The Chinese Herbal Mixture Tien-Hsien Liquid Augments the Anticancer Immunity in Tumor Cell–Vaccinated Mice

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Abstract

Background. The Chinese herbal mixture, Tien-Hsien liquid (THL), has been used as an anticancer dietary supplement for more than 20 years. Our previous studies have shown that THL can modulate immune response and inhibit tumor growth. In this study, we further evaluated the effect of THL on anticancer immune response in mice vaccinated with γ-ray-irradiated tumor cells.

Methods. The antitumor effect of THL was determined in mice vaccinated with low-tumorigenic CT-26-low colon cancer cells or γ-ray-irradiated high-tumorigenic CT-26-high colon cancer cells. The number of natural killer (NK) cells and T lymphocytes in the spleen was analyzed by flow cytometry. The tumor-killing activities of NK cells and cytotoxic T lymphocytes (CTLs) were analyzed by flow cytometry using YAC-1 and CT-26-high cells, respectively, as target cells. The levels of IFN-γ, IL-2, and TNF-α were determined by ELISA.

Results. THL suppressed the growth of CT-26-high tumor in mice previously vaccinated with low-tumorigenic CT-26-low cells or γ-ray-irradiated CT-26-high cells. THL increased the populations of NK cells and CD4+ T lymphocytes in the spleen and enhanced the tumor-killing activities of NK cells and CTL in mice vaccinated with γ-ray-irradiated CT-26-high cells. THL increased the production of IFN-γ, IL-2, and TNF-α in mice vaccinated with γ-ray-irradiated CT-26-high cells.

Conclusion. THL can enhance the antitumor immune responses in mice vaccinated with killed tumor cells. These results suggest that THL may be used as a complementary medicine for cancer patients previously treated with killed tumor cell vaccines, radiotherapy, or chemotherapy.

Keywords

Tien-Hsien liquid, herbal mixture, anticancer immunity, tumor suppression, natural killer cell, cytotoxic T lymphocyte, interferon-γ, interleukin-2, tumor necrosis factor α

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Introduction

The Chinese herbal cocktail Tien-Hsien liquid (THL; prepared by China-Japan Feida Union Co, Ltd, Hong Kong) has been used as an anti-cancer dietary supplement for more than 20 years and has been used by many cancer patients with favorable outcomes in more than 15 countries. THL is an aqueous preparation of herbal mixture consisting mainly of extracts from 14 Chinese medicinal herbs: Cordyceps sinensis (CS), Oldenlandia diffusa (OD), Indigo pulverata levis (also known as indigo naturalis), Polyporus umbellatus (PU), Radix astragali (RA), Panax ginseng (PG), Solanum nigrum L., Pogostemon cablin, Atractylodis macrocephalae rhizoma (AMR), Trichosanthes radix, Clematis radix, Ligustrum lucidum Ait (LLA), and Glycyrrhiza radix (GR). It is believed that a properly formulated herbal cocktail, which takes advantage of synergy and interactions among the myriad phytochemicals present in the different herbs, may achieve better therapeutic efficacy than single herbs. Our

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previous studies have shown that THL can induce apoptosis and inhibit metastasis and angiogenesis in a wide variety of human cancer cells.\textsuperscript{1,2} Other studies also showed that THL can induce apoptosis and inhibit the growth and metastasis of cancer cells by targeting various oncogenic signaling pathways and metastatic markers.\textsuperscript{3-5} More recently, Yao et al\textsuperscript{1} showed that THL could eliminate the cancer stem-like cells, accompanied by the suppression of stemness genes expression, colony formation, and tumorigenicity.\textsuperscript{6} Together, these studies suggest that THL has the potential to be used as a therapeutic agent for established tumors.

The enhancement of host immune response has been considered as an alternative strategy for the prevention and cure of cancers and as a possible means of inhibiting tumor growth without harming the host.\textsuperscript{7,8} Natural killer (NK) cells and cytotoxic T lymphocyte (CTLs) are the 2 major cytotoxic lymphocytes that are important in the defense against tumors.\textsuperscript{9,10} CTLs perform the surveillance function by recognizing and killing potentially malignant cells that express peptides derived from mutant cellular protein or oncogenic proteins, which are presented by major histocompatibility complex (MHC) class I molecules. Unlike CTLs, the killing by NK cells is not through antigen/MHC recognition. NK cells kill many types of tumor cells, especially cells that have reduced MHC class I expression and can escape killing by CTLs.\textsuperscript{11} Many in vitro and in vivo studies have suggested that tumor cells are recognized as NK cell targets.\textsuperscript{12} NK cells also act as regulatory cells to influence various other cells, such as dendritic cells, helper T-cells, CTLs, and B cells.\textsuperscript{13} Therefore, many studies for cancer immunotherapy were focused on enhancing the activity of NK cells and CTLs.\textsuperscript{14}

Immunotherapy using whole tumor cell vaccines has become an alternative strategy for cancer treatment.\textsuperscript{15,16} For example, granulocyte-macrophage colony-stimulating factor-expressing tumor cell vaccines are very efficient in inducing tumor-specific immune response in mice and in preliminary clinical trials.\textsuperscript{17,19} In addition, γ-ray-irradiated apoptotic tumor cell vaccines can induce a potent immune response in vivo probably through the cross-presentation of tumor antigens to CTLs by dendritic cells.\textsuperscript{20,21} Our previous studies have shown that THL has immunomodulating activity and can modulate the antigen-stimulated cytokine production by T-cells.\textsuperscript{22,23} Moreover, several major ingredients of THL have been reported to be able to modulate immune response.\textsuperscript{24,25} For instance, CS, RA, PG, and GR can increase the cytokotoxic activity of murine NK cells. OD can increase the cytokotoxic activity of murine CTLs. CS and GR can increase the secretion of interleukin (IL)-1 by murine macrophages. RA, PG, and GR can induce the secretion of interferon-γ (IFN-γ) by mouse spleen cells. CS, OD, PU, RA, PG, AMR, LLA, and GR can induce the secretion of IL-2 by mouse spleen cells. Together, these results suggest that THL can modulate antitumor immunity in tumor-bearing mice. In this study, we used γ-ray-irradiated apoptotic tumor cells as a vaccine to immunize mice and investigate whether THL could enhance the antitumor immunity in tumor cell–vaccinated mice. We found that THL could enhance the tumor-killing activities of NK cells and CTL and increase the production of IFN-γ, IL-2, and TNF-α in mice vaccinated with γ-ray-irradiated tumor cells.

### Materials and Methods

#### Cell Culture

The mouse colon carcinoma cell lines, CT-26 (including CT-26-low and CT-26-high), were established and provided by Dr Sheng-Hong Tseng (Department of Surgery, National Taiwan University Hospital, Taipei, Taiwan). Their tumorigenicity was confirmed, as shown in Table 1.

| Injected Cells | CT-26-Low Cells | CT-26-High Cells |
|---------------|-----------------|-----------------|
| 2 × 10⁴       | ND*             | 90%             |
| 5 × 10⁴       | ND              | 100%            |
| 1 × 10⁵       | ND              | 100%            |
| 3 × 10⁶       | 50%             | 100%            |

**Table 1.** The Tumorigenicity of CT-26-Low and CT-26-High Colon Cancer Cells in the Syngeneic BALB/c Mice.

Abbreviations: ND, not determined.

**Handling of THL**

THL (obtained from China-Japan Feida Union Co, Ltd, Hong Kong) is an aqueous preparation of herbal mixture and consists mainly of extracts from 14 Chinese medicinal herbs as mentioned previously. For the in vivo mice xenograft experiment, THL (200 µL/d) was orally given to mice directly. The dose of THL for each mouse was calculated as follows. The dosage of THL for a cancer patient (60 kg) is 1 mL/kg/d.\textsuperscript{26} The given dosage (D) of THL in mice was calculated according to the Meeh-Rubner conversion formula between human and mouse: \( D_{\text{mouse}} = D_{\text{human}} \times (K_{\text{mouse}}/K_{\text{human}}) \), where \( K \) is the conversion factor (\( K_{\text{mouse}} = 1 \) and \( K_{\text{human}} = 0.11 \)). The calculated dosage in mice (0.02 kg) was 9.09 µL/kg/d.
mL/kg/d, which equals 198 µL per day for each mouse. Therefore, a daily dose of 200 µL of THL per mouse was used in this study. This dose of THL did not cause toxic effects in the mice in our preliminary study.

**Preparation of Tumor Vaccines**

CT-26-high cells were irradiated with 72-Gy γ-rays, and the viability of the irradiated cells was analyzed by propidium iodide (PI) staining and flow cytometry. The majority of irradiated CT-26-high cells were found to be dead, and the irradiated cells were promptly inoculated subcutaneously on the back of BALB/c mice (1 × 10⁶ cells, suspended in 100 µL of phosphate-buffered saline [PBS]).

**Experimental Animals**

All animal experiments in this study were performed following the Guidelines for Animal Experiments in National Taiwan University and were approved by the Institutional Animal Care and Use Committee in College of Medicine, National Taiwan University (IACUC Approval No.: 20110101). BALB/c female mice (6-8 weeks old) were purchased from Laboratory Animal Center at College of Medicine, National Taiwan University (Taipei, Taiwan) and given food and water ad libitum. The mice were randomly divided into 2 groups for oral feeding with THL or water (200 µL; each day) throughout the experimental period. The experimental schedule for assessing the immune-enhancing effect of THL in tumor-bearing mice is summarized in Figures 1A and 2A. The body weight and tumor size of mice were measured at different time points following tumor implantation, and the tumor volume was calculated according to the following formula: 1/2 (Length × Width²).

**Preparation of Splenocytes**

Spleens were removed aseptically, placed in RPMI medium, gently homogenized, and passed through a 200-µm-mesh (Becton Dickinson Bioscience, San Jose, CA) to generate single-cell suspension. Erythrocytes were rapidly washed and lysed by the RBC lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA), and the splenocytes were resuspended at a density of 1 × 10⁷ cells/mL in RPMI medium.

**Flow Cytometry Analysis of NK Cells and T Lymphocytes in Spleen**

The lymphocytes from spleens were stained with fluorescence-labeled antibodies in PBS for 30 minutes, followed by 3 washes with PBS, and then analyzed by flow cytometry (Becton Dickinson Bioscience). Fluorescence-labeled antibodies against CD4, CD8a, and CD49b (pan-NK) were all purchased from Becton Dickinson Bioscience.
Assay of NK Cell– and CTL-Mediated Killing Activity

The mouse lymphoma YAC-1 cells and CT-26-high cells were used as the target cells of NK cells and CTL, respectively. Before the coincubation of effector and target cells, YAC-1 cells and CT-26-high cells were labeled with 200 nM CFSE (Molecular Probes, Eugene, OR) for 15 minutes in serum-free RPMI medium, washed once, and diluted in complete RPMI or DMEM medium. In the NK killing assay, lymphocytes and CFSE-labeled YAC-1 cells were cocultured in 5-mL Falcon tubes (Becton Dickinson Bioscience) at 25:1 or 50:1 of the effector cell versus target cell (E:T) ratio. After incubation at 37°C for 4 hours, the samples were resuspended in PI solution (0.25 µg/mL PI and 1% bovine serum albumin (BSA) in PBS) and analyzed by flow cytometry. The CFSE+/PI+ YAC-1 cells were considered as killed target cells. In the CTL-killing assay, lymphocytes were stimulated in vitro with 72-Gy γ-irradiated CT-26-high cells at a 40:1 ratio (lymphocytes vs γ-irradiated CT-26-high cells) and 1 ng/mL of IL-2. After incubation for 5 days, lymphocytes and CFSE-labeled viable CT-26-high cells were cocultured in 5-mL Falcon tubes at 10:1, 25:1, or 50:1 E:T ratios. After incubation at 37°C for 4 hours, the samples were resuspended in PI solution and analyzed by flow cytometry. The CFSE+/PI+ CT-26-high cells were considered as killed target cells.

Cytokine Production by Cultured Splenocytes and Tumor Xenografts

Isolated splenocytes (1 × 10⁶/mL) from water- or THL-treated mice were ex vivo stimulated with concanavalin A (Con A; Sigma-Aldrich, Inc, St Louis, MO) at 5 µg/mL for 24 hours. The levels of IFN-γ, IL-2, and tumor necrosis factor α (TNF-α) secreted in the culture supernatant were measured by ELISA (BioLegend, San Diego, CA). To measure cytokine levels in tumor xenografts, tumor tissues were taken, weighed, minced, and homogenized with tissue lysis buffer (1% Triton X-100; 50 mM HEPES, pH 7.4; 150 mM NaCl; 1.5 mM MgCl₂; 1 mM ethylene glycol tetraacetic acid (EGTA); 100 mM NaF; 1 mM Na₃VO₄; 10% glycerol; and 1× protease inhibitor cocktail from Roche Applied Science). The supernatant of the homogenized sample was obtained by centrifugation (12000 rpm for 10 minutes), and the levels of IFN-γ, IL-2, and TNF-α in the supernatant were assayed by ELISA (BioLegend).

Statistical Analyses

Data are presented as mean ± standard error. The significance of the difference between groups was evaluated with the Student’s t-test; *P < .05 was considered significant.

Results

THL Suppresses Tumor Growth in Tumor Cell–Vaccinated Mice

To investigate the antitumor activity of THL in mice, 2 murine CT-26 colon cancer cell lines, CT-26-low and
CT-26-high, which have low and high tumorigenic activity, respectively, were established. Subcutaneous injection of 5 \times 10^4 high-tumorigenic CT-26-high cells resulted in 100% tumor formation in syngeneic BALB/c mice. In contrast, in mice subcutaneously injected with 3 \times 10^4 low-tumorigenic CT-26-low cells, only about 50% of mice showed tumor formation (Table 1). We hypothesized that CT-26-low cell may be used as a tumor vaccine to elicit an antitumor immune response in mice. To test this possibility, we compared the tumor-inducing ability of CT-26-high cells in mice previously injected with CT-26-low cells with that in mice not injected with CT-26-low cells. We found that while injection of 5 \times 10^4 CT-26-high cells was enough to induce 100% tumor formation in mice not injected with CT-26-low cells previously, a much higher number (~5 \times 10^5) of CT-26-high cells was required to induce 100% tumor formation in mice previously injected with CT-26-low cells (data not shown). This result suggests that CT-26-low cell injection may elicit antitumor immune responses against CT-26-high cells. Next, we tested whether THL could suppress tumor growth in mice vaccinated with CT-26-low cell. To do this, mice were first injected with 3 \times 10^6 CT-26-low cells and then randomly divided into 2 groups: one orally fed with THL and the other orally fed with water. Then 56 days later, the number of tumor-free mice in the THL-treated group was 12, whereas that in the water-treated group was 5, indicating that administration of THL increased the frequency of tumor formation. The mice were then injected subcutaneously with 2 \times 10^6 CT-26-high cells as indicated in the experimental protocol shown in Figure 1A. Mice were fed with either THL or water throughout the entire experimental period (200 \muL; twice a day). At 42 days after second tumor-cell injection, 4 out of 12 THL-treated mice remained tumor free, whereas only 1 out of 5 water-treated mice was tumor free. The average tumor volume of THL-treated mice was about 50% that of water-treated mice at 42 days after second tumor-cell injection (Figure 1B). These data indicate that THL can suppress tumor growth in tumor cell–vaccinated mice, and this tumor-suppressing activity of THL may be, at least partly, mediated through its ability to promote antitumor immune responses.

To further study the enhancement of antitumor immunity by THL, we used \gamma-ray-irradiated CT-26-high cells to immunize syngeneic BALB/c mice. Briefly, BALB/c mice were vaccinated twice with 1 \times 10^5 \gamma-irradiated CT-26-high cells. One week after the second injection of the \gamma-irradiated cells, the mice were subcutaneously injected with 5 \times 10^6 viable CT-26-high cells, and the growth of tumor xenografts was monitored and measured for 21 days (see Figure 2A for the experimental protocol). As shown in Figure 2B, the tumor growth was slower in THL-treated mice than in water-treated mice. At day 21 after tumor cell implantation, the average tumor volume of the THL-treated mice was 0.55 \pm 0.1 cm^3, whereas that of the water-treated mice was 1.29 \pm 0.2 cm^3. In addition, THL did not cause body weight loss (Figure 2C) or other side effects, such as hair loss and lethargy, during the experimental period. These results further demonstrate that THL possesses potent tumor-suppressing activity, and this antitumor activity may, at least partly, be a result of its ability to enhance antitumor immune responses.

**THL Increases NK Cell and CTL Activities in Tumor Cell–Vaccinated Mice**

NK cells and CTLs represent 2 major populations of cytotoxic lymphocytes and play a vital role in antitumor defense.\(^9,10\) To elucidate the mechanisms for the anticancer efficacy of THL in tumor cell–vaccinated mice, splenocytes were isolated from the mice of the above experiment (see Figure 2), and the populations of NK cells (CD49b+) and T lymphocytes (CD8+ or CD4+) were analyzed by flow cytometry. As shown in Figures 3A and 3B, the populations of NK cells and CD4+ T lymphocytes were higher in the THL-treated mice than in the water-treated mice. To investigate whether the activities of NK cells and CTLs were increased following the THL treatment, ex vivo assays for tumor cell killing by NK cells and CTLs were performed. As shown in Figures 3C and 3D, the tumor-killing activities of NK cells and CTLs of the THL-treated mice were higher than those of the water-treated mice. Together, these results indicate that THL may inhibit tumor growth through the enhancement of the NK cell and CTL activities.

**THL Increases the Cytokine Secretion in Tumor Cell–Vaccinated Mice**

Cytokines, such as IFN-\(\gamma\), IL-2, and TNF-\(\alpha\), play an essential role in NK cell and CTL activation. We thus investigated the effect of THL on the production of cytokines IFN-\(\gamma\), IL-2, and TNF-\(\alpha\) from Con A–stimulated splenocytes isolated from mice vaccinated with \gamma-irradiated CT-26-high cells by ELISA. As shown in Figure 4A, splenocytes isolated from the THL-treated mice produced higher levels of IFN-\(\gamma\), IL-2, and TNF-\(\alpha\) than splenocytes isolated from the water-fed mice. In addition, the infiltrating cytokines in tumor xenografts were also examined by ELISA analyses. Similarly, the levels of IFN-\(\gamma\), IL-2, and TNF-\(\alpha\) were higher in THL-treated tumors than in water-treated tumors (Figure 4B). Together, these data indicate that THL increases the production of IFN-\(\gamma\), IL-2, and TNF-\(\alpha\) in tumor cell–vaccinated mice and suggest that THL may promote the activities of NK cells and CTLs by inducing cytokine production.

**Discussion**

THL is a Chinese herbal mixture that has been used as an anticancer dietary supplement for many years. It has been suggested that the antitumor activity of traditional Chinese
herbs is mediated through the augmentation of the immune responses.\textsuperscript{27} It also has been reported that THL can modulate immune responses.\textsuperscript{22,23,28} However, it is still not clear whether THL could suppress tumor growth through enhancing antitumor immunity. In this study, we investigated the immune-enhancing activity of THL. We found that THL can efficiently suppress the growth of CT-26-high tumors in syngeneic BALB/c mice previously vaccinated with γ-irradiated CT-26-high colon cancer cells. Mice from the experiment in Figure 2 were killed humanely at 21 days after viable CT-26-high cell injection. The splenocytes were then isolated and used to determine the amounts and cytotoxic activities of NK cells and CTLs. The populations of NK cells (A) and T lymphocytes (B) were determined by flow cytometry. Values represent mean ± standard error (SE); n = 9 mice per group. *$P < .05$ versus the water-treated group. The cytotoxicities of NK cells (C) and CTLs (D) were determined by flow cytometry as described in the Methods section. Values represent mean ± SE; n = 6 mice per group. $^\diamond P < .05$ versus the water-treated group.

Figure 3. Tien-Hsien liquid (THL) increased the populations and tumor-killing activities of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) in mice previously vaccinated with γ-irradiated CT-26-high colon cancer cells. Mice from the experiment in Figure 2 were killed humanely at 21 days after viable CT-26-high cell injection. The splenocytes were then isolated and used to determine the amounts and cytotoxic activities of NK cells and CTLs. The populations of NK cells (A) and T lymphocytes (B) were determined by flow cytometry. Values represent mean ± standard error (SE); n = 9 mice per group. *$P < .05$ versus the water-treated group. The cytotoxicities of NK cells (C) and CTLs (D) were determined by flow cytometry as described in the Methods section. Values represent mean ± SE; n = 6 mice per group. $^\diamond P < .05$ versus the water-treated group.

To investigate the ability of THL to induce antitumor immunity, we used viable low-tumorigenic CT-26-low cells or γ-irradiated CT-26-high cells as tumor vaccines to immunize syngeneic BALB/c mice. Both viable CT-26-low cells and γ-irradiated CT-26-high cells were capable of inducing antitumor immune responses because a much higher number of CT-26-high cells was required to induce tumor formation in mice previously injected with viable CT-26-low cells or γ-irradiated CT-26-high cells compared with mice not previously injected with these cells (data not shown).
The observation that γ-irradiated apoptotic tumor cells can induce a potent antitumor immune response in vivo has been reported before. We found that THL can effectively suppress the growth of CT-26-high tumor in mice previously vaccinated with CT-26-low cells or γ-irradiated CT-26-high cells (Figures 1 and 2). This tumor-suppressing effect of THL is most likely mediated by its ability to enhance antitumor immunity in tumor cell–vaccinated mice. 

Figure 4. Tien-Hsien liquid (THL) increased the level of cytokines in mice previously vaccinated with γ-irradiated CT-26-high colon cancer cells. Mice from the experiment in Figure 2 were killed humanely at 21 days after viable CT-26-high cell injection. The production of interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α from splenocytes isolated from mice (A) and in tumor xenografts (B) was determined. (A) Splenocytes were cultured with 5 μg/mL concanavalin A for 24 hours, and the levels of IFN-γ, IL-2, and TNF-α in the supernatant were measured by ELISA. Values represent mean ± standard error (SE); n = 6 mice per group. *P < .05; ***P < .001 versus the water-treated group. (B) The levels of IFN-γ, IL-2, and TNF-α in tumor xenografts were measured by ELISA. Values represent mean ± SE; n = 4 mice per group. *P < .05 versus the water-treated group.
mice, based on the following reasons. First, THL significantly increased the tumor-killing activities of NK cells and CTLs in tumor cell--vaccinated mice (Figure 3). Second, THL significantly increased the secretion of IFN-γ, IL-2, and TNF-α in tumor cell--vaccinated mice (Figure 4). Third, our preliminary data indicated that THL could only slightly inhibit the growth of CT-26-high tumor in nonvaccinated mice (statistically not significant). This result suggests that THL’s tumor-suppressing activity is largely mediated by its ability to enhance antitumor immunity rather than by its ability to kill CT-26-high tumor cells directly.

Our previous studies have shown that THL has immune-modulating activity and can modulate the antigen-stimulated cytokine production by T-cells. More recently, Kuo et al reported that in a phase IIa trial, THL showed immune-modulating effects in patients with refractory metastatic breast cancer. The levels of T lymphocytes (CD3+, CD4+, and CD8+), B lymphocytes (CD19+), and mature NK cells (CD16+/CD56+) in peripheral blood of THL-treated patients were elevated. In the present study, we further showed that THL could increase the tumor-killing activities of CTL and NK cells in tumor cell--vaccinated mice (Figure 3). This effect of THL may be derived from its ability to stimulate the production of IFN-γ, IL-2, and TNF-α in tumor cell--vaccinated mice (Figure 4). IFN-γ is known to play a central role in the induction of host defenses against tumors. Its antigen effects can be mediated directly through inhibition of tumor cell growth and/or indirectly by activation of CTLs, NK cells, and macrophages, which are involved in innate as well as adaptive antitumor immune responses. IFN-γ also facilitates antigen processing and presentation by both the MHC class I and II pathways in tumor cells and antigen-presenting cells, leading to tumor recognition and subsequent destruction by CTLs. IL-2 has long been considered as a T-cell growth factor and thought to play an important role in mediating clonal expansion of T-cells and in driving effector differentiation to CTLs. It can promote tumor-reactive lymphocyte proliferation and cytotoxicity. TNF-α is an important effector molecule in CTL and NK cell killing of immunogenic tumor cells. When expressed locally by immune cells, TNF-α can destroy tumor blood vessels and induce the apoptosis and necrosis of tumor cells. TNF-α has also been shown to be able to inhibit the growth of tumors through the recruitment of macrophages and NK cells. Together, these studies suggest that the above cytokines may exert their antitumor activities directly through inhibiting tumor growth or indirectly through modulating innate as well as adaptive immune responses against tumors. Our finding that THL can suppress tumor growth and promote the tumor-killing activities of NK cells and CTLs may be related to its ability to stimulate the secretion of IFN-γ, IL-2, and TNF-α.

In addition to the immune-enhancing and tumor-suppressing activities, depending on the tumor microenvironment, cytokines can also promote the initiation, progression, invasion, and metastasis of cancer through various mechanisms, including modulation of the immune system. For example, TNF-α has been shown to participate in the initiation, progression, and metastasis of cancer. A number of studies also showed that IFN-γ may be intimately involved in immunosuppressive mechanisms. In this study, we cannot exclude the possibility that cytokines stimulated by THL also display tumor-promoting activities. Further investigation is needed to clarify the exact roles of these cytokines in our tumor model.

Chemotherapy and radiotherapy are the major therapeutic modalities commonly used for treatment of a variety of cancer patients. However, in many cases, chemotherapy or radiotherapy alone cannot achieve a satisfactory therapeutic outcome because of severe adverse effects induced at therapeutic doses. Manipulation of the immune system has been considered an alternative strategy for the prevention and cure of cancers and as a promising means of suppressing tumor growth without harming the host. Here, we show that THL can enhance the antitumor immunity in mice previously injected with γ-irradiated tumor cells. Both chemotherapy and radiotherapy can induce tumor cell apoptosis. The tumor antigens in the apoptotic cells may be processed and presented by professional antigen-presenting cells and induce antigen-specific immune response in the presence of immune-enhancing agents such as THL. The observation that herbal mixtures can enhance the therapeutic efficacy of ionizing radiation and augment antitumor immune response in tumor-bearing mice has been reported before. THL has been shown to be a safe adjuvant regimen for patients with refractory metastatic breast cancer in a phase IIa trial and can effectively palliate cancer-related syndromes and improve quality of life. It is possible that THL can be used as a complementary medicine to enhance the therapeutic efficacy and lower therapeutic doses of chemotherapy or radiotherapy in cancer patients. Immunotherapy using γ-irradiated tumor cell vaccines is an alternative strategy for cancer treatment. Also, it is possible that THL may enhance the therapeutic efficacy of γ-irradiated tumor cell vaccines via augmenting antitumor immunity.

Conclusions

THL, a Chinese herbal mixture, has been shown to have various anticancer activities. In this study, we further show that THL can suppress tumor growth in mice previously vaccinated with γ-irradiated tumor cells. This function of THL may be related to its ability to enhance the tumor-killing activity of NK cells and CTLs and increase the secretion of IFN-γ, IL-2, and TNF-α in mice vaccinated with γ-irradiated tumor cells. Together, these results suggest that THL may be used as a complementary medicine for cancer patients previously treated with...
γ-irradiated apoptotic tumor cell vaccines, radiotherapy, or chemotherapy.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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