Article

Trichosanthin Induced Th2 Polarization Status

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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. *Cellular & Molecular Immunology*. 2006;3(4):297-301.

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.

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Received Jun 30, 2006. Accepted Jul 22, 2006.

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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 NH_4Cl, 10 mM $KHCO_3$, 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.

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

Primers		Sequences	Length
β-actin	F	5'-ATGGATGACGATATCGCT-3'	569 bp
	R	5'-ATGAGGTAGTCTGTCAGGT-3'	
IFN-γ	F	5'-AACGCTACACACTGCTTGG-3'	399 bp
	R	5'-GAGCTCATTGAATGCTTGG-3'	
IL-4	F	5'-TAGTTGTCATCCTGCTCTT-3'	359 bp
	R	5'-GTCTTTCAGTGATGTGGAC-3'	
IL-10	F	5'-AGCTGGACAACATACTGCTAAC-3'	301 bp
	R	5'-TCATTCATGGCCTTGTAGACAC-3'	
TGF-β	F	5'-GGCGGTGCTCGCTTTGTA-3'	424 bp
	R	5'-GCCCTGTATTCCGTCTCCTT-3'	

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 (A_{260}) and the ratio of A_{260}/A_{280}. 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 $MgCl_2$. 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.

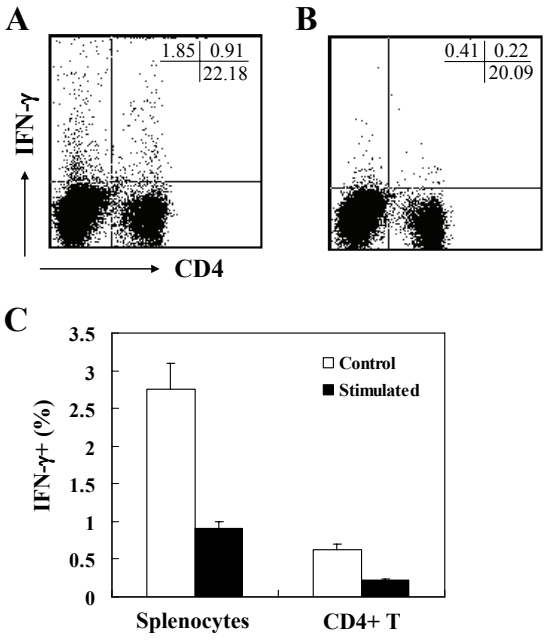

Figure 1. ***In vivo*** **TCS stimulation inhibited IFN-γ expression.** C57BL/6 mice were injected with 5 μg TCS. A week later, the mice were received another TCS injection. At day 5 after the second immunization, the mice were sacrificed and spleen cells were collected for intracellular IFN-γ staining. (A) The expression of IFN-γ in control $CD4^+$ T cells. (B) The expression of IFN-γ in $CD4^+$ T cells of TCS immunized mice. (C) The percentages of IFN-γ^+ cells in splenocytes and $CD4^+$ T cells of control and TCS immunized mice.

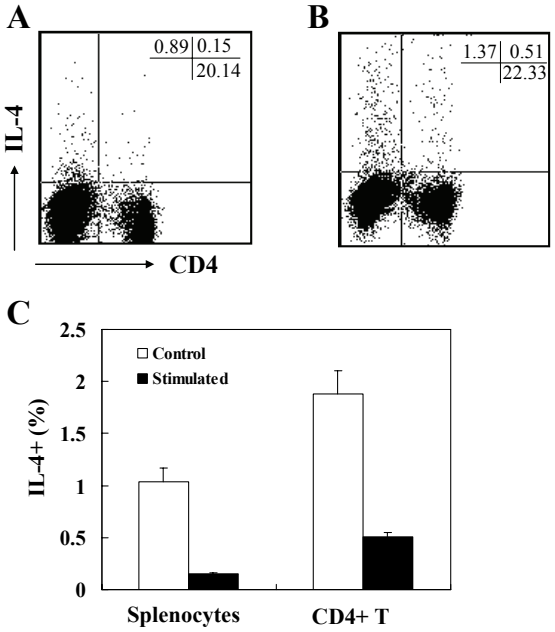

Figure 2. ***In vivo*** **TCS stimulation upregulated IL-4 expression.** C57BL/6 mice were injected with 5 μg TCS. A week later, the mice were received another TCS injection. At day 5 after the second immunization, the mice were sacrificed and spleen cells were collected for intracellular IL-4 staining. (A) The expression of IL-4 in control $CD4^+$ T cells. (B) The expression of IL-4 in $CD4^+$ T cells of TCS immunized mice. (C) The percentages of IL-4^+ cells in splenocytes and $CD4^+$ T cells of control and TCS immunized mice.

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 stained with FITC-anti-CD4, the samples (1×10^6 cells) were fixed with 2% (w/v) paraformaldehyde for 30 min at 4°C. After permeabilized 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

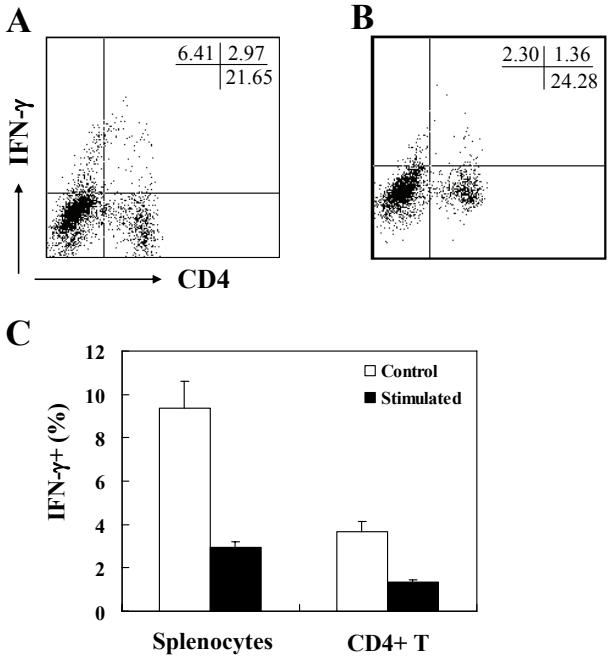

Figure 3. *In vitro* TCS stimulation inhibited IFN-γ expression. The splenocytes of normal C57BL/6 mice were treated with or without 0.1 μg/ml TCS for 5 h. Then the cells were stimulated with l mM inomycin and 30 ng/ml PMA in the presence of 2 μM momensin for 4 h. And the splenocytes were collected for intracellular IFN-γ staining. (A) The expression of IFN-γ in control $CD4^+$ T cells. (B) The expression of IFN-γ in $CD4^+$ T cells of TCS stimulated splenocytes. (C) The percentages of IFN-γ^+ cells in splenocytes and $CD4^+$ T cells of control and TCS stimulated splenocytes.

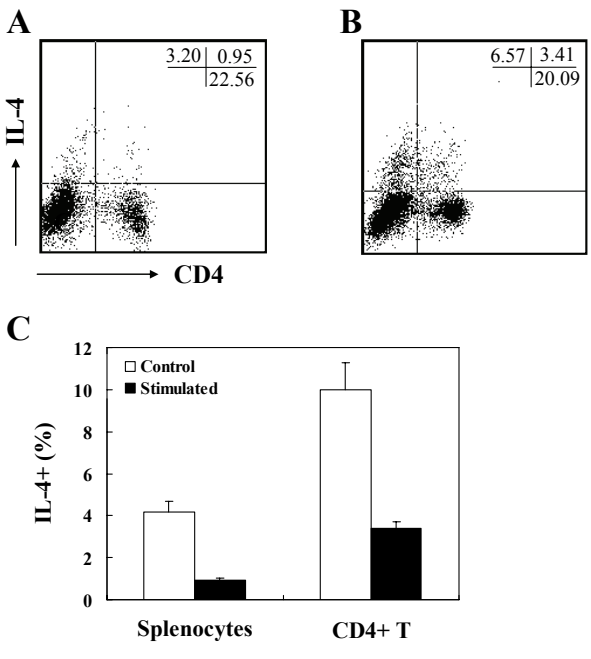

Figure 4. *In vitro* TCS stimulation upregulated IL-4 expression. The splenocytes of normal C57BL/6 mice were treated with or without 0.1 μg/ml TCS for 5 h. Then the cells were stimulated with l mM inomycin and 30 ng/ml PMA in the presence of 2 μM momensin for 4 h. And the splenocytes were collected for intracellular IL-4 staining. (A) The expression of IL-4 in control $CD4^+$ T cells. (B) The expression of IL-4 in $CD4^+$ T cells of TCS stimulated splenocytes. (C) The percentages of IL-4^+ cells in splenocytes and $CD4^+$ T cells of control and TCS stimulated splenocytes.

permeabilization buffer for l 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 $CD4^+$ Th cells (Figure 1). But the expressions of IL-4 were increased in both splenocytes and $CD4^+$ Th 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 $CD4^+$ Th 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 *in vitro*. Intracellular staining was used to evaluate IFN-γ and IL-4 expressions in cultured cells. As shown in Figure 3, the expression of IFN-γ was inhibited by TCS stimulation. The percentage of IFN-γ^+ cells was decreased from 9.38 to 3.66 in splenocytes and decreased from 2.97 to 1.36 in $CD4^+$ Th cells. But IL-4 was significantly higher after TCS stimulation. The percentage of IL-4^+ cells in splenocytes was increased from 4.15 to 9.98 and the percentage of IL-4^+ cells in $CD4^+$ Th cells was increased from 0.95 to 3.41 (Figure 4).

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

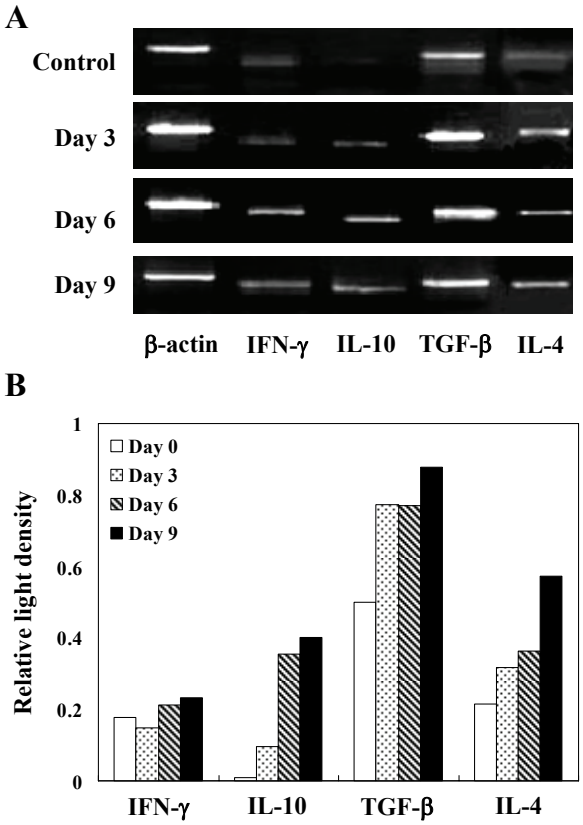

Figure 5. The kinetics of Th1-to-Th2 transition at mRNA level after second TCS immunization. The splenocytes were collected after second TCS immunization *in vivo* for total RNA extraction to analyze the expressions of IFN-γ, IL-10, TGF-β and IL-4. Gene expressions were measured by RT-PCR (A) and gene expression data were normalized to β-actin expression (B). The results were depicted as relative light intensities of detected cytokines against β-actin, assuming the light intensity of β-actin was 1.

later. The expressions of IL-4 and TGF-β were increased slowly. The change of IL-10 expression is quite significant. The expression of IL-10 was undetectable in control mice, but at day 9 after the second immunization, its relative light density was increased to 0.402 (Figure 5).

Discussion

Differentiation of Th0 to Th1 or Th2 is strictly regulated. Cytokine milieu plays a key role in this process. IL-12, a heterodimeric cytokine composed of two subunits of p35 and p40, is critical for the development of Th1 cells and the initiation of cell mediated immune response (16-18). TNF-α is also an important cytokine that mediates a wild range of biological functions and has been shown recently required for IL-12 induced development of Th1 cells (19). Conversely, some cytokines, such as IL-10, can inhibit antigen-specific activation and proliferation of Th1 cells, thus facilitating Th2 response (20, 21).

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.

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