Biochemical and Biophysical Research Communications **284,** 168–172 (2001)
doi:10.1006/bbrc.2001.4940, available online at http://www.idealibrary.com on IDEAL®

Inhibition of Th1- and Enhancement of Th2-Initiating Cytokines and Chemokines in Trichosanthin-Treated Macrophages

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Received May 1, 2001

Trichosanthin (TCS), the major effective component from Chinese herb *Trichosanthes Kirilowii Maxim*, is also a potent allergen. Our previous work has shown that TCS can upregulate interleukin-4 (IL-4) and interleukin-13 (IL-13) while inhibit interferon-γ (IFN-γ) in mesenteric lymph node cells after TCS immunization. Thus, TCS can arouse a T helper 2 (Th2) response in the draining lymph node. However, little is known about the early effects of TCS on antigen-presenting cells, the initiator of T cell response. In the current study, the effects of TCS on macrophage cytokines and chemokine expression were investigated. Peritoneal macrophages were treated with or without TCS in the presence of lipopolysaccharide (LPS). We found that TCS increased macrophage interleukin-10 (IL-10) and monocyte chemoattractant protein-1 (MCP-1) expression, whereas it decreased interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) expression. Our study clearly demonstrated that TCS, as an allergen, has differential effects on macrophage Th1/Th2 initiative factors, effects that are likely to facilitate its inducing of Th2 and immunoglobulin E (IgE) response. © 2001 Academic Press

Key Words: **trichosanthin; tumor necrosis factor-α; interleukin-12; interleukin-10; monocyte chemoattractant protein-1; macrophage; lipopolysaccharide; T helper 1; T helper 2; immunoglobulin E.**

Trichosanthin (TCS), the major effective component from Chinese herb *Trichosanthes Kirilowii Maxim*, was used in abortion. Recently it was also found to be effective in anti-cancer and anti-HIV therapy (1–3). However, it is a potent allergen and can induce allergic reaction in clinical treatment. Therefore it is necessary to clarify the mechanism of allergic reaction induced by TCS. We have established a mice model in which TCS alone without any adjuvant is able to induce high level of TCS-specific IgE (4). We also demonstrated that TCS could induce significant expression of interleukin-4 (IL-4) and IL-13, the major T helper 2 (Th2) cytokines, in mesenteric lymph node cells (5). However, the earlier events after TCS immunization that lead to a Th2 response and TCS-specific IgE production are still poorly known.

Differentiation of Th0 to Th1 or Th2 is strictly regulated. During this process, cytokine milieu play a key role. Interleukin-12 (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 (6–8). 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 (9). Conversely, some cytokines, such as interleukin-10 (IL-10), can inhibit antigen-specific activation and proliferation of Th1 cells, thus facilitating Th2 response (10, 11). The chemokine monocyte chemoattractant protein-1 (MCP-1) is a predominantly monocytic chemoattractant *in vivo*. It can stimulate IL-4 production in T cells and its overexpression is associated with defects in cell-mediated immunity while its deficient resulted in impaired Th2 responses, indicating that it might be involved in Th2 polarization (12–15). Could TCS enhance antigen presenting cells to secrete Th2-inducing cytokines/chemokine and/or inhibit their Th1-inducing cytokines/chemokines thus switch the balance toward Th2 and initiate an allergic response? In this paper, the effects of TCS on macrophage cytokine/chemokine productions were investigated, a try to mimic the early effects of TCS on antigen presenting cells *in vivo*. Macrophages obtained after thioglycollate injection were stimulated with or without TCS in the presence of LPS and the levels of cytokine mRNA were measured with RT-PCR, together with that of the intracellular staining of the IL-12 P40 protein level. We report here that

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0006-291X/01 $35.00

TCS enhanced IL-10 and MCP-1 expression but inhibited IL-12 and TNF-α expression of peritoneal macrophage, thus facilitating a Th2 response and IgE production.

MATERIALS AND METHODS

Animals. C57BL/6 mice, female, 9–12 weeks old, were obtained from the Animal Center of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and were bred according to the Institutional guidelines.

Cell culture. Peritoneal macrophages were obtained from mice 4 days after 2 ml of 5% thioglycollate solution injection as detailed in reference (16). After preincubation in the presence or absence of TCS (TCS injection solution produced by Jin-San Pharmaceutical Factory, Shanghai; 0.1 or 1 μg/ml) for 30 min, the peritoneal macrophages were stimulated with or without LPS (10 ng/ml, Sigma) for another 3 h. The total cellular RNAs were isolated from cultured cells to measure mRNA expression by RT-PCR. IL-12 protein synthesis was analyzed by intracellular staining in 12 h cultured macrophages stimulated with ionomycin (10 μg/ml, Sigma).

RT-PCR. Cells for RNA extraction were destroyed with TRIzol (Gibco BRL) directly on the cell culture plates as suggested by the producer. cDNA was synthesized using Gibco-BRL M-MLV Reverse Transcriptase: 10 μl 5× first-strand buffer, 0.01 M DTT, 1 mM of each dNTPs, 5 μM random hexamer primers, 4 μg RNA, and added distilled water to 48 μl. The mixture was denatured at 70°C for 10 min then chilled on ice for 2 min. After adding 200 u M-MLV, the mixture was bathed in 37°C water for 1 h then inactivated at 75°C for 5 min. PCR system: 5 μl 10× reaction buffer, 0.25 mM each dNTPs, 1 u *Taq* DNA polymerase (all from Promega) 0.75 pmol each sense and antisense primer for target genes, and 2 μl cDNA. Add ddH_2O to 50 μl. PCR condition: denaturation for 45 s at 94°C; annealing at 57°C for IL-12 P40, MCP-1, IL-10, GAPDH and 61°C for TNF-α, all for 45 s, and extension for 45 s at 72°C. Primers for each genes are GAPDH (sense, 5′-acgac ccctt cattg acc-3′; antisense, 5′-agaca ccagt agact ccacg-3′, 203 bp). IL-12 p40 (sense, 5′-tgttg tagag gtgga ctgg 3′, antisense, 5′-tggca ggaca ctgaa tactt-3′, 483 bp). IL-10 (sense, 5′-aaggg ttact tgggt tgc-3′; antisense, 5′-aagga gttgt ttccg tta-3′, 412 bp). TNF-α (sense, 5′-acaag cctgt agccc acg-3′; antisense, 5′-tccaa agtag acctg ccc-3′; 428 bp). MCP-1 (sense, 5′-agcac cagcc aactc tcac-3′; antisense, 5′-tctgg accca ttcct tcttg-3′, 301 bp). The PCR products were electrophoresed, stained with ethidium bromide and photographed. The bands' density were calculated by FURI SmartView 2000 (Shanghai).

Flow cytometry. Cells for intracellular staining were dissociated from the cell culture plates after 8 min of 0.5% trypsin at 37°C.

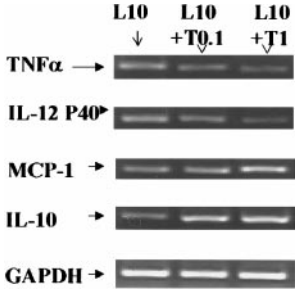

FIG. 1. RT-PCR assay of TNF-α, IL-12 p40, MCP-1, and IL-10 mRNA. Macrophages were pretreated with or without TCS 0.1 μg/ml (T0.1) or 1 μg/ml (T1) for 30 min and then cultured with 10 ng/ml LPS (L10) for another 3 h. Cells were then destroyed and the total RNA was extracted for RT-PCR analysis.

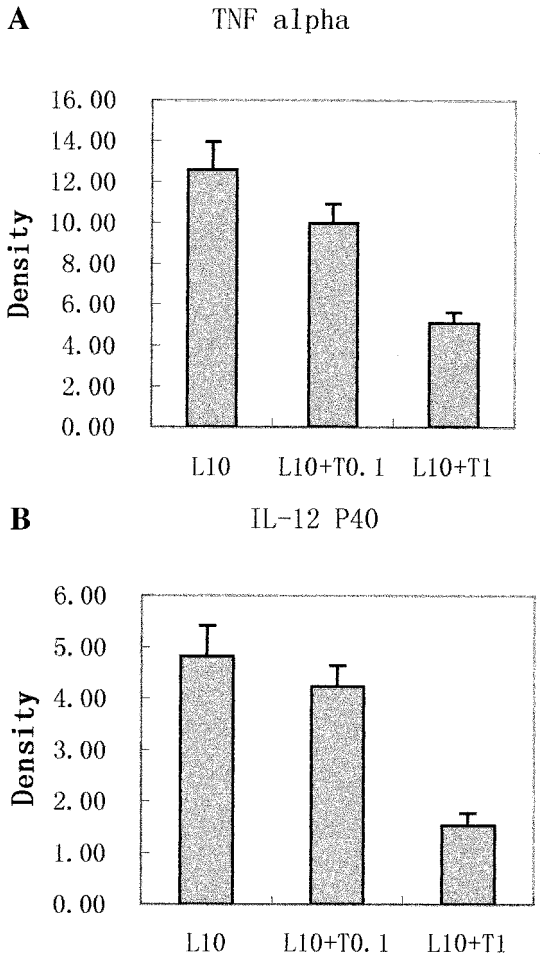

FIG. 2. TCS inhibited peritoneal macrophage TNF-α and IL-12 p40 production. Densitometric analysis of Fig. 1 results. (A) TNF-α. (B) IL-12 P40.

Briefly, add 200 μl 2% rat serum to block FcγR on macrophage cell surface at 4°C for 20 min, after wash with staining buffer, add 200 μl cytoFix/cytoperm reagent (PharMingen) to fix cells for 20–40 min at 4°C. Wash with 1 ml 1× Perm/Wash buffer for two times, then resuspended at 50 μl perm/wash buffer which contains PE-conjugated rat anti-mouse IL-12 p40/p70 monoclonal antibody (PharMingen, Jingmei Biotech Co., Ltd., Shanghai) at 4°C for 30 min in the dark. After washing with 1 ml perm/wash buffer for two times, resuspended at 400 μl staining buffer for flow cytometry analysis.

RESULTS

TCS Inhibited Peritoneal Macrophage TNF-α and IL-12 p40 Expression

Macrophages were pretreated with or without 0.1 or 1 μg/ml TCS for 30 min and then cultured with 10 ng/ml LPS for another 3 h. Cells were then harvested with TRIzol and total RNA was extracted and mRNA expressions were analyzed with RT-PCR. As shown in Fig. 1 and Fig. 2, TCS can effectively inhibited LPS stimulated TNF-α (Fig. 2A) and IL-12 p40 expression (Fig. 2B), in a very similar and dose dependent man-

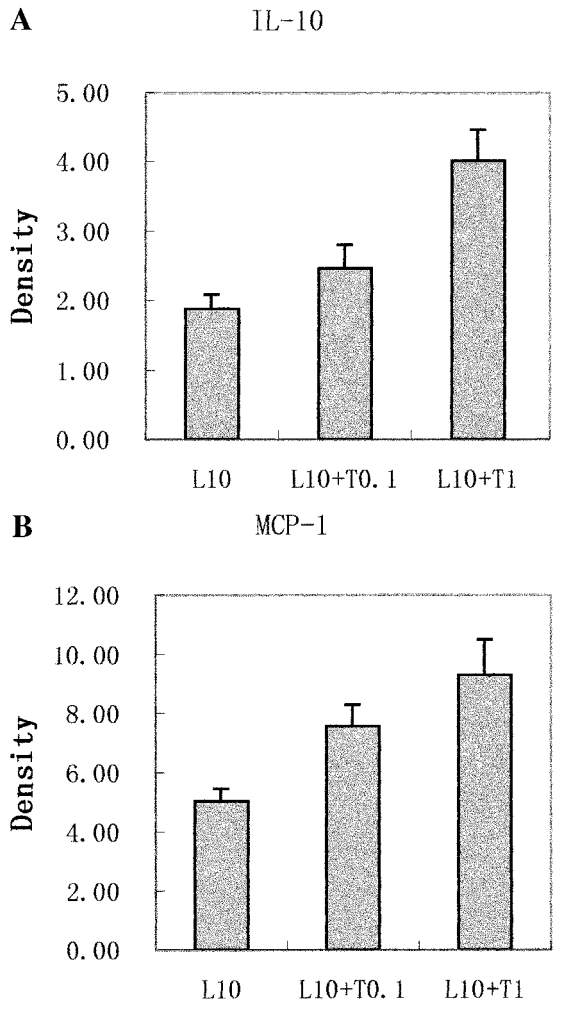

FIG. 3. TCS enhanced peritoneal macrophage IL-10 and MCP-1 production. Analytic conditions were similar to those in Fig. 2. (A) IL-10. (B) MCP-1.

ner. In all experiments, both TNF-α and IL-12 p40 mRNA expressions were undetectable in cultured cells without LPS stimulation. The data suggested that TCS was able to suppress TNF-α and IL-12 p40 mRNA expression.

TCS Enhanced Peritoneal Macrophage IL-10 and MCP-1 Expression

Next, the effects of TCS on IL-10 and MCP-1 expression were investigated. When macrophages were treated with TCS and LPS together, the IL-10 and MCP-1 expression was higher than that of the macrophages treated with LPS alone (Fig. 1, Fig. 3). This enhancement of TCS to IL-10 and MCP-1 expression was also dose dependent, TCS at concentration of 1 μg/ml increased both IL-10 and MCP-1 expression significantly higher than that of 0.1 μg/ml. In fact, when stimulated with TCS in the absence of LPS, macrophages could be induced to express significant high IL-10 and MCP-1 mRNA, though the level was fairly lower than that of stimulated with LPS (data not shown). The data suggested that IL-10 and MCP-1 mRNA expression was enhanced in TCS-treated macrophages.

TCS Inhibited LPS-Induced IL-12 P40 Protein Production

To evaluate IL-10, MCP-1, IL-12, and TNF-α protein synthesis in cultured cells, intracellular staining with their specific fluorescent monoclonal antibodies was determined by flow cytometry, but among them IL-10, MCP-1, and TNF-α were undetectable, perhaps due to the low dose of LPS used in our experiments. However, the intracellular staining of IL-12 p40 was significantly high, and the same inhibitory effects of TCS could be seen in this case as in mRNA level. When stimulated with 10 ng/ml LPS alone, there were 12.18% IL-12 p40 positively stained cells and their mean fluorescence was 190.17. If 1 μg/ml TCS added 30 min before the LPS, the percentage of positively stained cells decreased to 3.98%, and their mean fluorescence was only 137.39 (Fig. 4).

DISCUSSION

TCS is a potent allergen but the mechanisms of its allergenicity remain obscure. Cytokines and some of chemokines play important roles in Th1/Th2 differentiation and may be important in IgE mediated immediate hypersensitivity. We report here that TCS enhanced IL-10 and MCP-1 expression by peritoneal macrophages, whereas it inhibited IL-12 and TNF-α expression by these same cells. These differential effects of TCS on macrophage cytokines/chemokine expression likely contribute to its allergen properties.

TCS is an effective IgE inducer in our mice model, 5 μg TCS without any adjuvant can arouse IgE after the first immunization. This simplicity of our TCS-induced IgE model and together with the background studies, such as high-resolution 3-dimensional structure of TCS and TCS-specific IgE monoclonal antibodies make TCS an ideal candidate for the study of the mechanism of IgE response and Th1/Th2 differentiation.

In our previous study, TCS injection induced rapid IL-4 expression and inhibited IFN-γ expression at the same time in mesenteric lymph node cells (5), indicating the Th2-induction ability of TCS. Since antigen-presenting cells (APC) play a key role in Th response, we hypothesized that TCS may influence the phenotype of APC thus make them a Th2 response driver. However it is hard to elucidate this question in antigen presenting cells *in vivo.* To this end, LPS-stimulated macrophages that were able to produce significantly higher levels of both Th1 and Th2 initiators than their untreated counterparts were selected in this study.

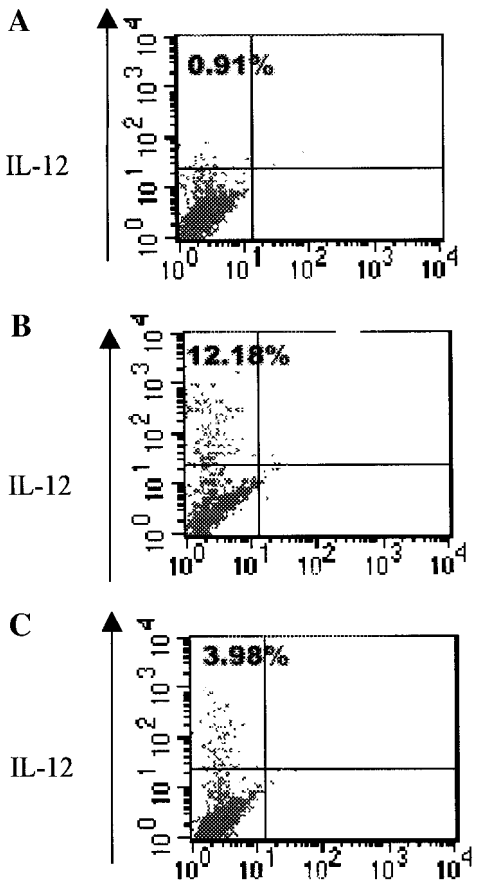

FIG. 4. TCS inhibited IL-12 P40 protein production as measured by flow cytometry. Cells pretreated with TCS for 30 min or not and then treated with LPS, together with the protein transfer inhibitor ionomycin, for another 12 h. (A) Control, with no TCS or LPS stimulation. (B) 10 ng/ml LPS. (C) 1 μg/ml TCS pretreated for 30 min, then 10 ng/ml LPS was added.

IL-12 is a cytokine that plays a critical role in the differentiation of naïve T cells to the Th1 cells while TNF-α has been shown recently required for this Th1-inducing ability of IL-12, thus it is of interest that TCS significantly inhibited both IL-12 and TNF-α expression. Since systematically administration of exogenous IL-12 directly or DNA vaccines that contain CpG can enhance IL-12 production *in vivo* and can both redirect a response from Th2 to Th1 (17, 18), this inhibitory effects of TCS on IL-12 and TNF-α may inhibit Th1 differentiation and facilitate Th2 differentiation.

IL-10 has been shown to down-regulate most cytokines produced by macrophages, including TNF-α, IL-12, IL-6, and IL-8 (10, 11). Recently Cong *et al.* demonstrated that cholera toxin could enhance IL-10 production and this enhancement of IL-10 result in the decreased IL-12 and TNF-α production (19). Since our system is somehow similar to their system, the same inhibitory relationship between IL-10 and IL-12, TNF-α may also exists. Thus, TCS increased IL-10 expression in macrophage may further inhibit Th1 and facilitate Th2 response.

MCP-1 is a predominantly monocytic chemoattractant *in vivo* but it is also important in Th1/Th2 differentiation (12–14). MCP-1-deficient mice are unable to mount TH 2 responses (15). In our current study, MCP-1 expression is also enhanced by TCS in peritoneal macrophage in a pattern similar to that of IL-10, but no further mechanisms can be explained.

In summary, our present study demonstrates that TCS significantly enhanced IL-10 and MCP-1 expression, but inhibited IL-12 and TNF-α expression in peritoneal macrophages. These effects of TCS partially explain its Th2- and IgE-inducing ability.

ACKNOWLEDGMENTS

This work was supported by 973 Project (G1999053907, People's Republic of China) and the National Natural Science Foundation of China (30070708). We are grateful to Hongwei Yu for technique help.

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