Trichosanthin Induced Th2 Polarization Status

Shuying Zhao¹, Yanyan Wang¹ and Haiming Wei¹, ²

Trichosanthin is extracted from the root tuber of Chinese medicinal herb Trichosanthes kirilowii maximowicz (Tian Hua Fen). TCS has abortifacient, anti-tumor, anti-HIV and immunoregulatory functions. It has been proved that it could inhibit immune response and arouse a T helper 2 response in the draining lymph node. In the current study the effect of TCS on mouse splenocytes was investigated. We stimulated C57BL/6 mice with TCS both in vivo and in vitro and analyzed the change of type 1 and type 2 cytokines in mouse splenocytes. The results showed that TCS could induce the expression of IL-4, one of the major T helper 2 (Th2) cytokines, and inhibit the expression of IFN-γ, an important Th1 cytokine in spleen lymphocytes both in vivo and in vitro. It is also shown the kinetics of Th1-to-Th2 transition after TCS stimulation in vivo in C57BL/6 mice. We found that type 2 cytokines, such as IL-10, TGF-β and IL-4 were increased regularly but IFN-γ was decreased at day 3 and then increased. However the mechanism for cytokine change is not clear.

Key Words: trichosanthin, IL-4, IFN-γ, Th

Introduction

Trichosanthin (TCS) is a 27 kD protein extracted from the root tuber of Chinese medicinal herb Trichosanthes kirilowii maximowicz (Tian Hua Fen). TCS has long been used medicinally in China for abortifacient purposes (1). Recent studies have shown that TCS also has anti-tumor, anti-HIV, anti-HSV and immunoregulatory functions (2-10).

It was reported that TCS immunization could upregulate interleukin-4 (IL-4) and IL-13 while inhibit interferon-γ (IFN-γ) gene expressions in mesenteric lymph node cells (11). And TCS increased IL-10 and monocyte chemoattractant protein-1 (MCP-1) expressions, whereas decreased IL-12 and tumor necrosis factor-α (TNF-α) expressions in peritoneal macrophages (12). However, little is known about the effect of TCS stimulation on mouse splenocytes. In this study, we demonstrated that TCS could induce the expression of IL-4, one of the major T helper 2 (Th2) cytokines, and inhibit the expression of IFN-γ, an important Th1 cytokine in spleen lymphocytes both in vivo and in vitro. It is also shown the kinetics of the trend of Th1-to-Th2 transition in C57BL/6 mice after in vivo TCS stimulation.

Materials and Methods

Animals

Female C57BL/6 mice, 4-6 weeks old, were obtained from Shanghai Experimental Center, Chinese Academy of Sciences, and maintained at our animal facility under the specific pathogen-free condition.

Reagents

Trichosanthin was purchased from Shanghai Jinshan Pharmaceutical Limited Company (Shanghai, PRC). FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse IL-4 and IFN-γ monoclonal antibodies were purchased from eBioscience.

Mouse immunization and cell preparation

C57BL/6 mice were injected with 5 μg TCS. A week later the mice were re-immunized with 5 μg TCS. After five days, the mice were sacrificed and the splenocytes were obtained by forcing tissues through stainless steel mesh. Before using, the erythrocytes were removed from the cell suspension by lysing solution (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA, and 170 mM Tris, pH 7.3). For semi-quantitative RT-PCR assay, the mice were sacrificed at day 3, day 6 and day 9 respectively after the second immunization to extract the total RNA of splenocytes. For in vitro stimulation, the spleen cells were separated from C57BL/6 mice under sterile conditions and used after erythrocyte lysing. Then the splenocytes were cultured in RPMI 1640 culture medium (Gibco) and stimulated with 0.1 μg/ml TCS for 5 h.
Semi-quantitative RT-PCR assay

Total RNA was extracted from splenocytes using TRIzol reagents (Life Technologies) according to the manufacturer’s instructions. Concentration and quality of the extracted RNA were determined by measuring light absorbance at 260 nm (A260) and the ratio of A260/A280. Reverse transcription (RT) was carried out with 4.5 μg total RNA using random hexamers as primers and M-MLV reverse transcriptase with 5× first strand buffer and 0.1 M DTT. The reaction system was incubated at 37°C for 50 min, and M-MLV was inactivated by heating at 70°C for 15 min. PCR was performed using 4× dNTP mixture and the Taq DNA polymerase with 10× reaction buffer and MgCl₂. The cytokine primers and PCR products’ sizes were shown in Table 1. The PCR reaction programs were optimized for each cytokine (13-15). Semi-quantitative RT-PCR was performed using β-actin as an internal control to normalize gene expression for the PCR templates. Results were obtained by electrophoresis and the relative light intensities of bands were analyzed by Scion Image (Release Beta 4.0.2 edition) software.

Flow cytometry

The splenocytes were treated as described above. Cells were then stimulated with 1 mM inomycin and 30 ng/ml PMA. Monensin (2 μM) was also added at the start of culture to inhibit cytokine secretion. After stimulation with FITC-anti-CD4, the samples (1 × 10⁶ cells) were fixed with 2% (w/v) paraformaldehyde for 30 min at 4°C. After permeabilization with 100 μl permeabilization buffer for 30 min at 4°C, the cells were incubated with rat serum for 30 min at 4°C. Then PE-anti-IFN-γ mAb or PE-anti-IL-4 mAb was added to the

Table 1. Specific primer sequences used in semi-quantitative RT-PCR assay

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Length</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>F 5’-ATGGATGACGATATCGCT-3’</td>
<td>569 bp</td>
</tr>
<tr>
<td></td>
<td>R 5’-ATGAGGTAGTCGTCAGGT-3’</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F 5’-AACGCTACACACTGCTTG-3’</td>
<td>399 bp</td>
</tr>
<tr>
<td></td>
<td>R 5’-GAGCTCATTGAATGCTTG-3’</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>F 5’-TAGTTGTCATCCTGCTCT-3’</td>
<td>359 bp</td>
</tr>
<tr>
<td></td>
<td>R 5’-GTCTTTTCAGTGATGGGAC-3’</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>F 5’-AGCGTGAAACACTGTCAAC-3’</td>
<td>301 bp</td>
</tr>
<tr>
<td></td>
<td>R 5’-TCATTCAAGGCCCTGAGACAC-3’</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>F 5’-GGGCCGGTGCTCGCTTTGTA-3’</td>
<td>424 bp</td>
</tr>
<tr>
<td></td>
<td>R 5’-GCCCTGTATTCCGCTCTCTT-3’</td>
<td></td>
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</table>
permeabilization buffer for 1 h at 4°C. The cells were washed with permeabilization buffer twice and with PBS once, and resuspended with 300 μl PBS for flow cytometric analysis. The stained cells were analyzed by FACSCalibur (Becton Dickinson) and the data were analyzed by WinMDI 2.8 software.

Results

**TCS inhibited IFN-γ but upregulated IL-4 expressions in vivo**

C57BL/6 mice were immunized with 5 μg TCS twice. Five days later after the second immunization, the splenocytes were collected, and intracellular staining was performed. The results showed that after TCS in vivo stimulation the expression of IFN-γ was decreased. The percentage of IFN-γ+ cells was decreased from 2.76 to 0.63 in splenocytes and decreased from 0.91 to 0.22 in CD4+ T cells (Figure 1). But the expressions of IL-4 were increased in both splenocytes and CD4+ T cells. The percentage of IL-4+ cells in splenocytes was increased from 1.04 to 1.88 and the percentage of IL-4+ cells in CD4+ T cells was increased from 0.15 to 0.51 (Figure 2).

**TCS inhibited IFN-γ but upregulated IL-4 expressions in vitro**

The splenocytes of normal C57BL/6 mice were treated with or without 0.1 μg/ml TCS for 5 h. Then the cells were washed with permeabilization buffer twice and with PBS once, and resuspended with 300 μl PBS for flow cytometric analysis. The stained cells were analyzed by FACSCalibur (Becton Dickinson) and the data were analyzed by WinMDI 2.8 software.

**The kinetics of Th1-to-Th2 transition after second TCS immunization**

C57BL/6 mice were immunized with 5 μg TCS twice and the mice were sacrificed at day 3, day 6 and day 9 respectively after the second immunization. The splenocytes were collected, total RNA was extracted and mRNA expressions of different cytokines were analyzed by RT-PCR. The results showed that after the second TCS immunization the expression of IFN-γ was decreased at day 3 but increased.
In the present study, C57BL/6 mouse immunization with TCS in vivo could cause IFN-γ (type 1 cytokine) decrease and IL-4 (type 2 cytokine) increase, and in vitro TCS stimulation also upregulated IL-4 but inhibited IFN-γ expressions in mouse splenocytes. In the investigation on the change of cytokines on temporal order we found that type 2 cytokines were increased regularly especially IL-10, which changed from undetectable to a quite high level. TGF-β, one of typical type 2 cytokines, was expressed highly in the normal C57BL/6 mice but it also had some change after the second immunization. The results of RT-PCR showed that the expression of IFN-γ was decreased at day 3 but increased later. The reason for this maybe is the invalidation of TCS in mice. It may also be concluded that this phenomenon of type 1 cytokines has something with the change of type 2 cytokines. However the mechanism for the change of these two types of cytokines after TCS stimulation is not quite clear. Maybe TGF-β is a necessary factor for this phenomenon. The particular explanation for this needs our future study.

References

19. Shibuya K, Robinson D, Zonin F, et al. IL-1α and TNF-α are required for IL-12-induced development of Th1 cells producing high levels of IFN-γ in BALB/c but not C57BL/6 mice. J Immunol. 1998;160:1708-1716.