

Enhancement of IL-2 and IFN- γ expression and NK cells activity involved in the anti-tumor effect of ganoderic acid Me *in vivo*

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Abstract

Ganoderic acid Me (GA-Me) is a lanostane triterpenoid purified from *Ganoderma lucidum* mycelia, one of the most widely used herbs for cancer treatment and prevention in east Asia. In the present study, it was demonstrated that GA-Me could inhibit both tumor growth and lung metastasis of Lewis lung carcinoma in C57BL/6 mice. Compared with the control group, Natural Killer (NK) cells activity was significantly enhanced by intraperitoneal administration of GA-Me (28 mg/kg). Results of ELISA assay and RT-PCR showed that the expressions of Interleukin-2 (IL-2) and Interferon- γ (IFN- γ) were also increased ($p < 0.05$). Additionally, the expression of Nuclear Factor- κ B (NF- κ B) was up-regulated after the treatment of GA-Me, which might be involved in the production of IL-2. In conclusion, the findings of this study implied that GA-Me could effectively inhibit tumor growth and lung metastasis through increasing immune function.

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Keywords: Ganoderic acid Me; NK cells activity; IL-2; IFN- γ ; NF- κ B; Lewis lung carcinoma

1. Introduction

Drugs enhancing anti-tumor activities and the condition of the immune system in tumor-bearing organisms are of current interest. Screening for products with specific health benefits for immunological activities has been a fast growing sector in cancer research. *Ganoderma lucidum* (*G. lucidum*), one of the most famous traditional Chinese medicines, has been obtaining considerable significance owing to its well-known pharmacological activities and anti-tumor effects [1]. Evidence has been accumulated concerning the medical application of *G. lucidum* in the treatment of various diseases, such as oncogenesis, cancer metastasis, hyper-

Abbreviations: GA-Me, Ganoderic acid Me; IL-2, Interleukin-2; IFN- γ , Interferon- γ ; NK cell, Natural Killer cell; NF- κ B, Transcription factor Nuclear Factor- κ B; LLC cell, Lewis lung carcinoma cell; MTT, 3-[4,5-thiazol-2-yl] 2,5-diphenyltetrazolium bromide; DMSO, Dimethyl sulfoxide; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; RT, Reverse Transcription.

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tension, arthritis, bronchitis, asthma, anorexia and immunological disorders [2,3]. *G. lucidum* has also been suggested to promote longevity and maintain the vitality of humans [4].

The potent bioactive compounds in *G. lucidum* are polysaccharides and ganoderic acids, a group of triterpenes [5–7]. Some investigations have been carried out that the natural mixtures of triterpenoids in *G. lucidum* can inhibit the proliferation of human and mouse carcinoma cell lines [3,8,9]. However, little work has been done on understanding the anti-tumor activity and immune regulation of purified triterpenes *in vivo*.

Ganoderic acid Me (GA-Me) is a lanostane triterpene purified from methanol extract of *G. lucidum* mycelia. Using C57BL/6 mice bearing Lewis lung carcinoma, the present study was undertaken to examine the anti-tumor effects of GA-Me. First, the anti-tumor effects of GA-Me through immunity stimulation were confirmed. Then this immunity effect was found to be related to the amelioration of IL-2, IFN- γ and NK cells. Furthermore, the results showed that GA-Me enhanced the expression of NF- κ B, and the up-regulation of NF- κ B might be involved in regulating the secretion of immunity-related cytokines.

2. Materials and methods

2.1. Chemicals

Methanol extract of the dried and powdered mycelia of fermented *G. lucidum* was conducted with chloroform. Chromatographic purification of the chloroform fraction yielded GA-Me. Endotoxin was removed by anion-exchange chromatography and ultra filtration in the present study. Afterwards, endotoxin concentration in GA-Me was measured using Limulus Amebocyte Lysate (LAL) assay kit (Shanghai Yihua Medical Science technology, China). The results showed that endotoxin activity in GA-Me was less than 0.05 endotoxin units/ml, indicating the LPS contamination which was negligible.

The purity and structure elucidation of these compounds are determined by Electrospray Ionization Mass Spectrometry, ^1H and ^{13}C Nuclear Magnetic Resonance, Infrared Spectroscopy respectively. The structure of the compound is shown in Fig. 1. Purity of GA-Me was over 99%. Stock solution of GA-Me was prepared in endotoxin-free dimethyl sulfoxide (DMSO) and stored at $-20\text{ }^\circ\text{C}$. The final DMSO concentration never exceeded 10% (v/v) in the medium. Cisplatin (DDP) was purchased from Sigma Chemical Corporation.

2.2. Cell line

YAC-1 cells have been classically used as targets for murine NK cells. YAC-1 cell line was purchased from Cell

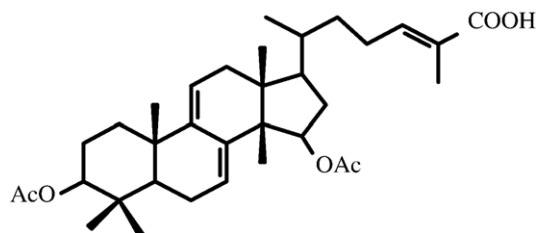


Fig. 1. Structure of ganoderic acid Me (GA-Me).

Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and was cultured in RPMI 1640 medium with 10% Fetal Bovine Serum (FBS), penicillin (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$). Cells were incubated in an atmosphere of 5% CO_2 and 95% air at $37\text{ }^\circ\text{C}$.

2.3. Animals and Lewis lung cancer model

C57BL/6 mice (male, $20\pm 2\text{ g}$, 8–10 weeks) were purchased from Shanghai Center of Experimental Animals, Chinese Academy of Sciences. The animals were maintained under specific-pathogen-free standards animal laboratory with $24\pm 2\text{ }^\circ\text{C}$, 50% humidity, and a 12-h light/dark cycle. The Institutional Animal Care and Use Committee of Shanghai Long Hua Hospital approved all of the experimental procedures. All the experiments were treated in accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

The cell line of Lewis lung carcinoma (LLC cells) was purchased from Shanghai Hutchison MediPharma Ltd. LLC cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). A single cell suspension was prepared in phosphate-buffered saline (PBS) by the passage of fresh tumor tissue through a sequential series of 18-, 22-, 27- and 30-gauge needles. 1×10^6 tumor cells suspended in 0.1 ml of PBS were injected subcutaneously (s.c.) in the right armpit region of C57BL/6 mice. Animals were randomized into four groups after tumor implantation. In the surgical groups, GA-Me were injected into mice at 28 mg/kg (HIGH) and 7 mg/kg (LOW) for 10 days and then observed for another 10 consecutive days. Control mice were injected with endotoxin-free saline at the same time points in an identical manner. In the positive surgical group, Cisplatin (DDP) administration (1 mg/kg, i.p.) was initiated for the first 4 days. At the end of the experiments, the mice were sacrificed and the tumors were harvested. The tumor weight was measured and the tumor growth inhibition rate was calculated according to the formula: tumor growth inhibition rate = [(mean tumor weight of the control group – mean tumor weight of the treatment group) / mean tumor weight of the control group] $\times 100\%$.

Lung tissues were also removed and the tumor nodes in the surface of lungs were counted under the microscope. The lung metastasis was calculated using the following formula: lung metastatic inhibition rate = [(mean lung metastatic

number of the control group—mean lung metastatic number of the treatment group)/mean lung metastatic number of the control group]×100%.

2.4. Detection of NK cells activity

All procedures were conducted under aseptic conditions by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [10]. The cell suspensions derived from spleens of the experimental mice were passed through a 200-mesh stainless steel sieve and then the tissue fragments were removed. The cells were freed of red blood cells by treatment with lysis buffer (0.15 M NH₄Cl, 0.01 M KHCO₃, and 0.1 mM Na₂EDTA, pH 7.4). To remove adherent cells such as macrophages, total spleen cells were incubated for 1 h in Petri dishes. The suspended cell populations collected were washed twice in cold RPMI 1640 medium and centrifuged at 1000 g for 20 min. After collection, the cells were washed three times and resuspended in RPMI 1640 medium. The viability of splenic lymphocytes determined by the trypan blue exclusion test was over 98%. The non-adherent lymphocytes were directly used as NK effector cells.

NK cells (in a concentration of 2×10⁶ cells/ml) together with YAC-1 cells (in a concentration of 1×10⁵ cells/ml) were incubated in the wells of a flat bottom 96 well sterile cell culture plate (Effector: Target ratio of 20:1) with a total volume of 100 μl in each well (ET). 100 μl of the same concentration of NK cells (E), 100 μl of the same concentration of YAC-1 cells (T), and 100 μl of RPMI medium (B) were incubated in different wells as controls and blank, respectively. The test was conducted in triplicates. After 4 h of incubation in a humidified incubator with 5% CO₂ at 37 °C, 10 μl of MTT solution (5 mg/ml in phosphate-buffered saline, PBS) was added to each well. After another 4 h of incubation, the plate was centrifuged at 200 g for 5 min and supernatants were removed and replaced by 100 μl DMSO. The optical density (OD) of each well was measured by an Automated Microplate Reader (Multiskan Ex, Lab systems, Finland). The percentage of cytotoxicity was determined by the formula: % cytotoxicity=1−[(absorbance at 540 nm of effector+target cells)−(absorbance at 540 nm of effector cells)]/[absorbance at 540 nm of target cells]×100.

2.5. Enzyme-linked immunosorbent assay (ELISA) for IL-2 and IFN-γ

The animals were anesthetized with Ether. Blood was collected by heart puncture. The production of cytokine (IL-2 and IFN-γ) in blood serum was measured by a standard sandwich cytokine ELISA procedure to assess the pharmaceutical effect on cytokine quantity. Standards (recombinant cytokine at 0, 15.63, 31.25, 62.5, 125, 250, 500, 1000 pg/ml concentrations) and samples were added in 100 μl/well. Cytokine quantities in the samples were calculated from standard curves of recombinant cytokines using a regression

linear method. The assay was performed according to the instructions of mouse IL-2 and IFN-γ kits from R&D Systems (Minneapolis, MN). Absorbance results were assessed using an ELISA microplate reader set (Multiskan Ex, Lab Systems, Finland).

2.6. Reverse Transcription PCR of IL-2 and IFN-γ gene expression

Spleens were removed immediately after mice sacrifice. Half of the spleens were restored in liquid nitrogen. Total RNA was isolated using TRIzol™ reagent (Promega Corporation) and then reverse-transcribed using Reverse Transcription System (TAKARA Corporation). An aliquot (4 μl) of RT product was used for PCR amplification in a total volume of 50 μl. IL-2 cDNA (243 bp) was amplified using the sense primer 5'-GAC ACT TGT GCT CCT TGT CA-3' and the antisense primer 5'-TCA ATT CTG TGG CCT GCT TG-3'. IFN-γ cDNA (427bp) was amplified using the sense primer 5'-TCTGA GACAA TGAAC GCTAC-3' and the antisense primer 5'-GAGTA GGCTC ACCAG GTG-3' and β actin was used as the load control. The thermal cycle profile used in this study was (1) denaturing for 30 s at 94 °C (2) annealing primers for 30 s at 55 °C and (3) extending the primers for 2 min at 72 °C. PCR amplification was performed for 35 cycles and an aliquot (10 μl) of the PCR mixture was visualized by electrophoresis in agarose gel. The gel was photographed and then quantitatively measured by scanning densitometry.

2.7. Western blotting for NF-κB protein expression

After sacrifice, mice spleen tissues were harvested and prepared in liquid nitrogen. Frozen tissues (0.2 to 0.25 g) were homogenized in a pestle homogenizer. 1 ml of lysis buffer containing 0.5% NP-40 and 0.5% sodium deoxycholate, in 10 mM Tris buffer (pH 7.5) was added. The homogenate was put on ice to swell for 10 min. After centrifugation at 1000 ×g for 8 min, the supernatant was extracted in lysis buffer (10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 5 mM leupeptin, and 10 mg·ml⁻¹ aprotinin) for 30 min on ice. The lysates were centrifuged to remove insoluble materials, normalized according to their protein content. Lysates cleared of debris and nuclei were resolved on 10% gels. Equal amounts of proteins (30 μg) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Amresco). After blocking for 1 h at room temperature using PBST buffer (PBS buffer plus 0.1% (v/v) Tween-20) containing 3% (m/v) BSA, the filter was applied sequentially with anti-NF-κB (diluted 1:1500; Santa Cruz Biotechnology), and appropriate secondary antibody conjugated alkaline phosphatase (diluted 1:2000; Santa Cruz Biotechnology). The blots were then visualized with Nitro Blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate

(Sigma Co.) detection. Band intensities were measured using image analysis software (NIH Image).

2.8. Statistical analysis

Statistical analysis was performed using the Student's *t*-test to evaluate the significance of differences between groups. In all the graphs, * indicated significantly different than untreated groups, $p < 0.05$; ** indicated significantly different than untreated groups, $p < 0.01$; *** indicated significantly different than untreated groups, $p < 0.001$. All data points represented the mean of triplicates.

3. Results

3.1. Effects of GA-Me on the inoculated tumor growth and lung metastasis

To determine whether GA-Me has an anti-tumor effect *in vivo*, a Lewis lung carcinoma model was established by subcutaneously injecting LLC cells into C57BL/6 mice. As shown in Table 1, the inhibition rate of tumor growth and lung metastasis in GA-Me-treated group (28 mg/kg) was 43.23% ($p < 0.05$) and 54.89% ($p < 0.05$) respectively. No discernible loss of the body weight was observed in the tumor-bearing mice (Fig. 2).

Table 1

Effects of GA-Me on the growth of the inoculated tumor and lung metastasis

A. Tumor growth inhibition effect of GA-Me <i>in vivo</i>				
Groups	N	Dose (g/kg)	Tumor weight (g)	Tumor growth inhibition rate (%)
Control group	8		1.768±0.556	
DDP	8	0.001	1.023±0.154*	42.15
GA-Me low	8	0.007	1.270±0.582	28.17
GA-Me high	8	0.028	1.004±0.259*	43.23
B. Lung metastatic inhibition effect of GA-Me <i>in vivo</i>				
Groups	N	Dose (g/kg)	Number of lung metastases	Lung metastatic inhibition rate (%)
Control group	8		7.600±2.102	
DDP	8	0.001	1.400±0.894**	81.58
GA-Me low	8	0.007	4.500±2.726	40.79
GA-Me high	8	0.028	3.429±1.902*	54.89

Tumors were harvested 10 days after GA-Me administration. (A) Tumor weight growth inhibition rate: (control group tumor weight – treatment group tumor weight)/control group tumor weight × 100%. Lung tissues were also removed and the tumor nodes in the surface of lungs were counted under the microscope. (B) Lung metastatic inhibition rate: (control group lung metastatic number – treatment group lung metastatic number)/control group lung metastatic number × 100%. Data are presented as the mean ± SEM of triplicate samples of a representative experiment, similar results were obtained in three independent experiments. Significant differences from untreated control are indicated by * $p < 0.05$; ** $p < 0.01$.

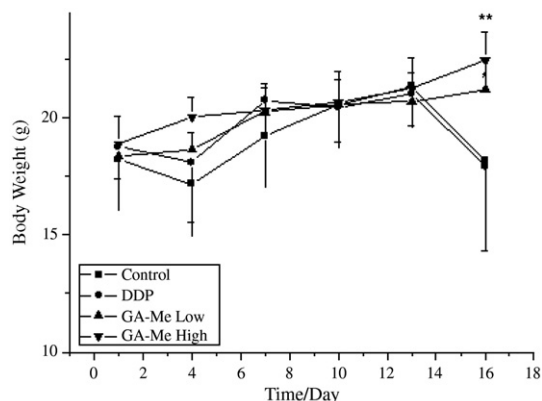


Fig. 2. Body weights of mice bearing Lewis lung carcinoma. Data are presented as the mean ± SEM of triplicate samples of a representative experiment, similar results were obtained in three independent experiments. Significant differences from untreated control are indicated by * $p < 0.05$; ** $p < 0.01$.

3.2. Roles of GA-Me on NK cells activity

It has been reported that NK cells play pronounced roles in the innate immunity against infections and tumor development. In this study, the effect of GA-Me on the cytotoxicity of spleen NK cells against YAC-1 cells was investigated. The activity of NK cells in the spleens of the experimental mice was significantly ($p < 0.05$) enhanced after GA-Me administration (Table 2). This indicated that GA-Me had immunomodulatory effects on NK cells *in vivo*.

3.3. Effects of GA-Me on cytokines production *in vivo*

IL-2 and IFN- γ levels in blood serum specimens of the mice were examined through the ELISA procedure. In Fig. 3, IL-2 and IFN- γ concentrations in GA-Me high-dose-treated group (28 mg/kg) were higher than that in GA-Me low-dose-treated group (7 mg/kg). Compared with the

Table 2

Roles of GA-Me on NK cells activity in spleen

Groups	N	Dose (g/kg)	NK cells activity (%)
Control group	8		32.64 ± 6.10
DDP	8	0.001	25.79 ± 12.05
GA-Me low	8	0.007	39.70 ± 9.02
GA-Me high	8	0.028	51.63 ± 14.01*

Cytotoxicities of spleen NK cells against YAC-1 tumor cells were determined as described in Materials and methods. Data are presented as the mean ± SEM of triplicate samples of a representative experiment, similar results were obtained in three independent experiments. Indicated significant difference between control and experimental, * $p < 0.05$.

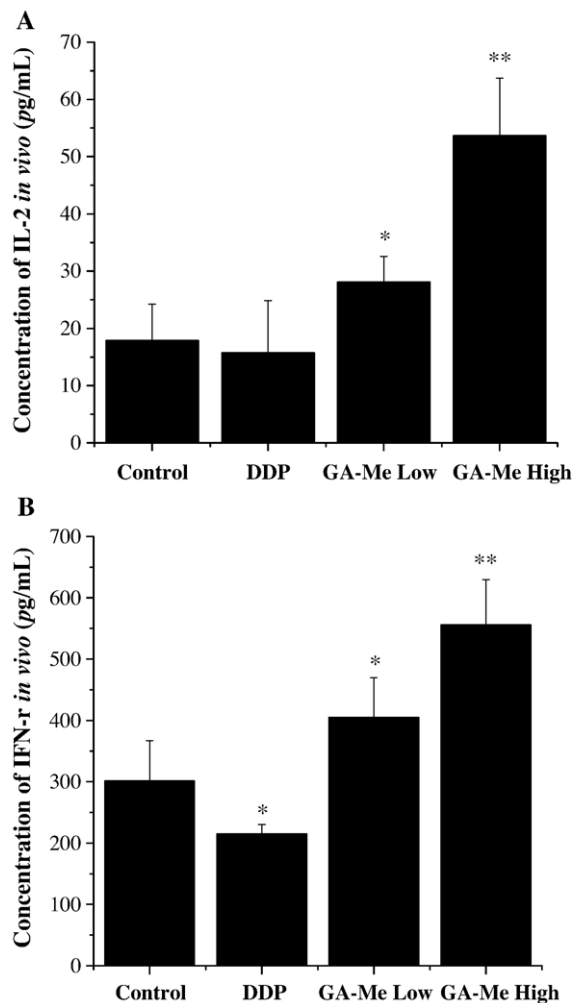


Fig. 3. Effects of GA-Me on cytokine production *in vivo*. The serum concentrations of IL-2 and IFN- γ were tested by ELISA assay. (A) Concentration of IL-2 *in vivo*. (B) Concentration of IFN- γ *in vivo*. Data are presented as the mean \pm SEM of triplicate samples of a representative experiment, similar results were obtained in three independent experiments. Indicated significant difference between control and experimental, * $p < 0.05$, ** $p < 0.01$.

control group, IL-2 and IFN- γ concentrations were augmented ($p < 0.01$) in the GA-Me-treated group. That is, GA-Me treatment improved the serum concentrations of IL-2 and IFN- γ *in vivo*.

3.4. Enhancement of IL-2 and IFN- γ in the mRNA level

To explore the mechanism of GA-Me action on cytokines production, IL-2 and IFN- γ mRNA levels in C57BL/6 mice bearing Lewis lung carcinoma after treatment with different concentrations of GA-Me were assayed with RT-PCR. As shown in Fig. 4, the cytokine mRNA levels were higher in GA-Me-treated group than that in control group. The results obviously indicated that GA-Me possessed the ability of up-

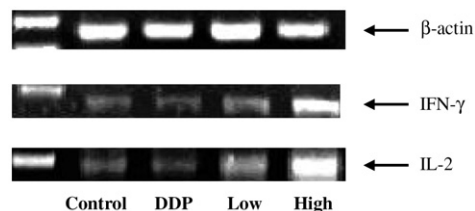


Fig. 4. Influences of GA-Me on the gene expression of IL-2 and IFN- γ in spleen.

regulating the expression of IL-2 and IFN- γ mRNA level *in vivo*.

3.5. Effects of GA-Me on regulation of NF- κ B

To further understand the mechanisms of GA-Me in immune regulation, western blotting was performed to detect the level of NF- κ B in both the cytoplasm and the nucleus of mice spleen tissues. Fig. 5 indicated that administration of GA-Me enhanced the protein expression of NF- κ B in the nucleus. These observations suggested that the signal transduction of NF- κ B pathway was involved in mediating immune regulation *in vivo*.

4. Discussion

In recent years, increasing the human body's immunity in defending against tumors has been very popular [11,12]. A major challenge to tumor therapy is to find novel chemical entities with less toxicity and greater effectiveness, especially to explore the immune-enhancing effect of possible dietary substances. Owing to the well-known anti-tumor properties of ganoderic acids, investigations on these chemicals are of current interest. Historical data has shown that natural mixtures of ganoderic acids demonstrated an inhibitive effect on proliferation of human and mouse carcinoma cell lines [3,13]. However, the prevention of tumor growth is through diverse mechanisms, including tumor cell apoptosis and immune-mediated cancer regression. To elucidate the anti-tumor and immunological effects of ganoderic acids, GA-Me treatment was conducted in a Lewis lung carcinoma mice model. The present results

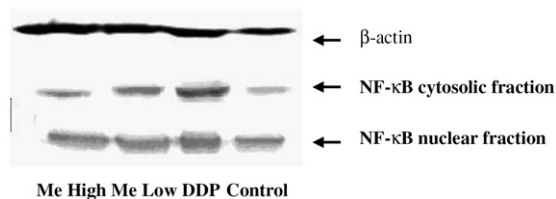


Fig. 5. Effects of GA-Me on protein expression of NF- κ B *in vivo*.

showed that GA-Me inhibited the tumor growth and lung metastasis *in vivo* and increased the NK cells activity ($p < 0.05$). The administration of GA-Me enhanced the expression of T-helper type 1 (Th1) cytokines, including IL-2 and IFN- γ ($p < 0.01$), and augmented the protein expression of NF- κ B. To our knowledge, the anti-tumor and anti-metastatic ability of GA-Me was proved *in vivo* for the first time.

Cytokines play critical roles in regulating the outcome of antigen-specific T-cell responses, and thus have been a major focus in the study of the pathogenesis of autoimmunity. When a T-cell responds to an antigen, its cytokine profile indicates certain T-helper cell pathway stimulated by the antigen-presenting cell. Th1 responses are identified primarily by the presence of IL-2, IFN- γ and IL-12. Many investigations have given evidence that IL-2 and IFN- γ play an important role in specific immunological reactions to tumor cells growth and promoting innate and adaptive immune responses [14]. Therefore, this study investigated the synthesis of IL-2 and IFN- γ to test the hypothesis that GA-Me increases T lymphocyte activation by modulating cytokines IL-2 and IFN- γ . In this study, as shown in Fig. 3, the serum concentrations of IL-2 and IFN- γ were enhanced in C57BL/6 mice bearing Lewis lung carcinoma, which suggested that GA-Me could be a potent inducer of Th1-type cytokines.

NK cells participate in the nonspecific anti-virus and anti-tumor defense. Several reports have indicated that NK cells contributed to the elimination of transformed tumor cells and exhibited cytotoxic activity against cancer cells [15]. NK cells also play a supportive role in the induction in Ag-specific type 1 immunity. The currently demonstrated activity of NK cells may support the development of Th1 dominated type 1 immunity and may have implications for cancer immunotherapy [16,17]. Herein, the results implied that GA-Me increased NK cells activity ($p < 0.05$). This adds to a better understanding of the effectiveness of GA-Me against cancer through increasing immune responses. Cytokines regulate the innate immune system and increase NK cells activity. NK cells also regulate the adaptive immune system and responses to produce cytokines. This result was consistent with other reported data, which proved the abilities of NK cells to respond to IL-2 and IFN- γ [18,19]. Thus, it is proposed that GA-Me elicits its anti-tumor effect by promoting a Th1-dominant state and killer activities.

IL-2 is an autocrine growth factor from T lymphocyte and the transcription of IL-2 is an important step in T-cells activation. To further understand the mechanisms of IL-2 enhancement, measurement of the protein level

of NF- κ B in spleen tissues by western blotting assay was then conducted. In this work, it was proved that NF- κ B was up regulated in Lewis lung carcinoma bearing mice after the treatment of GA-Me. It has been well established that NF- κ B participates in the regulation of many aspects of innate and adaptive immunity. NF- κ B transcription factors are activated by a surprising variety of signaling pathways involved in immune function and development [20]. Our results provided evidence that NF- κ B may act as a regulatory step in the cytokines cascade. This suggested that the biochemical events induced by GA-Me were possibly associated with NF- κ B. Activation of NF- κ B may bind to the κ B regulating element, which stimulated the transcription of downstream genes. However, the activating mechanism of NF- κ B *in vivo* is not clear and the actual target of NF- κ B in the transcription of IL-2 is also unknown, which require further studies.

In conclusion, results from the present study demonstrated that GA-Me elicits pronounced immuno-stimulating activities, leading to the significant anti-tumor effect in C57BL/6 mice bearing Lewis lung carcinoma. As it has not yet been completely verified, further work needs to be done to investigate the signal pathway involved in T lymphocyte activation induced by GA-Me. The results suggest that GA-Me is a promising candidate for cancer immunotherapeutic agent and a potentially valuable substance for pharmacological uses.

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