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Molecules and Cells

Tacrolimus Differentially Regulates the Proliferation of Conventional and Regulatory CD4$^+$ T Cells

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Tacrolimus is a widely used T cell targeted immunosuppressive drug, known as a calcineurin inhibitor. However, the exact pharmacological effects of tacrolimus on CD4$^+$ T cells have yet to be elucidated. This study investigated the effects of tacrolimus on CD4$^+$ T cell subsets. Mouse or human CD4$^+$ T cells were cultured with immobilized anti-CD3/CD28 antibodies in the presence of tacrolimus. The cell division of CD4$^+$ T cells was analyzed using a flow cytometer according to the expression of Foxp3. The gene expression patterns of tacrolimus-exposed T cells were examined by quantitative PCR. In the case of conventional CD4$^+$ T cells (Tconv cells), tacrolimus inhibited T cell receptor stimulation-induced cell division. In contrast, the cell division of regulatory CD4$^+$ T cells (Treg cells) was even promoted in the presence of tacrolimus, especially in humans. Tacrolimus did not promote conversion of Tconv to Treg cells in mice. Furthermore, tacrolimus modified the expression levels of Foxp3-regulated T cell receptor signal related-genes, PTPN22 and Itk, in human Treg cells. Immunosuppressive effect of tacrolimus may be attributed to the relatively enhanced proliferation of Treg cells in association with altered gene expression levels of TCR signaling molecules.

INTRODUCTION

Tacrolimus (FK506) is an immunosuppressive drug, which is now widely used to treat not only transplant recipients but also the patients with autoimmune diseases, including rheumatoid arthritis (RA) (Yocum et al., 2004). Tacrolimus is a macrolide compound from the filamentous bacterium *Streptomyces tsukubaensis* found in Japan in 1987. The immunosuppressive function of tacrolimus depends on the inhibition of T cell activation.

T cells receive activation signals from T cell receptors (TCR). Signals from TCR induce intracellular Ca^{2+} release and activate a Ca^{2+}-activated enzyme called calcineurin. Then, calcineurin dephosphorylates nuclear factor of activated T cells (NFATc). Dephosphorylated NFATc is allowed to move into the nucleus and work as a transcription factor, coupled with another transcription factor, AP-1 (Kiani et al., 2000). Tacrolimus binds to FK-binding protein (FKBP), and creates a complex. This complex binds to calcineurin, preventing its activation and blocking the dephosphorylation of NFATc. The failure of dephosphorylation prevents NFATc from entering nucleus and activating the transcription of various genes (Dumont, 2000; Granelli-Piperno et al., 1990; Kiani et al., 2000). As a result, tacrolimus reduces the expression of several cytokine genes, including IL-2, which is an important growth factor for T cells, and inhibits T cell activation (Tocci et al., 1989). However, the precise mechanisms of immune suppression by tacrolimus remain unclear, for example a previous report showed that tacrolimus prevents the induction of T cell anergy in *in vitro* culture.

Recently, CD4$^+$CD25$^+$ regulatory T cells (Treg cells) have emerged as a critical player in regulation, or suppression, of the immune system (Baecher-Allan and Hafler, 2004). Treg cells control the immune response by producing inhibitory cytokines, such as transforming growth factor-β and interleukin-10, or by cell-cell interaction with antigen presenting cells (Sakaguchi, 2004). The lack of Treg cells results in severe systemic inflammation in Scurfy mouse (Brunkow et al., 2001) and human IPEX syndrome (Bennett et al., 2001; Wildin et al., 2001). The effects of tacrolimus on Treg cells are still unknown. Thus, the present study hypothesized that tacrolimus not only reduces the activities of conventional T cells (Tconv cells) but also enhances the regulatory activities of Treg cells when tacrolimus functions as an immunosuppressant.

Here, it was investigated how tacrolimus affects CD4$^+$ T cells, particularly focusing on Treg cells. The proliferation of Tconv cells was reduced in the presence of tacrolimus. Notably, the proliferation of Treg cells was decreased less or even promoted in the presence of tacrolimus, compared with Tconv cells. Interestingly, tacrolimus suppressed PTPN22 expression in Treg but not in Tconv cells. PTPN22 is a lymphoid specific phosphatase involved in inactivating TCR signaling. In addition, Itk, another molecule for positive TCR signaling, expression in Treg cells was increased in the presence of tacrolimus. The immunosuppressive effects of tacrolimus may be attributed to the relatively enhanced proliferation of Treg cells in association with

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the altered gene expression of the TCR signaling molecules.

MATERIALS AND METHODS

Mice

Balb/c, DBA1, and C57BL/6 (B6) Thy1.2$^+$ congenic mice were obtained from Japan SLC (Shizuoka, Japan). B6 Thy 1.1$^+$ congenic mice were obtained from the Jackson Laboratory (USA). All mice were used at 6-8 weeks of age. All animal experiments were conducted in accordance with institutional and national guidelines.

Tacrolimus and culture medium

Tacrolimus was provided by Astellas Pharma Inc. (Japan). Tacrolimus was dissolved in methanol and was diluted by culture medium just before the experiments.

Cells were incubated in RPMI1640 medium with 10% of fetal bovine serum, 2 mM γ-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

Antibodies and flow cytometry

Monoclonal antibodies against mouse CD4-APC, CD8-biotin, CD11c-biotin, CD19-biotin, CD25-PE, and Thy1.2-APC were obtained from BD Biosciences (USA). A monoclonal antibody to mouse CD4-Qdot was obtained from Invitrogen (USA). Monoclonal antibodies to mouse GITR-biotin and human CD4-APC were obtained from eBioscience (USA). PE-conjugated human and mouse anti-Foxp3 antibodies were obtained from eBioscience, and cells were stained in accordance with the manufacturer's manual. Flow cytometry analysis was performed using an EPICS Elite flow cytometer (Beckman Coulter, USA). In some experiments, cell sorting was performed using FACSVantage (USA).

Murine CD4$^+$ T cell culture

Murine CD4$^+$ T cells were purified from splenocytes by negative selection using a MACS system with biotin-conjugated anti-CD8, anti-CD11c, and anti-CD19 antibodies and streptavidin-conjugated magnetic beads (Miltenyi Biotec, Germany). Negatively selected CD4$^+$ cells were further divided into CD25$^+$ cells and CD25$^-$ cells using the MACS system with PE-labeled anti-CD25 antibody and anti-PE magnetic beads (Miltenyi Biotec). In some experiments, CD4$^+$GITR$^-$ cells, which were isolated from C57BL/6 mice (Thy1.2), and CD4$^+$CD25$^+$ cells, isolated from Thy1.1 mice, were co-cultured in the same proportion.

Human peripheral CD4$^+$ T cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of healthy volunteers by density centrifugation using Ficoll-Paque plus (GE Healthcare, UK) and Leucosep (Greiner bio-one, Germany).

CD4$^+$ T cells were isolated from PBMCs by negative selection using the MACS system (Miltenyi Biotec). PBMCs were labeled with biotin-conjugated antibodies against human CD8, CD14, CD16, CD19, CD36, CD56, CD123, and TCRγ/δ. The cells were then incubated with anti-biotin magnetic beads and loaded onto MACS separation columns (Miltenyi Biotec). In some experiments, the CD4$^+$ T cells were further labeled with PE-conjugated anti-human CD25 mAb (Miltenyi Biotec), incubated with anti-PE magnetic beads (Miltenyi Biotec), and CD4$^+$CD25$^+$ T cells were obtained by positive selection. CD4$^+$CD25- T cells were obtained by negative selection.

CD4$^+$ T cell proliferation assay

Isolated CD4$^+$ T cells were stained by Carboxyfluorescein succinimidylester (CFSE). In some experiments, human CD4$^+$ CD25$^+$ cells were stained with CFSE and co-cultured with unla beled CD4$^+$CD25$^-$ cells. These cells were cultured with plate-bound anti-mouse or anti-human CD3/CD28 antibodies (pre-coated with 5 μg/ml of antibodies) (BD Biosciences) in the presence or absence of various concentrations of tacrolimus (0.1, 1, 10 ng/ml) for 72 h. T cell proliferation was analyzed, and certain cell populations were sorted by flow cytometry after culture.

cDNA synthesis and quantitative real-time PCR

Total mRNA from sorted cells was extracted using an RNeasy Micro Kit and RNeasy Mini kit (Qiagen) in accordance with the manufacturer's protocol. cDNA was reverse-transcribed from mRNA using Superscript III reverse transcriptase and random primers (Invitrogen) as described by the manufacturer. Quantitative real-time PCR analysis was performed using an iCycler (Bio-Rad). The PCR mixture consisted of 25 μl of SYBR Green Master Mix (Qiagen), 15 pmoles of forward and reverse primers, and cDNA samples for a total volume of 50 μl. The results of real-time PCR are shown in terms of relative expression compared with GAPDH. The forward and reverse primers for GAPDH used in this study were as follows: 5′-GCTCTCCAG-AACATCATCCCTGCC-3′, and 5′-CGTTGTCATACCAGGAA-ATGAGCTT-3′. The primers for human PTPN22 were as follows: 5′-ACCAAGCAAGCCTACAGAACGTG-3′, and 5′-CCAG-AGGTGCGTTACATATTCCAAG-3′. The primers for human Itk were obtained from Applied TAKARA BIO (Japan).

Statistical analysis

Data are expressed as means $\pm$ standard deviation. All results were obtained from at least three independent experiments. Statistical significance was determined by Mann-Whitney U test, and p-values less than 0.05 were considered significant.

RESULTS

Differential effects of Tacrolimus on the proliferation of mouse Treg cells

To test the effects of tacrolimus on CD4$^+$ T cells proliferation, purified mouse splenic CD4$^+$ T cells were cultured in various concentrations of tacrolimus, which were similar to the therapeutic serum concentrations after oral administration of tacrolimus in humans (Fig. 1). Isolated CD4$^+$ T cells were stained using CFSE, and CFSE dilution of the CD4$^+$ T cells stained with anti-Foxp3 antibody was assayed by flow cytometry after 72 h incubation. Cell division of CD4$^+$Foxp3$^-$ T cells was suppressed in the presence of tacrolimus. This inhibition was observed in a dose-dependent manner, especially for < 1 ng/ml of tacrolimus. Cell division of CD4$^+$Foxp3$^+$ cells was also inhibited in the presence of tacrolimus. However, greater numbers of Foxp3$^+$ cells still divided even in the presence of high concentrations of tacrolimus, compared with Foxp3$^-$ cells. The Foxp3 protein expression itself was not increased significantly in the presence of tacrolimus.

Tacrolimus did not induce cell conversion to Treg cells

Previous reports demonstrated that CD4$^+$Foxp3$^-$ cells are converted to Foxp3$^+$ cells in the peripheral tissues or *in vitro* culture, and TGF-β plays an important role in this conversion (Chen et al., 2003). Because this study was investigated the proliferation of CD4$^+$Foxp3$^+$ cells after 72 h incubation, it was possible that part of the dividing Foxp3$^+$ cells in the presence of tacrolimus was converted from Foxp3$^-$ cells. To examine whether tacrolimus induces this conversion of Foxp3$^-$ to Foxp3$^+$ cells, Thy1.1 congenic mice were used (Fig. 2A). Because GITR is a

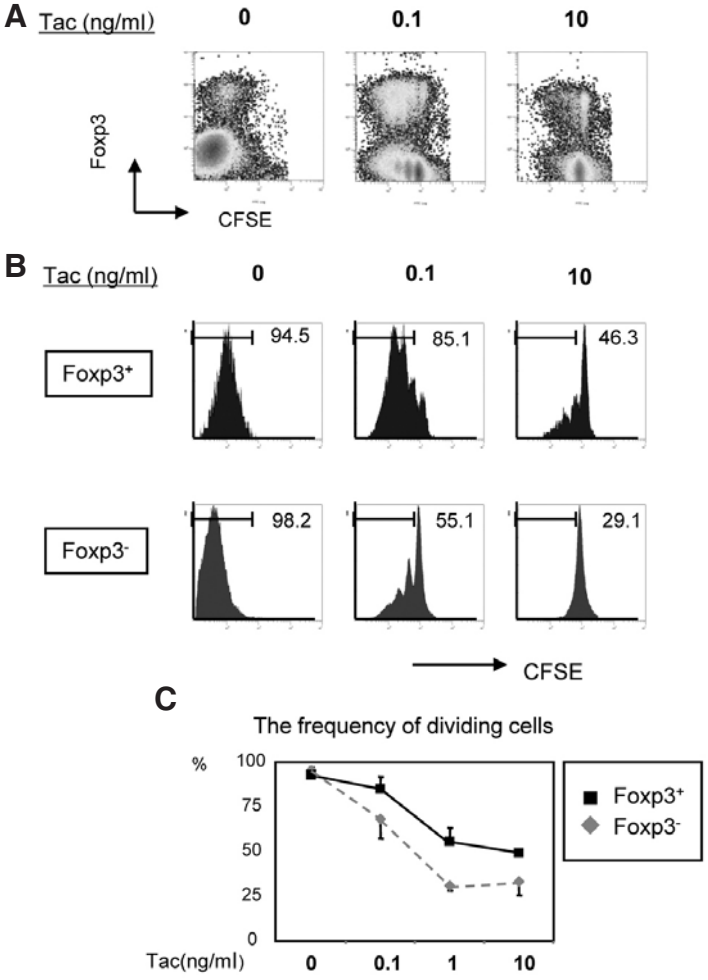

Fig. 1. Influence of tacrolimus on murine $CD4^+$ T cells *in vitro*. Purified splenic $CD4^+$ T cells were CFSE labeled and stimulated for 72 h with plate-coated anti-CD3/CD28 antibodies in the presence of the indicated concentrations of tacrolimus. Cells were stained for CD4 and Foxp3. (A) CD4 gated dot-plot of the indicated conditions. (B) The extent of proliferation was determined by CSFE dilution. (C) Plot of the frequency of dividing cells. Data are representative of at least three experiments.

marker for $CD4^+Foxp3^+$ Treg cells as well as CD25 cells (Shimizu et al., 2002), $Thy1.2^+CD4^+$ T cells were negatively selected with anti-GITR antibody to obtain $CD4^+Foxp3^+$ Treg-depleted $CD4^+$ T cells. Anti-GITR-depleted $Thy1.2^+CD4^+$ T cells contained less than 0.2% of $CD4^+Foxp3^+$ cells (Fig. 2B and data not shown). The same numbers of both $Thy1.2^+CD4^+$ $GITR^-$ cells and $Thy1.1^+CD4^+$ cells were co-cultured in the presence or absence of tacrolimus (Fig. 2B). After 72 h incubation, $Thy1.2^+Foxp3^+$ cells did not appear, and tacrolimus had no effect on cell conversion from $CD4^+Foxp3^-$ cells to $CD4^+Foxp3^+$ cells. Therefore, the difference in the dividing cell rates between $Foxp3^+$ and $Foxp3^-$ cells in the presence of tacrolimus observed in Fig.1 was not due to cell conversion from Tconv to Treg cells.

Tacrolimus promoted cell proliferation in human Treg cells

Then, the effects of tacrolimus on the proliferation of human peripheral $CD4^+$ T cells were examined. Human $CD4^+$ T cells were isolated from peripheral blood mononuclear cells and cultured with tacrolimus *in vitro*. It was reported that human $CD4^+CD25^-Foxp3^-$ cells up-regulate Foxp3 after strong *in vitro* stimulation (Allan et al., 2007; Walker et al., 2003; 2005). In the present experiments, most of the total $CD4^+$ T cells stimulated with anti-CD3/28 antibodies showed intermediate- and high-levels of Foxp3 protein expression (Fig. 3A). Previous reports showed that the amount of Foxp3 protein per cell in activated $CD4^+CD25^-$ cells was always significantly lower than levels in Treg cells exposed to equivalent activating conditions (Allan et al., 2007; Tran et al., 2007). It was also observed here that the Foxp3 expression levels of stimulated $CD4^+CD25^-$ cells were relatively confined to intermediate levels (data not shown). Therefore, most of the $CD4^+$ T cells with high-levels of Foxp3 ($CD4^+$ $Foxp3^{high}$ cells) were thought to be derived from $CD4^+CD25^+$ Treg cells and most of the $CD4^+$ T cells with intermediate-levels of Foxp3 ($CD4^+Foxp3^{int}$ cells) were regarded as activated Tconv cells.

In the presence of tacrolimus, cell division of $CD4^+Foxp3^{int}$ cells was clearly decreased (Figs. 3A and 3B). In contrast, cell division of $CD4^+Foxp3^{high}$ cells was maintained or even increased in the presence of tacrolimus. The differences in percentage in $CD4^+$ T cells induced by 10 ng/ml of tacrolimus were significant between $CD4^+Foxp3^{high}$ and $CD4^+Foxp3^{int}$ cells (Fig. 3C). In this way, tacrolimus plays different roles in the proliferation of $CD4^+$ T cells between Tconv and Treg cells.

Tacrolimus modified PTPN22 and Itk expression in human Treg cells

To determine the modification mechanisms of tacrolimus for the cell proliferation, the changes in gene expression levels induced by tacrolimus were analyzed in human Treg cells. It was hypothesized that modification of the gene expression levels regulated by Foxp3 was involved in the effect of tacrolimus on the differential division of human Treg cells. The present study

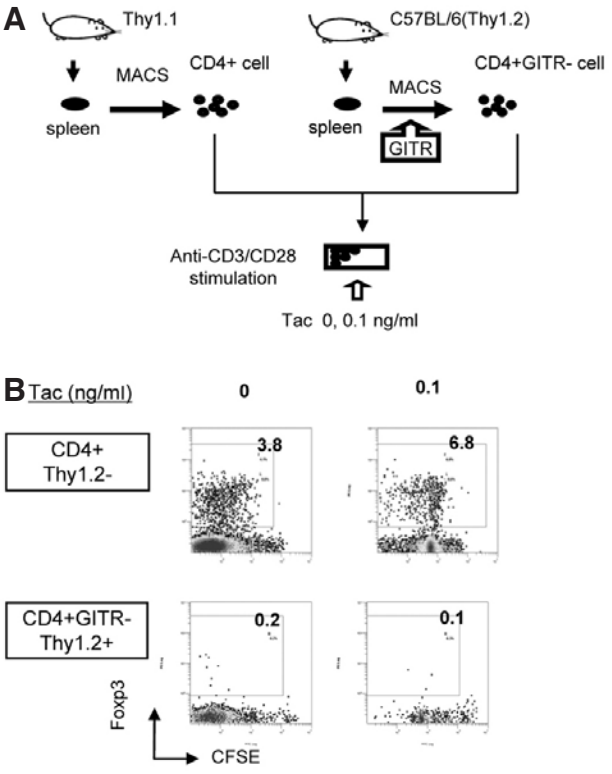

Fig. 2. Analysis of conversion from murine $CD4^+Foxp3^-$ to $CD4^+Foxp3^+$ T cells in the presence of tacrolimus. Equal numbers of $CD4^+Thy1.1^+$ and $CD4^+GITR^-Thy1.2^+$ T cells were mixed and stimulated for 72 h with plate-coated anti-CD3/CD28 antibodies in the presence of the indicated concentrations of tacrolimus. Cells were stained with anti-Thy1.2, anti-Foxp3, and anti-CD4 antibodies. (A) The experimental scheme. (B) $CD4^+Thy1.2^+$ and $CD4^+Thy1.2^-$ T cells were analyzed for Foxp3 expression and CFSE dilution. Data are representative of at least three experiments.

focused on the expression of TCR signal-related genes, particularly PTPN22, Itk, ZAP70, and Jak2. Foxp3 was reported to bind to the promoter lesions of these four genes and directly controls their expression (Marson et al., 2007). MACS purified and CFSE-labeled human $CD4^+CD25^+$ cells were mixed with $CD4^+CD25^-$ cells and stimulated by plate-bound anti-CD3/CD28 antibodies in the presence or absence of 10 ng/ml of tacrolimus. After 72 h, $CD4^+CFSE^+$ cells and $CD4^+CFSE^-$ cells were sorted separately with flow cytometry for gene expression analysis. Quantitative PCR assays demonstrated that tacrolimus down-regulated PTPN22 expression and up-regulated Itk expression in $CD4^+CD25^+$ cells (Fig. 4). While up-regulation of Itk expression was also observed in $CD4^+CD25^-$ cells, down-regulation of PTPN22 was not observed in the CD25 negative population. No significant change due to tacrolimus was observed in the expression of ZAP70 or Jak2 (data not shown). PTPN22 is an inhibitory molecule of proximal TCR signaling, and expression of the PTPN22 gene was reported to be suppressed by Foxp3. The expression of Itk was also controlled by Foxp3. Itk consists of a proximal TCR signaling element and activation signals proceed from TCR by phosphorylation of PLC-γ (Gomez-Rodriguez et al., 2007; Schaeffer et al., 1999). In the present experiments, tacrolimus modified the PTPN22 and Itk expression levels in Treg cells, which could promote cell division of Treg cells in the presence of tacrolimus.

DISCUSSION

Tacrolimus is an immunosuppressive drug, and its pharmacological mechanism is attributed to the inhibition of calcineurin by binding to FKBP in T cells. Tacrolimus suppresses the activation and proliferation of T cells and also reduces interleukin-2 (IL-2) production. Although IL-2 plays a principle role in the activation and expansion of T cells, IL-2 deficient mice develop lymphoid hyperplasia and autoimmune diseases (Sadlack et al., 1993). Therefore, IL-2 also plays a suppressive role in T cell function. Indeed, IL-2 is necessary for the maintenance of the peripheral Treg cell pool (Setoguchi et al., 2005). Moreover, calcineurin-inhibitor also inhibits anergy induction of T cells *in vitro*, which would be a controversial effect of tacrolimus. The precise mechanism of immune regulation by tacrolimus remains unclear.

Foxp3 is an essential transcription factor of Treg cells. Severe inflammatory lesions develop in Foxp3-deficient mice, and forced expression of Foxp3 induces Treg phenotypes and suppressive functions (Hori et al., 2003). Foxp3 can associate with other transcription factors, such as NFAT and AML1, to confer Treg phenotypes by means of gene expression modification. Though rapamycin induces the expansion of $CD4^+CD25^+Foxp3^+$ Treg cells *in vitro* via modification of the AKT-mTOR axis (Battaglia et al., 2005; Haxhinasto et al., 2008), cyclosporin, another class of calcineurin-inhibitor, inhibits Foxp3 promoter activity and protein expression (Mantel et al., 2006).

In the present experiment, Foxp3 protein expression itself was not increased in the presence of tacrolimus, as is the case with cyclosporin. It was demonstrated that TCR-mediated cell division of Treg cells was less suppressed by tacrolimus than that of Tconv cells in both mouse and human. Moreover, tacrolimus even promoted cell division in human Treg cells. The differential effect of tacrolimus to Tconv and Treg cells may affect the balance of Tconv and Treg cells at inflammation sites and account for one of the important mechanisms of immunosuppression by tacrolimus.

Recently, chromatin immunoprecipitation (ChIP) combined with DNA microarray analysis revealed that 4 TCR signal-related genes, PTPN22, Itk, ZAP70, and JAK2, were under the control of Foxp3. It was demonstrated that tacrolimus modifies the expression levels of these T cell receptor signal-related genes in human Treg cells. In the presence of tacrolimus, PTPN22 expression was decreased, and Itk expression was increased in human Treg cells. PTPN22 has an inhibitory effect on TCR signaling, whereas Itk plays an important role in enhancing TCR signals. The PTPN22 1858T allele is associated with a number of autoimmune diseases, including rheumatoid arthritis (RA) (Begovich et al., 2004; Gregersen and Batliwalla, 2005), systemic lupus erythematosus (SLE) (Orozco et al., 2005), type 1 diabetes mellitus (T1D) (Bottini et al., 2004), and Grave's diseases (Yu et al., 2007). Recent findings have revealed that the PTPN22 risk-associated variant results in a gain of PTPN22 phosphatase activity in T-cells (Gregersen et al., 2006). Because the PTPN22 risk-associated variant results in a gain of phosphatase activity in T-cells and increasing sensitivity to autoimmune diseases, down-regulation of the PTPN22 expression with tacrolimus may be a legitimate correction of the genetic variant.

The present results suggested that tacrolimus promotes the antigen-specific proliferation of Treg cells at the lymphoid and peripheral organs. Immunosuppressive effect of tacrolimus may be attributed to the relatively enhanced proliferation of Treg cells in association with altered gene expressions of the TCR signaling molecules.

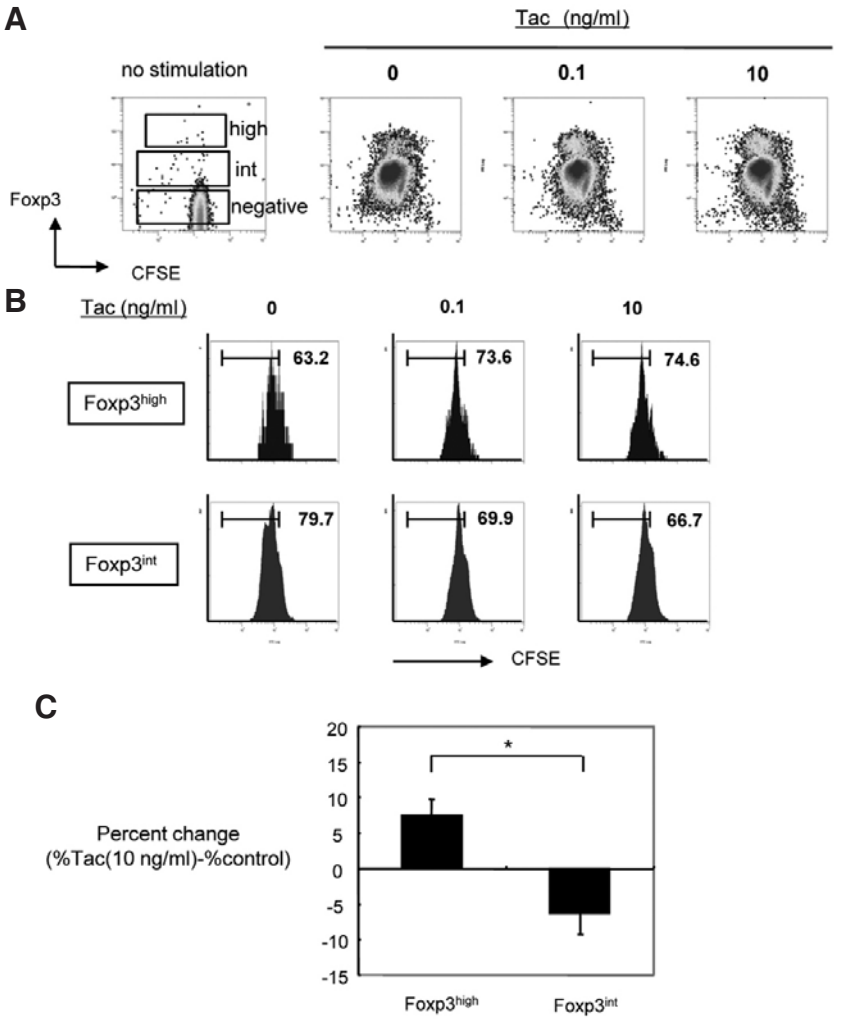

Fig. 3. Influence of tacrolimus on human $CD4^+$ T cells *in vitro*. Purified human $CD4^+$ T cells from peripheral blood were CFSE labeled and stimulated for 72 h with plate-coated anti-CD3/CD28 antibodies in the presence of the indicated concentrations of tacrolimus. Cells were stained with anti-CD4 and anti-Foxp3 antibodies. (A) A CD4 gated dot-plot of unstimulated cells is shown in the left-hand panel. Regions of Foxp3-high, Foxp3-intermediate, and Foxp3-negative expression are indicated. A CD4 gated dot-plot of stimulated cells in the presence of indicated concentration of tacrolimus is shown in the right-hand panel. (B) The extent of proliferation was determined using the histogram of CSFE dilution. Data are representative of four experiments. (C) The differences in the percentages of $CD4^+$ T cells induced by 10 ng/ml of tacrolimus are shown for $CD4^+Foxp3^{high}$ and $CD4^+Foxp3^{int}$ cells. Data represent mean ± SE of four individuals. *, indicates significant difference ($p < 0.05$).

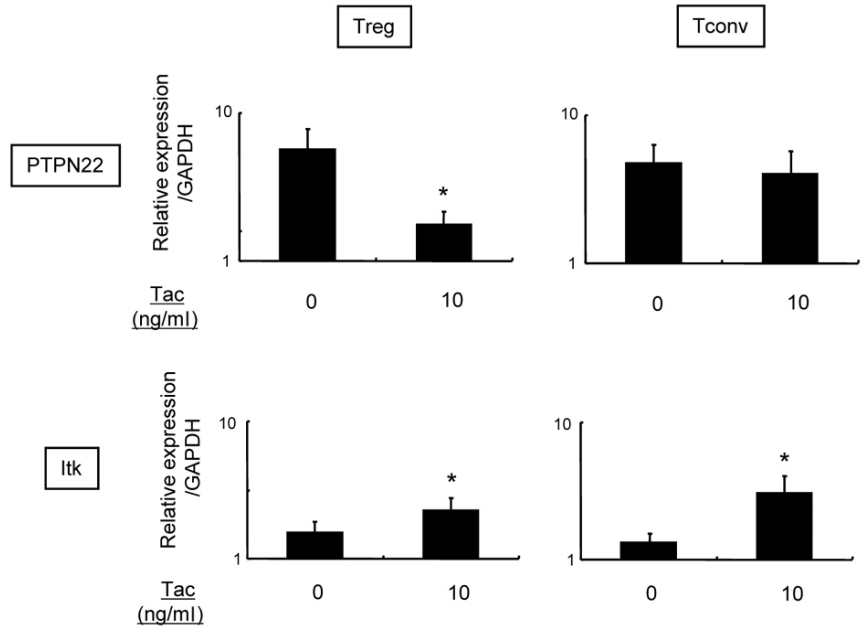

Fig. 4. Alteration of PTPN22 and Itk expression levels in human Treg and Tconv cells by tacrolimus. MACS Purified human $CD4^+CD25^-$ (Tconv) cells and CFSE-labeled $CD4^+CD25^+$ (Treg) cells were mixed and stimulated with plate-coated anti-CD3/CD28 antibodies in the presence of 10 ng/ml of tacrolimus. After 72 h, $CD4^+CFSE^+$ (Treg) cells and $CD4^+CFSE^-$ (Tconv) cells were sorted separately with flow cytometry for gene expression analysis. Messenger RNA expression levels were analyzed by quantitative PCR. Data are representative of at least three experiments. *, indicates significant difference ($p < 0.05$).

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