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Therapeutic Injection of a C-Type CpG ODN Induced an Antitumor Immune Response in C57/BL6 Mice of Orthotopically Transplanted Hepatocellular Carcinoma

Huijie Jia,*†[1] Tiesuo Zhao,‡§[1] Di Zou,¶ Xiaolong Jia,§ Ji Gao,# and Xiangfeng Song‡§

*Department of Pathology, Xinxiang Medical University, Xinxiang, Henan, China
†Laboratory of Transplantation and Immunology, Xuzhou Medical University, Xuzhou, Jiangsu, China
‡Department of Immunology, Xinxiang Medical University, Xinxiang, Henan, China
§Henan Collaborative Innovation Center of Molecular Diagnosis and Laboratory Medicine, Xinxiang Medical University, Xinxiang, Henan, China
¶Department of Nephrology, Affiliated Hospital of Changchun University of Traditional Chinese Medicine, Changchun, Jilin, China
#Department of Urinary Surgery, China-Japan Union Hospital, Jilin University, Changchun, Jilin, China

Synthetic CpG oligodeoxynucleotides (ODNs), as TLR9 agonists, have been found to play a possible role in antitumor effect. In order to determine the effect of YW002, known as a C-type CpG ODN, on the treatment of hepatocellular carcinoma (HCC), which is one of the most aggressive carcinomas, we chose to inject YW002 at the doses of 12.5 μg and 25 μg per mouse 7 days post-tumor challenge. The survival rate of mice was recorded every day. On day 14 postinjection, five mice in each group were bled and randomly sacrificed. The level of IFN-γ or TNF-α in the serum was detected and lymphocyte infiltration in the tumor tissue; the ratios of $CD8^+$ T cells and $CD4^+$ T cells in the spleen of mice were also analyzed. The results indicated that treatment with YW002 could raise the survival rate and delay tumor growth in the mice with orthotopically transplanted HCC. Furthermore, the treatment improved the antitumor immune response through increasing the T-cell infiltration in tumor and the ratio of $CD4^+$, $CD8^+$, and NK cells in the spleen. In addition, the concentration of IFN-γ was raised, and the level of TGF-β was depressed. Our data suggested that CpG ODN might be a proper medicament in a monotherapeutic regimen for treatment of HCC.

Key words: CpG oligodeoxynucleotides (ODNs); Hepatocellular carcinoma (HCC); Immune cells; Cytokines

INTRODUCTION

Hepatocellular carcinoma (HCC), as one of the most aggressive carcinomas, significantly threatens public health (1). Multidisciplinary collaboration is necessary for treating HCC. For example, surgery is combined with other therapeutic options, including microwave ablation, radiofrequency ablation, molecular targeted therapy, radiotherapy, and immunotherapy (2,3). It has been proven that the immune response of antitumor was suppressed in the tumor patient. As reported, in many tumor patients, the host immune response elicits ineffectively (4). Therefore, improving the antitumor immune response is crucial for treating HCC patients (5–7).

CpG ODNs, synthetic oligodeoxynucleotides containing CpG motifs, which can activate the innate immune system through Toll-like receptor 9, have been proven to play a role in the antitumor effect in extensive studies (8,9). CpG ODNs can induce innate or adaptive T-cell immunity whether they are used as a monotherapy or an adjuvant (10–14).

According to the different stimulation effects in vitro, CpG ODNs can be divided into three classes: class A, class B, and class C. Class A CpG ODNs can stimulate plasmacytoid dendritic cells (pDCs) to secrete a large amount of IFN-α and activate NK cells or CTL. Class B CpG ODNs have the greatest effect on B cells, proliferating and activating NK cells, secreting IL-6 and IL-12. Class C CpG ODNs have effects of both class A and class B (8,15).

It has been proven that CpG ODNs can inhibit growth of orthotopic tumors in a mouse model of breast cancer

[1]These authors provided equal contribution to this work.
Address correspondence to Xiangfeng Song, Department of Pathology, Xinxiang Medical University, Jinsui Street 601, Xinxiang, Henan 453000, China. Tel/Fax: +86-03733831939; E-mail: xfsong@xxmu.edu.cn *or* Ji Gao, Department of Urinary Surgery, China-Japan Union Hospital, Jilin University, Xiantai Street 126, Changchun, Jilin 130033, China. E-mail: gaoji2015long@sina.com

combining brain metastasis, but there was no effect on the brain metastasis (16). In addition, a CpG ODN combination with IFN-α could result in enhanced antitumor activity in the murine model of B16 melanoma by enhancing cytotoxicity and activating NK cells (17). Research proved that CpG ODNs could inhibit Treg production and downregulate TGF-β levels in the peripheral blood of non-small cell lung cancer patients (18). Furthermore, Yang et al. found that a C-type CpG ODN could extend the survival time and delay the growth of tumors in a model of breast cancer (8).

In this study, we detected an antitumor effect in a model of orthotopically transplanted HCC. We found that treatment with YW002 could raise the survival rate and improve the antitumor immune response in the mice orthotopically transplanted with HCC. These data suggested that CpG ODN might be a proper medicament in a monotherapeutic regimen for treatment of HCC.

MATERIALS AND METHODS

Cells and Mice

H22 cells were provided by Professor Xuejian Zhao (Department of Pathophysiology, Prostate Diseases Prevention and Treatment Research Centre, Norman Bethune College of Medicine, Jilin University, Changchun). C57/BL6 mice, 6–8 weeks old, were used for establishing the orthotopic transplant HCC model. The mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice had free access to food and water for experiments in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

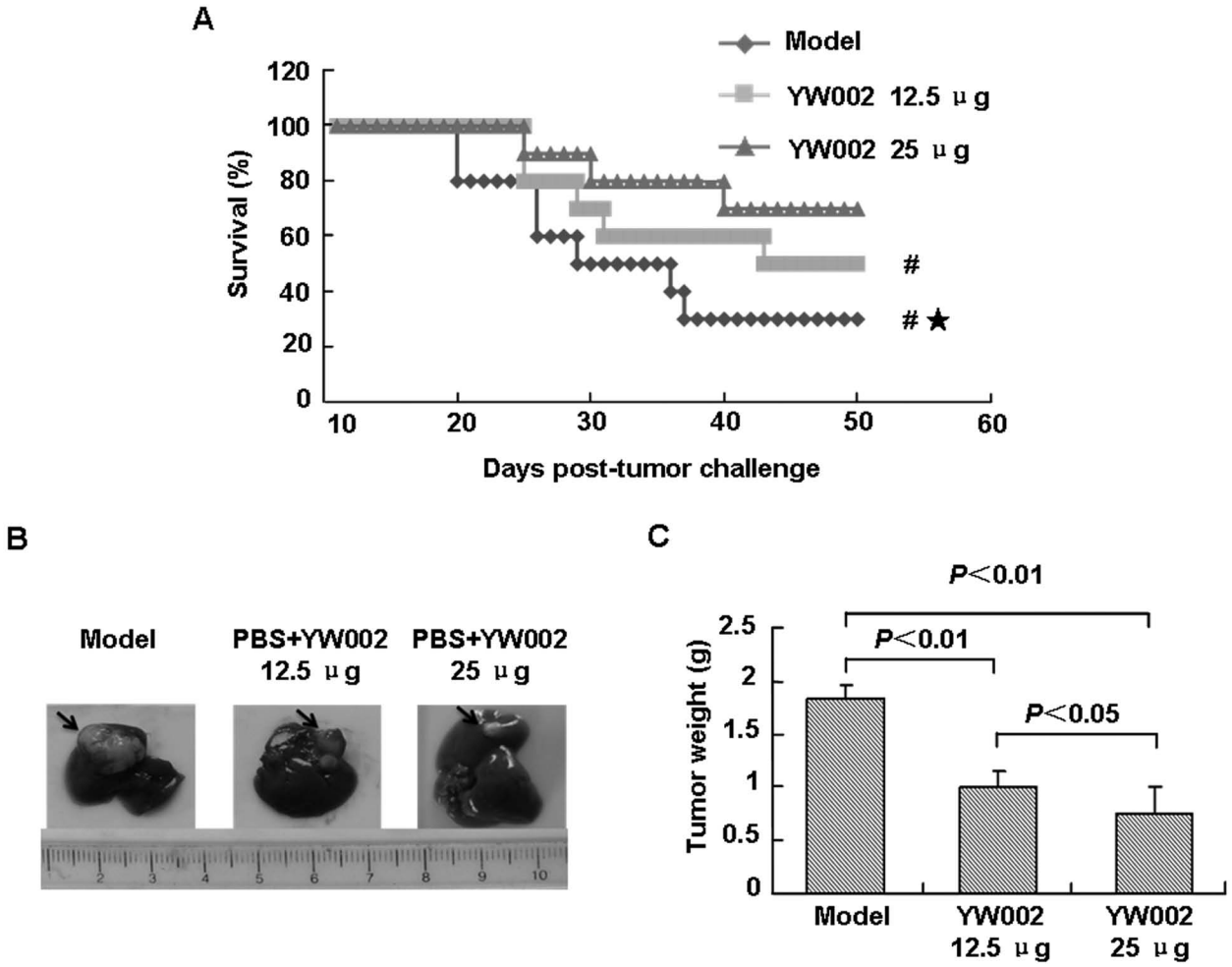

Figure 1. Survival rate and tumor inhibition induced by CpG ODN 0800 at different doses. On day 7 after the tumors were implanted, mice were injected with PBS, YW002 at a dose of 12.5 μg and 25 μg per mouse six times in 1-day intervals from 7 days post-tumor challenge by recording for survival every day. Five mice of each group were sacrificed randomly on day 14 after the injection. The tumors were isolated and weighed. #$p < 0.05$ versus that in the Model group; ★$p < 0.05$ versus that in the YW002 12.5 μg group. (A) Survival curve. (B) Size of tumors. (C) Average tumor weight. Data are presented as mean ± SD.

CpG ODN

YW002, a C-type CpG ODN, with the sequence 5′-tcgc gaacgttcgccgcgttcgaacgcgg-3′, was synthesized in Sangon Biotech (Shanghai) Co. Ltd. and diluted in PBS buffer.

Establishment of Orthotopic Transplant HCC

The orthotopic transplant HCC model was established as described previously (19). Briefly, mice were anesthetized, and a laparotomy was performed. Then the fragment of tumor was transplanted into the right liver lobes with a 3-mm-long sinus tract. Seven days after the operation, the mice were randomly separated into three groups: PSB group, YW002 12.5 μg group, and YW002 25 μg group. The survival rate of mice was recorded every day. In addition, in order to detect the effect of YW002 on the mice, five mice of each group were sacrificed randomly on day 14 after the injection. The tumor tissue, spleen, and serum were collected in order to do the next research.

Immunohistochemistry Assay

Each tumor tissue was isolated from the mice for immunohistochemical analyses. The protocol was carried out according to methods described previously (20,21). The antibodies of CD4 and CD8 were purchase from BD Bioscience. The immunostaining scores were calculated according to the following criteria: 0, no staining or the staining is detected in less than 10% of the cells; 1+, the staining is observed in more than 10% of the cells but only stained in part of the membrane; 2+, the moderate complete staining is detected in more than 10% of the tumor cells; 3+, the strong complete staining is detected in more than 10% of the tumor cells. The scores of 0 and 1+ are designated as negative expression, while the scores of 2+ and 3+ are represented as weakly positive and strongly positive, respectively.

Flow Cytometry Analysis

The NK1.1-PE monoclonal antibody (mAb) was purchased from eBioscience; other mAbs were from BD Bioscience. Each procedure was executed according to the manufacturer's protocol and previously described (8). Briefly, the spleen tissues were homogenized immediately after the isolation. Next, the red blood cell lysis using ACK buffer (Invitrogen, Carlsbad, CA, USA) was mixed with the spleen cell suspension. The cells were washed, counted, and stained with the fluorescence-labeled antibodies. The mixtures were incubated at 4°C for 30 min. The flow cytometric data were acquired on a FACS (BD Bioscience) and analyzed using WinMDI29 (Becton Dickinson).

Cytokine Detection

On day 14 after the treatment, the mice were bled and the serum was separated. The levels of IFN-γ and TGF-β1 were detected using the Mouse ELISA Kit (ExCell

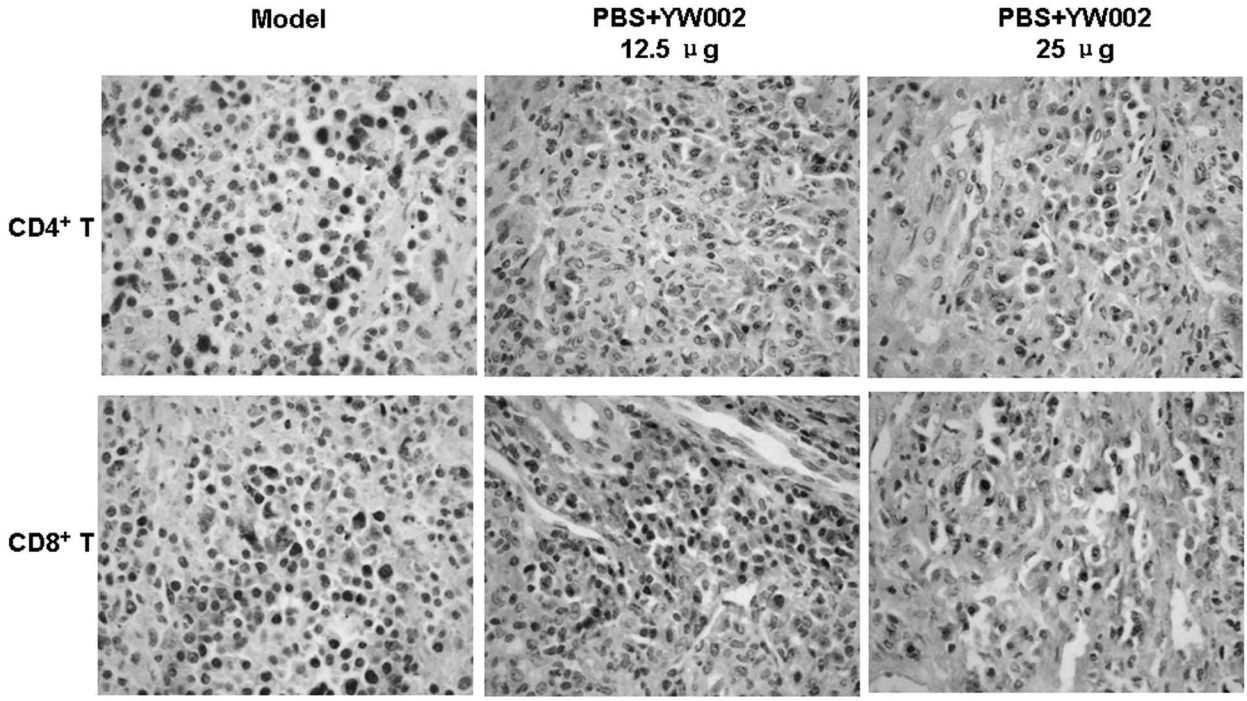

Figure 2. Enhancement of immune cell infiltration in the tumor tissue. On day 10 after the last injection, five mice of each group were sacrificed randomly on day 14 after the injection. The tumors were separated and detected.

Biology, Shanghai, China), according to the manufacturer's directions.

Statistical Analysis

The Kaplan–Meier test was used for analyzing the difference of mouse survival in different groups, and other data analysis was done using the ANOVA test. Statistical analysis was performed using the SPSS software. Differences were considered statistically significant for a value of $p < 0.05$.

RESULTS

The Antitumor Effect of YW002 on Mice With Orthotopically Transplanted HCC

First, in order to identify the antitumor effect of YW002 on the C57/BL6 mice with orthotopically transplanted HCC, we chose two doses of 12.5 μg and 25 μg per mouse to inject the mice according to the report of Yang et al. (8). As shown in Figure 1A, YW002 at both doses of 12.5 μg and 25 μg per mouse displayed a significant anti-HCC effect. By day 40 after the tumor challenge, 70% (7/10) of the mice in the model group were dead. In contrast, 50% (5/10) and 70% (7/10) of mice injected with YW002 at doses of 12.5 μg and 25 μg were still alive ($p < 0.01$).

In addition, the tumor growth was also inhibited in mice injected with YW002 at doses of 12.5 μg and 25 μg (Fig. 1B). YW002 also could induce the inhibition of tumor development. The averages of tumor weight were 1.02 g and 0.75 g in mice injected with YW002 at doses of 12.5 μg and 25 μg compared with 1.83 g in mice injected with PBS (Fig. 1C).

Treatment With YW002 Increased T-Cell Infiltration in Tumor Tissue

Next, in order to investigate the intratumoral lymphocyte infiltrates, we detected the $CD4^+$ and $CD8^+$ T-cell

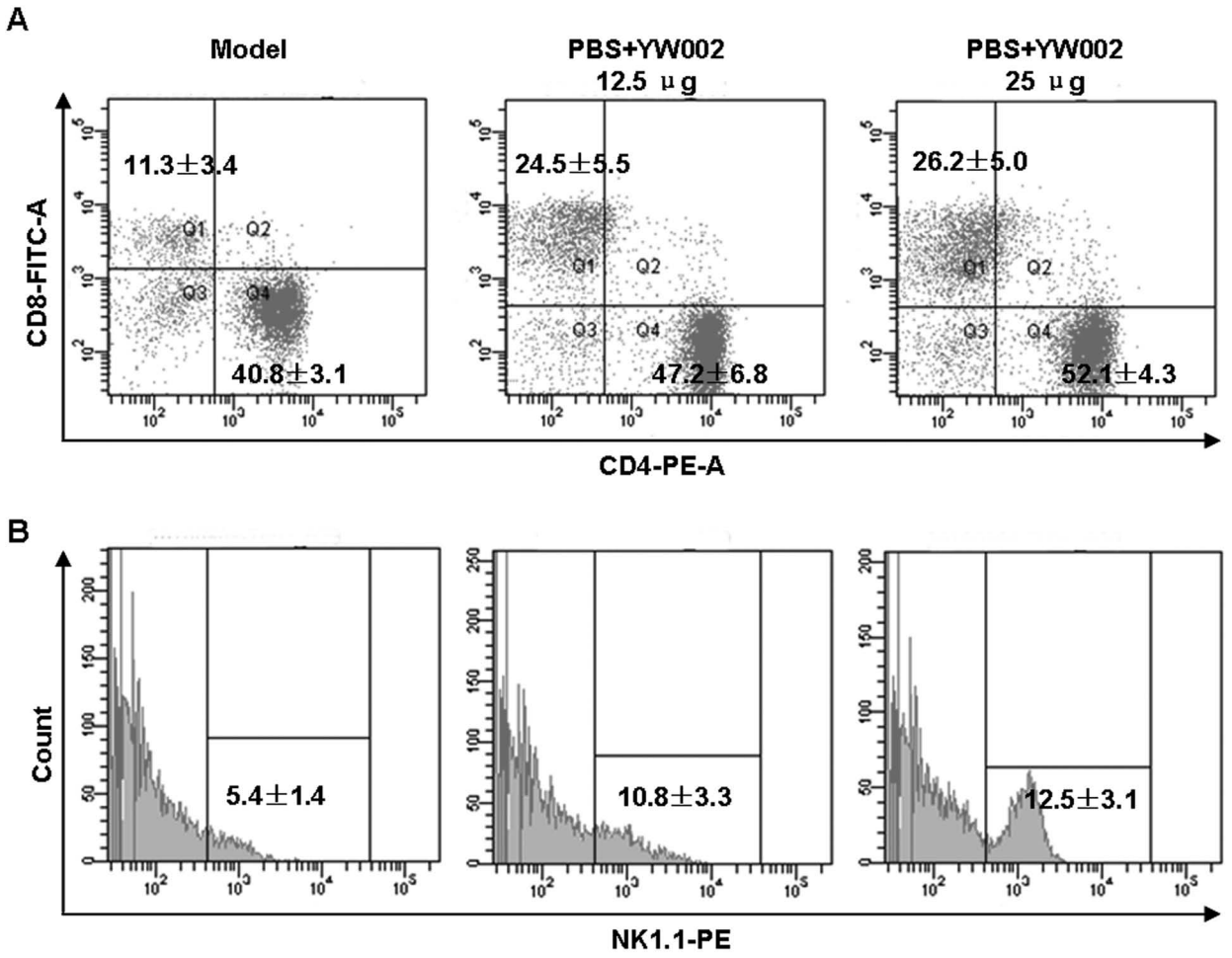

Figure 3. Antitumor effect of CpG ODN YW002 activates the T lymphocyte and NK cells on HCC mice. (A) NK cell expression in the spleen, as analyzed by flow cytometry. (B) Responses of $CD4^+$ and $CD8^+$ T lymphocytes in the spleen were analyzed by flow cytometry. Numbers showed the percentages of cytokine-positive cells in the gates within the total population.

infiltration in the tumor. Fourteen days after the last injection, tumor tissues were obtained from the mice with orthotopically transplanted HCC and then subjected to analysis by immunohistochemistry. As shown in Figure 2, compared with the mice in the PBS group, the YW002 group significantly increased $CD4^+$ and $CD8^+$ T-cell infiltration in the tumors.

Effect of YW002 on the Ratio of $CD4^+$ and $CD8^+$ T Cells in the Spleen

It is known that the spleen plays a central role in cell immunity. We next detected whether YW002 changed the ratio of $CD4^+$ and $CD8^+$ T cells in the mouse spleen using flow cytometry (Fig. 3). Interestingly, the ratio of $CD8^+$ and $CD4^+$ T cells in the spleen of mice injected with YW002 was markedly increased compared with PBS, especially for $CD8^+$ T cells.

Treatment With YW002 Influenced the Secretion of Cytokine in Serum

In addition, cytokine secretion also affects the tumor growth. We next detected the concentration of IFN-γ and TNF-α in the serum of mice. The results showed that compared with PBS treatment, YW002 treatment resulted in significantly higher levels of IFN-γ and TNF-α, especially treatment with a dose of 25 μg (Fig. 4). It is coincident with the tumor weight.

DISCUSSION

It has been proven that YW002, a C-type CpG ODN, could play a role against breast cancer (8). In this study, we detected whether treatment with YW002 could promote the antitumor immune response in mice transplanted with HCC. Our data showed that treatment with YW002 inhibited tumor growth through increasing the immune cell infiltration in the tumor tissue, activating the T lymphocyte and NK cells in the spleen and increasing the levels of IFN-γ and TNF-α in the serum. It is suggested that YW002 is a potent anti-HCC agent through improving the antitumor immune response in mice transplanted with HCC.

According to the previous report, we chose YW002 at the dose of 12.5 μg and 25 μg to inject the mice with orthotopically transplanted HCC. We also detected whether YW002 at dose of 3.25 μg per mouse could exert an antitumor effect. But we found that it did not improve the survival rate of mice (data not shown), which is consistent with the previous study. Thus, we thought that the antitumor effect was related with the dose of YW002.

In this study, we found that treatment with YW002 induced a dramatic increase in $CD8^+$ T cells and $CD4^+$ T cells in tumor tissue. The increasing cells might be the source of the spleen because we also found that both ratios of $CD8^+$ T cells and $CD4^+$ T cells were raised in the spleen of mice treated with YW002. As is known, as the TLR9 agonist, CpG ODNs are the potent activators of innate immunity (22). It has been proven that YW002 could stimulate mouse splenocyte proliferating to $CD8^+$ T cells. This might lead to increasing $CD8^+$ T-cell infiltration in the tumor tissue. $CD8^+$ T cells have the ability to directly kill tumor cells. In addition, CpG ODNs have the ability to induce the Th1 immune response, which could exert an effect on tumor growth (14,23). Therefore, treatment with YW002 not only prompted $CD8^+$ infiltration in the tumor, but also increased $CD4^+$ T-cell infiltration. Both types of T cells could contribute to inhibition of tumor growth.

It has been shown that some cytokines, such as IFN-γ and TNF-α, could have an antitumor effect in the body. IFN-γ could induce tumor cell senescence in mice and tumor cells (24). Likewise, TNF-α also played an antitumor role on HCC tumor-bearing mice (25). Our data show that the levels of IFN-γ and TNF-α in serum were raised through injecting YW002. All of these induced a stronger antitumor effect on the mice treated with YW002.

In brief, in this study, we found that YW002, a C-type CpG ODN, has an effect against tumor growth and improves the antitumor immune response in the

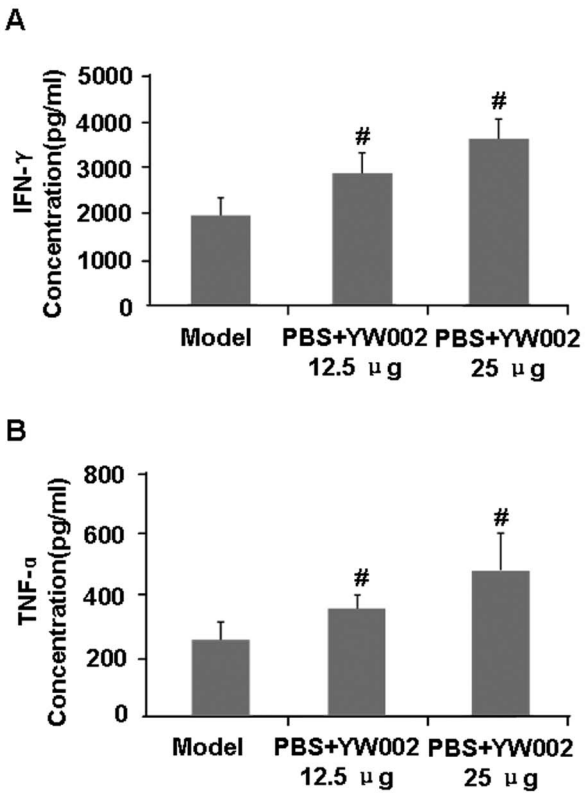

Figure 4. Effect of CpG ODN 0800 on cytokine secretion in HCC mice. (A) The serum level of IFN-γ. (B) The serum level of TNF-α. #$p < 0.05$ versus that in the Model group. Data are presented as the mean ± SD.

orthotopically transplanted HCC. These results indicate that YW002 might be an immunotherapeutic regimen for the treatment of HCC in the clinic.

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