

The modulative effect of Cyperi Rhizoma on Th1/Th2 lineage development

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ABSTRACT

To evaluate the direct effects of Cyperi Rhizoma (CR), a plant water extract, on Th1/Th2 lineage development in vitro, this study was conducted. Sorted CD4⁺ T cells obtained from the splenocytes of BALB/c mice were activated with anti-CD3/anti-CD28 and then cultured in medium that contained CR medium under Th1 inducing or Th2 inducing conditions. Subsequently, IFN- γ or IL-4 secreting cells were quantitated using flow cytometry analysis. In addition, IFN- γ and IL-4 protein secretions were detected by ELISA analysis, after which, IFN- γ and T-bet transcripts, key players in the Th1 immune function, and also, IL-4 and GATA-3, which are primary components in the Th2 immune mechanism, were quantitated by real-time RT-PCR. CR had no mitogenic effects on un-stimulated CD4⁺ T cells, however, it increased the CD4⁺ T cell population. Th1/Th2 polarization experiments revealed that CR enhanced IFN- γ secretion in Th1 cells, but reduced the IL-4 in Th2 cells, and this occurred in a dose-dependent manner and showed significances. In addition, under Th1/Th2 skewed conditions, the transcription levels of IFN- γ and T-bet were considerably increased, while the expressions of IL-4 and GATA-3 were relatively decreased with CR treatment. These findings suggest that CR enhances Th1 lineage development by increasing Th1 specific cytokine expression and secretion and reduces Th2 lineage development by repressing Th2 specific cytokine productions. Therefore, CR extract may be useful for preventing the onset of allergies or improving allergic symptoms.

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1. Introduction

When CD4⁺ T helper (Th) cells encounter microbial antigens that are presented by antigen presenting cells, they are activated and differentiate into two functionally distinct subsets [1,2]. The functional differences between these Th cell subsets are explained primarily through the activities of their secreted cytokines. For example, interferon (IFN)- γ , which is secreted by Th1 cells, is known to induce differentiation of naive CD4⁺ T cells into Th1 cells, and to inhibit the proliferation of Th2 cells [3]. Conversely, IL-4 which is secreted from Th2 cells, are known to induce the differentiation of naive CD4⁺ T cells into Th2 cells and to inhibit the function of Th1 cells [1,4–6].

The functions of Th1 and Th2 cells correlate well with their distinctive cytokines. Th1 cells are involved in cell-mediated inflammatory functions and Th1 clones induce delayed-type hypersensitivity reactions and macrophage activation [1,7]. Th2 cytokines encourage antibody production [8,9], particularly IgE responses, and also

enhance eosinophil proliferation and function. Accordingly, Th2 cytokines are commonly found to be associated with strong antibody and allergic responses [1,7,10].

The Th1/Th2 classification has been useful in relating the overall patterns of cytokine production to clinical outcomes in a variety of pathological states [1], and it has been demonstrated that the Th1/Th2 cell response is shifted to a predominantly Th1 cell response in autoimmune diseases, which results in increased IFN- γ production by Th1 cells in organisms affected with those diseases [11]. In order to better understand these pathogenic advances in Th1/Th2 deviation, the development of specific agents affecting helper T cell differentiation has drawn special attention in the last decade.

Many natural products are reported as a good agent for modulating the immune response. For example, it has been suggested that *Atractylodes macrocephala* Koidz. showed the ability to induce the preferential stimulation of Th1 type [12] and orally administered *Bambusae Caulis* in *Taeniam* modulates Th2 immune response in OVA-induced asthmatic BALB/c mice [13].

Cyperi Rhizoma (CR), the root of *Cyperus rotundus* L., is a traditional herbal medicine that is widely used as a traditional medicine for the treatment of spasms, bowel and stomach disorders, candidiasis, malarial fever, and inflammatory diseases in India, China, Japan, and Korea [14,15]. In this study, we investigated the possible immune modulatory effects of CR on Th1/Th2 lineage development

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in vitro and found that CR appears to decrease Th2 lineage development.

2. Methods

2.1. Preparation of CR

The water extracts powder CR was purchased from Sun Ten Pharmaceutical (Taipei, Taiwan), and deposited in the herbarium at the Department of Oriental Medicine in Kyung Hee University. It was weighed to 0.1 g and then extracted by adding to 10 ml of DW and stirring over night at room temperature to ensure that the powder dissolved completely. The sample was then centrifuged for 10 min at 3000 rpm. The supernatant was sterilized by passing it through a 0.22 μ m syringe filter and then used as a stock CR solution for the experiments conducted in this study.

2.2. Antibodies

The antibodies used were anti-CD3 mAb (clone: 145-2C11), anti-CD28 mAb (clone: 37.51), anti-IL-4 (BVD4-1D11), anti-IL-12 (C17.8), FITC-conjugated anti-CD4⁺ antibody, and recombinant mouse IL-2 (rIL-2), rIL-12, rIL-4 (BD Pharmingen, CA, USA). In addition, magnetic cell sorting CD4⁺ (L3T4) microbeads, a mouse IL-4 secretion assay detection Kit, a mouse IFN- γ secretion assay detection Kit (Mitenyi Biotec, CA, USA), an OptEIA mouse IFN- γ set and an OptEIA mouse IL-4 set (BD Pharmingen, CA, USA).

2.3. Chemicals

HPLC grade reagents (J.T. Baker Co., Ltd., Phillipsburg, NJ, USA), eugenol (Sigma-Aldrich, Inc., St. Louis, USA). All other chemicals used were of analytical grade.

2.4. Quantitative analysis of eugenol in *Cyperi Rhizoma*

CR powder was accurately weighed to 1.03 g and then dissolved in 10 ml of 50% methanol. The sample was then subjected to ultrasound for 15 min, after which it was centrifuged at 15,000 rpm for 10 min. The supernatant of the sample was then filtered through a 0.45 μ m syringe filter (PVDF, GELMAN, USA). To allow a quantitative analysis of CR, eugenol was purchased from Sigma-Aldrich, Inc., USA and used as a reference material. Eugenol was weighed to 1 mg and then dissolved in 1 ml of acetonitrile. The dissolved standard solution was then diluted to 0.1, 0.25, and 0.5 mg/ml, respectively. HPLC analysis was then performed to evaluate the CR. HPLC analysis was conducted using a Waters system (Waters Co., Milford, MA, USA) with a 717+ autosampler, 2487 dual λ absorbance detector, and 1525 binary HPLC pump. A Waters Millennium³² System (Waters Co., Milford, MA, USA) was used for data acquisition and integration. The samples were analyzed by reverse phase (C₁₈) analysis HPLC (XTerraTM RP₁₈, 4.6 $\times$ 250 mm i.d., 5 μ m, flow rate: 1 ml/min, UV detection: 250 nm) using a gradient solvent system comprised of AcN–MeOH–H₂O (1:1:2 $\rightarrow$ 1:1:0, 20 min). The relationship between the concentration and the peak area was measured by the minimum square method (R^2 value).

2.5. Splenocytes preparation and CD4⁺ T cell isolation

Splenocytes of 8-week-old female BALB/c mice (Orient Bio, Seoul, Korea) were prepared by disrupting the spleen with a syringe in complete medium (RPMI 1640 with 10% fetal bovine serum, 1% penicillin–streptomycin, 10 mM HEPES). After a 10 min centrifugation at 300g to separate the debris, the cells were washed in RPMI medium, followed by lysis of the erythrocytes using ammo-

nium chloride reagent (BD Pharmingen, CA, USA). The cells were then counted. Subsequently, CD4⁺ T cells were isolated from splenocytes by positive selection using MACS CD4⁺ (L3T4) microbeads (Mitenyi Biotec, Germany) following the manufacturer's protocols. Briefly, spleen cells were resuspended at a concentration of 10⁷ total cells per 90 μ l of medium. Ten microliters of MACS CD4⁺ microbeads were then added to the mixture, which was subsequently incubated for 15 min at 4 °C. After adding a sufficient amount of medium, cells were centrifuged for 10 min at 300g and the supernatant was then removed. The cell pellet was then resuspended in 500 μ l of medium and a positive selection column was chosen and placed in the magnetic field of the MACS separator (Mitenyi Biotec, Germany). The cell suspension was then applied to the column, after which it was removed from the separator and placed on a collection tube. The positively fractionated cells were then flushed out of the column using the plunger supplied.

2.6. Survival and proliferation assay

To determine the survival and proliferation capacity of the splenocytes and CD4⁺ T cells, a Cell Titer 96TM non-radioactive cell proliferation assay (Promega, WI, USA) was used according to the manufacturer's protocols. Briefly, cells were plated at 4 $\times$ 10⁵ cells/well in a total volume of 100 μ l. To activate the CD4⁺ T cells, the cells were placed onto anti-CD3 coated plates, and then anti-CD28 Ab was added. CR (0, 0.01, 0.1, 1, 10, 100, and 1000 μ g/ml) containing medium was then added and the cells were cultured for 48 h at 37 °C under 5% CO₂.

2.7. In vitro Th1 cell polarization

CD4⁺ T cells (2 $\times$ 10⁶ cells/ml) were resuspended in complete medium (RPMI 1640 with 10% fetal bovine serum, 1% penicillin–streptomycin, 10 mM HEPES) and activated with plate-bound 10 μ g/ml anti-CD3 (BD Pharmingen, CA, USA) and 2 μ g/ml anti-CD28 (BD Pharmingen, CA, USA) for 24 h. Naive CD4⁺ T cells were incubated with 10 ng/ml rIL-12 (BD Pharmingen, USA), 10 μ g/ml anti-IL-4 (BD Pharmingen, CA, USA) and 50 U/ml rIL-2 to induce Th1 differentiation. Under both culture conditions, two times the original volume of medium containing the corresponding antibodies and cytokines was added to the culture on Day 5, at which time the cells were harvested, washed, and re-stimulated (at 10⁶ cells/ml) with plate-bound 10 μ g/ml anti-CD3 (BD Pharmingen, CA, USA) and 2 μ g/ml anti-CD28 (BD Pharmingen, CA, USA) for 24 h.

2.8. In vitro Th2 cell polarization

CD4⁺ T cells (1 $\times$ 10⁶ cells/ml) were resuspended in complete medium (RPMI 1640 with 10% fetal bovine serum, 1% penicillin–streptomycin, 10 mM HEPES) and activated with plate-bound 10 μ g/ml anti-CD3 (BD Pharmingen, CA, USA) and 2 μ g/ml anti-CD28 (BD Pharmingen, CA, USA) for 24 h. Naive CD4⁺ T cells were incubated with 10 ng/ml rIL-4 (BD Pharmingen, USA), 10 μ g/ml anti-IL-12 (BD Pharmingen, CA, USA) and 50 U/ml rIL-2 to induce Th2 differentiation. Under both culture conditions, two times the original volume of medium containing the corresponding antibodies and cytokines was added to the culture on Day 4, at which time the cells were harvested, washed, and re-stimulated (at 10⁶ cells/ml) with plate-bound 10 μ g/ml anti-CD3 (BD Pharmingen, CA, USA) and 2 μ g/ml anti-CD28 (BD Pharmingen, CA, USA) for 24 h.

2.9. Cytokine secretion assays

A cytokine secretion assay was performed using a Mouse secretion assay detection kit according to the manufacturer's protocols

(Mitenyi Biotec, Germany). Briefly, sorted CD4⁺ T cells (10⁶ cells) were washed twice by adding 2 ml of cold buffer (PBS, 1% FBS, NaN₃), then centrifuged at 300g for 10 min at 4 °C. The cells were then resuspended in 270 µl of cold medium, after which Mouse Catch Reagent (30 µl) was added and the solution was incubated on ice for 5 min. After adding 10× volume of warm medium, cells were incubated in a tube for 45 min at 37 °C while inverting the tube every 5 min to resuspend cells that had settled. The cells were then washed once and resuspended in cold buffer at a concentration of 10⁶ cells per 270 µl of buffer. After PE or FITC-conjugated antibodies were added to the mixture, samples were incubated for 10 min on ice. The cells were then analyzed using a Becton–Dickinson FACScan.

2.10. ELISA

An ELISA was conducted to detect IFN-γ and IL-4. Briefly, flat bottomed, 96-well plates were coated overnight at 4 °C with IFN-γ or IL-4 mAbs. Next, the primary mAbs were discarded and the plates were blocked with Assay Diluent (BD Pharmingen, CA, USA) for 1 h at room temperature. The plates were then washed with wash buffer (0.05% Tween 20 in PBS) and blotted on a paper towel. Next, diluted IFN-γ and IL-4 standards and samples were added in triplicate and the plates were then incubated for 2 h at room temperature, after which the supernatant was discarded and the wells were washed with wash buffer. IFN-γ and IL-4 detecting mAb plus avidin HRP was then

added to the plates, which were subsequently incubated for 1 h at room temperature. Next, the plates were washed, and tetramethylbenzidine substrate solution (BD Pharmingen, CA, USA) was added. The color was allowed to develop for 30 min in the dark before the reaction was quenched with a stop solution (0.2 M H₂SO₄). The plates were then read at 450–570 nm and the sample concentrations were determined with the help of a standard curve.

2.11. Isolation of RNA, cDNA synthesis, and real-time RT-PCR analysis

CD4⁺ T cells and Th2 cells were lysed in 500 µl of Trizol reagent (Gibco Life-Technologies, NY, USA), and the total RNA was then isolated according to the manufacturer's protocol. The integrity of the RNA was confirmed by using denaturing agarose gel electrophoresis (data not shown), and the concentration of total RNA was then quantified by determining the optical density at 260 nm (OD₂₆₀). The total RNA was analyzed by reverse transcription PCR by mixing 2 µg of RNA and 7.5 µl of DEPC-DW with 1 U of DNAase I and 10× DNAase I reaction buffer. This solution was then incubated for 15 min at room temperature, after which 1 µl of 25 mM EDTA was added to the tubes, which were then incubated at 70 °C for 10 min. Oligo(dT) primer (0.5 µg; Table 1) was then added to this mixture, which was then incubated at 70 °C for 5 min. Next a solution containing 5 µl of M-MLV 5× reaction buffer, 1 µl of 10 mM dNTPS, 20 U of RNAase inhibitor, and 5.5 µl of DEPC-DW was added and the tubes were incubated at 25 °C for 2 min. The tubes then received 200 U of M-MLV reverse transcriptase and were incubated at 42 °C for 50 min. Finally, the tubes were heated for 15 min at 70 °C. The samples were then stored at –20 °C until they were needed.

Real-time quantitative PCR was performed using a GeneAmp 5700 Sequence detection system (PE Applied Biosystems, USA) by employing SYBR Green I as the dsDNA-specific binding dye for continuous fluorescence monitoring. Amplification was carried out in a total volume of 25 µl that contained 5 µM of each cytokine-specific primer (Table 1), 2× PCR Master Mix (Applied Biosystems, USA) and 2 µl of 1:4 diluted cDNA. The mixture was then subjected to 40 cycles of denaturation (95 °C, 15 s) and annealing and extension (60 °C, 1 min). The data were analyzed using the GeneAmp 5700 SDS software (PE Applied Biosystems, USA).

Table 1
Sequences of primer used for real-time RT-PCR.

Gene	Oligonucleotide sequence
GAPDH	RV: 5'-GGC ATG GAC TGT GGT CAT GA-3' FW: 5'-TTC ACC ACC ATG GAG AAG GC-3'
IL-4	FW: 5'-ACA GGA GAA GGG ACG CCA T-3' RV: 5'-GAA GCC CTA CAG ACG AGC TCA-3'
IFN-γ	FW: 5'-TCA AGT GGC ATA GAT GTG GAA-3' RV: 5'-TGG CTC TGC AGG ATT TTC ATG-3'
T-bet	FW: 5'-GCC AGG GAA CCG CTT ATA TG-3' RV: 5'-GAC GAT CAT CTG GGT CAC ATT-3'
GATA-3	FW: 5'-GAA GGC ATC CAG ACC CGA AAC-3' RV: 5'-ACC CAT GGC GGT GAC CAT GC-3'

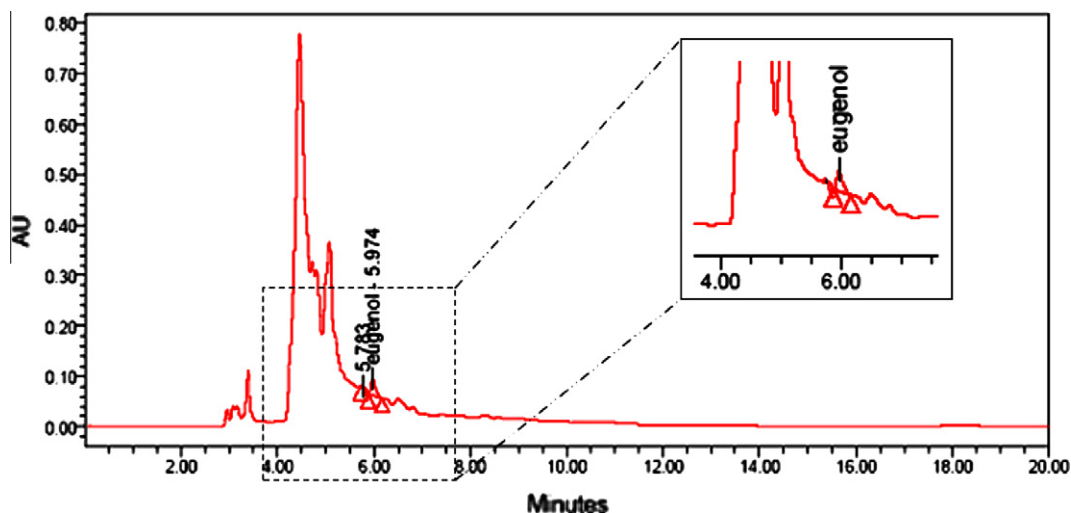


Fig. 1. HPLC chromatograms of eugenol and CR. HPLC chromatography for determining the amount of eugenol in CR. The relationship between the concentration and peak area was measured by the minimum square method (R^2 value).

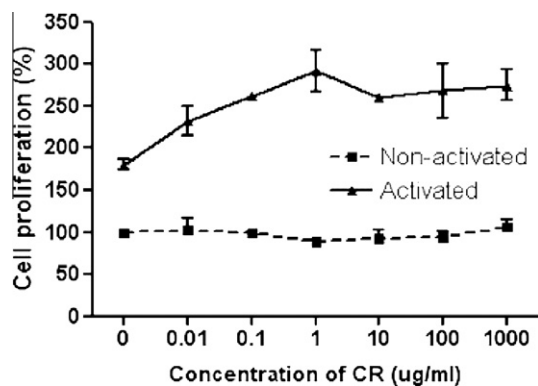


Fig. 2. Proliferation of CD4⁺ T cells in medium containing various concentrations of CR extract after 48 h of incubation. Sorted CD4⁺ T cells were treated with anti-CD3/anti-CD28 antibodies to activate them. Cell proliferation was quantified by an MTS assay. Error bars indicate SEM.

2.12. Statistical analysis

All data were analyzed by one-way ANOVA followed by a post hoc test (Newman–Keuls Multiple Comparison Test). Results were considered significant when a $p < 0.05$ was obtained.

3. Results

3.1. HPLC spectrum of CR

The standard calibration curve for eugenol was determined to be $Y = 24051520X + 263306.9$ ($R^2 = 0.998$). The average content of

eugenol in CR was calculated to be 0.015 mg/g ($n = 3$) using the above formula (Fig. 1).

3.2. The effect of CR extract on the viability of CD4⁺ T cells

In order to determine if CR extract has an influence on the proliferation of CD4⁺ T cells without antigen presenting cells, we isolated CD4⁺ T cells using magnetic anti-CD4 microbeads in mice spleen lymphocytes. Sorted CD4⁺ T cells were then treated with anti-CD3/anti-CD28 antibodies for 48 h to activate T cells in the presence of various concentrations of CR extract or in medium alone. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay revealed that the viability of CD4⁺ T cells that were not stimulated was not influenced by CR extract, suggesting that CR does not behave as an antigenic stimulator (Fig. 2). However, CR extract treatment did tend to enhance CD4⁺ T cell proliferation in a dose-dependent manner, but this enhancement was not statistically significant.

3.3. The effect of CR extract on Th1/Th2 lineage development in vitro

To evaluate the direct effects of CR on Th1/Th2 differentiation, CD4⁺ T cells were activated with anti-CD3/anti-CD28 and then cultured in medium that contained CR under Th1 inducing or Th2 inducing conditions. Subsequently, IFN- γ or IL-4 secreting cells were quantitated using flow cytometry analysis to evaluate the Th1/Th2 polarized status after CR treatment (0, 0.1, and 1 μ g/ml). The results demonstrated that CR treatment (0, 0.1, and 1 μ g/ml) shows significantly increasing fashion of IFN- γ secreting CD4⁺ T

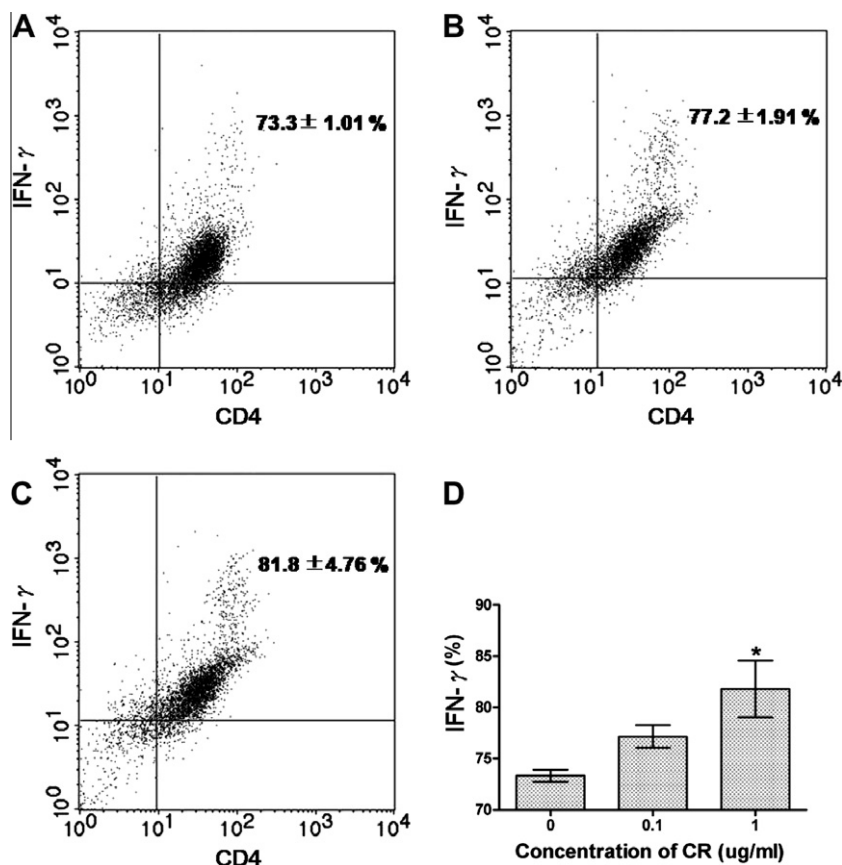


Fig. 3. Analysis of the IFN- γ secreting CD4⁺ T cell population in a Th1 skewed condition in vitro. Sorted CD4⁺ T cells were treated with rIL-12, rIL-2, and anti-IL-4 antibodies to polarize the CD4⁺ T cells toward Th1. Cells were then stained with FITC-conjugated anti-IFN- γ and PE-conjugated anti-CD4 antibodies. (A) Cells were incubated in medium without CR as a control. (B and C) Cells were incubated in medium containing 0.1 and 1 μ g/ml CR extract, respectively. (D) Data are presented as means $\pm$ SEM. Data were analyzed by one-way ANOVA followed by a post hoc test (Newman–Keuls Multiple Comparison Test). * $p < 0.05$ compared to the control group.

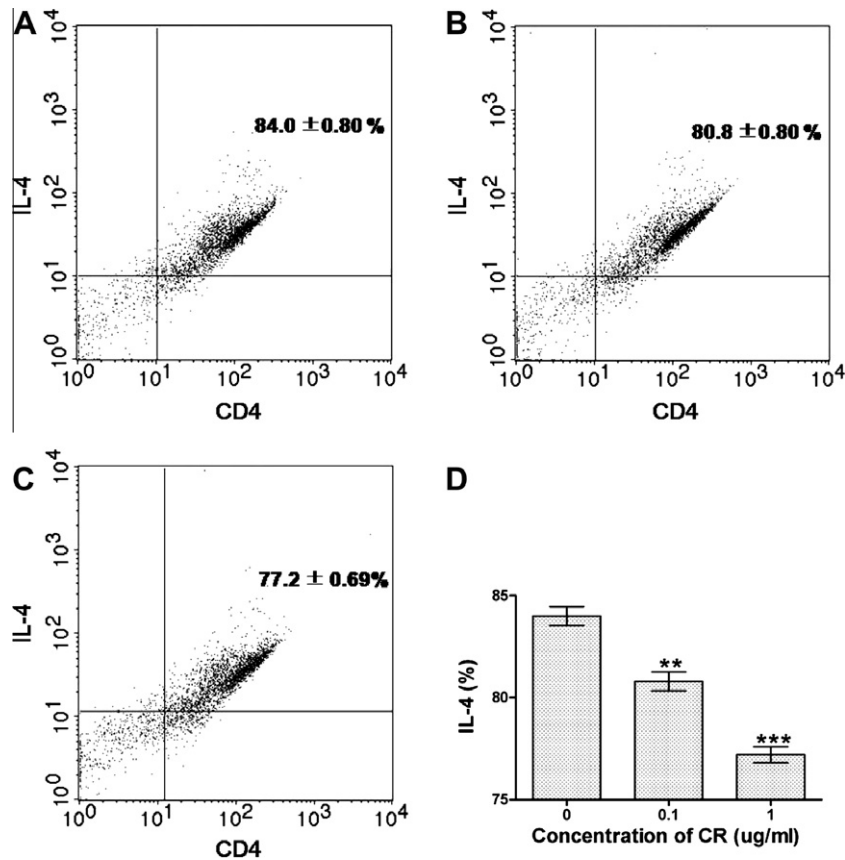


Fig. 4. Analysis of the IL-4 secreting CD4⁺ T cell population in a Th2 skewed condition in vitro. Sorted CD4⁺ T cells were treated with rIL-4, anti-IL-12 antibody, and rIL-2 to polarize CD4⁺ T cells toward Th2. Cells were then stained with FITC-conjugated anti-IL-4 and PE-conjugated anti-CD4 antibodies. (A) Cells were incubated in medium without CR as a control. (B and C) Cells were incubated in medium containing 0.1 and 1 µg/ml CR extract, respectively. (D) Data are presented as means ± SEM. Data shown are the means ± SEM. Data were analyzed by one-way ANOVA followed by a post hoc test (Newman–Keuls Multiple Comparison Test). ***p* < 0.01, ****p* < 0.001 compared to the control group.

cells from 73.3 ± 1.01% to 77.2 ± 1.91% and 81.8 ± 4.76% in the Th1 skewed condition, whereas IL-4 secreting cells were significantly decreasing pattern from 84.0 ± 0.80% to 80.8 ± 0.80% and 77.2 ± 0.69% in the Th2 skewed condition (Figs. 3 and 4). In addition, ELISA showed that CR increased the amount of IFN-γ in Th1 polarizing cultures (Fig. 5). Conversely, the secretion of IL-4 was significantly decreased in the Th2 polarizing culture (Fig. 6).

3.4. The effect of CR extract on the expressions about Th1/Th2 specific cytokines and transcription factors under Th1/Th2 skewed conditions

To investigate whether the regulations of Th1/Th2 immune responses was due to the adjusted Th1/Th2 specific cytokines and transcription factors gene expression, we measured the mRNA expressions level of IFN-γ, T-bet, IL-4, and GATA-3 utilizing real-

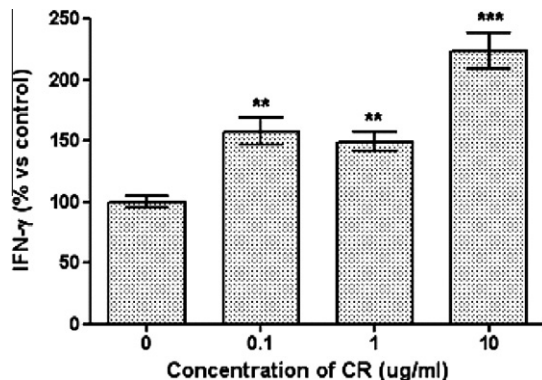


Fig. 5. Quantitation of IFN-γ secretion from CD4⁺ T cells in Th1 skewed condition. Sorted CD4⁺ T cells were treated with rIL-12, anti-IL-4 antibody, and rIL-2 to induce Th1 differentiation. The supernatants were then analyzed by an ELISA to determine the amount of IFN-γ produced. CR; cells were incubated in medium containing 0, 0.1, 1, and 10 µg/ml extract. Data were analyzed by one-way ANOVA followed by a post hoc test (Newman–Keuls Multiple Comparison Test). ***p* < 0.01, ****p* < 0.001 vs control group.

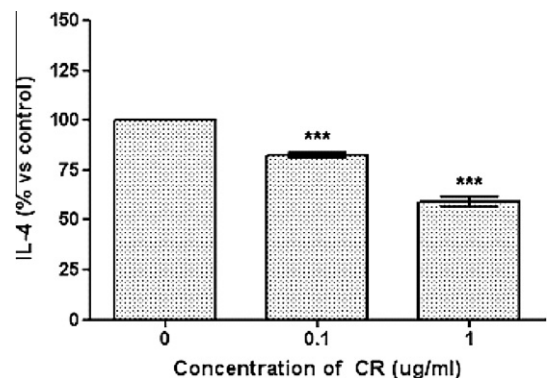


Fig. 6. Quantitation of IL-4 secretion from CD4⁺ T cells in a Th2 skewed condition. Sorted CD4⁺ T cells were treated with rIL-4, anti-IL-12 antibody, and rIL-2 to induce Th2 differentiation. The supernatants were then analyzed by an ELISA to determine the amount of IL-4. CR; cells were incubated in medium containing 0, 0.1, and 1 µg/ml extract. Data were analyzed by one-way ANOVA followed by a post hoc test (Newman–Keuls Multiple Comparison Test). ****p* < 0.001 vs control group.

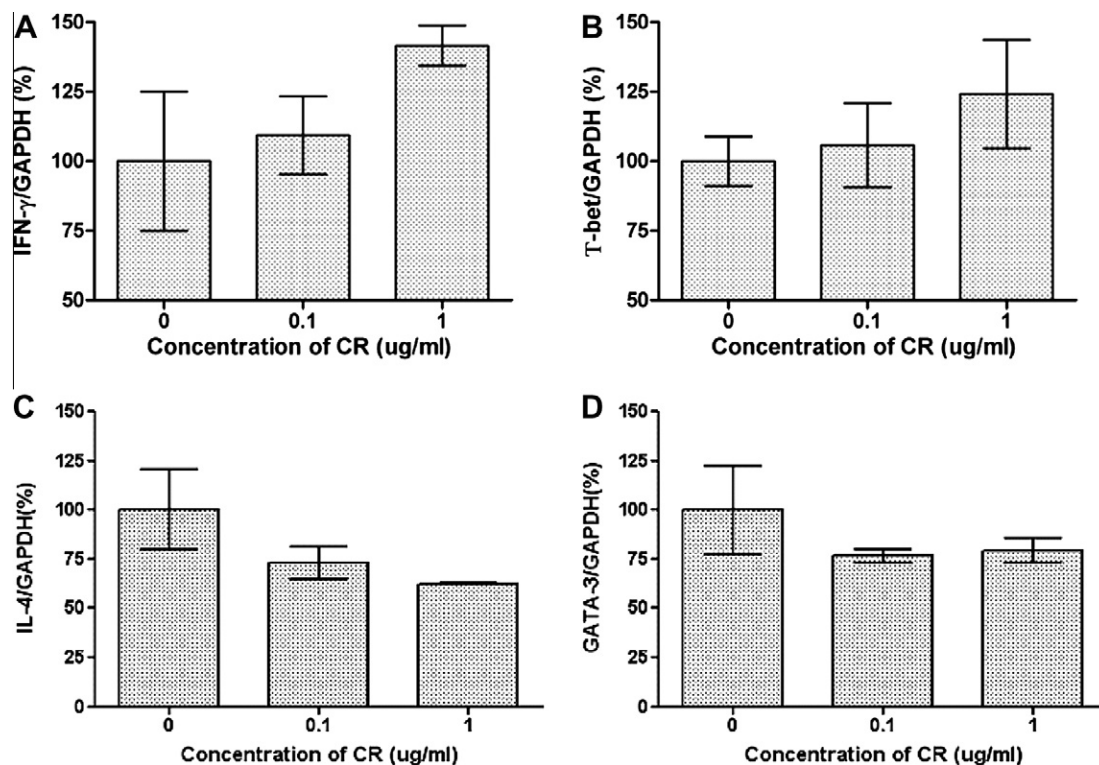


Fig. 7. The effect of CR on IFN- γ , IL-4, T-bet, and GATA-3 mRNA expression in Th1/Th2 skewed conditions by real-time PCR analysis. Naive CD4⁺ T cells were incubated with rIL-12 and anti-IL-4 Ab for Th1 differentiation (A and B). For Th2 cell differentiation, rIL-4 and anti-IL-12 Ab were added to the culture medium in the presence of rIL-2 (C and D). The cells were then incubated in the absence or presence of 0, 0.1, and 1 μ g/ml CR. In both culture conditions, total RNA was isolated and real-time RT-PCR was performed as described in Section 2. Data normalization was performed by dividing by the amount of GAPDH gene expression. (A and B) The amount of IFN- γ /T-bet expression was determined in the Th1 skewed condition. (C and D) The amount of IL-4/GATA-3 expression in the Th2 skewed condition. Data shown are the means $\pm$ SEM. Data were analyzed by one-way ANOVA followed by a post hoc test (Newman–Keuls Multiple Comparison Test).

time PCR. Although not statistically significant, in the Th1 skewed condition, the transcript of IFN- γ was increased (Fig. 7A), while IL-4 gene expression was reduced in cells cultured in the Th2 skewed condition (Fig. 7C). Similarly, the major transcription factor responsible for inducing Th1 lineage programming, T-bet, was induced upon CR treatment (Fig. 7B), conversely, the expression of GATA-3, a transcription factor controlling Th2 lineage commitment, was slightly diminished, but not significant (Fig. 7D).

4. Discussion

A key component of the immune defense against pathogens is mediation by CD4⁺ T cells that can differentiate into functionally distinct subsets. The Th1 and Th2 cytokine patterns have now been implicated in several immune responses concerning infections, allergies, and autoimmunity [7]. In addition, many experimental models shown that the diversity of clinical or pathologic features of immune related diseases are correlated with the heterogeneity of the immune responses involved [16]. IFN- γ secreted from Th1 cells is known to induce the differentiation of naïve CD4⁺ T cells into Th1 cells and to inhibit the proliferation of Th2 cells [17,18]. Conversely, IL-4 secreted from Th2 cells is known to induce the differentiation of naïve CD4⁺ T cells into Th2 cells and to inhibit the function of Th1 cells [6,19,20]. Therefore, each Th1 cytokine or Th2 cytokine controls the differentiation and function of helper T cells.

The results of our MTS assay showed that CR extract did not influence the viability of un-stimulated CD4⁺ T cells, which implies that CR does not behave as an antigenic stimulator or a mitogenic activator (Fig. 2). Upon CR treatment, IL-4 secreting CD4⁺ T cells shows decreasing fashion significantly in Th2 condition, while

IFN- γ secreting cells were increased significantly in Th1 skewed condition (Figs. 3 and 4). In addition, to confirm the effect of CR on the Th1/Th2 lineage development, Th1/Th2 specific cytokine profiles were examined. Upon CR treatment, IL-4 secretion was significantly decreased in dose dependant manner, and higher levels of the IFN- γ secretion were observed significantly in CR treated cells that were incubated under skewed Th1/Th2 conditions (Figs. 5 and 6). These results demonstrated that CR has abilities to promote selective helper T cell lineage development without affecting the integrity of naïve CD4⁺ T cells. In significant cytokines of Th2 cells, IL-4 is primarily responsible for switching on B-cell production of IgE and is therefore a key trigger of IgE-dependent reactions [1,7,21]. Therefore, our observation implies that the CR extract could be effective at treating allergic diseases, such as asthma, by diminishing Th2 cell development. To confirm whether the induction level of IFN- γ secretion was corresponding to the increasing level of IFN- γ mRNA expression in the Th1 skewed condition, and also the reduction level of IL-4 secretion was corresponding to the decreasing level of IL-4 mRNA expression in cells in the Th2 skewed condition, real-time PCR was performed. Although not statistically significant, the amount of IFN- γ mRNA was induced and also the IL-4 mRNA was diminished upon CR treatment (Fig. 7A and C). Currently, several transcription factors that control the differentiation of Th1/Th2 cells have been identified. For example, T-bet is specifically correlates with IFN- γ [3], and GATA-3 is selectively induced by IL-4 through STAT6 during the early stages of Th2 development [22,23]. In addition, c-maf, NIP45 have also been shown to induce Th2 cytokine production, although only c-maf and GATA-3 are expressed selectively in Th2 cells [7]. To investigate the intracellular events that regulate Th1/Th2 cell development by CR, we examined the amount of T-bet and GATA-3

mRNA, which is the one of main regulators in the Th1/Th2 immune response. Although not statistically significant, the expression level of T-bet mRNA increased upon CR treatments, while the expression level of GATA-3 mRNA slightly decreased by CR treatments (Fig. 7B and D). These results suggest that CR may directly regulate T-bet and GATA-3 gene expressions. However, we cannot exclude the involvement of other transcription factors regulating Th1/Th2 lineage commitment such as Stat6, c-maf and NIP45, since the differences of T-bet and GATA-3 expression level were not significant.

In conclusion, the present study demonstrated that CR directly influences CD4⁺ T cells and has effect on the improvement of Th cell responses, from a Th2-dominant to a Th1-dominant pattern by increasing the expression of IFN- γ and decreasing IL-4. Therefore, CR extract may be useful material for preventing the onset of allergies or improving allergic symptoms. These findings suggest that CR with its immune-modulatory activities is useful for the correction of Th2-dominant pathological disorders.

Acknowledgments

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