4% in the thymus of LEF-treated mice, particularly from non-BMT mice, whereas LEF only slightly altered the population of $CD4^+CD8^-$ T cells ($P < 0.001$, Fig. 5a–c). Furthermore, although the ratio of $CD4^+CD25^+$ Treg cells to $CD4^+$ T cells was significantly increased in the thymus of non-BMT mice after LEF treatment (from 2.96 to 5.45%, $P < 0.01$, Fig. 6a), there was no change observed in the thymus of BMT mice under the same condition (Fig. 6a). A similar result was also obtained for the ratio of $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells in the thymus (from 2.66 to 5.21%, $P < 0.001$, Fig. 6b). However, the total numbers of $CD4^+CD25^+Foxp3^+$ Treg cells were significantly reduced, due to the marked decrease in total thymocyte number in LEF-treated mice without BMT ($P < 0.05$, Fig. 6c, d).

Discussion

$CD4^+CD25^+Foxp3^+$ Treg cells are pivotal for the maintenance of self-tolerance in hosts [27]. The balance between Teff cells and $CD4^+CD25^+Foxp3^+$ Treg cells principally controls the direction and quality of the host immune responses. Recent studies have revealed that $CD4^+CD25^+Foxp3^+$ Treg cells, especially expressing CD62L, are involved in transplantation tolerance and play an important role in preventing GVHD and graft rejection, and that adoptive transfer of Treg cells prevents GVHD in the absence of any post-transplant immunosuppressive therapies [28–31]. Clinical applications require higher numbers of $CD4^+CD25^+Foxp3^+$ Treg cells. Interestingly, it has been reported that rapamycin could contribute to the development of transplantation tolerance by promoting the induction of functional $CD4^+CD25^+Foxp3^+$ Treg cells [32]. In this study, we investigated the effects of LEF on immune cells in a BMT animal model. Surprisingly, we found that the impact of LEF on $CD4^+$ T subset cells was different in the immune tissues we examined (PBL, spleens and LNs). Firstly, LEF treatment increased the percentage of $CD4^+$ T cells in the peripheral lymphocytes of LEF-treated C57BL/6 mice. Secondly, the thymus masses in both non-BMT and BMT mice were decreased after treatment with LEF. As a result, the total number of thymocytes in LEF-treated mice was dramatically reduced. Interestingly, the percentages of $CD4^+CD25^+$ Treg cells and $CD4^+CD25^+Foxp3^+$ Treg cells were significantly higher in the thymus of LEF-treated mice than in those of control mice. Thirdly, the ratios of $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells were significantly decreased in the spleens but not in PBLs and LNs after LEF treatment.

In the transplant setting, alloreactive T cells are crucial in the initiation of a rejection response. It has been known that alloantigen-specific $CD4^+CD25^+Foxp3^+$ Treg cells arising in vivo both from naturally occurring $CD4^+CD25^+Foxp3^+$ Treg cells and from $CD4^+$ T cells can suppress the acute and chronic rejection of donor

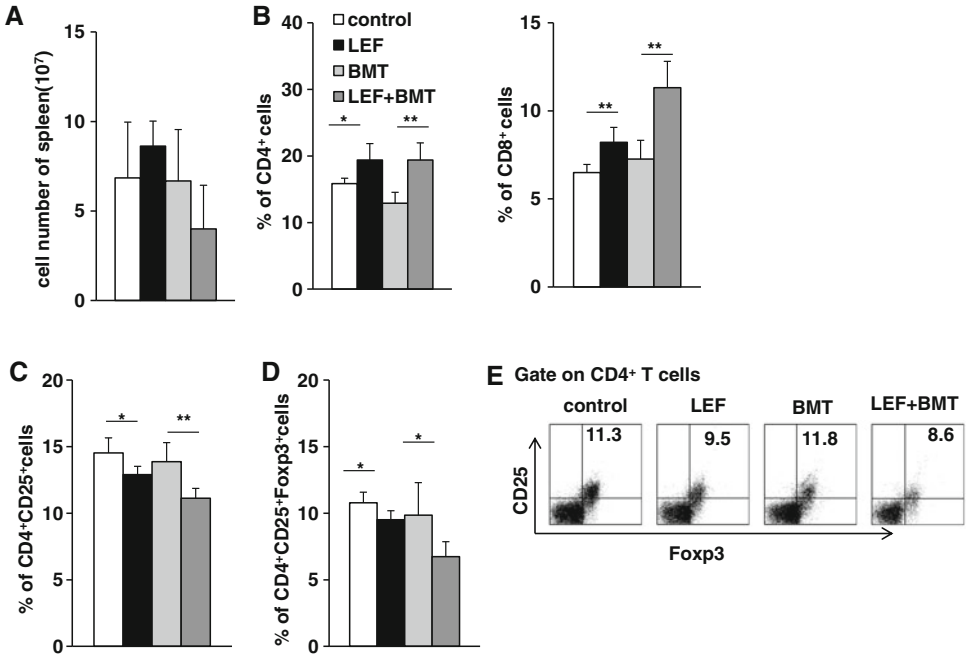

Fig. 2 Enhanced CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in the spleen of mice treated with or without LEF. The cells in the spleens of control and LEF-treated mice were analyzed for the percentages of CD4$^+$ T and CD4$^+$CD25$^+$Foxp3$^+$ Treg cells by FCM. **a** The total cell number of spleen in mice treated with LEF and/or BMT. The proportion of CD4$^+$ T cells (**b**), the ratio of CD4$^+$CD25$^+$ T cells to CD4$^+$ T cells (**c**), and the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in CD4$^+$ T cells (**d**) in the spleen of control and LEF-treated mice were shown. **e** One representative of staining for Foxp3 and CD25 in gated CD4$^+$ cells. Data are presented as mean ± SD ($N = 5$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the indicated control mice. The data are one representative of three separated experiments

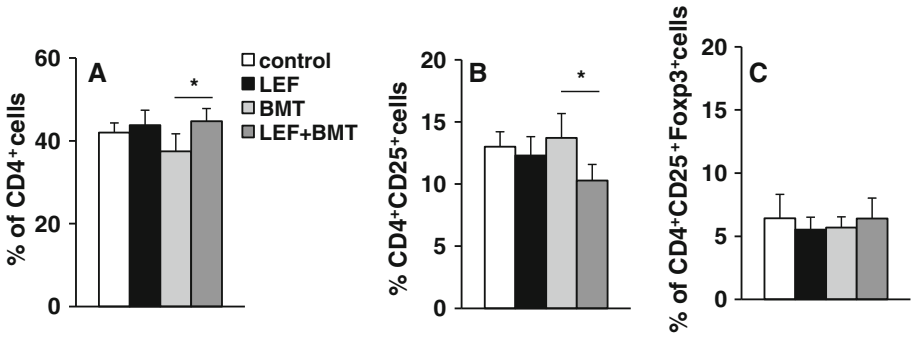

Fig. 3 Percentages of different T cells in the LNs of mice treated with or without LEF. The cells in the LNs of control or LEF-treated mice were analyzed for the expression of CD4$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ Treg cells by FCM. The proportion of CD4$^+$ T cells (**a**), the ratio of CD4$^+$CD25$^+$ T cells to CD4$^+$ T cells (**b**), and the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in CD4$^+$ T cells (**c**) in the LNs of control and LEF-treated mice are shown. Data are shown as mean ± SD ($N = 5$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the indicated groups. The data are one representative of three separated experiments

allografts mediated by recipient CD4$^+$ or CD8$^+$ T cells [33, 34]. It is reported that CD4$^+$CD25$^+$Foxp3$^+$ Treg cells might also play a important role in the protection of allografts from ischemia/reperfusion injury (IRI) [35]. Thus, the ideal immunosuppressive drug for the treatment of autoimmune diseases or a graft reaction should have a strong inhibiting ability towards Teff cells, but would also be able to promote CD4$^+$CD25$^+$Foxp3$^+$ Treg cells [36]. Obviously, not all conventional immunosuppressive drugs currently used in the clinic meet this standard. For example, FK506 and CsA target calcineurin, which is involved in a common step associated with T-cell activation and IL-2 induction, which are also found to affect other immune cells such as B cells and antigen-presenting cells such as dendritic cells (DCs) [37–40]. CsA has different effects on the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in the central and peripheral immune tissues. CsA markedly reduced the percentage and function of

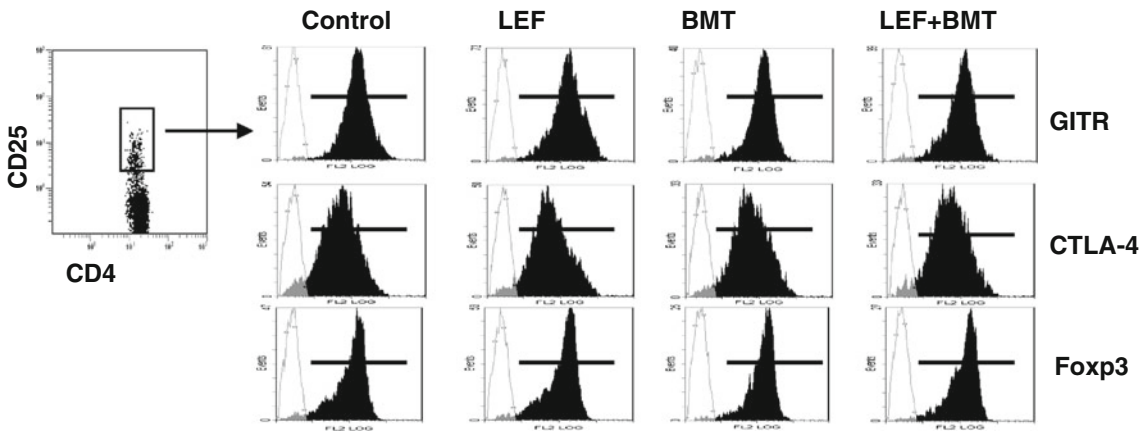

Fig. 4 The expression of GITR, CTLA-4 and Foxp3 molecules in $CD4^+CD25^+$ Treg cells in LEF-treated mice. Splenocytes from control and LEF-treated mice were stained with anti-CD4 and anti-CD25 mAbs and washed. The cells were then stained with anti-GITR mAb or fixed and stained with anti-CTLA-4 and Foxp3 mAbs as described in "Materials and methods". One representative of the gated $CD4^+CD25^+$ cells was shown. More than four mice in each group showing identical results were studied

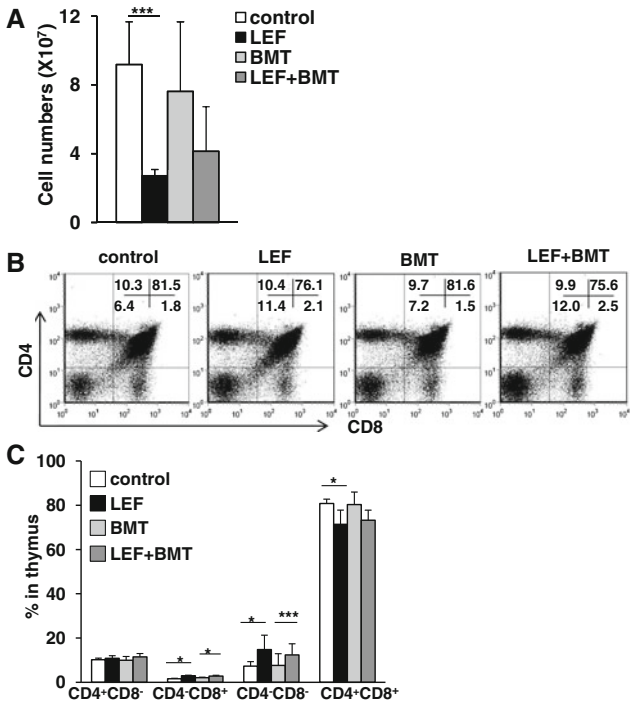

Fig. 5 T-cell subtypes in the thymus of LEF-treated mice. Thymocytes were stained with PE-Cy5-labeled anti-CD4 mAb, PE-labeled anti-CD25 mAb, and FITC-labeled anti-CD8 mAb, and then analyzed by FCM after injection of LEF for 2 weeks. **a** The total cell numbers in the thymus of mice with different treatments. **b** One representative for the staining with Cy5-labeled anti-CD4 mAb and PE-labeled anti-CD8 mAb is shown. **c** The percentages of different thymocyte subsets in the thymus mice treated with LEF or not. $*P < 0.05$; $**P < 0.01$ compared with control mice. Results were shown as mean ± SD ($N = 5$), which is one representative of three independent experiments

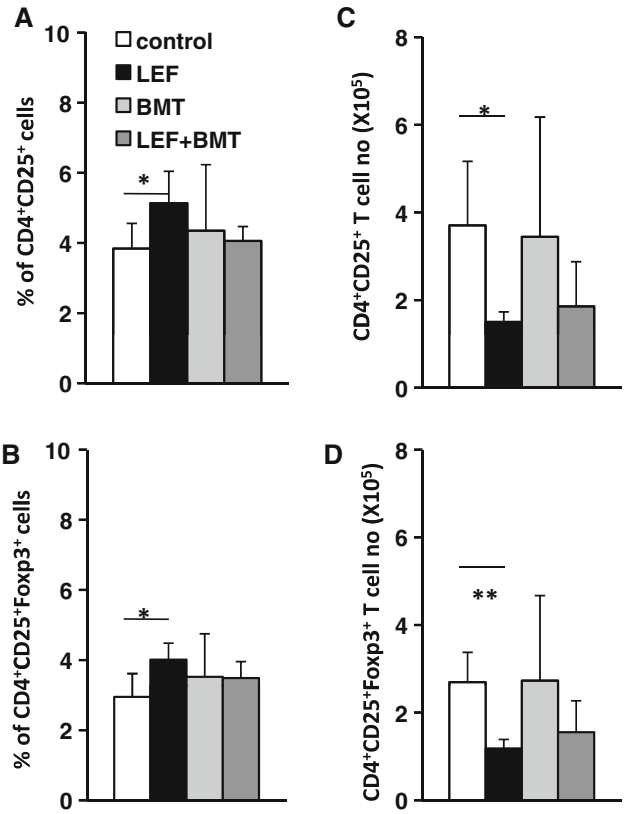

Fig. 6 The enhanced percentages of $CD4^+CD25^+$Treg cells in the thymus of LEF-treated mice. Thymocytes were stained with PE-Cy5-labeled anti-CD4 mAb, PE-labeled anti-CD25 mAb, and FITC-labeled anti-CD8 mAb, and analyzed by FCM after injection of LEF for 2 weeks. The percentages of $CD4^+CD25^+$ T cells (**a**) and $CD4^+CD25^+Foxp3^+$ Treg cells (**b**) in $CD4^+CD8^-$ T cells in mice treated with LEF or not are summarized. The cell numbers of $CD4^+CD25^+$ T cells (**c**) and $CD4^+CD25^+Foxp3^+$ Treg cells (**d**) in mice treated with LEF or not are summarized. $*P < 0.05$; $*P < 0.01$ compared with the indicated groups. Results were shown as mean ± SD ($N = 5$), which is one representative of three independent experiments

$CD4^+CD25^+Foxp3^+$ Treg cells in peripheral tissues while significantly enhancing the percentages of $CD4^+CD28^-Foxp3^+$ Treg cells in the thymus in mice [41]. Rapamycin is a novel macrolide immunosuppressive drug which has been widely used in preventing clinical allograft rejection and in some autoimmune diseases [42, 43]. Rapamycin binds to FKBP12 and the complex formed inhibits the function of the mammalian target of rapamycin, which in turn reduces protein phosphorylation and cell cycle progression [44]. In rapamycin-treated mice, the ratio of $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells was significantly enhanced after rapamycin treatment [45].

In our study, we observed that the effects of LEF on $CD4^+CD25^+Foxp3^+$ Treg cells in different tissues were somewhat distinct. The differential effects of LEF on $CD4^+CD25^+Foxp3^+$ Treg cells in different tissues might be due to different tissue and cellular responses to the treatment of LEF. Our data collectively indicated that $CD4^+CD25^+Foxp3^+$ Treg cells were resistant to LEF in the thymus and were sensitive to LEF in the spleen, compared to $CD4^+CD25^-$ cells. It has been reported that LEF promoted the differentiation of spleen lymphocytes into $CD4^+CD25^+Foxp3^+$ Treg cells in collagen-induced arthritis (CIA) rats [46]. Although LEF also could suppress contact hypersensitivity (CHS) and adoptive transfer of leukocytes from LEF-treated mice into naïve mice resulted in a loss of CHS responsiveness, Foxp3 expression in $CD4^+$ T cells was not enhanced after LEF treatment [47]. It was also reported that LEF was a powerful agent for preventing GVHD in rats [48]. The reasons for the different response of $CD4^+CD25^+Foxp3^+$ Treg cells in different tissues or in different animal models are unclear at present, but are possibly related to different doses and treatment time courses of LEF, different tissues and different animal models. The nonsignificant changes of Treg cells caused by LEF indicate that LEF may not be ideal for tolerance induction, althoug the anti-inflammatory effects of LEF may still be helpful for tolerance induction directly.

In summary, differential changes of $CD4^+CD25^+Foxp3^+$ Treg cells in different peripheral immune tissues or organs were observed after LEF treatment in mice with and without BMT. Although LEF enhanced the ratio of $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells in the thymus, it reduced the ratio in the spleen, and there was little change in the ratio of $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells in the peripheral blood and LNs. In any case, LEF failed to induce $CD4^+CD25^+Foxp3^+$ Treg cells in naïve or BMT mice, indicating it may not be an ideal choice for the purpose of immune tolerance induction.

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References

1. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol. 2005;6:345–52.
2. Billiard F, Litvinova E, Saadoun D, Djelti F, Klatzmann D, Cohen JL, Marodon G, Salomon BL. Regulatory and effector T cell activation levels are prime determinants of in vivo immune regulation. J Immunol. 2006;177:2167–74.
3. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, Nochy D, Debre P, Piette JC, Gorochov G. Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol. 2005;175:8392–400.
4. Paust S, Cantor H. Regulatory T cells and autoimmune disease. Immunol Rev. 2005;204:195–207.
5. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes. 2005;54:92–9.
6. Gartner D, Hoff H, Gimsa U, Burmester GR, Brunner-Weinzierl MC. CD25 regulatory T cells determine secondary but not primary remission in EAE: impact on long-term disease progression. J Neuroimmunol. 2006;172:73–84.
7. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003;4:330–6.
8. Sakaguchi S. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. J Clin Invest. 2003;112:1310–2.
9. Zhang L, Yi H, Xia XP, Zhao Y. Transforming growth factor-beta: an important role in CD4+CD25+ regulatory T cells and immune tolerance. Autoimmunity. 2006;39:269–76.
10. Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. Nat Rev Immunol. 2007;7:305–10.
11. Ikehara S. Bone marrow transplantation: a new strategy for intractable diseases. Drugs Today (Barc). 2002;38:103–11.
12. Nakagawa T, Nagata N, Hosaka N, Ogawa R, Nakamura K, Ikehara S. Prevention of autoimmune inflammatory polyarthritis in male New Zealand black/KN mice by transplantation of bone marrow cells plus bone (stromal cells). Arthritis Rheum. 1993;36:263–8.
13. Rezvani K, Mielke S, Ahmadzadeh M, Kilical Y, Savani BN, Zeilah J, Keyvanfar K, Montero A, Hensel N, Kurlander R, Barrett AJ. High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. Blood. 2006;108:1291–7.
14. Strauss L, Czystowska M, Szajnik M, Mandapathil M, Whiteside TL. Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. PLoS One. 2009;4:e5994.
15. Schattenkirchner M. The use of leflunomide in the treatment of rheumatoid arthritis: an experimental and clinical review. Immunopharmacology. 2000;47:291–8.
16. Johansen A, Jensen JE, Horn HC, Nielsen B, Kemp E. Effect of leflunomide and cyclosporine on graft survival and changes in lymphocyte phenotypes in a rat heart allotransplantation model. Transpl Immunol. 1998;6:48–52.
17. He C, Lu X, Yan Z, Wu M, Liu S, Yu Y, Luo P (2010) Therapeutic effect of leflunomide on the development of experimental lupus nephritis in mice. Rheumatol Int [Epub ahead of print] doi: 10.1007/s00296-010-1630-z

18. Snydman DR. Leflunomide: a small step forward in meeting the urgent need for treatment of drug-resistant cytomegalovirus infection. Transplantation. 2010;90:362–3.
19. Orden AO, Chuluyan JC, Colombini AC, Barbera RF (2011) Leflunomide use in a cytomegalovirus infection of a patient with dermatomyositis. Rheumatol Int [Epub ahead of print] doi: 10.1007/s00296-010-1786-6
20. Avery RK, Mossad SB, Poggio E, Lard M, Budev M, Bolwell B, Waldman WJ, Braun W, Mawhorter SD, Fatica R, Krishnamurthi V, Young JB, Shrestha R, Stephany B, Lurain N, Yen-Lieberman B. Utility of leflunomide in the treatment of complex cytomegalovirus syndromes. Transplantation. 2010;90:419–26.
21. Liacini A, Seamone ME, Muruve DA, Tibbles LA. Anti-BK virus mechanisms of sirolimus and leflunomide alone and in combination: toward a new therapy for BK virus infection. Transplantation. 2010;90:1450–7.
22. Hail N Jr, Chen P, Bushman LR. Teriflunomide (leflunomide) promotes cytostatic, antioxidant, and apoptotic effects in transformed prostate epithelial cells: evidence supporting a role for teriflunomide in prostate cancer chemoprevention. Neoplasia. 2010;12:464–75.
23. Xu X, Williams JW, Bremer EG, Finnegan A, Chong AS. Inhibition of protein tyrosine phosphorylation in T cells by a novel immunosuppressive agent, leflunomide. J Biol Chem. 1995;270:12398–403.
24. Williamson RA, Yea CM, Robson PA, Curnock AP, Gadher S, Hambleton AB, Woodward K, Bruneau JM, Hambleton P, Moss D, Thomson TA, Spinella-Jaegle S, Morand P, Courtin O, Sautes C, Westwood R, Hercend T, Kuo EA, Ruuth E. Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. J Biol Chem. 1995;270:22467–72.
25. Kutluana U, Oruc N, Nart D, Kaptanoglu B, Yonetci N, Ozutemiz O. Leflunomide: is a new oral agent in treatment of acute pancreatitis? Pancreas. 2010;39:237–42.
26. Sun Z, Zhao L, Wang H, Sun L, Yi H, Zhao Y. Presence of functional mouse regulatory CD4+CD25+ T cells in xenogeneic neonatal porcine thymus-grafted athymic mice. Am J Transplant. 2006;6:2841–50.
27. Li XC, Rothstein DM, Sayegh MH. Costimulatory pathways in transplantation: challenges and new developments. Immunol Rev. 2009;229:271–93.
28. Giorgini A, Noble A. Blockade of chronic graft-versus-host disease by alloantigen-induced CD4+CD25+Foxp3+ regulatory T cells in nonlymphopenic hosts. J Leukoc Biol. 2007;82:1053–61.
29. Velasquez-Lopera MM, Eaton VL, Lerret NM, Correa LA, Decresce RP, Garcia LF, Jaramillo A. Induction of transplantation tolerance by allogeneic donor-derived CD4(+)CD25(+)Foxp3(+) regulatory T cells. Transpl Immunol. 2008;19:127–35.
30. Edinger M. Regulatory T cells for the prevention of graft-versus-host disease: professionals defeat amateurs. Eur J Immunol. 2009;39:2966–8.
31. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, Del Papa B, Zei T, Ostini RI, Cecchini D, Aloisi T, Perruccio K, Ruggeri L, Balucani C, Pierini A, Sportoletti P, Aristei C, Falini B, Reisner Y, Velardi A, Aversa F, Martelli MF. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood. 2011;117:3921–8.
32. Bocian K, Borysowski J, Wierzbicki P, Wyzgal J, Klosowska D, Bialoszewska A, Paczek L, Gorski A, Korczak-Kowalska G. Rapamycin, unlike cyclosporine A, enhances suppressive functions of in vitro-induced CD4+CD25+ Tregs. Nephrol Dial Transpl. 2010;25:710–7.
33. Francis RS, Feng G, Tha-In T, Lyons IS, Wood KJ, Bushell A. Induction of transplantation tolerance converts potential effector T cells into graft-protective regulatory T cells. Eur J Immunol. 2011;41:726–38.
34. Semple K, Yu Y, Wang D, Anasetti C, Yu XZ. Efficient and selective prevention of GVHD by antigen-specific induced Tregs via linked-suppression in mice. Biol Blood Marrow Transpl. 2011;17:309–18.
35. Kinsey GR, Sharma R, Huang L, Li L, Vergis AL, Ye H, Ju ST, Okusa MD. Regulatory T cells suppress innate immunity in kidney ischemia-reperfusion injury. J Am Soc Nephrol. 2009;20:1744–53.
36. Zhao Y. The different effects of cyclosporin A and rapamycin on regulatory CD4+CD25+ T cells: potential relationship with transplant tolerance induction. Expert Rev Clin Immunol. 2007;3:245–9.
37. Friedman J, Weissman I. Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. Cell. 1991;66:799–806.
38. Tajima K, Amakawa R, Ito T, Miyaji M, Takebayashi M, Fukuhara S. Immunomodulatory effects of cyclosporin A on human peripheral blood dendritic cell subsets. Immunology. 2003;108:321–8.
39. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, Negrin RS. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. Blood. 2006;108:390–9.
40. Vincenti F, Jensik SC, Filo RS, Miller J, Pirsch J. A long-term comparison of tacrolimus (FK506) and cyclosporine in kidney transplantation: evidence for improved allograft survival at five years. Transplantation. 2002;73:775–82.
41. Wang H, Zhao L, Sun Z, Sun L, Zhang B, Zhao Y. A potential side effect of cyclosporin A: inhibition of CD4(+)CD25(+) regulatory T cells in mice. Transplantation. 2006;82:1484–92.
42. Antin JH, Kim HT, Cutler C, Ho VT, Lee SJ, Miklos DB, Hochberg EP, Wu CJ, Alyea EP, Soiffer RJ. Sirolimus, tacrolimus, and low-dose methotrexate for graft-versus-host disease prophylaxis in mismatched related donor or unrelated donor transplantation. Blood. 2003;102:1601–5.
43. Busca A, Locatelli F, Moscato D, Falda M. Sirolimus-related toxicity in stem cell transplantation. Biol Blood Marrow Transpl. 2005;11:647–9.
44. Breslin EM, White PC, Shore AM, Clement M, Brennan P. LY294002 and rapamycin co-operate to inhibit T-cell proliferation. Br J Pharmacol. 2005;144:791–800.
45. Qu Y, Zhang B, Zhao L, Liu G, Ma H, Rao E, Zeng C, Zhao Y. The effect of immunosuppressive drug rapamycin on regulatory CD4+CD25+Foxp3+ T cells in mice. Transpl Immunol. 2007;17:153–61.
46. Wang TY, Li J, Li CY, Jin Y, Lu XW, Wang XH, Zhou Q. Leflunomide induces immunosuppression in collagen-induced arthritis rats by upregulating CD4+CD25+ regulatory T cells. Can J Physiol Pharmacol. 2010;88:45–53.
47. Weigmann B, Jarman ER, Sudowe S, Bros M, Knop J, Reske-Kunz AB. Induction of regulatory T cells by leflunomide in a murine model of contact allergen sensitivity. J Invest Dermatol. 2006;126:1524–33.
48. Mrowka C, Thoenes GH, Langer KH, Bartlett RR. Prevention of the acute graft-versus-host disease (GVHD) in rats by the immunomodulating drug leflunomide. Ann Hematol. 1994;68:195–9.