Antitumor and Immunomodulatory Effects of Weikangfu Granule Compound in Tumor-Bearing Mice

Xiaohua Nie, PhD1; Baojun Shi, PhD2; Yuting Ding, PhD1; and Wenyi Tao, PhD3

1College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou, People’s Republic of China; 2Guangdong VTR Bio-Tech Co., Ltd., Zhuhai, People’s Republic of China; and 3College of Biotechnology, Southern Yangtze University, Wuxi, People’s Republic of China

ABSTRACT

Background: Weikangfu granule compound (WKC) is a drug preparation based on a clinical prescription drug, Weikangfu-tang, which has been found to have therapeutic effects on gastric cancer. WKC comprises 7 components, including polysaccharides, saponin, flavonoids, and essential oil.

Objective: The purpose of this study was to assess the antitumor and immunomodulatory effects of WKC in a tumor-bearing rodent model.

Methods: Male and female Kuming mice weighing ~20 g were subcutaneously implanted with sarcoma 180 (S180) tumor cells and randomly assigned to 1 of 5 treatment groups: oral WKC 175, 350, or 525 mg/kg · d, isotonic saline (negative control), or intraperitoneal cyclophosphamide 25 mg/kg · d (positive control). All treatments were administered daily for 10 days. After euthanization on day 11, the mice, tumors, and spleens were weighed. Lymphocyte proliferation and cytotoxic T lymphocyte (CTL) activity were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cellular viability assay method. Macrophage phagocytosis was identified using a yeast test.

Results: Fifty mice were included in the study (10 mice were assigned to each group). The tumors of the mice administered WKC 175, 350, and 525 mg/kg · d were significantly regressed, as determined using MICs, compared with those in the negative-control group (P < 0.05, P < 0.01, and P < 0.01, respectively), and the inhibitory rates were 30.43%, 46.72%, and 54.35%, respectively. Compared with those in the negative-control group, CTL activities and lymphocyte proliferations in the presence of concanavalin A were significantly greater in the WKC-treated groups at all doses (CTL activities: P < 0.05, P < 0.01, and P < 0.01, respectively; lymphocyte proliferations: P < 0.05, P < 0.01, and P < 0.01, respectively). In the groups receiving WKC 175, 350, and 525 mg/kg · d, the phagocytic rates were 1.5- to 2.0-fold those in the negative-control group (P < 0.05, P < 0.01, and P < 0.01, respectively). In the groups receiving WKC 175, 350,
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and 525 mg/kg • d, the phagocytic indexes were 3.7- to 5.0-fold those in the negative-control group (all, \( P < 0.01 \)). In contrast, lymphocyte proliferation in the positive-control group was significantly less compared with that in the negative-control group (\( P < 0.01 \)), but no significant differences were found in CTL activities or macrophage phagocytosis between these 2 groups.

**Conclusion:** The results of this study in a rodent model suggest that WKC exhibited antitumor and immunomodulatory activities in S180-bearing mice, and that WKC improved nonspecific and specific immune functions in mice, such as lymphocyte proliferation, CTL activity, and macrophage phagocytosis.


**Key words:** Weikangfu granule compound, S180 tumor, antitumor activity, immunomodulation activity.

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**INTRODUCTION**

Chinese herbal medicines (CHMs) have been widely used in many countries for thousands of years, and recently have been used clinically to treat cancer or as adjuvant therapy. Some CHMs, including prescription CHMs, are thought to have marked antitumor and antimetastatic effects, with a low risk for toxicity in normal tissue, and immunomodulatory properties. In recent years, the medical potential of CHMs has drawn increasing attention to scientists. Some CHMs and their bioactive components have been reported to have chemotherapeutic effects. For example, in a study of the anticancer effects of 3-day treatment with the CHM *Scutellaria baicalensis* in human cell lines of head and neck squamous cell carcinoma, Zhang et al found that the drug was associated with anticancer activity in vitro and in vivo (dose- and time-dependent growth inhibition [median inhibitory concentration in both cell lines: 150 \( \mu \)g/mL], dose-dependent significant suppression of proliferation cell nuclear antigen expression at 150 \( \mu \)g/mL in both cell lines [both, \( P < 0.05 \)], and significant inhibition of cyclooxygenase-2 expression in both cell lines at 1.5–150 \( \mu \)g [both, \( P < 0.05 \)]. In a study of the chemotherapeutic effects of 2- to 3-day treatment with the CHM *Euphorbia fischeriana* in a human prostate cancer cell line, Liu et al found that the drug was associated with inhibitory effects (regulation of proliferation, neuroendocrine differentiation, and apoptosis) in vitro. In a study of the anticancer effects of 0.5- to 2.0-hour treatment with eugenol, a component of the essential oil isolated from *Eugenia caryophyllata*, on human promyelocytic leukemia cells, Yoo et al found that the drug was associated with apoptosis (cytotoxicity, increased DNA fragmentation, generation of reactive oxygen species, loss of mitochondrial transmembrane potential).

Weikangfu granule compound (WKC) is a drug preparation partly based on a clinical prescription medication, Weikangfu-tang. Weikangfu-tang consists of 4 herbs: rhizome of *Curcuma wenyujin*, root of *Astragalus membranaceus*, root of *Glycyrrhiza inflata*, and sclerotium of *Poria cocos*. Previous studies in humans have reported that Weikangfu-tang was associated with therapeutic effects on
gastric cancer, with significantly effective and total effective rates of 52.46% and 90.16%, respectively (significantly effective rate, \( P < 0.01 \); total effective rate, \( P < 0.05 \) and \( P < 0.01 \)). WKC has 7 components:

- Essential oil of *Curcuma wenyujin*
- Polysaccharides of *Astragalus membranaceus* (Fisch.) Bge. var *mongholicus*
- Saponins of *Astragalus membranaceus* (Fisch.) Bge. var *mongholicus*
- Polysaccharides of *Glycyrrhiza inflata* Bat.
- Glycyrrhizic acid of *Glycyrrhiza inflata* Bat.
- Flavonoids of *Glycyrrhiza inflata* Bat.
- Poriaatin of *Poria cocos* (Schw.) Wolf

In this study, the purpose was to assess the antitumor and immunomodulatory activities of WKC in a tumor-bearing rodent model. To do so, the proliferative responses of spleen lymphocytes with or without T cell mitogen, the activity of cytotoxic T lymphocytes (CTLs), and phagocytosis of peritoneal macrophages were examined.

**MATERIALS AND METHODS**

**Preparation of Weikangfu Granule Compound**

The components of WKC were obtained from Nantong Sihai Plant Extract Co., Ltd. (Nantong, People’s Republic of China [PRC]). The purity of each component was >70%. The components were mixed according to a certain proportion.

**Animals and Cells**

Male and female Kuming mice aged ~4 weeks and weighing ~20 g were purchased from the Animal Research Center, Center for Disease Control and Prevention of Jiangsu Province, PRC. The mice were randomly assigned to 1 of 5 groups: oral WKC 175, 350, or 525 mg/kg · d, isotonic saline (negative control), or intraperitoneal cyclophosphamide (provided by Ruiheng Co., Ltd., Suzhou, PRC) 25 mg/kg · d (positive control). The mice were housed in plastic cages with wood-chip bedding in an animal room with a 12-hour light–dark cycle at room temperature (25°C [2°C]), and allowed free access to a standard laboratory diet and water. Animal handling was conducted in accordance with the regulation for the use of laboratory animals, and the study protocol was approved by the ethics committee at the university.

Mouse sarcoma 180 cells (S180) and colon 26 carcinoma cells (C26) were provided by the Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, PRC. C26 cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS) (Shanghai Institute of Cell Biology) at 37°C in a humidified 5% CO₂ incubator. S180 cells were maintained in the peritoneal cavities of the mice.
Assay of Antitumor Activity and Spleen Weight

Six-day-old S180 ascites tumor cells (~2 × 10^6 cells; 0.2 mL) were subcutaneously implanted into the right hind groin of the mice. One day after inoculation, the study drugs were administered. All treatments were administered for 10 days. On day 11, the mice were euthanized, and the mice, tumors, and spleens were weighed. The inhibitory rate was calculated as follows:

\[
\text{Inhibitory rate (\%) = \left( \frac{A - B}{A} \right) \times 100}
\]

where \(A\) was the mean tumor weight of the negative-control group, and \(B\) was that of the WKC-treated or positive-control group. Relative spleen weight was measured as the ratio of the spleen weight (in milligrams) to body weight (in grams).

Histopathology and Morphologic Observations

The histopathology of S180 tumor was tested as described by Yang et al. \(^{16}\) After euthanization, portions of tumor from each group were randomly selected and coded. Each tumor was fixed in 10% formalin, dehydrated by graded ethanol, embedded with paraffin, cut into small pieces, fixed in Heidenhain’s Suela Fluid (HgCl₂, 4.5 g; NaCl, 0.5 g; distilled water, 80.0 mL; formalin, 20.0 mL; acetic acid, 4.0 mL; trichloroacetic acid, 2.0 mL), stained with hematoxylin and eosin, and examined and photographed under an optical microscope (Olympus Corporation, Tokyo, Japan; magnification, 100× and 400×). Personnel involved in these procedures were blinded to treatment assignment.

Preparation of Mouse Splenocytes

Splenocytes were prepared as described by Lee et al. \(^{2}\) Spleens were aseptically extirpated from the mice and placed in phosphate-buffered saline (PBS) (0.01 M; pH 7.2). Splenocytes were obtained in PBS by forcing spleen fragments through stainless mesh, treated with a hypotonic solution to lyse erythrocytes, and washed twice with PBS. The viability of the splenocytes was >95%, as assessed by the trypan blue dye exclusion method described by Cheng. \(^{17}\) The cells were resuspended in RPMI 1640 medium supplemented with 10% FBS. The concentration of splenocytes was adjusted to 2 × 10^6 cells/mL.

Assessment of Lymphocyte Proliferation

To investigate the effect of WKC on the cellular immune response, we evaluated spleen lymphocyte proliferation and CTL activity in S180-bearing mice. The proliferation of spleen lymphocytes was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular viability assay method (Sigma Diagnostics, St. Louis, Missouri). \(^{17,18}\) Two hundred microliters of splenocyte suspension (2 × 10^6 cells/mL) was placed into 96-well microplates and cultured with or without concanavalin A (ConA) 5 μg/mL (Sigma Diagnostics) at 37°C in a humidified 5% CO₂ incubator for 68 hours. Ten micro-
liters of MTT solution (5 mg/mL) was added to each well and incubated for an additional 4 hours for MTT cellular viability assay. All determinations were conducted in triplicate. Proliferation activity was expressed as the absorbance at 570 nm.\textsuperscript{19}

Assays of Cytotoxic T Lymphocytes

Splenocytes from each mouse were prepared as described previously and used as effector cells. To determine CTL activity (percentage of target cells killed), MTT assay was conducted according to previous reports.\textsuperscript{20,21} C26 cells were used as the target cells. The cells were plated into 96-well microplates at a density of 5 × 10\textsuperscript{3} cells/well in RPMI 1640 medium, and cultured for 24 hours at 37°C in a humidified 5% CO\textsubscript{2} incubator. Then splenocytes were added at 5 × 10\textsuperscript{5} cells/well. The cell ratio of effector-to-target was 100:1. After 68-hour incubation, 10 \muL MTT solution was added to each well and cultured for an additional 4 hours. RPMI 1640 medium and splenocytes were removed, and C26 cells were washed twice with PBS. One hundred fifty microliters of dimethyl sulfoxide was added to each well. The absorbance was detected at 570 nm using an enzyme-linked immunosorbent assay plate reader (EL × 800, BioTek, Winooski, Vermont). All determinations were conducted in triplicate. To determine the CTL activity, the following equation was used:

\[
\text{CTL activity (\%)} = \frac{(T - M)}{T} \times 100
\]

where \(T\) was the mean of the absorbance of target cells incubated alone, and \(M\) was the mean of the absorbance of target cells incubated with effector cells.

Assay of Macrophage Phagocytosis

Yeast was suspended in isotonic saline, heated for 10 minutes at 100°C to remove the impurity, and dried at 70°C. Then the dried yeast (1 g) was suspended in 100 mL of isotonic saline before the experiment.

After WKC administration for 10 days, the mice were intraperitoneally injected with 3 mL sterile isotonic saline before euthanization on day 11. Then ascites (1 mL) was drawn out 2 minutes later, seeded on a microscope slide, and incubated for 50 minutes at 37°C to allow macrophage adhesion. Nonadherent cells were washed away with isotonic saline. The remaining adherent cells were used as the monolayer of peritoneal macrophages. The monolayer of peritoneal macrophages was co-incubated with yeast suspension for 40 minutes at 37°C. The macrophages were rinsed with isotonic saline to remove non-cell-ingested yeast, fixed with glutaraldehyde fixative, and stained with 1% methylene blue solution (Sigma Diagnostics). For each microscope slide, ~100 macrophages were examined with the Olympus microscope using a 400× objective lens. All determinations were conducted in triplicate. The phagocytic rate was calculated as follows:\textsuperscript{22}
Phagocytic rate = \frac{\text{No. of macrophages containing yeast cells}}{\text{Total macrophages counted}} \times 100

The phagocytic index was calculated as follows\textsuperscript{23}:

\text{Phagocytic index} = \frac{\text{No. of yeast cells inside macrophages}}{\text{No. of macrophages phagocytosing}} \times 100

**Statistical Analysis**

The data obtained were analyzed statistically using the analysis of variance method for repeated measurements. Significance of any differences between WKC-treated groups and the negative-control group was assessed using the Student \( t \) test. \( P < 0.05 \) was considered statistically significant. Statistical analysis was performed using SPSS version 9.0 (SPSS Inc., Chicago, Illinois). All data were expressed as mean (SD).

**RESULTS**

Fifty Kuming mice were studied (mean [SD] weight, 20.0 [2.0] g; 10 mice per study group).

**Tumor and Spleen Weight**

The effects of WKC on S180-bearing mice are shown in Table I. WKC 175, 350, and 525 mg/kg \( \cdot \) d were found to be associated with significant growth inhibition of S180 tumor compared with the negative-control group (\( P < 0.05, P < 0.01, \) and \( P < 0.01 \), respectively). WKC 175, 350, and 525 mg/kg \( \cdot \) d were found to be asso-

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Increase in Body Weight, Mean (SD), g</th>
<th>Relative Spleen Weight, Mean (SD), mg/g</th>
<th>Tumor Weight, Mean (SD), g</th>
<th>Inhibitory Rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control*</td>
<td>6.0 (1.8)</td>
<td>5.43 (0.70)</td>
<td>0.92 (0.35)</td>
<td>-</td>
</tr>
<tr>
<td>Positive control\textsuperscript{f}</td>
<td>-0.2 (2.1)\textsuperscript{f}</td>
<td>3.16 (0.39)\textsuperscript{f}</td>
<td>0.34 (0.26)\textsuperscript{f}</td>
<td>63.04</td>
</tr>
<tr>
<td>WKC 175 mg/kg ( \cdot ) d</td>
<td>5.9 (2.9)</td>
<td>6.19 (0.41)</td>
<td>0.64 (0.34)\textsuperscript{f}</td>
<td>30.43</td>
</tr>
<tr>
<td>350 mg/kg ( \cdot ) d</td>
<td>6.6 (2.5)</td>
<td>6.34 (0.73)\textsuperscript{f}</td>
<td>0.49 (0.22)\textsuperscript{f}</td>
<td>46.72</td>
</tr>
<tr>
<td>525 mg/kg ( \cdot ) d</td>
<td>6.2 (1.4)</td>
<td>6.21 (0.68)\textsuperscript{f}</td>
<td>0.42 (0.40)\textsuperscript{f}</td>
<td>54.35</td>
</tr>
</tbody>
</table>

*This group received physiologic saline.
†This group received cyclophosphamide 25 mg/kg \( \cdot \) d.
\footnote{\( p < 0.05 \) versus negative control (Student \( t \) test).}
\footnote{\( p < 0.01 \) versus negative control (Student \( t \) test).}
associated with significant inhibition of S180 tumor growth in a dose-dependent manner compared with the negative-control group, with inhibitory rates of 30.43%, 46.72%, and 54.35%, respectively. In addition, relative spleen weights in the groups treated with WKC 350 and 525 mg/kg · d were significantly greater compared with those in the negative-control group (P < 0.01 and P < 0.05, respectively). No significant differences in body weight were found between the groups treated with WKC and the negative-control group. On the other hand, cyclophosphamide was associated with a significant decrease in body and spleen weights (P < 0.05 and P < 0.01, respectively), although it was also associated with significant growth inhibition compared with the negative-control group (63.04%; P < 0.01).

**Histopathologic and Morphologic Assessments**

On histopathology of S180 tumor from the WKC-treated groups (Figures 1 and 2), the border between tumor and fatty tissue was distinct, and the tumor cells had nucleus pycnosis and necrosis areas in different degrees. In contrast, tumor in the negative-control group invaded into fatty tissue and grew vigorously.

**Cellular Immunity**

As shown in Figure 3, treatment with WKC resulted in a statistically significant increase in lymphocyte proliferation with or without T cell mitogenic stimuli, and

![Figure 1. Invasion of S180 tumor into fatty tissue after 10-day oral administration of Weikangfu granule compound (WKC). (A) Negative control; (B) WKC 175, (C) 350, (D) 525 mg/kg · d. (Hematoxylin and eosin; magnification, 100 ×.)](image)
Figure 2. Mean morphologic appearance after 10-day oral administration of Weikangfu granule compound (WKC). (A) Negative control; (B) WKC 175, (C) 350, (D) 525 mg/kg·d. (Hematoxylin and eosin; magnification, 400 ×.)

The levels of lymphocyte proliferation in the presence of ConA were significantly greater at 175, 350, and 525 mg/kg·d compared with that in the negative-control group (P < 0.05, P < 0.01, and P < 0.01, respectively). However, lymphocyte proliferation in the positive-control group was significantly decreased compared with negative controls (P < 0.01). Furthermore, CTL activities were significantly enhanced in S180-bearing mice with WKC at the doses of 175, 350, and 525 mg/kg·d (P < 0.05, P < 0.01, and P < 0.01, respectively), in a dose-dependent manner (Figure 4).

**Macrophage Phagocytosis**

As summarized in Table II, the phagocytic rates and indexes of the WKC-treated groups were 1.5- to 2-fold and 3.7- to 5-fold those of the negative-control group, respectively. Compared with those in the negative-control group, the phagocytic rates and indexes were significantly higher in the groups treated with WKC 175, 350, and 525 mg/kg·d (for phagocytic rates: P < 0.05, P < 0.01, and P < 0.01, respectively; for phagocytic indexes: all, P < 0.01).

**DISCUSSION**

The results of this study suggest that WKC could activate peritoneal macrophage and enhance peritoneal macrophage phagocytosis in S180-bearing.
Figure 3. Spleen lymphocyte proliferation in S180-bearing mice after oral administration of Weikangfu granule compound (WKC). Spleen lymphocytes were obtained and cultured with or without concanavalin A (ConA). Proliferation activity was expressed as the absorbance at 570 nm. *$P < 0.01$ versus negative-control group (Student $t$ test); †$P < 0.05$ versus negative-control group (Student $t$ test). §This group received isotonic saline. ¶This group received cyclophosphamide 25 mg/kg · d.

Figure 4. Mean cytotoxic T lymphocyte (CTL) activity in S180-bearing mice after oral administration of Weikangfu granule compound (WKC). Spleen lymphocytes were obtained and cultured with C26 cells for 72 hours. CTL activity was expressed as $\frac{(T - M)}{T} \times 100$, where $T$ is the mean of the absorbance of target cells incubated alone, and $M$ is that of target cells incubated with effector cells. *$P < 0.05$ versus negative-control group (Student $t$ test); †$P < 0.01$ versus negative-control group (Student $t$ test). §This group received isotonic saline. ¶This group received cyclophosphamide 25 mg/kg · d.
Table II. Effects of Weikangfu granule compound (WKC) on macrophage phagocytosis in S180-bearing mice (n = 10 mice per study group).

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Phagocytic Rate, Mean (SD), %</th>
<th>Phagocytic Index, Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control*</td>
<td>30.12 (7.28)</td>
<td>0.95 (0.22)</td>
</tr>
<tr>
<td>Positive control†</td>
<td>17.22 (6.83)</td>
<td>0.65 (0.18)</td>
</tr>
<tr>
<td>WKC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>175 mg/kg • d</td>
<td>45.80 (4.74)†</td>
<td>3.76 (0.86)§</td>
</tr>
<tr>
<td>350 mg/kg • d</td>
<td>63.02 (8.05)§</td>
<td>5.08 (1.02)§</td>
</tr>
<tr>
<td>525 mg/kg • d</td>
<td>58.79 (7.96)§</td>
<td>4.59 (1.35)§</td>
</tr>
</tbody>
</table>

*This group received isotonic saline.
†This group received cyclophosphamide 25 mg/kg • d.
§P < 0.05 versus negative control (Student t test).
¶P < 0.01 versus negative control (Student t test).

mice. The relationship between the occurrence, growth, and decline of tumor and immune states is the essential problem of tumor immunology. The discovery of new antitumor drugs that can potentiate immune function has become an important goal of research in immunopharmacology and immunotherapy.19 In this study, a tumor-bearing animal model was used to research the antitumor and immunomodulatory activities of WKC.

The results of the present study suggest that WKC significantly inhibited S180 tumor formation in a dose-dependent manner, without causing a significant decrease in body or spleen weight in mice. Results on histopathology suggest that WKC prevented the tumor invasion into fatty tissue. With 10-day WKC treatment, the relative spleen weights in S180-bearing mice were significantly greater compared with those of the 2 control groups. The relative spleen weight is an important indicator of nonspecific immunity.24 In a study of the effects of 10-day treatment with 3 doses of polysaccharide L-II on cellular immune response in S180-bearing mice, Zheng et al24 found that use of immunopotentiators might increase spleen and thymus weights, and that use of immunosuppressive agents might decrease spleen and/or thymus weight and/or lead to a decline in immune function. Based on those results, WKC might have immune potentiator properties.

Protective immunity against tumor comprises cellular and humoral immunity. Immune responses, especially cellular immunity, play an important role in the elimination of locally growing and circulating tumor cells and thus result in the inhibition of growth and metastasis of tumors.25 In the present study, cell-mediated immune defense seemed to be mediated specifically by T cells, including cytotoxic T cells. T cells can kill tumor cells and produce many lymphocytic factors, including macrophage mobile factor, lymphotoxin, transfer
factor, and interferon, which can enhance macrophage phagocytosis and the capacity of killing target cells. The results of the present study suggest that WKC at all doses significantly enhanced spleen lymphocyte proliferation and CTL activity with or without stimulation by T cell mitogen (ConA).

Macrophages have diverse functions, including phagocytosis, tumor cytotoxicity, cytokine secretion, and antigen presentation. They represent a defense line against pathogens and tumor cells, recognizing and destroying them. The enhancement of phagocytic function is applicable for therapy of cancer, because phagocytes act as regulator and effector cells in the immune system, and phagocytosis represents the indispensable step of the immunologic defense system. Therefore, stimulation of macrophages might be a major target for therapeutic application. This study also found that oral administration of WKC was associated with significant improvement in macrophage phagocytic function in S180-bearing mice.

**Study Limitations**

The present study had some limitations, including the lack of a pharmacodynamic test of each independent component in the formulation. In the treatment of S180-bearing mice, each effective component might exert a different function. Some might induce main effects, whereas others might not. It is necessary to assess the pharmacodynamic function of each component in the medication in future research. In addition, although WKC is composed of effective components extracted from natural drugs, the adverse effects of WKC (eg, hepatotoxicity and renal toxicity) remain to be studied.

**CONCLUSION**

The results of this study in a rodent model suggest that WKC exhibited antitumor and immunomodulatory activities in S180-bearing mice, and that WKC improved nonspecific and specific immune functions in mice, such as lymphocyte proliferation, CTL activity, and macrophage phagocytosis.

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**Address correspondence to:** Wenyi Tao, PhD, College of Biotechnology, Southern Yangtze University, Wuxi, People’s Republic of China. E-mail: niexiaohua2000@126.com