# FK506 attenuates thymic output in patients with myasthenia gravis

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## Abstract

**Introduction:** Myasthenia gravis (MG) is an antibody-mediated, T-cell-dependent autoimmune disease. The symptoms are caused by high-affinity IgG against the muscle acetylcholine receptor (AChR) at the neuromuscular junction. The production of these antibodies in B-cells depends on AChR-specific $CD4^+$ T-cells and the thymus gland seems to play a significant role in the pathogenesis of MG. Altered thymic T-cell export seems to be associated with a pathological mechanism in myasthenia gravis. Tacrolimus (FK506) has recently been used to treat MG.
**Material and methods:** We examined the effects of tacrolimus on thymic T-cell export in patients with MG. Sixteen patients with nonthymomatous and/or thymectomized MG were treated with oral administrations of tacrolimus. To assess the effect of tacrolimus on the thymic output, we assayed the levels of T-cell receptor excision circle (TREC), a molecular marker of thymus emigrants.
**Results:** T-cell receptor excision circle was not significantly different from those in age-matched controls before tacrolimus therapy, but they were partially decreased 4 months after tacrolimus therapy. T-cell receptor excision circle levels were significantly decreased in the thymomatous group ($p < 0.05$), but not in the nonthymomatous group. Tacrolimus treatment significantly attenuated TREC levels in cultured $CD4^-CD8^+$ cells ($p < 0.05$), but total cell counts were not significantly changed.
**Conclusions:** These results indicate that TREC levels may become a marker of the curative effect of tacrolimus therapy for thymomatous MG, and that tacrolimus suppresses not only activating T-lymphocytes, but also naïve T-cells.

**Key words:** T cell receptor excision circle, T-cell receptor excision circle, lymphocytes, cell culture.

## Introduction

Myasthenia gravis (MG) is an antibody-mediated, T-cell-dependent autoimmune disease. The symptoms are caused by high-affinity IgG against the muscle acetylcholine receptor (AChR) at the neuromuscular junction [1–3]. The production of these antibodies in B-cells depends on AChR-specific $CD4^+$ T-cells [4, 5]. The thymus gland seems to play a significant role in the pathogenesis of MG. Thymic abnormalities exist in a high proportion (approximately 75%) of myasthenic patients [1]. Of these patients, 85% have hyperplasia and the rest thymomas [1]. Although no pathological basis for the association between thymomas and an autoimmune

mechanism has been identified, some evidence suggests that myasthenic patients with thymoma harbor different immunoregulatory abnormalities from those without it [6, 7]. Recent evidence indicates that lymphocyte composition of peripheral blood differs between patients with thymomatous MG and those with nonthymomatous MG [1, 3, 7]. Thymoma is the only tumor proven to generate mature T-cells from immature precursors. In thymomatous MG, mature T-cells with autoreactive activities may leave the thymus for the bloodstream and persist in the periphery for more than 10 years [6, 8]. Phenotypic and T-cell receptor excision circle (TREC) analysis confirmed a thymic origin of the expanded naïve T-cell subset. An analysis of the T-cell receptor repertoire showed the reconstitution of an overall broader clonal diversity, and TREC was used as a marker of thymic output. T-cell receptor excision circle levels were significantly raised in peripheral lymphocytes in thymomatous MG patients and decreased after thymectomy [6, 8, 9]. Recent observations indicate that altered thymic T-cell export may be associated with a pathological mechanism in some autoimmune diseases [10–17].

During the past two decades, the outlook for MG patients has improved dramatically as a result of advances in treatment. The most important methods used in the treatment of MG include anticholinesterase agents, thymectomy, immunosuppression with glucocorticoids, and plasma exchange [1]. Tacrolimus (FK506) has recently been used to treat MG [13–17]. It is an immunosuppressive agent similar to cyclosporin A that inhibits the action of calcineurin, a serine/threonine phosphatase, thereby suppressing interleukin-2 production [18, 19] and T-cell proliferation [20]. We herein report the effects of tacrolimus on thymic T-cell export in patients with MG.

## Material and methods

### Clinical assessment

The subjects of this study were 16 patients with myasthenia gravis. All were selected from Japanese patients who had been treated at Tokushima University Hospital from April 2006 to January 2008 and who agreed to participate in the study. The 16 patients fulfilled the following criteria at pretreatment: (1) a muscle weakness in ocular and limb muscle groups that was aggravated by exercise and relieved by rest; (2) a significant rise in anti-AChR titers; (3) a significant decrement (over 10%) on repetitive nerve stimulation; (4) a positive edrophonium chloride test; (5) a negative history of administration with penicillamine. The classification of MG was performed according to Myasthemia Gravis Foundation of America (MGFA) [21], and all cases were classified into types I, II, or III. Twelve patients underwent extended thymectomy with a transsternal approach. Thymoma was found in 6 patients, hyperplasia in two, and the examination was unremarkable in the others. Invasive thymoma was not found in the present patients. All patients received oral administrations of tacrolimus (3 mg/day; 1 mg was taken three times a day). Other therapies, such as anticholinesterase and corticosteroids, remained unchanged during the tacrolimus therapy. Seven patients received corticosteroid therapy. The patients were evaluated 2 and 4 months after treatment by measurement of the quantified myasthenia gravis disability score (MG score) ranging from 0 to 39 [22] and an anti-acetylcholine receptor (AchR) antibody titer. For measurement of the MG score, muscle strength of all the patients was assessed by a neurologist who was blinded to their clinical information. Laboratory examinations were also performed before and after treatment, including peripheral blood cell count, transaminases, cholesterol, and blood sugar. Plasma concentrations of tacrolimus were assayed 2 and 4 months after the therapy.

Control samples were obtained from 12 age-matched volunteers (5 men and 7 women). Their age was 60.5 ±5.2 years (mean ± SE). They did not have diseases except hypertension or family history of neuromuscular disorders. After obtaining approval from the Tokushima University Hospital Ethics Committee following their ethical consideration, we gave an oral explanation about Vague, and written agreement was obtained.

### Separation of $CD4^+$ and $CD8^+$ cell populations

Separation of $CD4^+$ and $CD8^+$ cell populations was carried out according to the previous report [6]. In brief, mononuclear cells were separated from peripheral blood using Lymphoprep (Nycomed, UK). Magnetic cell-sorting (MACS®) (Miltenyi-Biotec, Surrey, UK) columns were used as per the manufacturer's protocol to positively select $CD8^+$ and then $CD4^+$ lymphocytes so that any double positive $CD4^+/CD8^+$ cells were included in the $CD8^+$ fraction. After the two step of affinity columns, the lymphocytes were classified in four fractions: $CD4^+/CD8^+$, $CD4^-/CD8^+$, $CD4^+/CD8^-$, and $CD4^-/CD8^-$. The cells were counted using a hemocytometer. Some cells were used for primary culture, and the remaining cells were stored at –80°C until PCR analysis.

### Quantification of T-cell receptor excision circle (TREC) DNA

Genomic DNA was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For quantification of TRECs, we used a Light Cycler PCR and detection system (Roche Diagnostics, Mannheim, Germany)

according to the previous report [23]. Primers (5'-CTC TCC AAG GCA AAA TGG G, 5'-GTG ACA TGG AGG GCT GAA C) were used to detect a defined region of the δRec-ψJα signal joint TREC, which is generated after the TCR-$^{L}$ gene rearrangement. For the amplicon detection, the Light Cycler DNA Fast-Start Master Hybridization Probes Kit was used. Two different oligonucleotides were hybridized to a specific sequence of the δRec-ψJα signal joint TREC. One probe was labeled at the 5'-end with the Light Cycler-Red 640 fluorophore (5'-AAA ACC AGA GGT GTC AGC ATG GT p), the other at the 3'-end with fluorescein (5'-CCA CAG GAG TGG GCA CCT TTA C X). The polymerase chain reaction (PCR) was run under standard conditions with 5 mM magnesium chloride and 12.5 pmol primers performing an initial 9 min at 95°C for FastStart Taq activation followed by 45 cycles of repeated denaturation (1 s at 95°C), annealing (15 s at 57°C) and enzymatic chain extension (25 s at 72°C). External standardization was performed using a plasmid containing the δRec-ψJα signal joint TREC break point. Concentrations of δRec-ψJα signal joint TREC DNA in the blood specimens were calculated comparing the cycle numbers of the log-linear phase of the samples with the cycle numbers of the external standards. All samples were run in duplicate and if there was greater than 10% discrepancy between duplicates, the assay was repeated. No-template controls were always included and never produced measurable fluorescence. T-cell receptor excision circle levels are expressed as TREC DNA copies per microgram of DNA. Cell lysates were checked for consistency of DNA content using β-actin PCR; interassay variability was found to be less than 10% of the mean for the same sample in different assays.

### Mononuclear cell culture

Single-positive cells ($CD4^+CD8^-$ and $CD4^-CD8^+$ cells) were resuspended in RPMI 1640 containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin, according to the previous report [24]. The cells were incubated for 72 h in 5% $CO_2$/air at 37°C in a humidified chamber in the presence (10 ng/ml and 1000 ng/ml) or absence of FK506. The cell count was measured with a hemocytometer prior to plating cells, and 72 h after FK506 treatment.

### Statistical analysis

We used StatView for Windows (version 5.0) for statistical analysis. Nonpaired data and paired data were analyzed by the nonparametric Mann-Whitney *U* test and Wilcoxon's signed rank test, respectively.

## Results

This study included 16 patients (Table I). The ages ranged from 33 to 84 years, and the mean (± SE) age was 63.6 (± 3.2) years. Disease duration was from 2 to 33 years, and mean duration was 14.1 (± 10.0) years. Figure 1 shows the profiles of titers

**Table I.** Summary of patients

| Patient | Age | Gender | MGFA | Duration [years] | PSL [mg/dl] | AChR Ab [nmol/l] | Thymectomy | Thymoma |
|---|---|---|---|---|---|---|---|---|
| 1 | 63 | M | IIA | 4 | 7.5 | 1.1 | | |
| 2 | 64 | F | IIA | 6 | | 24 | + | |
| 3 | 60 | F | IIA | 31 | 7.5 | 77 | + | + |
| 4 | 61 | M | I | 17 | | 0.3 | | |
| 5 | 81 | F | IIIA | 31 | 10 | 8.2 | + | |
| 6 | 63 | M | IIA | 10 | | 24 | + | |
| 7 | 72 | F | IIA | 33 | | 10 | + | + |
| 8 | 67 | F | IIA | 6 | 15 | 4.2 | + | + |
| 9 | 72 | F | IIIA | 20 | | 480 | + | |
| 10 | 47 | F | IIA | 10 | | 17 | + | |
| 11 | 65 | F | IIA | 19 | 7.5 | 21 | + | |
| 12 | 48 | F | IIIA | 8 | 2.5 | 1.8 | + | + |
| 13 | 74 | F | IIB | 10 | 7.5 | 22 | | |
| 14 | 33 | F | IIB | 3 | | 120 | | |
| 15 | 84 | M | IIA | 12 | | 40 | + | + |
| 16 | 63 | M | IIA | 7 | | 65 | + | + |

*MGFA – myasthenia gravis foundation of America, PSL – prednisolone, AChR Ab – acetylcholine receptor antibody*

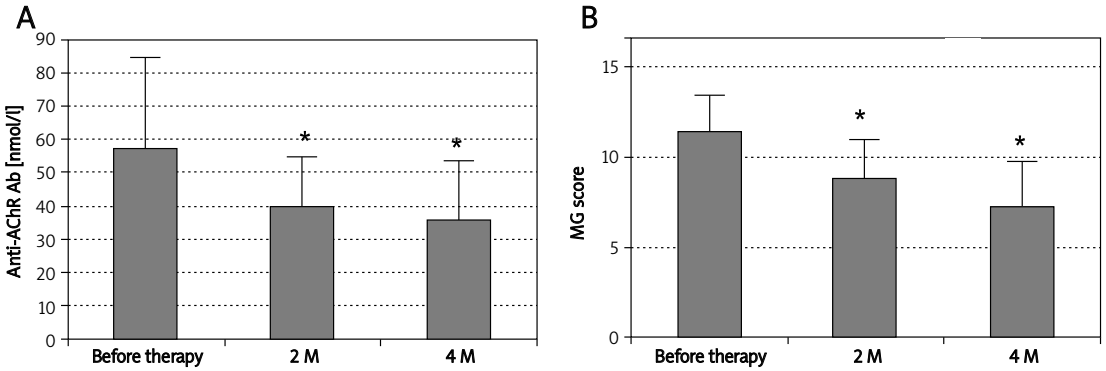


**Figure 1.** Titers of antiacetylcholine receptor (anti-AChR) antibody (**A**) and MG (**B**) scores in patients with myasthenia gravis 2 months (2 M) and 4 months (4 M) after oral administration of tacrolimus (3 mg/day). After tacrolimus therapy, titers of anti-AChR and MG scores significantly improved
**p < 0.05*

of anti-AChR antibody and MG scores. Titers of anti-AChR antibody (0 M, 57.23 ±29.3; 2 M, 39.5 ±18.3; 4 M, 35.8 ±17.7 (mean ± SE)) and MG scores (0 M, 11.7 ±1.9; 2 M, 8.2 ±2.1; 4 M, 7.2 ±2.3 (mean ± SE)) were significantly decreased after tacrolimus therapy ($p < 0.05$).

We assessed TREC levels in single double-positive cells ($CD4^+CD8^+$), positive cells ($CD4^+CD8^-$ and $CD4^-CD8^+$ cells), and double-negative cells ($CD4^-CD8^-$). Control samples were obtained from age-matched volunteers ($n = 12$). As shown in Figure 2 A, the TREC levels of MG patients (CD4+CD8+, 1515 ±436 copy/µg DNA; $CD4^+CD8^-$, 1725 ±515 copy/µg DNA; $CD4^-CD8^+$, 2322 ±772 copy/µg DNA; $CD4^-CD8^-$, 813 ±230 copy/µg DNA) were not significantly different from those of controls ($CD4^+CD8^+$, 1585 ±351 copy/µg DNA; $CD4^+CD8^-$ 1488 ±511 copy/µg DNA; $CD4^-CD8^+$, 1634 ±776 copy/µg DNA; $CD4^-CD8^-$, 608 ±261 copy/µg DNA). However, the levels in single-positive cells and double-negative cells were significantly decreased 4 months after tacrolimus therapy ($p < 0.05$) ($CD4^+CD8^+$, 658 ±240 copy/µg DNA; $CD4^+CD8^-$ 509 ±245 copy/µg DNA; $CD4^-CD8^+$, 550 ±156 copy/µg DNA; $CD4^-D8^-$, 310 ±127 copy/µg DNA).

We further examined the effect of thymoma or corticosteroid therapy on TREC levels in patients

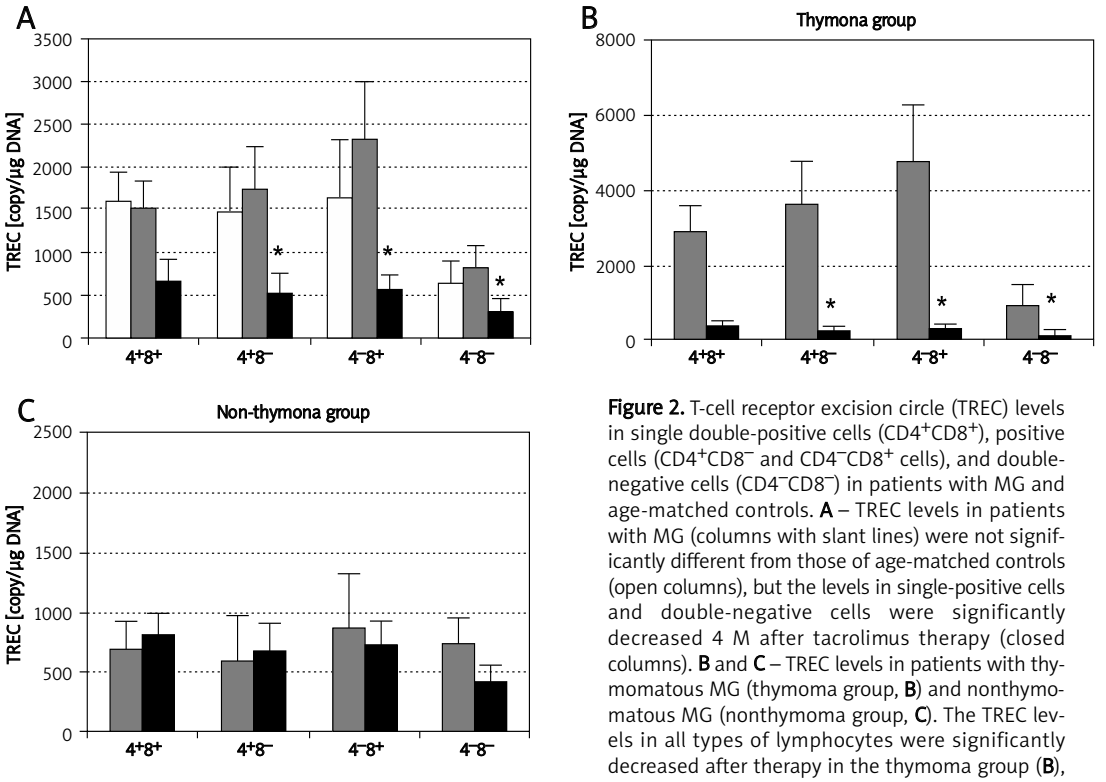


**Figure 2.** T-cell receptor excision circle (TREC) levels in single double-positive cells ($CD4^+CD8^+$), positive cells ($CD4^+CD8^-$ and $CD4^-CD8^+$ cells), and double-negative cells ($CD4^-CD8^-$) in patients with MG and age-matched controls. **A** – TREC levels in patients with MG (columns with slant lines) were not significantly different from those of age-matched controls (open columns), but the levels in single-positive cells and double-negative cells were significantly decreased 4 M after tacrolimus therapy (closed columns). **B** and **C** – TREC levels in patients with thymomatous MG (thymoma group, **B**) and nonthymomatous MG (nonthymoma group, **C**). The TREC levels in all types of lymphocytes were significantly decreased after therapy in the thymoma group (**B**), but not in the nonthymoma group (**C**)
**p < 0.05*

with MG. In the thymoma group, TREC levels ($CD4^+CD8^+$, 2884 ±592 copy/µg DNA; $CD4^+CD8^-$, 3613 ±1338 copy/µg DNA; $CD4^-CD8^+$, 4760 ±1268 copy/µg DNA; $CD4^-CD8^-$, 940 ±372 copy/µg DNA) were significantly decreased after tacrolimus therapy ($CD4^+CD8^+$, 398 ±81 copy/µg DNA; $CD4^+CD8^-$, 243 ±108 copy/µg DNA; $CD4^-CD8^+$, 266 ±65.3 copy/µg DNA; $CD4^-CD8^-$, 133 ±43.5 copy/µg DNA) ($p < 0.05$, Figure 2 B). In the nonthymoma group, TREC levels ($CD4^+CD8^+$, 694 ±154 copy/µg DNA; $CD4^+CD8^-$ 592 ±145 copy/µg DNA; $CD4^-CD8^+$, 859 ±203 copy/µg DNA; $CD4^-CD8^-$, 737 ±224 copy/µg DNA) were not different after tacrolimus therapy ($CD4^+CD8^+$, 813 ±280 copy/µg DNA; $CD4^+CD8^-$, 685 ±283 copy/µg DNA; $CD4^-CD8^+$, 721 ±235 copy/µg DNA; $CD4^-CD8^-$, 416 ±132 copy/µg DNA) (Figure 2 C). In the corticosteroid group, TREC levels ($CD4^+CD8^+$, 1964 ±1167 copy/µg DNA; $CD4^+CD8^-$ 2953 ±1237 copy/µg DNA; $CD4^-CD8^+$, 4080 ±2033 copy/µg DNA; $CD4^-CD8^-$, 882 ±483 copy/µg DNA) were not different after tacrolimus therapy ($CD4^+CD8^+$, 508 ±160 copy/µg DNA; $CD4^+CD8^-$, 290 ±117 copy/µg DNA; $CD4^-CD8^+$, 494 ±153 copy/µg DNA; $CD4^-CD8^-$, 104 ±20 copy/µg DNA) (Figure 2 C). In the noncorticosteroid group, TREC levels ($CD4^+CD8^+$, 1166 ±442 copy/µg DNA; $CD4^+CD8^-$, 762 ±383 copy/µg DNA; $CD4^-CD8^+$, 954 ±445 copy/µg DNA; $CD4^-CD8^-$, 760 ±475 copy/µg DNA) also were not different after tacrolimus therapy ($CD4^+CD8^+$, 774 ±416 copy/µg DNA; $CD4^+CD8^-$, 698 ±283 copy/µg DNA; $CD4^-CD8^+$, 594 ±219 copy/µg DNA; $CD4^-CD8^-$, 470 ±254 copy/µg DNA) (Figure 2 C). The patients with myasthenia gravis took FK506 for at least 6 months. During FK506 treatment, laboratory data and clinical findings did not show any side effects including infection, liver and kidney dysfunction, hyperkalemia, hyperglycemia, and diabetes mellitus.

Next we examined the direct effect of tacrolimus on cultured T-lymphocytes from patients with MG. Single-positive cells were used in this study, since double-negative/-positive cells were unable to secure sufficient cell counts. Tacrolimus treatment did not significantly change TREC levels (Figure 3 A) or total cell counts (Figure 3 C) in $CD4^+CD8^-$ cells. On the other hand, tacrolimus treatment significantly attenuated TREC levels of $CD4^-CD8^+$ cells (0 ng/µl, 1062 ±551 copy/µg DNA; 10 ng/µl, 694 ±112 copy/µg DNA; 1000 ng/µl, 171 ±107 copy/µg DNA; $p < 0.05$) (Figure 3 B), but total cell counts were not significantly changed (Figure 3 D).

## Discussion

The thymus gland, an indispensable organ for the deletion of autoreactive T-cells, seems to play a significant role in the pathogenesis of MG. Recent

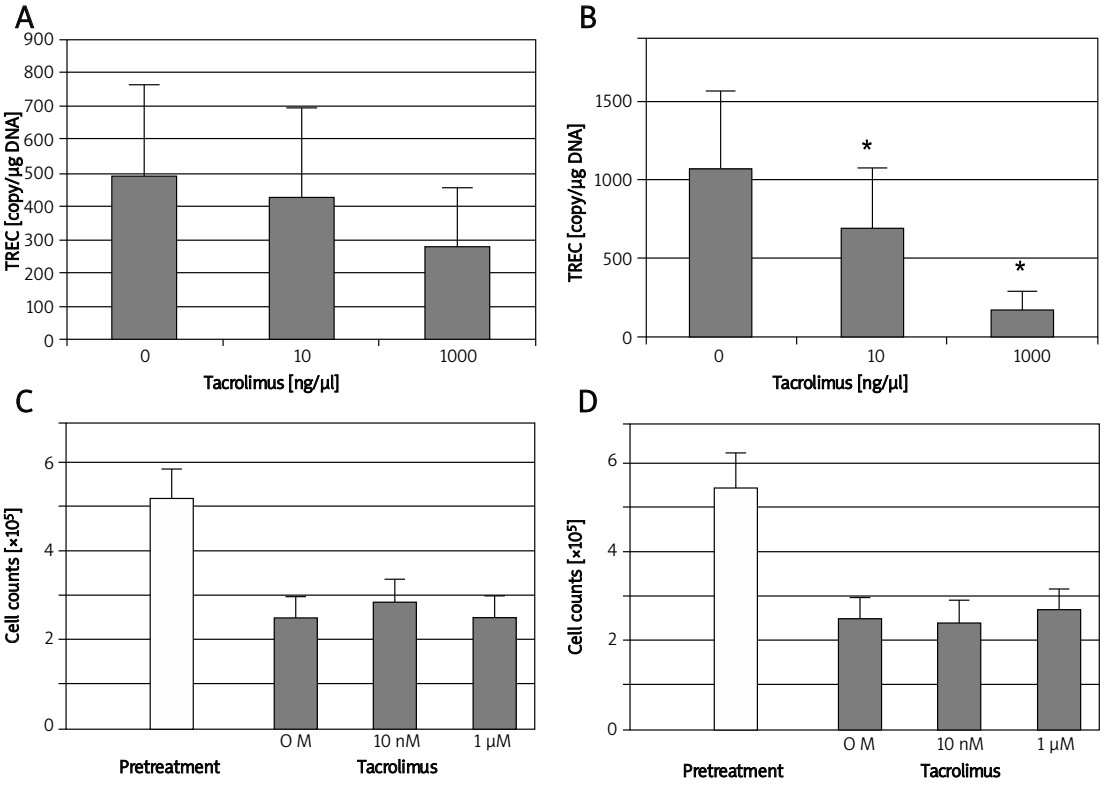


**Figure 3.** The effect of tacrolimus on cultured $CD4^+CD8^-$ cells (**A** and **C**) and $CD4^-CD8^+$ cells (**B** and **D**). Tacrolimus treatment (72 h) did not significantly change T-cell receptor excision circle levels in $CD4^+CD8^-$ cells (**A**), but it significantly decreased the levels in $CD4^-CD8^+$ cells (**B**). Cell counts were not changed by tacrolimus treatment
**p < 0.05*

evidence indicates that lymphocyte composition of peripheral blood differs between patients with thymomatous MG and those with nonthymomatous MG [1, 3, 7]. In thymomatous MG, naïve T-cells as well as TRECs were increased in the peripheral bloodstream, and the TREC levels decreased after thymectomy [6, 8, 9]. Given these facts, we find that the enhanced thymopoiesis is quite likely to be related to the development of the autoimmune mechanism. However, it has not been clarified whether this hypothesis is true in nonthymomatous MG or whether TREC becomes a general disease marker of MG [6, 9]. In the present study, we investigated whether tacrolimus therapy alters TREC levels in patients with MG. It is well known that TREC levels depend on aging; they remain high for the first 20 years of life and gradually decrease thereafter as the thymus atrophies [25]. We compared TREC levels between patients with MG and age-matched controls. Although TREC levels in patients were not significantly different from those in age-matched controls, we found that tacrolimus therapy significantly decreased TREC levels in patients with MG. Taken together, titers of anti-acetylcholine receptor antibody and MG scores were significantly decreased. It is noteworthy that tacrolimus therapy significantly attenuated TREC levels in the thymomatous group, but not in the nonthymomatous group. The present study included 6 thymomatous patients who underwent thymectomy. We previously reported that tacrolimus has an especially beneficial effect on clinical symptoms in thymomatous MG [13]. These results indicate that TREC levels may become a marker of the curative effect of the tacrolimus in thymomatous MG.

T-cell receptor excision circle production seems to play important roles in the development of autoimmune diseases, but the precise mechanism is not postulated and may be complicated; TREC levels are reportedly increased in patients with autoimmune thyroid disease [10] and with thymomatous MG [6, 8, 9] and decreased in patients with multiple sclerosis [26] and juvenile arthritis [27]. We found that corticosteroid therapy did not modulate the effect of tacrolimus therapy on TREC levels. This is concordant with the previous report that TREC levels might not depend on the immunosuppressive therapy using prednisone and azathioprine [9]. In the present study, we examined the direct effect of tacrolimus on cultured T-lymphocytes. It did not show the toxic effect on them, but it significantly decreased the TREC levels in $CD4^{-}CD8^{+}$ cells. It is well known that tacrolimus inhibits the activation of T-lymphocytes via the suppression of calcineurin [20]. It is confirmed in many institutions that low-dose FK506 is effective in myasthenia gravis. Recently Minami *et al.* reported data from 9 patients with steroid-dependent generalized MG treated with low-dose tacrolimus (2–3 mg/day) for 5 years [28]. Following treatment with tacrolimus, the mean MG activities of daily living score improved and mean dose of prednisolone could also be reduced. Zhao *et al.* reported that low-dose tacrolimus (3 mg/day) for 24 weeks reduced steroid dosage and levels of cytokines, such as interferon-γ (IFN-γ), interleukin 2, 10 and 13 (IL-2, IL-10, and IL-13) [29]. The present results suggest that it suppresses not only activating T-lymphocytes, but also naïve T-cells. We could not identify the molecular mechanism, but the suppressive effect of tacrolimus on naïve T-cells and thymic output may suggest the unique immunosuppressive action of this drug.

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