Chung-Yeul-Gue-Soup-Sa-Gan-Tang, Traditional Korean Medicine, Enhances CD4⁺ T Cell Activities and Modulates Th1/Th2 Lineage Development

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Abstract. Chung-Yeul-Gue-Soup-Sa-Gan-Tang (CYT), a traditional Korea herbal medicine, has been widely used in Korea for the treatment of various immunological disorders, including allergic asthma. In this study, CYT was examined in vitro and tested for possible immunological effects. The results demonstrated that CYT had no mitogenic effects on unstimulated CD4⁺ T cells, but rather increased CD4⁺ T cell proliferation upon activation with anti-CD3/CD28 antibody. Under the Th0 condition, CYT also enhanced expression of interleukin (IL)-2 in purified murine CD4⁺ T cells assayed by real-time PCR, suggesting that CYT moderately increases the activity of helper T cells upon T cell receptor ligation under the neutral condition. However, the Th1 cells were overpopulated following CYT treatment under the Th1 condition, while Th2 cells were under-populated in the Th2 driven condition. In addition, under Th1/Th2-skewed conditions, the levels of IL-4 were considerably decreased, while the expression of T-bet and interferon-γ were increased with CYT treatment. Thus, CYT enhances Th1 lineage development from naive CD4⁺ T cells both by increasing Th1 specific cytokine secretion and repressing Th2 specific cytokine production. These results suggest that CYT is a desirable agent for the correction of Th2 dominant pathological disorders.

Keywords: Th1, Th2, CD4⁺, interleukin-4, interferon-γ

Introduction

Naive CD4⁺ T cells are divided into two subpopulations, Th1 and Th2 cells, according to differences in their cytokine expression profiles (1). CD4⁺ T cells recognize specific MHC class II molecules on antigen presenting cells (APC) via T cell receptor interactions, thereby providing the first signal required for activation (2 – 4). The second, co-stimulatory signal, is provided by accessory molecules expressed on APCs, such as the widely studied B7 family of proteins (5). The combination of these two signals induces a cascade of events: interleukin (IL)-2 synthesis and secretion, IL-2 receptor expression, followed by clonal expansion and differentiation of precursor CD4⁺ T cells into effector T helper (Th) cells (4). The functional differences between Th cell subsets are explained primarily through the activities of their secreted cytokines. Interferon (IFN)-γ secreted from Th1 cells is known to induce differentiation of naive CD4⁺ T cells into Th1 cells, and to inhibit the proliferation of Th2 cells (3). On the other hand, IL-4 and IL-10, 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 (6, 7). These distinct subsets of helper T cell are responsible for specific immune functions; Th1 cells contribute to cell-mediated inflammatory immunity, while Th2 cells are responsible for humoral responses (8, 9). Actually, this regulation occurs in response to
numerous environmental factors that directly or indirectly influence the decision of a naive CD4⁺ T cell to become a Th1 or Th2 effector cell; and this occurs in autoimmune disease and allergies, respectively (5). Therefore, the Th1/Th2 classification scheme is useful when relating overall cytokine production patterns to clinical outcomes in a variety of pathological states (2).

It is well documented that the Th1/Th2 cell response is shifted to a predominantly Th2 cell response in IgE medicated allergic diseases, and thus IL-4 production from Th2 cells is increased in these diseases (10). To solve these pathogenic advances in Th1 from Th2 cells is increased in these diseases (10). To solve these pathogenic advances in Th1/Th2 deviation, the development of specific agents affecting helper T cell differentiation have drawn special attention in the last decade. Chung-Yeul-Gue-Soup-Sa-Gan-Tang (CYT) as an herbal prescription was first used by You et al., while at Kyunghee Oriental Medical Center of Seoul, Korea, during the late 1960s. It was based on Teum-Hwa-Sa-Gan-Tang and is still used for treating various immunological disorders, especially atopic dermatitis, one of the typical Th2 dominant diseases. To investigate the possible immune modulatory effects of CYT on helper T cells, we isolated CD4⁺ T cells from the splenocytes of mice and observed their immune function, with intense focus on the Th1/Th2 lineage development.

Materials and Methods

Mice

Eight-weeks-old female BALB/c mice were purchased from Taconic (O San, Korea), and they were maintained in temperature- and humidity-controlled, pathogen-free conditions.

Preparation of CYT extract

CYT, which contains 30 species of medicinal plants (Table 1), was purchased from Korean Association of Crude Medicinal Herbs (Seoul, Korea) and washed with distilled water. Every herb of CYT was ground to a fine powder and mixed with amount of 800 g as the ratio in the Table 1. These were extracted with 70% ethanol (Duksan Pharmaceutical Co., Ltd. (Ahn-San, Korea); technical reagent) (v/v) by using an ultra-sonicator (Branson, Danbury, CT, USA) for 10 min at room temperature and then gradually extracted with 80%, 90%, and 100% ethanol using the same method. The alcoholic solution extract was evaporated at 60°C and then freeze-dried. The yield was 97.68 g (12.21%). CYT extracts were dissolved in ultra-pure distilled water and then sterilized by passing through 0.22-μm syringe filter.

The HPLC analysis of standard materials to test samples

The dried ethanol extract, about 240 mg of CYT, was accurately weighed; then it was put in test tube, and dissolved in 4 ml of 50% methanol (HPLC reagent; J.T. Baker Co., Ltd., Phillipsburg, NJ, USA) followed by filtering using 0.45-μm syringe filter (PVDF; Waters Co., Milford, MA, USA). The following marker substances (standard materials), used for the quantitative analysis to CYT, were purchased from the indicated sources: quercetin, glycyrrhizic acid, and scopoletin (Sigma, St. Louis, MO, USA); (-)-epicatechin and baicalin (Aldrich, St. Louis, MO, USA); aesculin and homogentistic acid (Fluka, Buchs, Switzerland); atracylenolide III, chlorogenic acid, magnolol, alisol B acetate, saikosaponin A, C, D, p-coumaric acid, paeonol, (+)-corydaline, and geniposidic acid (Wako, Osaka). The standard materials weigh 10 mg each and were dissolved in according to the analysis condition of standard materials. The dissolved standard solution was diluted to 0.1, 0.5, 1.0, 1.5, 2.0 mg/ml, respectively; and then we obtained the standard HPLC chromatogram. The relationship between the concentration and the peak-area was measured by the minimum square method (R² value). The HPLC apparatus was a Waters Breeze System (717+ Autosampler, 2487 dual λ absorbance detector, 1525 binary HPLC Pump; Waters Co., and Waters Breeze System (Ver. 3.20, Waters Co.) was used for data acquisition and integration.

The quantity of standard materials contained in CYT was calculated by the following formula:

\[
\text{The amount (mg) of standard materials} = \left( \frac{\text{[the quantitative amount (mg) of standard materials} \times \text{AT} / \text{AS}} \right) / n \quad (n = 3)
\]

(c.f., AT: the peak-area of test samples containing standard materials, AS: the peak-area of standard materials).

The standard materials for quantitative analysis of each herb medicine containing CYT are shown in Table 1. From the result of the standard calibration curve, R² values of all marker substances are between 0.991 and 0.999. The amounts of standard materials in CYT are indicated in Table 1.

Antibodies

The antibodies used were anti-CD3ε mAb (145-2C11); anti-CD28 mAb (37.51); anti-IL-4; anti-IL12; PE-conjugated anti-CD69; FITC-conjugated anti-CD4⁺; and recombinant IL-2 (rIL-2), rIL-12, and rIL-4 (BD Biosciences, San Diego, CA, USA) and magnetic cell sorting CD4⁺ (L3T4) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Splenocytes preparation and CD4⁺ T cell isolation

Splenocytes 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 300 × g to separate cells from debris, the cells were washed in RPMI medium, followed by lysis of erythrocytes using ammonium chloride lysing reagent (BD Biosciences). Cells were counted and viability was determined by trypan blue exclusion. Subsequently, CD4+ T cells were isolated from splenocytes by positive selection using MACS CD4+ (L3T4) microbeads (Mitenyi Biotec) as described by the manufacturer. In brief, spleen cells were resuspended in 90 μl of medium per 10^7 total cells. Ten microliters of MACS CD4+ microbeads was added to the cell suspension and incubated for 15 min at 4°C. After adding sufficient amounts of medium, cells were centrifuged for 10 min at 300 × g and the supernatant was removed. The cell pellet was resuspended in 500 μl of medium. A positive selection column was chosen and placed in the magnetic field of the MACS separator (Mitenyi Biotec). After applying the cell suspension onto the column, it was removed from the separator and placed on a collection tube. The positively fractionated cells were flushed out of the column using the plunger supplied with the column.

**Survival and proliferation assay**

To determine the survival and proliferative capacity of the splenocytes and CD4+ T cells, the Cell Titer 96™ non-radioactive cell proliferation assay (Promega, Madison, WI, USA) was used, following the directions provided by the manufacturer. Cells were plated at 4 × 10^5 cells/well in a total volume of 100 μl. For activation of CD4+ T cells, cells were placed onto anti-
CD3 coated plates, followed by addition of anti-CD28 Ab, and cultured in CYT (0, 1, 2, 5, 20 μl/ml) containing medium. Cells were incubated for 48 h at 37°C in 5% CO2.

Analysis of cell surface marker CD69 expression

For cell surface staining, cells were cultured in 24-well plates (3 x 10^6 cells/well). Cells were placed onto anti-CD3 Ab-immobilized 24-well plates, followed by addition of anti-CD28 Ab and cultured in CYT (0, 1, 2, 5 μg/ml) containing medium for 48 h. Staining for cell surface markers was performed as described by the instructions of the manufacturer. In brief, cells were harvested and centrifuged 300 x g for 5 min. After removing the supernatant, the pellet was resuspended in cold wash buffer (PBS, 0.1% NaN3, 1% FBS); centrifuged; and resuspended in 100 μl of wash buffer and stained with FITC-conjugated CD4+ and PE-conjugated CD69 and incubated at 4°C in the dark for 40 min. After washing twice, the cells were analyzed with a FACSscan (BD Biosciences, Becton Dickinson, Flanklin Lakes, NJ, USA).

In vitro Th1 and Th2 cell polarization

CD4+ T cell (3 x 10^6 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 μg/ml anti-CD3 (Pharmigen) and 2 μg/ml anti-CD28 (Pharmigen) for 24 h. Naive CD4+ T cells were incubated with 5 ng/ml rIL-12 (Pharmigen), 10 μg/ml anti-IL-4 (Pharmigen), and 50 U/ml rIL-2 for Th1 differentiation. For Th2 cell differentiation, 10 ng/ml rIL-4 (Pharmigen) and 10 μg/ml anti-IL-12 (Pharmigen) were added to the culture medium in the presence of 50 U/ml of rIL-2. In both type of culture conditions, two-fold of the original volume of medium containing corresponding antibodies and cytokines were added to the culture on day 3 and then incubated for another for 4 days. On day 7, cells were harvested, washed, and restimulated (at 10^6 cells/ml) with plate-bound 10 μg/ml anti-CD3 (Pharmigen) and 2 μg/ml anti-CD28 (Pharmigen) for 24 h.

Cytokine secretion assays

Cytokine secretion assays were performed using a Mouse Secretion Assays detection kit following the user’s manual (Mitenyi Biotec). Briefly, sorted CD4+ T cells (10^6 cells) were washed twice by adding 2 ml of cold buffer (PBS, 0.5% BSA, 2 mM EDTA) and then centrifuged at 300 x g for 10 min at 4°C. The cells were resuspended in 90 μl of cold medium. Mouse Catch Reagent (10 μl) was added and incubated for 5 min on ice. After adding 10 x 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 settled cells. Then the cells were washed once and then resuspended in 90 μl of cold buffer per 10^6 cells. After PE or FITC-conjugated antibodies were added to the mixture, samples were incubated for 10 min on ice. Finally the cells were analyzed with a Becton Dickinson FACScan.

Isolation of RNA, cDNA synthesis, and Real-Time PCR analysis

CD4+ T cell and Th1/Th2 cell were lysed in 500 μl Trizol reagent (Gibco Invitrogen, Carlsbad, CA, USA) and total RNA was isolated exactly according to the instructions of the manufacturer. The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis (data not shown), and the concentration of total RNA was quantified by determination of optical density at 260 nm (OD260). The total RNA was used for reverse transcription. Reverse transcription was performed by mixing 2 μg of RNA, 7.5 μl DEPC-DW with 1 U DNase I, and 10 x DNase I reaction buffer. This solution was incubated for 15 min at room temperature. Tubes were added with 1 μl of 25 mM EDTA and heated for 10 min at 70°C. Then 0.5 μg oligo (dT) primer was added and the mixture was incubated at 70°C for 5 min. Then a solution containing 5 μl of M-MLV 5x reaction buffer, 1 μl of 10 mM dNTPs, 20 U of RNase inhibitor, and 5.5 μl of DEPC-DW was added and tubes were incubated for 2 min at 25°C. M-MLV (200 U) reverse transcriptase was added and incubated for 50 min at 42°C. Finally the tubes were heated to 15 min at 70°C. Samples were stored at –20°C until further use.

Real-time quantitative PCR was performed on a GeneAmp 5700 Sequence detection system (Applied

Table 2. Sequences of primer used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>RV: 5’-GGC ATG GAC TGT GGT GAT CA-3’</td>
</tr>
<tr>
<td></td>
<td>FW: 5’-TTC ACC ACC ATG GAG AGC GC-3’</td>
</tr>
<tr>
<td>IL-2</td>
<td>RV: 5’-GTC TCT CGT GTC AAC AGC GC-3’</td>
</tr>
<tr>
<td></td>
<td>FW: 5’-GAG CTT TAT GTT TGG TAA GC-3’</td>
</tr>
<tr>
<td>IL-4</td>
<td>FW 5’-ACA GGA GAA GGG AGC CCA T-3’</td>
</tr>
<tr>
<td></td>
<td>RV 5’-GAA GCC CTA CAG AGC AGC TCA-3’</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>FW 5’-TCA AGT GGT GGT GAT GTG GAA-3’</td>
</tr>
<tr>
<td></td>
<td>RV 5’-TTG CTC TGC AGG ATT TTC ATG-3’</td>
</tr>
<tr>
<td>T-bet</td>
<td>FW 5’-GCC AGG CAG CTT ATA TG-3’</td>
</tr>
<tr>
<td></td>
<td>RV 5’-GAC GAT CAT CTG GTG CAC ATT-3’</td>
</tr>
<tr>
<td>GATA-3</td>
<td>FW 5’-GAA GGC ATC CAG ACC CGA AAC-3’</td>
</tr>
<tr>
<td></td>
<td>RV 5’-ACC CAT GGC GGT GAC CAT GC-3’</td>
</tr>
</tbody>
</table>
Biosystems, Warrington, UK) by using SYBR Green I as dsDNA-specific binding dye and continuous fluorescence monitoring. Amplification was carried out in a total volume of 25 μl containing 5 μM of each cytokine-specific primer (Table 2), 2 × PCR Master Mix (PE Applied Biosystems), and 2 μl of 1:4 diluted cDNA. The PCR reactions were cycled 40 times, denaturation (95°C, 15 s) and annealing (60°C, 1 min). Data was analyzed by GeneAmp 5700 SDS software (Applied Biosystems). The dissociation curves from all data show only one distinct peak, implicating that there are only one PCR product in the reaction. Additionally, the standard curves also demonstrate that the R² values are between 0.98 and 0.999, which show high reliability of the data.

ELISA assay for detecting tumor necrosis factor (TNF)-α and IL-5

Flat-bottomed, 96-well plates were coated overnight at 4°C with TNF-α mAb or IL-5 mAbs. The primary mAbs were discarded and the plates were blocked with Assay Diluent (Pharmlingen) for 1 h at room temperature. The plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS) and blotted on a paper towel. Diluted samples or TNF-α and IL-5 standards were added in triplicate. The plates were incubated for 2 h at room temperature, the supernatant was discarded, and the wells were washed 5 times with wash buffer. TNF-α or IL-5 detecting mAb plus avidin-HRP was added and incubated for 1 h at room temperature. After washing, tetramethylbenzidine substrate solution (Pharmlingen) 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 read at 450 – 570 nm and the sample concentrations were determined with the help of a standard curve.

Statistical analysis

Statistical analysis of data was carried out using the Prism 3.02 (GraphicPad Software Inc., San Diego, CA, USA). All experiments have been repeated at least three times and revealed comparable results. Values are depicted as the mean ± S.E.M.

Results

CYT directly influences CD4⁺ T cell activities

The direct effect of CYT on purified CD4⁺ T cells was measured under in vitro conditions. CYT treatment increased the proliferation rate of sorted CD4⁺ T cells upon CD3/CD28-mediated activation in a dose-dependent manner up to 5 μg/ml. The maximum response was achieved at a concentration of 2 μg/ml of CYT (Fig. 1A). In addition, T cell activation surface markers on CD69-positive cells were slightly increased with CYT treatment upon T cell receptor (TCR) ligation, although this result was not statistically significant (Fig. 1: B and C). We also determined the expression of IL-2, the major cytokine of CD4⁺ T cells, using the method of Real Time PCR. These results indicate that IL-2 expression increased in CD4⁺ T cells treated with CYT (Fig. 1D). These results imply that CYT directly enhances CD4⁺ T cell activation without antigen presenting cells being present.

CYT effect on the modulation of Th1/Th2 cell polarization

To investigate the effect of CYT on helper T cell development, CD4⁺ T cells were activated with anti-CD3/anti-CD28 and then cultured in CYT-containing medium under Th1-inducing or Th2-inducing conditions. After 7 days, the cells were washed and re-stimulated for 24 h in the absence of cytokines and blocking antibodies. To directly show the effect of CYT on Th1/Th2-specific cytokine gene expressions, we performed a Real Time RT-PCR reaction using RNAs from the activated CD4⁺ T cells. IL-4- or IFN-γ-secreting cells were quantitated using flow cytometry analysis to evaluate the Th1/Th2 polarized status following CYT treatments. Under the Th1 skewed condition, the T cell population secreting IFN-γ is slightly increased in cells treated with CYT. On the other hand, IL-4 secreting cells are dramatically under-represented in CYT treated cells under the Th2 polarized condition (Fig. 2). Similar patterns are observed at the transcription level under Th1/Th2 skewed conditions (Fig. 3). In addition, the secretion of another Th1 cytokine, TNF-α, was dramatically increased under the Th1 condition. However, IL-5, a Th2 cytokine, was not altered in its secretion level (Fig. 3). Interestingly, these immune modulatory effects by CYT seem to be mediated by a Th1-specific transcription factor called T-bet, since the expression of T-bet was significantly enhanced with CYT treatment. However, the major transcription factor responsible for inducing Th2 lineage programming, called GATA-3, was not affected by CYT, suggesting that CYT may modulate Th2 development independent of GATA-3 action.

These results strongly suggest that CYT specifically induces the immune response to change from the Th2 cell dominated pattern to the Th1 cell dominated pattern.

Discussion

Many experimental models have correlated the diversity of clinical or pathological features in immune-
related diseases with the heterogeneity of the immune responses involved (11). IFN-$\gamma$, secreted from Th1 cells, is known to induce differentiation of naive CD4$^+$ T cells into Th1 cells and to inhibit the proliferation of Th2 cells (12). On the other hand, IL-4 secreted from Th2 cells is known to induce the differentiation of naive CD4$^+$ T cells into Th2 cells and to inhibit the function of Th1 cells (6, 7). Thus, it was reported that each Th1 cytokine or Th2 cytokine controlled the differentiation and function of helper T cells. It was reported that the Th1/Th2 cell response was shifted to a predominantly Th2 cell response in allergic diseases, and thus IgE production from B cells was increased under these conditions (2).

Numerous published reports describe the biological action of traditional Chinese herbal medicine on immune human responses. Among several Chinese herbal medicines, So-Cheong-Ryong-Tang (SCRT or Xiao-Qing-Long-Tang in China) is probably the most frequently prescribed herbal medicine for treating allergic asthma and rhinitis. The effects of SCRT have been investigated in vivo, in vitro, and at the clinical level. A broad
range of pharmacological evidence supports a role for SCRT, including decreasing antigen-induced eosinophil infiltration in guinea pigs (13), suppressing allergen-induced bronchial inflammation in mite-sensitized mice (14), and decreasing serum IgE level in allergic rhinitis patients (15, 16). All of these studies consistently demonstrate the beneficial effect of SCRT on these allergic diseases. On the other hand, although CYT is another herbal medicine that has been prescribed as much as SCRT for treating allergic diseases in Korea, the effects of CYT are poorly studied and not well understood. To our knowledge, this is the first study to report the beneficial effects of CYT in the treatment of these allergic diseases.

The most difficult obstacle in developing an anti-allergic medicine is the general immune suppressive effect seen when using cortisol or cyclosporin A. Our study demonstrated that CYT does not suppress the T cell immune reaction, but rather enhances the TCR-triggered T cell response and IL-2 expression, suggesting that CYT is not a general immune suppressor. These results also indicate that CYT extract restores Th cell responses from a Th2-dominant to a Th1-dominant pattern, suggesting that CYT extract has the capacity to suppress IgE mediated allergic reactions. However, it should be noted that the mouse strain used in this study, BALB/c, often develops overwhelming Th2 responses, so we cannot guarantee that the observations made here will occur in the general genetic background. Further studies should be performed to support the implication.
drawn from this report by using other mouse strains such as C57BL/6 that develop strong Th1 responses.

Currently, several transcription factors have been identified that control the differentiation of Th1/Th2 cells; GATA-3, as well as c-Maf, are involved in the Th1 transition while T-bet is upregulated in Th1 cells. Ectopic expression of T-bet enhances endogenous IFN-\(\gamma\) production and induces polarized Th2 cells to redirect their development into Th1 cells (3). These results strongly suggest that T-bet initiates the Th1 lineage development change from naive Th cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs. Moreover, T-bet knock-out mice manifest multiple physiological and inflammatory features characteristic of asthma, suggesting that a T-bet deficiency itself may induce allergic asthma in the absence of allergen (17). In this study, CYT strongly induced the expression of T-bet while the GATA3 expression remained unchanged. These results imply that the anti-allergic effect of CYT arises from direct regulation of helper T cell lineage programming via T-bet regulation. However, the involvement of T-bet alone cannot explain the effect of CYT, since the over-expression of T-bet in Th2 cells usually suppresses not only IL-4 but also IL-5 (3). These results do not agree with the present results showing an unchanged secretion of IL-5. Investigating the role of other transcription factors associated with helper T cell development such as Stat6, Stat4, and c-Maf in the presence of CYT will elucidate the detailed mechanisms of CYT on Th1/Th2 lineage development.

In conclusion, the present study demonstrates that
CYT directly influences CD4\(^+\) T cell immune activity without APCs and has an effect on the improvement of Th cell responses, from a Th2-dominant to a Th1-dominant pattern by increasing the expression of IFN-\(\gamma\) and decreasing IL-4. Therefore, CYT extract, with its immuno-modulatory activities, is useful for preventing the onset of allergies or improving allergic symptoms.

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**References**