So-Cheong-Ryong-Tang, Traditional Korean Medicine, Suppresses Th2 Lineage Development

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The present study was designed to evaluate the effect of So-Cheong-Ryong-Tang (SCRT, also called Sho-Seiryu-To or Xiao-Qing-Long-Tang) on helper T cell development by monitoring Th1/Th2-specific cytokine secretion patterns in artificially induced Th1 or Th2 polarized conditions. The results demonstrated that Th2 cells were dramatically underpopulated in the Th2-driven condition triggered by SCRT treatment, while the Th1 cells were not altered in the Th1-skewed condition. Furthermore, under Th2-skewed conditions the levels of interleukin-4 were considerably decreased with SCRT treatment. The expression of GATA-3, a transcription factor that plays a pivotal role in Th2 lineage programming, did not change with SCRT treatment, while the expression of another Th2 transcription factor, c-Maf, was dramatically suppressed. These data suggest that SCRT modulates Th2 development by suppressing c-Maf expression. This study implies that the SCRT effect on CD4+ T cells is a key pharmacologic point of effect for treating IgE-mediated allergic asthma. These results also suggest that SCRT might be a useful agent for the correction of Th2-dominant pathologic disorders.

Key words Th1; Th2; So-Cheong-Ryong-Tang; interleukin-4

Naive CD4+ T cells are divided into two subpopulations termed Th1 and Th2, according to differences in their cytokine expression profiles.1) The functional differences between Th subsets are explained through the activities of their secreted cytokines. Interferon-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.2) On the other hand, interleukin (IL)-4 and IL-10, secreted from Th2 cells, are known to induce the differentiation of naive CD4+ T cells to Th2 cells and to inhibit the function of Th1 cells.3,4) These distinct subsets of helper T cells are responsible for specific immune functions; Th1 cells contribute to cell-mediated inflammatory immunity, while Th2 cells are responsible for humoral responses.5,6) 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. Malfunctions of this regulation occur in immune disorders such as autoimmune disease and allergies, respectively.7)

Therefore, the Th1/Th2 classification has been most useful when relating overall patterns of cytokine production to clinical outcomes in a variety of pathological states.8) The well-documented Th1/Th2 cell response is shifted to a predominantly Th2 cell response in IgE-mediated allergic diseases, and thus IL-4 production from Th2 cells is increased in these diseases.9) To resolve 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.

So-Cheong-Ryong-Tang (SCRT) also called Xiao-Qing-LongTang or Sho-Seiru-To, contains eight species of medicinal plants and has been an herbal medicine used to treat diseases such as allergic rhinitis and asthma for hundreds of years in Asian countries. However, its pharmacologic mechanism is poorly understood. Previously, we demonstrated that SCRT decreases the expression of the IL-4 mRNA that plays a pivotal role in Th2 cell development, while it increases IFN-γ expression in the Th0 condition. That study strongly implied that SCRT can correct the Th2-dominant condition by directly affecting CD4+ T cell development. To investigate further the possible immuno modulatory effects of SCRT on helper T cells, we isolated CD4+ T cells from the splenocytes of mice and observed the immune function with the focus on the Th1 or Th2 lineage development.

MATERIALS AND METHODS

Mice Eight-week-old female BALB/c mice were purchased from Taconic, Korea, and maintained under temperature and humidity-controlled pathogen-free conditions.

Preparation of SCRT Extract SCRT, containing eight species of medicinal plants (Table 1) was purchased from the Korean Association of Crude Medicinal Herbs and washed using distilled water. Every herb in SCRT was ground to a fine powder and mixed with the others in a total amount of 800 g in the ratios in shown Table 1. These were extracted with 70% ethanol (Duksan Pharmaceutical Co. Ltd., Korea, technical reagent) (v/v) using an ultrasonicator (Branson, U.S.A.) for 10 min at room temperature, and then gradually extracted with 80%, 90%, and 100% ethanol using the same method. The alcoholic solution extracted was evaporated at 60 °C and then freeze-dried. The yield was 97.68 g (12.21%). SCRT extracts were dissolved in ultrapure distilled water and sterilized by passing through a 0.22-μm syringe filter.

HPLC Analysis of Standard Materials to Test Samples The dried ethanol extract of about 240 mg of SCRT was accurately weighed, placed in a test tube, and dissolved in 4 ml of 50% methanol (HPLC reagent, J.T. Baker Co. Ltd., U.S.A.), followed by filtering using a 0.45-μm syringe filter (PVDF, Waters, U.S.A.). Each marker substance (standard

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In brief, spleen cells were resuspended in 90% 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 on the column, it was removed from the separator and placed in a collection tube. The positively fractionated cells were flushed out of the column using the plunger supplied with the column.

**In Vitro Th1 and Th2 Cell Polarization**

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

**Isolation of RNA, cDNA Synthesis, and Real-Time PCR Analysis**

Real-time PCR was performed on a GeneAmp 5700 Sequence detection system (PE Applied Biosystems, U.S.A.) using SYBR Green I as a dsDNA-specific binding dye for continuous fluorescence monitoring. Amplification was carried out in a total volume of 25 μl containing 5 μl of each of the cytokine-specific primers (5′-TTCACCCACCATGGAAGGCG-3′ and 5′-GGCATGGA-CGTGGTCATGA-3′ for GAPDH, 5′-ACAGGAGAAAGGCACCTCAG-3′ and 5′-GAAGCCCTTACAGCGGCTC-3′ for IL-4, 5′-GAAGGCCATCCAGGACCC-3′ and 5′-ACCACCATGCGTCCTACG-3′ for GATA-3, 5′-ATCCGACTGAAGCAGGG-3′ and 5′-TCCTTGAGCCC-3′ for c-Maf), 2×PCR Master Mix (Applied Biosystems, U.S.A.) 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 were analyzed using GeneAmp 5700 SDS software (Applied Biosystems). The dissociation curves from all data showed only one distinct peak, implying that there is only one PCR product in the reaction (data not shown). Additionally, the standard curves also demonstrate that the R² values were between 0.98 and 0.99.

### Table 1. Contents of SCRT and Amounts of Standard Materials in SCRT Extract (ex) Mixture

<table>
<thead>
<tr>
<th>Herbal medicine</th>
<th>Raw material amount, g (%)</th>
<th>Standard materials (SM)</th>
<th>SM amount, mg/ex. 1 g (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epiphragmi Herba</td>
<td>6.0 (14.3)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Paeoniae Radix Alba</td>
<td>6.0 (14.3)</td>
<td>Paeoniflorin</td>
<td>34.42 ± 1.367 (3.44)</td>
</tr>
<tr>
<td>Schizandreae Fructus</td>
<td>6.0 (14.3)</td>
<td>Schizandrin</td>
<td>2.39 ± 0.010 (0.24)</td>
</tr>
<tr>
<td>Pinelliae Rhizoma</td>
<td>6.0 (14.3)</td>
<td>Homogentistic acid</td>
<td>17.07 ± 0.318 (1.71)</td>
</tr>
<tr>
<td>Asari Herba Cum Radice</td>
<td>6.0 (14.3)</td>
<td>α-Ararone</td>
<td>0.18 ± 0.029 (0.018)</td>
</tr>
<tr>
<td>Zingiberis Rhizoma</td>
<td>4.0 (9.5)</td>
<td>6-Gingerol</td>
<td>2.11 ± 0.251 (0.21)</td>
</tr>
<tr>
<td>Cinnamomi Ramulus</td>
<td>4.0 (9.5)</td>
<td>Cinnamaldehyde</td>
<td>1.84 ± 0.438 (0.18)</td>
</tr>
<tr>
<td>Glycyrrhizae Radix</td>
<td>4.0 (9.5)</td>
<td>Glycyrrhizic acid</td>
<td>15.39 ± 0.283 (1.54)</td>
</tr>
</tbody>
</table>

N.A., not applied.
0.999, which show high reliability of the data (data not shown).

**Cytokine Secretion Assays** The cytokine secretion assay was performed using a mouse secretion assay detection kit following the user's manual (Mitenyi Biotec, Germany). Briefly, sorted CD4$^+$ T cells ($10^6$ cells) were washed twice by adding 2 ml of cold buffer (phosphate-buffered saline, 0.5% bovine serum albumin and 2 mM EDTA) then centrifuged at $300 \times g$ for 10 min at 4 °C. The cells were then resuspended in 90 μl of cold medium. Mouse Catch Reagent (10 μl) was added and incubated for 5 min on ice. After adding a 10-fold 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 re-suspended 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.

**RESULTS**

To verify that SCRT extract has an influence on CD4$^+$ T cells without APCs, we isolated CD4$^+$ T cells from mice spleen lymphocytes using magnetic anti-CD4 microbeads. Sorted CD4$^+$ T cells were activated with anti-CD3ε/anti-CD28 and cultured in SCRT containing medium under Th1-inducing or Th2-inducing conditions. After 7 d, the cells were washed and restimulated for 24 h in the absence of cytokines and blocking antibodies. Subsequently, IL-4- or IFN-γ-secreting cells were quantitated using flow cytometry analysis to evaluate the Th1/Th2-polarized status after SCRT treatment. In the Th1-skewed condition, the T cell population secreting IFN-γ did not change markedly in cells treated with SCRT. On the other hand, the number of IL-4-secreting cells dramatically decreased in SCRT-treated cells in the Th2-polarized condition (Fig. 1). To investigate the possible direct effect of SCRT on Th1/Th2-specific cytokine gene expressions, we performed real time RT-PCR using RNAs from activated CD4$^+$ T cells. In the Th2-skewed condition, the transcript levels of IL-4 decreased, which agreed with the flow cytometry data (Fig. 2). The major transcription factor responsible for inducing Th2 lineage programming, GATA-3, was not affected by SCRT, while the expression of another Th2 transcription factor called c-Maf was dramatically suppressed (Fig. 2). These data imply that SCRT modulates Th2 development by suppressing c-Maf expression. Together, these results strongly suggest that SCRT suppresses Th2 cell lineage development.

![Fig. 1](image_url)  
**Fig. 1.** Effects of SCRT on Helper T Cell Differentiation in Th1- or Th2-Skewed Conditions  
Isolated CD4$^+$ T cell (3 x $10^5$ 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 anti-CD3 10 μg/ml and anti-CD28 2 μg/ml. Naive CD4$^+$ T cells were incubated with rIL-12 and anti-IL-4 antibodies for Th1 differentiation (A and B). For Th2 cell differentiation, rIL-4 and anti-IL-12 antibodies were added to the culture medium in the presence of rIL-2 (C and D). The cells were incubated in the absence (A and C) or presence of SCRT 1 μg/ml (B and D). In both types of culture conditions, 2-fold of the original volume of medium containing corresponding antibodies and cytokines in the presence or absence of SCRT were added on day 3, then incubated another for 4 d. On day 7, cells were harvested, washed, and restimulated with plate-bound anti-CD3 and anti-CD28 for 24 h. Cells were stained with FITC-conjugated anti-CD4$^+$ antibodies and either PE-conjugated anti-IFN-γ antibodies (A and B), or anti-IL-4 antibodies (C and D). The flow cytometric data shown here are representative data from three independent analyses.
SCRT is the most frequently prescribed 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 pharmacologic evidence includes decreasing antigen-induced eosinophil infiltration in guinea pigs, suppressing allergen-induced bronchial inflammation in mite-sensitized mice, and decreasing serum IgE levels in allergic rhinitis patients. All of these studies consistently demonstrated a beneficial effect of SCRT in allergic diseases. The most difficult obstacle in developing antiallergic agents medicine is probably the general immuno suppressive effects as seen with cortisol or cyclosporin A. On the other hand, our previous study demonstrated that SCRT does not suppress the T cell immune reaction, but rather enhances the TCR-triggered T cell response and IL-2 expression, suggesting that SCRT is not a general immune suppressor. Furthermore, the present results also indicate that SCRT extract has selective immuno modulatory effects on Th2 cells but not on Th1 cells. Several transcription factors have been identified control the differentiation of Th1/Th2 cells; GATA-3 and c-Maf are involved in the Th2 transition, while T-bet is unregulated in Th1 cells. One line of study demonstrated that GATA-3 expression is increased within the bronchial mucosa of allergic asthmatic patients and the nasal mucosa of allergic rhinitis patients. In addition, ectopic expression of dominant-negative mutants of GATA-3 successively inhibits the allergic inflammation in a murine model of asthma. However, SCRT suppresses Th2 cell development by regulating factors other than GATA-3, since SCRT does not directly affect GATA-3 expression (Fig. 2). One candidate gene should be c-Maf because SCRT dramatically suppressed c-Maf expression in Th2 cells. C-Maf is a protooncogene that is produced predominantly in Th2 cells but not in Th1 cells. Overexpression of c-Maf transactivates the IL-4 promoter in Th2 cells and subsequently enhances Th2 lineage development. Interestingly, Kim et al. demonstrated that c-Maf knockout mice were markedly deficient in IL-4 production but that other Th2 cytokine levels are normal. Therefore it is necessary to examine the other Th2 cytokine production profiles with SCRT treatment before drawing any firm conclusions regarding the involvement of c-Maf regulation by SCRT. Investigating the role of other transcription factors associated with helper T cell development such as T-bet, Stat6 and Stat4 in the presence of SCRT will elucidate the detailed mechanisms of SCRT in Th1/Th2 lineage development.

In conclusion, the present study demonstrated that SCRT directly influences CD4+ T cells and ameliorates Th2 cell lineage development by decreasing IL-4 expression. Therefore SCRT extract may be useful for preventing the onset of allergies or improving allergic symptoms.

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