# Effect of tyrosine hydroxylase gene silencing in CD4+ T lymphocytes on differentiation and function of helper T cells

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## Abstract

**OBJECTIVES:** We explored effect of gene silencing of tyrosine hydroxylase (TH), a rate-limiting enzyme for synthesis of catecholamines (CAs), in CD4+ T cells on differentiation and function of helper T (Th) cells to provide more evidence for functional significance of lymphocyte-derived CAs.

**METHODS:** CD4+ T lymphocytes were isolated and purified from the mesenteric lymph nodes of mice. Recombinant TH miRNA expression vector (pcDNA6.2-GW/EmGFPmiR-TH) was constructed and transfected into concanavalin A (Con A)-activated CD4+ T lymphocytes using nucleofection technology. After incubated for 48 h, these cells were detected for TH gene and protein expression and CA content. Simultaneously, percentage of interferon-γ (IFN-γ)- and interleukin-4 (IL-4)-producing cells and levels of IL-2, IFN-γ, tumor necrosis factor (TNF), IL-4 and IL-5 in culture supernatants of Con A-stimulated CD4+ T cells were examined by flow cytometric analysis.

**RESULTS:** CD4+ T lymphocytes with TH RNAi expressed less TH mRNA and protein and synthesized less CAs including norepinephrine, epinephrine and dopamine than control cells with mock transfection. The silencing of TH gene in CD4+ T lymphocytes reduced percentage of IL-4-producing cells and elevated ratio of IFN-γ-producing cells to IL-4-producing cells, although it did not alter proportion of IFN-γ-producing cells. The Th1 cytokines, IL-2, IFN-γ and TNF, were increased, but the Th2 cytokines, IL-4 and IL-5, were decreased in the culture supernatants of Con A-stimulated CD4+ T lymphocytes that were transfected with TH miRNA.

**CONCLUSION:** TH gene silencing attenuates TH expression and CA synthesis in CD4+ T lymphocytes and promotes polarization of differentiation and function towards Th1 cells.

ORIGINAL ARTICLE

## INTRODUCTION

It has been well known that tyrosine hydroxylase (TH), a rate-limiting enzyme for synthesis of catecholamines (CAs), is expressed in neurons and endocrine cells. Therefore, the nervous and endocrine systems can synthesize and release CAs, which as neurotransmitters or endocrine hormones, regulate many functions including immune response (Pacheco-López *et al.* 2003; Oberbeck 2006; Webster-Marketon & Glaser 2008). Over recent decades, a new finding that lymphocytes can also express TH and synthesize and secrete CAs, including norepinephrine (NE), epinephrine (E) and dopamine (DA), has been presented (Bergquist *et al.* 1994; Musso *et al.* 1996; Qiu *et al.* 2004). These lymphocytes synthesizing and releasing CAs include T, B and natural killer (NK) cells (Cosentino *et al.* 2007). The lymphocyte-derived CAs modulate many functions of the immune system (Cosentino *et al.* 2007, 2009). Accordingly, the immune system probably becomes the third catecholaminergic system besides the nervous and endocrine systems (Leposavić *et al.* 2008). Since immunomodulation by lymphocyte-derived CAs is more direct and quicker than neuron- or endocrine-originated CAs, the findings of the third catecholaminergic system have important significance for immunomodulation and immune homeostasis.

We have reported that activated T lymphocytes increase TH expression and CA synthesis and secretion in comparison with resting lymphocytes, suggesting that T cell-derived CAs are related to T cell function (Qiu *et al.* 2004, 2005). Subsequently, we show that T lymphocyte-endogenous CAs inhibit T cell proliferation and accelerate T cell apoptosis, demonstrating a functional significance of T cell-derived CAs (Jiang *et al.* 2007, 2009). However, $CD4^+$ T lymphocytes, a largest class of lymphocyte subsets accounting for 60% of peripheral T lymphocytes, still need to be further explored about their expression of TH and synthesis of CAs.

$CD4^+$ T lymphocytes function mainly by helper T (Th) cells. By antigen stimulation, Th0 cells are mostly differentiated into Th1 and Th2 cells. Th1 cells are mainly involved in cellular immune response by secretion of cytokines, such as interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor (TNF). These cytokines activate inflammatory cells and therefore lead to tissue damage (D'Ambrosioa *et al.* 2000). Th2 cells mainly mediate humoral immune response and have anti-inflammatory effect by secretion of IL-4, IL-5, IL-6, and IL-10 (D'Ambrosioa *et al.* 2000). In the body, there is fine regulation of Th1- and Th2-type immune responses to maintain normal immune balance (Kidd 2003). Shift of Th1/Th2 balance is a root of certain dysfunctions and diseases, such as microbial infection, tumors, and autoimmune diseases (Infante-Duarte & Kamradt 1999; Ruschpler & Stiehl 2002; Dolff *et al.* 2011; Bleotu *et al.* 2012; Block *et al.* 2011). Exogenous CAs from the sympathetic and adrenal tissues regulate $CD4^+$ T cells, with the effects of decrease in secretion of pro-inflammatory cytokines and increase in release of anti-inflammatory cytokines by the $CD4^+$ T lymphocyte (Elenkov & Chrousos 2002; Sanders *et al.* 2002; Salicru *et al.* 2007). These findings suggest that CAs derived from nervous and endocrine systems promote a shift of differentiation and function of Th1 cells towards Th2 type. Nevertheless, there still is no direct evidence to show effect of $CD4^+$ T cell-derived CAs on differentiation and function of Th cells. Thus, we in the present study explored this effect by using TH gene interference in $CD4^+$ T cells to better understand functional significance of CAs derived from $CD4^+$ T cells.

## MATERIALS AND METHODS

*Purification and culture of $CD4^+$ T lymphocytes*

$CD4^+$ T cells were purified from the mesenteric lymph nodes of 4 to 6 week-old mice by using $CD4^+$ T-cell isolation kit according to the manufacturer's instructions (BD Biosciences, USA). The purity of the isolated cells was more than 98%, as determined by staining and flow cytometry (data not shown). $CD4^+$ T lymphocyte were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin, at the final concentration of $1.25\times10^6$ cells/ml. Concanavalin A (Con A) was added into the cultures with the concentration of 5 μg/ml to induce the cell proliferation. The cultures were incubated at 37 °C in a moist atmosphere with 5% $CO_2$ for 24 h.

*Construction of recombinant plasmid*

The recombinant TH miRNA expression vector (pcDNA6.2-GW/EmGFPmiR-TH, pcDNA/miR-TH) that targets mouse TH gene was constructed by using BLOCK-iT Pol II miR RNAi expression vector kit with EmGFP according to the manufacturer's instruction (Invitrogen, USA). We cloned four double-stranded oligonucleotides with TH miRNA target sequence into vectors containing Pol II promoters. For each double-stranded oligonucleotide, we firstly designed two single-stranded DNA oligonucleotides (32 bp for each), and then they were annealed to generate a double-stranded oligonucleotide (64 bp). The double-stranded oligonucleotides were cloned into pcDNA6.2-GW/EmGFPmiR (Invitrogen, USA), which constituted TH-targeting miRNA expression vector with green fluorescent protein. Sequence analysis showed that all of the four double-stranded oligonucleotides with TH miRNA were correctly cloned into pcDNA6.2-GW/EmGFPmiR vectors.

*Transfection of TH miRNA expression vector*

The four TH miRNA expression vectors (pcDNA/miR-TH) were transfected into $CD4^+$ T lymphocytes using nucleofection technology (Amaxa Biosystems, Germany). By detection of interference

efficiency of the four vectors, we found the most effective one and therefore it was used in the present study. The sequences of the single-stranded oligonucleotides were as follows: 5'-TGCTGATAGGAAGACAGCAGCCCTGCGTTTTGGCCACTGACTGACGCAGGGCTGGTCTTCCTAT-3' for top chain and 5'-CCTGATAGGAGGACAAGCCCTGCGTCAGTCAGTGGCCAAAACGCAGGGCTGCTGTCTTCCTATC-3' for bottom chain. The transfection protocol followed the instructions of mouse T cell nucleofector kit (Amaxa Biosystems, Germany). Briefly, after incubated with Con A for 24 h, $CD4^+$ T cells were resuspended in 100 µl of T cell nucleofector solution. Plasmid of 4 µg was added to 100 µl of $5\times10^6$ $CD4^+$ T cell suspension. The mixtures were subsequently transferred to an electroporation cuvette with aluminum electrodes and placed in the nucleofection device (Amaxa Biosystems, Germany). Nucleofection of these cells were accomplished using X-001 program, and the samples were immediately transferred to 12-well plates containing 2 ml pre-warmed media. Control experiments were performed by processing $CD4^+$ T cells in the same way, but without adding plasmid to the cells. The efficiency of TH miRNA transfection in $CD4^+$ T cells was determined by GFP-tagged recombinant plasmid under a fluorescence microscope (Leica, Germany). Number of GFP-labeled cells was approximately 50% to 70% (data not shown).

*Real-time quantitative PCR*

Total RNA were extracted from $CD4^+$ T lymphocytes, which had been transfected and incubated for 48 h, with Trizol reagent (Bio Basic Inc., Canada), as recommended by the manufacturer. After RNA content was determined by spectrophotometric analysis at 260 nm, 2 µg of total RNA was used for cDNA synthesis with M-MLV reverse transcriptase (Invitrogen, USA). The PCR reaction was performed in a Rotor-Gene 3000 Real-Time Cycler (Corbett Research, Australia) and the detection was made by measuring the binding of the fluorescence dye SYBR Green I (Molecular Probe) to double-stranded DNA. Each 20 µl reaction mixture contained 1 µl cDNA, 2 µl PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of oligonucleotide primer and 1 U Taq DNA polymerase. The sequences of oligonucleotide primers for amplification of specific TH gene (130 bp, NM_009377.1) were 5'-CGGAAGCTGATTGCAGAGAT-3' (sense) and 5'-GGGTAGCATAGAGGCCCTTC-3' (antisense).

The reaction procedures were as follows: an initial step at 95 °C for 5 min, 40 cycles of 94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min. Analysis was performed by standard curve method. To verify the specificity of the amplification reaction, melting curve analysis was performed. Relative expression level of the target gene was expressed as a ratio to value of β-actin, a house keeping gene. The primer sequences for β-actin gene (218 bp, NM_007393) were 5'-CTGTCCCTGTATGCCTCTG-3' (sense) and 5'-ATGTCACGCACGATTTCC-3' (antisense).

*Western blot analysis*

Total protein was extracted from $CD4^+$ T cells having transfected with TH miRNA for 48 h. Cells were homogenized in lysis buffer and centrifuged at 4 °C and 12,000 g for 10 min. The supernatants containing 20 µg of total cellular protein were mixed with sample buffer and boiled for 5 min. Then the cellular protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine difluoride membrane (Pall, Port Washington) using a semi-dry transfer apparatus. After blocking nonspecific binding with 5% (w/v) nonfat dry milk, the membranes were probed for rat anti-mouse TH monoclonal antibody (1:900; Sigma, USA) at room temperature for 4 °C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody and visualization with electro-chemi-luminescence reagent. Then the chemiluminescence was exposed to X-ray film. Blots were reprobed with β-actin antibody (1:5000; Sigma, USA) to confirm equal protein loading. The molecular weight and relative quantity of the protein bands were determined by an image analysis system (SynGene GeneTools software, UK).

*High performance liquid chromatography with electrochemical detection*

For detection of CAs in $CD4^+$ T lymphocytes having transfected with TH miRNA expression vector, the cultures were centrifuged and the cell pellets were resuspended in 0.2 ml of 0.4 N $HClO_4$ and disrupted by ultrasonication. The mixtures were then centrifuged for 15 min at 12,000 g at 4 °C. The supernatants were recovered, filtered and stored at −80 °C until analysis. Each extract of 20 µl was injected into a high performance liquid chromatography (HPLC) apparatus equipped with a reverse-phase column (Altantis TM dC18, 5 µm, 100 Å; Waters) to quantify DA, E and NE. The mobile phase was pumped at a flow rate of 1.0 ml/min. NE, E and DA in the samples were quantified by using the peak areas of a standard curve.

*Measurement of IFN-γ- and IL-4-producing cells*

$CD4^+$ T cells were introduced with phorbol myristate acetate (PMA, 10 ng/ml) and ionomycin (1 µM) in the presence of 1 µM monensin for 6 h in order to promote production of cytokines by these cells. The cultured cells were washed twice in perm/wash buffer (BD, Biosciences, USA) and then 250 µl fixation/permeabilization solution was added into the pellets, which were resuspended for 20 min at 4 °C. After two additional washes in perm/wash buffer, the cells were resuspended in perm/wash buffer to a concentration of $2\times10^5$ cells/ml. PE-conjugated anti-IFN-γ or anti-IL-4 monoclonal antibodies were added to the cells at a concentration of 1 µg/ml, which was incubated for 30 min

at room temperature. Analysis was performed with FACS Calibur flow cytometer equipped with an argon laser. Acquisition was analyzed with CellQuest software (BD, Biosciences, USA).

*Cytometric bead array*

CD4+ T cells were introduced with PMA (10 ng/ml) and ionomycin (1 μM) for 6 h to promote production of cytokines by these cells. The culture supernatants were collected after the cultures were centrifuged. These cytokines, IL-2, IFN-γ, TNF, IL-4, and IL-5, in the supernatants were measured using a cytometric bead array (CBA) kit (BD, Biosciences, USA). Briefly, the supernatants of 50 μl were mixed with 50 μl of mouse Th1/Th2 PE detection reagent consisting of PE-conjugated anti-mouse IL-2, IL-4, IL-5, TNF, and IFN-γ antibodies and 50 μl of mixed capture beads. The mixtures were incubated at room temperature for 3 h in the dark. They were then washed once and resuspended in 300 μl of wash buffer. Sample data were acquired using flow cytometer and were analyzed with CBA analysis software (BD Biosciences, USA).

*Statistical analysis*

Data were expressed as mean ± standard deviation (M ± SD). Statistical analysis was performed with Statistics Package for Social Science (SPSS, 12.0). The data were submitted to Student-t test and differences were considered statistically significant at $p<0.05$.

## RESULTS

*TH gene silencing in CD4+ T lymphocytes reduces TH expression and CA content in these cells*

RNAi expression plasmid targeting TH gene was constructed and then transfected into CD4+ T lymphocytes that had been activated by Con A. Expression of TH mRNA and protein in CD4+ T lymphocytes transfected with RNAi expression plasmid was markedly downregulated relative to that in control cells with mock transfection (Figure 1). Moreover, content of CAs, including NE, E and DA, in Con A-activated CD4+ T lymphocytes was significantly reduced by the TH gene silencing compared with that of control cells with mock transfection (Figure 1). These data showed that TH gene silencing had an evident suppression of TH expression and CA synthesis in Con A-induced CD4+ T lymphocytes.

*TH gene interference in CD4+ T lymphocytes decreases percentage of IL-4-producing cells and increases ratio of IFN-γ+/IL-4+ cells*

After incubated with Con A for 24 h, CD4+ T cells were transfected with TH miRNA expression vector. The TH gene silencing in CD4+ T cells remarkably decreased percentage of IL-4+ cells in the presence of PMA and ionomycin when compared with mock-transfected control cells (Figure 2). It indicated that TH gene silencing reduced proportion of IL-4-producing cells. But the TH miRNA did not significantly alter the percentage of

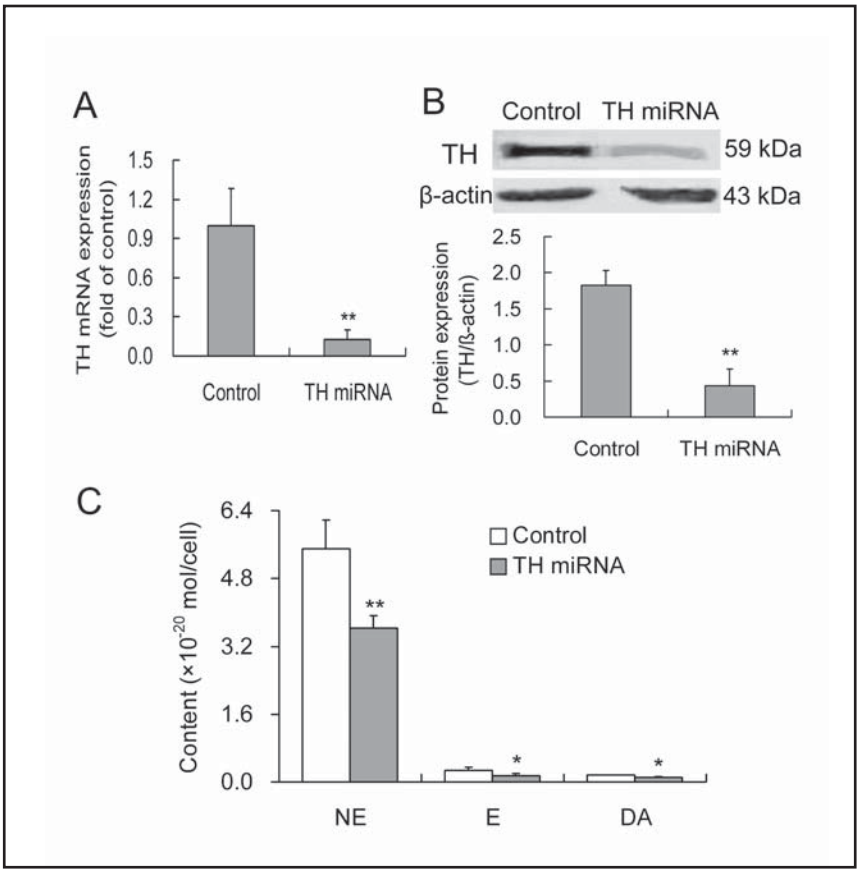


**Fig. 1.** Effects of TH gene silencing on TH expression and CA synthesis in CD4+ T lymphocytes. CD4+ T cells from the mesenteric lymph nodes were purified and activated with Con A, and then these cells were transfected with TH miRNA expression vector. After incubated with the TH miRNA for 48 h, the CD4+ T cells were measured for expression of TH gene (A) and protein (B) and for content of CAs including NE, E, and DA (C). The data are mean and standard deviation of four repeated experiments. *$p<0.05$, **$p<0.01$, compared with control cells with mock transfection.

IFN-γ-producing cells in CD4+ T cells. Therefore, the ratio in number of IFN-γ+ to IL-4+ cells was notably more in CD4+ T cells with TH gene interference than in control cells with mock transfection (Figure 2).

*TH gene interference in CD4+ T cells elevates Th1-cytokine but reduces Th2-cytokine secretion*

In culture supernatants of CD4+ T lymphocytes transfected with TH miRNA expression vector, concentrations of Th1 cytokines including IL-2, IFN-γ and TNF were all higher than in those of mock-transfected control cells (Figure 3). On the contrary, the Th2 cytokines, IL-4 and IL-5, were lower in culture supernatants of CD4+ T cells with TH miRNA interference than in those of CD4+ T cells with mock transfection (Figure 3). These data indicated that TH gene silencing in CD4+ T cells elevated Th1-cytokine but reduced Th2-cytokine secretion.

## DISCUSSION

Studies from the other and our laboratories have shown that human and rat lymphocytes can express TH and synthesize and secrete CAs (Bergquist *et al.* 1994; Qiu *et al.* 2004). The synthesis and secretion of CAs by lymphocytes are raised in response to antigen or mitogen (Cosentino *et al.* 2002a, 2002b; Qiu *et al.* 2004, 2005). Accordingly, in the current study we employed Con A to activate CD4+ T cells and to elevate basal levels of CAs in these cells. We found that Con A-activated CD4+ T cells expressed TH and synthesized CAs and that the expression and synthesis were both reduced by TH miRNA in these cells. The results not only add evidence for synthesizing CAs using the enzyme TH by CD4+ T cells but also show the effectiveness of TH gene interference with the TH miRNA expression vector on reduction of CA synthesis.

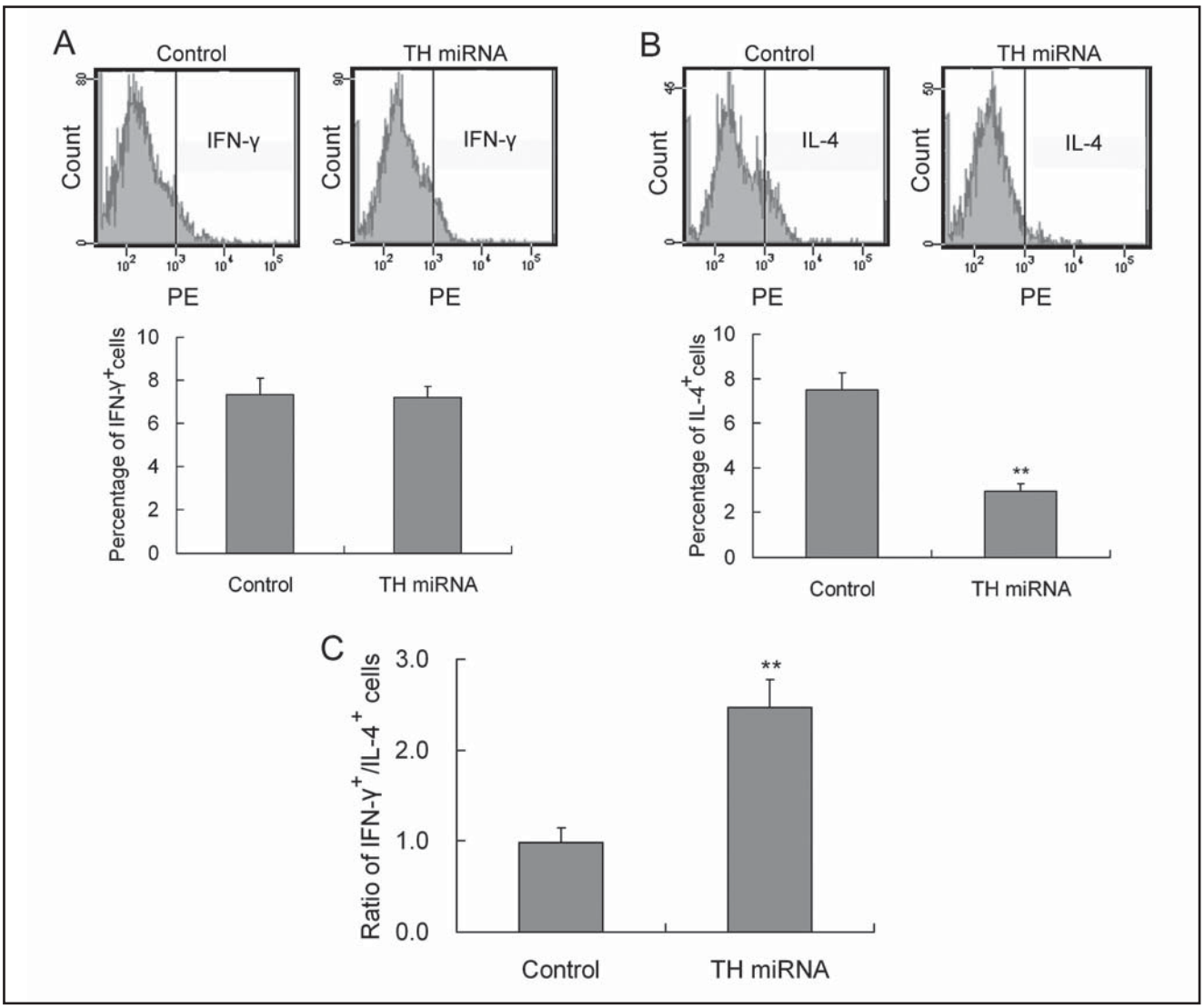


**Fig. 2.** Influences of TH gene interference on percentage of INF-γ-producing and IL-4-producing cells in CD4+ T lymphocytes. CD4+ T cells isolated from the mesenteric lymph nodes and stimulated with Con A were transfected with TH miRNA expression vector. After incubated with the TH miRNA for 48 h, the CD4+ T cells were determined for percentage of INF-γ+ cells (A) and IL-4+ cells (B) by flow cytometric analysis. (C) denotes the ratio in number of INF-γ+ cells to IL-4+ cells. The data are mean and standard deviation of four separate experiments. **$p$<0.01, compared with control cells with mock transfection.

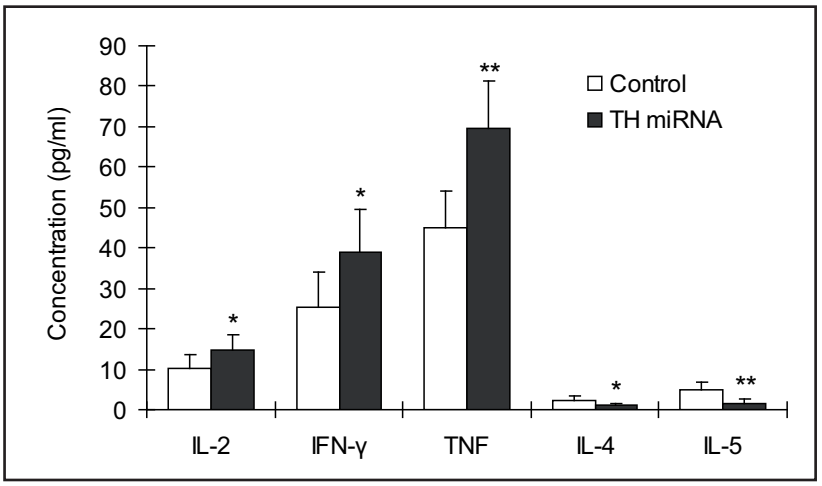


**Fig. 3.** Effects of TH gene silencing on secretion of Th1- and Th2-type cytokines by $CD4^+$ T cells. $CD4^+$ T cells were isolated from the mesenteric lymph nodes of mice and stimulated with Con A. These cells were then transfected with TH miRNA expression vector, which was incubated for 48 h. Concentrations of Th1-type cytokines, including IL-2, INF-γ and TNF and Th2-type cytokines, including IL-4 and IL-5, were examined in the culture supernatants of $CD4^+$ T cells by CBA analysis. The data are mean and standard deviation of eight respective experiments. *$p<0.05$, **$p<0.01$, compared with control cells with mock transfection.

The silencing of TH gene in $CD4^+$ T cells led to a decrease in percentage of IL-4-producing cells and an increase in ratio of IFN-γ- to IL-4-producing cells, although the number of IFN-γ-producing cells was not significantly affected by the TH gene interference. Since IFN-γ-producing and IL-4-producing cells represent Th1 and Th2 cells, respectively, the reduction of IL-4-producing cells and the augment of IFN-$\gamma^+$/IL-$4^+$ cells demonstrate a shift of Th cell differentiation towards Th1 cells by the TH gene silencing in $CD4^+$ T cells. These results also imply that normally synthesized CAs by $CD4^+$ T cells can promote Th cell differentiation towards Th2 direction. It has been reported that NE exogenously added to lymphocyte cultures suppresses IFN-γ and TNF-α production by $CD3^+$ cells (Takayanagi & Osawa 2012) and shifts Th1/Th2 cytokine balance towards a predominant Th2 profile (Sanders & Straub 2002; Loza *et al.* 2006). These effects of exogenous NE are similar to the present results obtained from lymphocyte-endogenous CAs. Therefore, it is possible that the effect of exogenous NE also reflects an action of lymphocyte-derived NE, because lymphocytes have been reported to secrete CAs out of the cells into extracellular fluid by autocrine/paracrine pathways to exert their regulation of lymphocytes (Musso *et al.* 1998; Cosentino *et al.* 2000a).

In the support of these results, we found that concentrations of IL-2, IFN-γ and TNF in culture supernatants of Con A-activated $CD4^+$ T cells with TH gene silencing were significantly higher but IL-4 and IL-5 levels were lower than those in control supernatants. The concentrations of cytokines in culture supernatants represent secreted levels by $CD4^+$ T cells. Consequently, the secretion elevation of Th1-type cytokines, IL-2, IFN-γ and TNF, indicates an enhancement of Th1 cell function, and the secretion reduction of Th2-type cytokines, IL-4 and IL-5, shows an attenuation of Th2 cell function. These results reveal that TH gene interference in $CD4^+$ T cells brings a functional bias towards Th1 cells. It is also proposed that normally synthesized CAs by $CD4^+$ T cells can facilitate Th cell function towards Th2 polarization. These data are consistent with those obtained at the profile of Th cell differentiation. Thus, the present findings strongly show that CAs in $CD4^+$ T cells regulate differentiation and function of Th cells and accordingly they are actively involved in the modulation of Th1/Th2 balance. CAs derived from non-lymphocytes cause selective suppression of cellular immunity and a shift towards Th2-mediated humoral immunity (Elenkov *et al.* 2000; Kin & Sanders 2006). These similar effects on Th cell differentiation and function by lymphocyte-derived and non-lymphocyte-derived CAs suggest a fine regulation of Th1/Th2 balance to maintain homeostasis of cellular and humoral immune responses. This regulation is probably more important by endogenous CAs derived from lymphocytes than by exogenous CAs arising from neurons or endocrine cells, because lymphocyte-derived endogenous CAs have closer and more direct contact with immune cells.

The Th1/Th2 balance is crucial in maintaining homeostasis of immune system function. Some autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, type 1 diabetes mellitus, autoimmune thyroid disease, and Crohn's disease, are closely related to Th1/Th2 imbalance. In most states of these diseases, the balance is skewed towards Th1 cells and an excess of production of pro-inflammatory cytokines, whereas Th2 activity and production of anti-inflammatory cytokines are deficient (Maini et al.,1994; Wilder 1995; Elenkov *et al.* 1997; Horwitz *et al.* 1998; Segal *et al.* 1998; Lorton *et al.* 2003). The shift of Th1/Th2 balance towards Th2 polarization by CAs in $CD4^+$ T cells presented in this study explains a mechanism, by which CAs alleviate autoimmune diseases. Therefore, a new therapeutic strategy focusing on CAs derived from $CD4^+$ T cells will be significant for treatment of autoimmune diseases.

In summary, the TH gene silencing in $CD4^+$ T cells by transfection of TH miRNA expression vector results in a downregulation of TH mRNA and protein expres-

sion and a reduction of synthesis of CAs including NE, E and DA. Simultaneously, the TH gene interference facilitates a shift of Th cell differentiation and function towards Th1 cell polarization. These findings show that CAs derived from CD4+ T cells modulate Th1/Th2 balance in differentiation and function and also suggest that normally synthesized CAs in CD4+ T cells have an effect promoting the shift of Th1/Th2 balance towards Th2 predominance.

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