



# *Ganoderma lucidum* spore oil synergistically enhances the function of cyclophosphamide in the prevention of breast cancer metastasis

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## Abstract

**Background:** *Ganoderma lucidum* (*G. lucidum*) is a traditional Chinese herbal medicine that has shown potential as an alternative adjuvant therapy for cancer patients. However, the mechanisms and adjuvant therapeutic effects of *G. lucidum* in cancer treatment remain unclear.

**Methods:** In this work, *G. lucidum* spore oil (GanoOil), a newly developed oily *G. lucidum* spore extract was used to investigate the mechanisms and adjuvant therapeutic effects of GanoOil in conjunction with the chemotherapeutic drug cyclophosphamide (CTX) for preventing breast cancer metastasis.

**Results:** In the model of lung metastasis, orally administered GanoOil increased the population of CD8<sup>+</sup> T cells and interleukin (IL)-6 cytokine levels in mouse blood, whereas also enhancing the activity of natural killer cells in the spleen. Furthermore, the combination of GanoOil and CTX effectively suppressed the lung metastasis of circulating breast cancer cells, alleviated CTX-induced weight loss, and reduced the ratio of lung and spleen weight to body weight in mice. Moreover, high concentrations of GanoOil exhibited no significant toxicity or side effects in both in vitro and in vivo experiments.

**Conclusion:** In conclusion, GanoOil is a safe drug that can enhance immune activity in mice to achieve therapeutic effects on cancer, and can also synergistically inhibit tumor metastasis with CTX.

**Keywords:** Cyclophosphamide; IL-6; Natural killer cells; Spleen; Spore

## 1. INTRODUCTION

By 2022, it is estimated that there will be 287 850 new cases of breast cancer and 61 350 cancer-related deaths attributed to breast cancer, making it the cancer with the highest incidence rate and the second-highest mortality rate.<sup>1</sup> Distant metastases account for most cancer-related deaths in patients with cancer.<sup>2</sup> Breast cancer exhibits a distinct metastatic pattern, commonly involving the bones, liver, lungs, and brain.<sup>3</sup> The metastasis of cancers, including breast cancer, is a serious problem in clinical tumor therapy. The traditional treatments for breast cancer and its metastases include surgery, chemotherapy, and radiotherapy. These treatments are associated with many adverse effects and

have no effective preventive effect on tumor metastasis. As a result, there is an urgent need to identify a safe and effective strategy for the prevention of tumor metastasis.

*Ganoderma lucidum* (*G. lucidum*) *Leys. ex Fr.* Karst, a mushroom known as Ling-zhi in China and Reish in Japan, is a traditional Chinese medicine that is widely used as a dietary supplement or over-the-counter product for health promotion in Asia.<sup>4-6</sup> More than 600 active compounds, including amino acids, polysaccharides, fatty acids, triterpenoids, and trace elements, have been isolated from *G. lucidum* fruiting bodies, mycelia, and spores.<sup>7</sup> Many components of *G. lucidum* have significant anti-obesity, antiaging, anti-inflammatory, anticancer, and immunomodulatory activities.<sup>8,9</sup> The main components isolated from *G. lucidum* fruiting bodies or spores are polysaccharides, triterpenoids, unsaturated fatty acids, and other components that play vital roles in cancers.<sup>10-13</sup> According to Xu et al,<sup>11</sup> extraction of *G. lucidum* fruiting body using high-pressure supercritical CO<sub>2</sub> could prevent hepatoma by inhibiting the Ras/Raf/MEK/ERK signaling pathway.<sup>14</sup> *G. lucidum* spore (GLS) oil (GanoOil) is extracted from GLS powder via CO<sub>2</sub> supercritical extraction. GanoOil has been identified as an immunomodulatory product that mainly contains lipid components, such as ergosterol, triglycerides, fatty acids, and triterpenes.<sup>15</sup> Notably, GanoOil has been widely studied for its role in the prevention of tumor.<sup>15,16</sup> Previous study revealed that GLS oil prevented murine sarcoma S180 and murine hepatoma H22 tumor growth,

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ultimately inducing G1 cell cycle arrest.<sup>16,17</sup> In addition to the anticancer function of *G. lucidum*, its immune-enhancing effects have garnered increasing attention from researchers worldwide.<sup>15,18</sup> *G. lucidum* was found to significantly enhance natural killer (NK) cell activity in patients with advanced cancer.<sup>18</sup> The sporoderm-breaking spore power of *G. lucidum* prevented 4T1 cell growth in a breast cancer xenograft mouse model and significantly increased the cytotoxic T cell (Tc) population and ratio of Tc in the peripheral blood of mice. According to a comprehensive microbiome and metabolomic analysis, GLS oil enhances the phagocytosis of macrophages and cytotoxicity of NK cells in normal mice.<sup>15</sup> *G. lucidum* polysaccharides were also found to significantly inhibit tumor growth and increase the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> Tcs in a mouse Lewis lung cancer (LLC) model.<sup>19</sup> Our previous research indicated that *G. lucidum* extract promoted tumor cell pyroptosis and inhibited metastasis in breast cancer.<sup>20</sup>

Although *G. lucidum* and its extracts have many advantages, the mechanism of action of GanoOil as an adjuvant therapy for cancer and its effects on the immune system remain unclear. In this study, we revealed the negligible cytotoxicity of GanoOil in vitro and in vivo, and proved the immune promoting function of GanoOil. Moreover, the combination of GanoOil and cyclophosphamide (CTX) effectively suppressed the lung metastasis of circulating breast cancer cells.

## 2. METHOD

### 2.1. Preparation and analysis of GanoOil

*G. lucidum* was obtained from Fujian Xianzhilou Biotechnology Co. Ltd. The GLS powder was collected during the ripening period of *G. lucidum*, and dried at 65°C overnight. The sporoderm-broken GLS powder was extracted using supercritical CO<sub>2</sub> carbon dioxide extraction (SFE24L × 3) to produce GanoOil. The following conditions were used: fixed feeding amount, 100g; CO<sub>2</sub> flow rate, 20L/h; and pressure and temperature of the separation kettle II and storage tank, 4MPa and 36°C, respectively, for 2 hours.

### 2.2. High performance liquid chromatography analysis of GanoOil

The standard ergosterol (CAS:57-87-4) was purchased from Sigma and examined using high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) (Waters, Milford, CT, and Alltech 2000ES, Chicago, Illinois, USA). HPLC analyses of the ergosterol reference substance and GanoOil were performed using an ODS-2 HYPERSIL column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of trichloromethane (Merco, Burlington, MA, USA) and methanol (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) (volume ratio at 1:1). The flow rate was set to 1 mL/min. The injection volume was 10 μL and column temperature was 35°C.

The triglyceride composition of GanoOil was determined using HPLC-ELSD. Trilinolein (CAS:33-1820), 1,2-linolein-3-oleic (CAS:34-1866), 1,2-linolein-3-palmitin (CAS:34-1862), 1,2-olein-3-linolein (CAS:34-1827), 1-palmitin-2-olein-3-linolein (CAS:34-3012), triolein (CAS:34-1810), 1,2-olein-3-palmitin (CAS:34-1821), and 1,2-olein-3-stearin (CAS:34-1822) were purchased from Larodan Fine Chemicals AB company (Shanghai, China). These eight components were mixed and their concentrations were 28.70, 146.8, 50.75, 279.6, 242.1, 613.2, 341.6, and 69.15 μg/mL, respectively, based on HPLC-ELSD. HPLC analyses of GLS oil triglycerides were performed using a Waters ACCGROM Unitary-C<sub>18</sub> column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of acetonitrile (Merco, USA) and isopropanol (Sinopharm Chemical Reagent Co. Ltd., Shanghai,

China) (volume ratio at 51:49). The flow rate was set to 1.0 mL/min. The injection volume was 20 μL and column temperature was 30°C.

### 2.3. Determination of total triterpenoids in GanoOil

The reference substance of ursolic acid was obtained from the China Institute for Food and Drug Control (CAS, 77-52-1). The total triterpene content in GanoOil was measured by using ultraviolet-visible light spectrophotometry, with ursolic acid as the calculated reference. The sample was dissolved in ethyl acetate and subjected to drying using a water bath at 100°C. Subsequently, the sample was treated with a solution containing 5% vanillin-glacial acetic acid and perchloric acid, followed by heating in a water bath at 60°C for 45 minutes. The total triterpene content of the sample was then determined using a spectrophotometer after adding glacial acetic acid. Preparation of the reference solution and generation of the standard curve involved the following steps: A quantity of 10 mg of ursolic acid was weighed and placed in a 100 mL volumetric flask. It was then dissolved in ethyl acetate, subjected to ultrasonication for 30 minutes, and prepared as a reference solution with a concentration of 0.1 mg/mL. The standard concentrations were set as follows: 0, 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mg/mL. The standard curve of concentration and absorbance was generated based on the aforementioned procedure. A GanoOil solution with a concentration of 1.0 mg/mL was used to determine the total triterpene content. The total triterpenes in GanoOil were determined and calculated by following the aforementioned procedure and utilizing the standard curve of ursolic acid.

### 2.4. Cell culture

The MCF-7 breast cancer, HT-29 colon cancer, 4T1 mouse breast cancer, LO2 normal human liver, and YAC-1 mouse lymphoma cell lines were procured from the Cell Bank of the Chinese Academy of Sciences in Shanghai, China. RPMI-1640 medium (Roswell Park Memorial Institute) supplemented with 10% foetal bovine serum (FBS) (Gibco, Carlsbad, CA) and 1% (v/v) penicillin/streptomycin (Gibco) was used for culturing the MCF-7, 4T1, LO2, and YAC-1 cells. McCoy's 5A medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin was used for culturing HT-29 cells. The cells were incubated at 37°C with 5% CO<sub>2</sub> and harvested using 0.25% trypsin (GenView) before usage.

### 2.5. Cell viability assay

The toxic effect of GanoOil on MCF-7, HT-29, LO2, and 4T1 cells was assessed using CCK-8 (Cell Counting Kit-8). Tumor cells were harvested, seeded at a density of 1 × 10<sup>4</sup> cells/well in 96-well plates, and incubated for 24 hours. One gram per milliliter solution of GanoOil was prepared by dissolving it in ethyl acetate (EtOAc) (Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China, CAS:141-78-6), and then further diluted to a required concentration using the same medium before usage. EtOAc was utilized as the solvent for GanoOil and served as the control group. Cells were exposed to varying concentrations of GanoOil (0, 10, 25, 50, 100, 150, 200, 500, 1000 μg/mL). After 24 hours, 10 μL of CCK-8 reagent was added to each well. The absorbance of each well was measured at 450 nm using a microplate reader (Tecon, Männedorf, Switzerland) after 1 hour.

### 2.6. Safety study of GanoOil

Six-week-old female BALB/c mice were randomly divided into two groups, each consisting of six mice (n = 6). The GanoOil group received daily oral administration of GanoOil at a dose of 0.5 g/kg, whereas the control group was treated with an equal amount of physiological saline. Mouse weights were recorded every alternate

day. After 28 days, the mice were euthanized, and their heart, liver, spleen, lung, and kidney were collected and subjected to hematoxylin and eosin (H&E) staining to assess the potential organ toxicity of GanoOil. Additionally, blood samples were collected and processed to measure the monocyte levels. Briefly, 500  $\mu$ L of blood samples were collected from the mice and anticoagulated with 100  $\mu$ L of 10<sup>6</sup> mM sodium citrate in a test tube. Monocyte levels were measured using a fully automated hematology analyzer (HF-3800, Han Fang Medical Instrument Co., Ltd., Jinan, China). The levels of white blood cells (WBC), lymphocytes (LYM), granulocytes (GRA), red blood cells (RBC), hemoglobin (HGB), and platelets (PLT) in the blood samples were assessed. Subsequently, the remaining blood samples were mixed with erythrocyte lysate at a ratio of 10:1 and incubated at 37°C for 10 minutes. The blood solution was centrifuged at 400  $\times$  g for 10 minutes, and the resulting cell pellet was collected and resuspended in 100  $\mu$ L of 1% FBS-PBS. The cell concentration was adjusted to 2 $\times$ 10<sup>6</sup>/mL, and 5  $\mu$ L of Per-CP-Cy5.5-CD8 and PE-CD4 Tc antibodies (Multisciences Biotech, CO., Ltd., Hangzhou, China) were added to each 100  $\mu$ L of the sample. Subsequently, the cells were incubated at 4°C for 30 minutes, and the proportion of CD8<sup>+</sup> and CD4<sup>+</sup> Tcs was determined using flow cytometry (BD FACS AriaIII, BD Biosciences, San Jose, CA, USA).

The function of immune cells and immune organs in mice was measured. The spleen of mice from different groups was collected aseptically, placed in a small plate containing an appropriate amount of sterile Hank's solution, and gently ground to obtain a single spleen cell suspension. The cells were filtered through 200 mesh screens, washed twice with Hank's solution, and centrifuged twice for 10 minutes (1000 r/min). The cells were resuspended in RPMI-1640 culture medium containing 10% FBS to obtain the splenic cell suspension. After that, 100  $\mu$ L of the spleen cell suspension (1 $\times$ 10<sup>7</sup> cells/mL) from different groups was added to 96-well plates. Each treatment was dispensed in three parallel wells, and 75  $\mu$ L Concanavalin A (Con A) solution (equivalent to 7.5  $\mu$ g/mL Con A) was added to each well to verify its effect on LYM-stimulated proliferation. In addition, control wells were set in each group without adding Con A. The culture plate was incubated at 37°C with 5% CO<sub>2</sub> for 72 hours. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Methyl Thiazolyl Tetrazolium (MTT) assay reagent was added to each well. After 4 hours, splenocyte proliferation was measured using a microplate reader. LYM proliferation was calculated as follows: Splenic LYM proliferation ratio (OD) =  $\frac{OD_{Con\ A\ pour} - OD_{control\ pour}}{OD_{Maximum\ release\ pore} - OD_{Natural\ release\ pore}}$

NK cell activity measurement: the ratio of the effector cells (BALB/c mouse splenocytes, 1 $\times$ 10<sup>6</sup> cells/well) to target cells (YAC-1) was 100:1 or 50:1, and these mixtures were added to 96-well plates. For comparison, the natural release well only contained YAC-1 cells, whereas the maximum release well contained both YAC-1 cells and 2.5% Triton. After 24 hours of culture, NK cell activity was detected using the MTT assay and expressed as the killing rate (%). NK cell activity was calculated as follows:

$$NK\ killing\ rate\ (\%) = \frac{OD_{Reaction\ pore} - OD_{Natural\ release\ pore}}{OD_{Maximum\ release\ pore} - OD_{Natural\ release\ pore}} \times 100\% \quad (1)$$

## 2.7. Mouse lung metastasis model and mouse tissue treatment

All animal experiments were conducted following the approved animal protocol procedures by the Institutional Animal Care and Use Committee (IACUC) of Minjiang University (20210519R). Six-week-old healthy female BALB/c mice were divided into four groups, each consisting of six mice (n = 6), based on the intended treatments as shown in Table 1. Each mouse was orally administered 100  $\mu$ L (60 mg/mL) of GanoOil daily. The 4T1 tumor cells were i.v. injected into mouse 3 days after the administration of GanoOil, and their weights were measured every alternate day. Each mouse in the CTX-treated group received 100  $\mu$ L of the CTX solution (dissolved in physiological saline, 10 mg/mL, equivalent to 1 mg) via intraperitoneal injection, which was administered three times in total. Following 21 days of treatment, the mice were euthanized by cervical dislocation under isoflurane anesthesia (3%). The weights of the lungs and spleens were measured to calculate the lung-to-body weight (L/W) ratio and spleen-to-body weight (S/W) ratio. The lung tissues were stained with Brinell's solution for 24 hours and then photographed. Metastasis was assessed quantitatively by counting the number of tumor nodules on the lung surface using a dissecting microscope. The heart, liver, spleen, lungs, and kidney tissues from each mouse were harvested, and stained with H&E. Blood samples were collected from the mice to investigate the impact of GanoOil or CTX on immune cell function and cytokine levels, following the previously described methodology.

The blood samples were centrifuged at 300  $\times$  g for 15 minutes. The supernatant was collected and the expression levels of IL-6 (Kit No. RK00008) and tumor necrosis factor (TNF)- $\alpha$  (Kit No. RK000027) were determined using an ELISA kit (ABclonal Technology Co., Ltd., Wuhan, China). The standard curves of IL-6 and TNF- $\alpha$  were performed according to the same method.

## 2.8. Statistical analysis

Statistical differences were analyzed using Statistical Product Service Solutions (SPSS) based on one-way analysis of variance (ANOVA) (for comparisons among three groups and more than three samples per group) or unpaired Student's *t* tests (for comparisons between two groups and more than three samples per group). A value of *p* < 0.05 was considered statistically significant. Significant differences in all results were marked as <sup>ns</sup>*p* > 0.05, \**p* < 0.05, \*\**p* < 0.01, or #*p* < 0.05.

## 3. RESULTS

### 3.1. Characterization of GanoOil

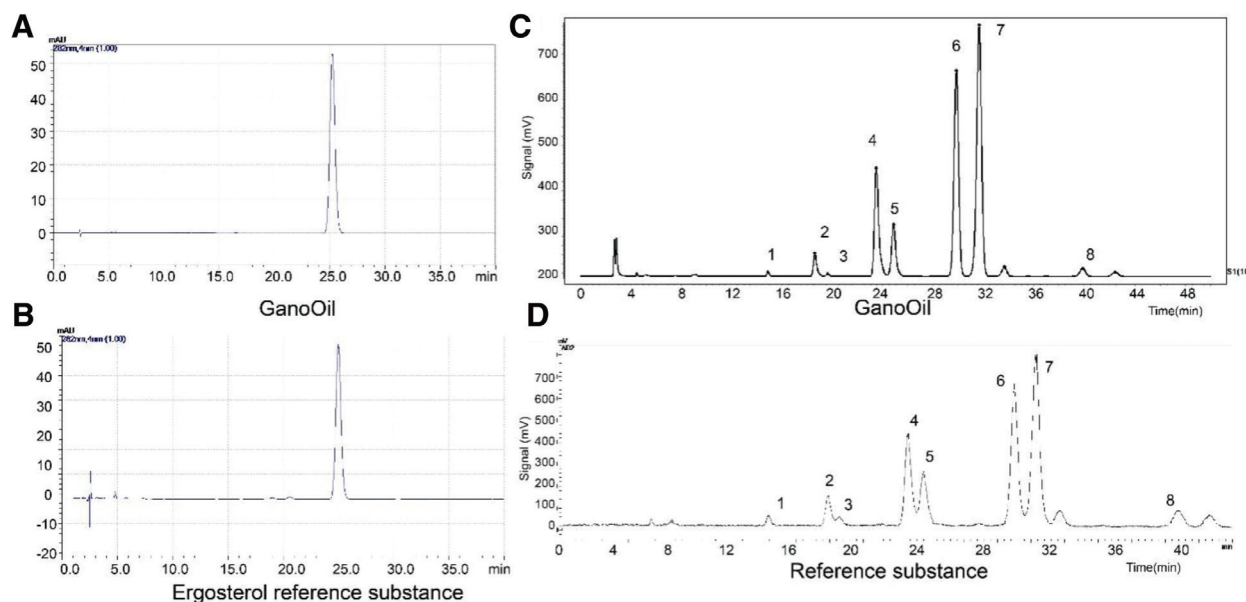
GanoOil contains a variety of chemical components, including triglycerides, triterpenoids, and sterols. The concentration of ergosterol in GanoOil was determined using HPLC-ELSD, yielding a concentration of 231.6 $\pm$ 1.9 mg/100g (refer to HPLC chromatogram in Fig. 1A, B and content data in Table S1, <http://links.lww.com/JCMA/A229>). Ultraviolet-visible spectrophotometry (UV-Vis) was used to measure the total triterpenoid content in GanoOil, using

**Table 1**

**Administration and treatment methods of four groups of mice**

Administration treatment in mice	Control group (group C)	4T1 group (4T1)	GanoOil group (GanoOil)	CTX group (CTX)	GanoOil assisted CTX group (CTX + GanoOil)
4T1 cell injection	—	5 $\times$ 10 <sup>4</sup>	5 $\times$ 10 <sup>4</sup>	5 $\times$ 10 <sup>4</sup>	5 $\times$ 10 <sup>4</sup>
CTX intraperitoneal administration, g/kg (3 times in total)	—	—	—	0.05	0.05
Oral GanoOil, g/kg (everyday)	—	—	0.3	—	0.3

CTX = cyclophosphamide.



**Fig. 1** Components analysis of GanoOil based on the HPLC chromatograms. A, Sample of GanoOil. B, Ergosterol reference substance. C, Sample of GanoOil. D, Reference substance. (1) trilinolein, (2) 1,2-linolein-3-oleic, (3) 1,2-linolein-3-palmitin, (4) 1,2-olein-3-linolein, (5) 1-palmitin-2-olein-3-linolein, (6) triolein, (7) 1,2-olein-3-palmitin, (8) 1,2-olein-3-stearin. HPLC = high performance liquid chromatography.

ursolic acid as the reference substance. GanoOil exhibited a total triterpenoid content of  $34.45\% \pm 0.51\%$  (refer to Table S2, <http://links.lww.com/JCMA/A229>). Importantly, GanoOil exhibited a high content of triglycerides, including trilinolein, 1,2-linolein-3-oleic acid, 1,2-linolein-3-palmitin, 1,2-linolein-3-linolein, 1-palmitin-2-olein-3-linolein, triolein, 1,2-linolein-3-palmitin, and 1,2-linolein-3-stearin, as determined by HPLC analysis (refer to Fig. 1C, D).

### 3.2. Safety of GanoOil in vitro and in vivo

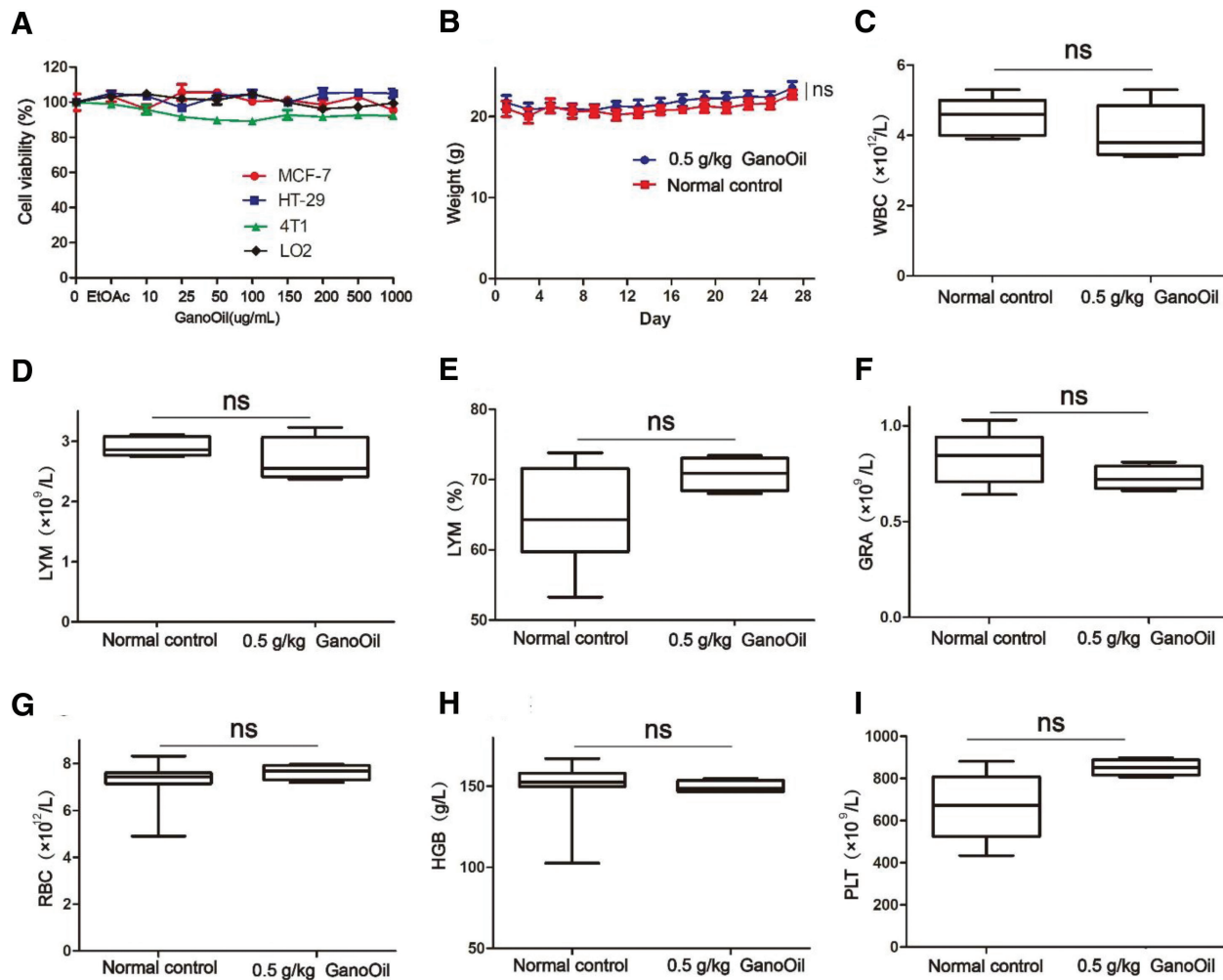
Following the determination of GanoOil's composition, its cytotoxic effects on human MCF-7, HT-29, LO2, and mouse 4T1 cells were assessed using a CCK-8 assay kit. The CCK-8 results indicated that GanoOil showed no cytotoxic effects on MCF-7, HT-29, LO2, and 4T1 cells even at a high concentration of 1000  $\mu\text{g/mL}$ . Moreover, the viability of MCF-7, HT-29, LO2, and 4T1 cells remained around 90% (Fig. 2A), and no significant differences were observed among the treatment groups. These findings indicate that GanoOil is nontoxic to normal cells in vitro and does not exert direct cytotoxic effects on tumor cells. Subsequently, the safety of GanoOil in BALB/c mice was investigated, and various parameters were measured, including WBC count, LYM count and percentage, GRA count, RBC count, HGB concentration, and PLT count. BALB/c mice were treated with GanoOil (0.5 g/kg) once daily for 28 days, and their weights were recorded every other day. Fig. 2B demonstrates that there was no significant difference in mouse weight between the control group and the group treated with 0.5 g/kg GanoOil ( $^{ns}p > 0.05$ ). Moreover, no significant differences were observed in WBC count, LYM count and percentage, GRA count, RBC count, HGB concentration, and PLT count between the control group and the GanoOil treatment group ( $^{ns}p > 0.05$ , Fig. 2C–I). It is worth noting that, although not significantly different among the groups, the percentage of LYM showed a slight increase in the group treated with 0.5 g/kg GanoOil (Fig. 2E). Heart, liver, spleen, lung, and kidney tissues were collected from the mice for H&E staining, which indicated no adverse effects on these organs following daily administration of 0.5 g/kg GanoOil (Fig. S1, <http://links.lww.com/JCMA/A229>). These findings demonstrate the safety of GanoOil in BALB/c mice, even at high doses.

### 3.3. GanoOil promotes immune activity in vivo

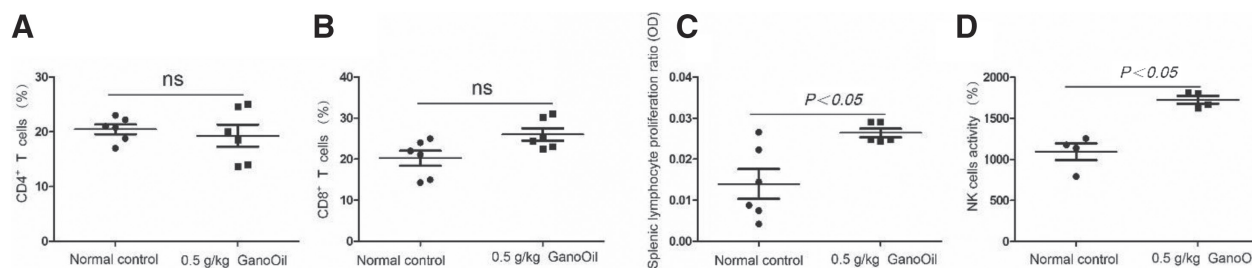
Following the assessment of GanoOil's toxicity in both in vivo and in vitro settings, we investigated its impact on the immune cells and organs of BALB/c mice. In brief, flow cytometry was used to evaluate the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> Tcs in blood samples. The results indicated no significant difference in the percentage of CD4<sup>+</sup> Tcs between the control group and the group treated with 0.5 g/kg GanoOil. However, a slight increase in CD8<sup>+</sup> Tcs was observed in the 0.5 g/kg GanoOil-treated group (Fig. 3A, B). Furthermore, the 0.5 g/kg GanoOil-treated group exhibited significantly higher proliferation of splenic LYM and activity of NK cells compared to the control group (Fig. 3C, D).

### 3.4. GanoOil synergizes with CTX to prevent tumor metastasis

CTX remains one of the most potent antitumor drugs; however, it can induce leukopenia and thrombocytopenia, particularly neutropenia. Our objective was to investigate whether GanoOil could enhance CTX's ability to prevent tumor metastasis. In brief, the mice were randomly divided into five groups, and the administration and treatment protocols are detailed in Table 1. The mouse lungs were collected and photographed, as depicted in Fig. 4A, where the red boxes indicate lung metastases. The CTX + GanoOil-treated group exhibited a significant reduction in the number of lung metastases compared to the control, GanoOil-treated, and CTX-treated groups. Statistical analysis (Fig. 4C) demonstrated a significant difference in the number of lung metastatic nodules between the CTX-treated group and the CTX + GanoOil-treated group ( $p < 0.05$ ). H&E staining (Fig. 4B) revealed that GanoOil can significantly aid CTX in reducing the number and size of metastatic nodules. H&E staining (Fig. S2, <http://links.lww.com/JCMA/A229>) further demonstrated the absence of evident tumor metastasis in the heart, liver, spleen, or kidney in all three groups. Notably, the administration of GanoOil alone resulted in a slight prevention of tumor metastasis to the lungs. Furthermore, GanoOil effectively



**Fig. 2** In vitro and in vivo safety of GanoOil. A, Viability of MCF-7, HT-29, LO2, and 4T1 cells after incubation with GanoOil (0, 10, 25, 50, 100, 150, 200, 500, and 1000 µg/mL) for 24 h. ( $^{ns}p > 0.05$ ,  $n = 6$ .) EtOAc was used as the GanoOil solvent and served as the control. B, Weight changes of mice in two treatment groups over 28 d. C-I, Numbers of WBC, LYM, LYM (percentage), GRA, RBC, HGB, and PLT in blood samples from two treatment groups ( $^{ns}p > 0.05$ ,  $n = 6$ ). C group, mouse blank control; 0.5 g/kg GanoOil group, mice were administered 0.5 g/kg GanoOil daily. EtOAc = ethyl acetate; GRA = granulocyte; HGB = hemoglobin; LYM = lymphocyte; ns = no significant; PLT = platelet; RBC = red blood cell; WBC = white blood cell.

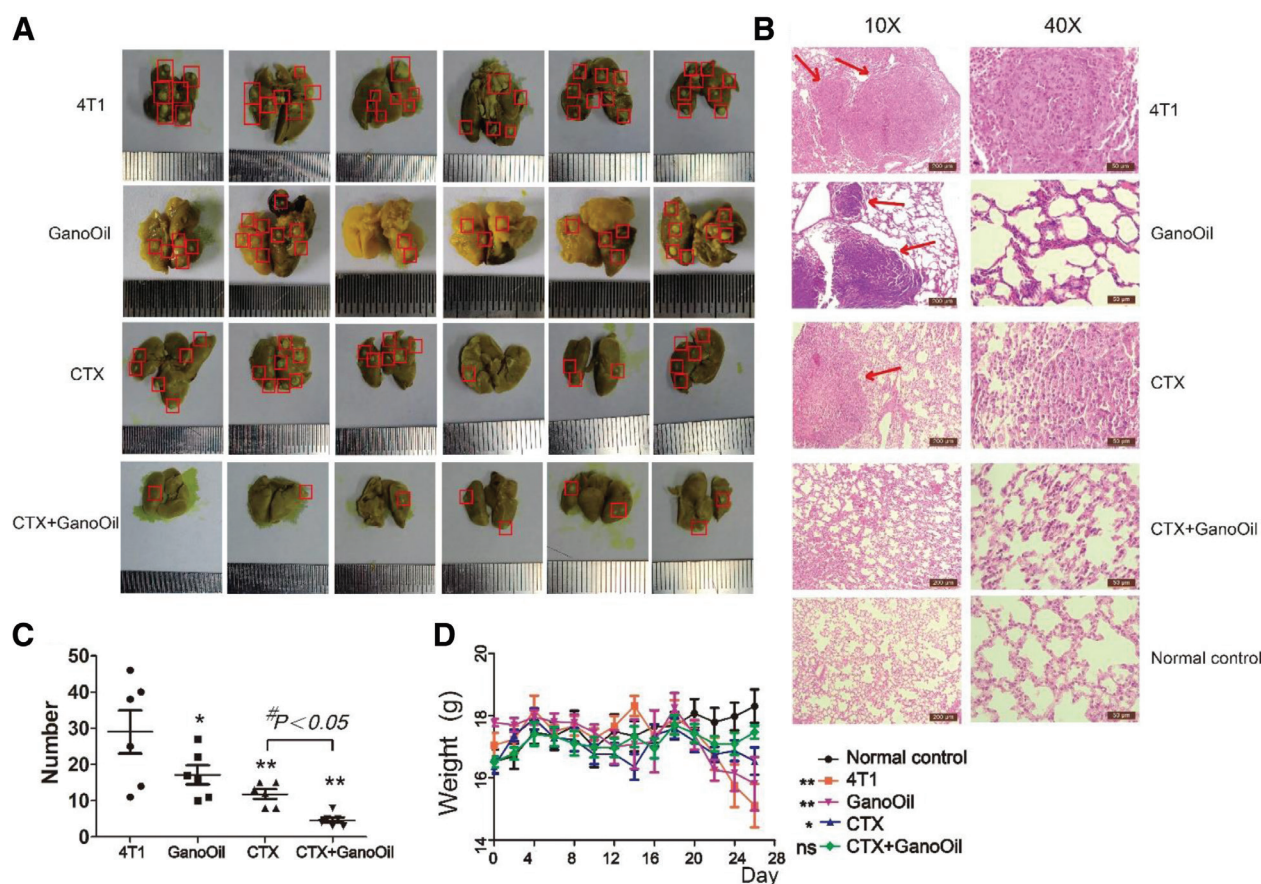


**Fig. 3** GanoOil promotes mouse splenic lymphocyte proliferation and NK cell activity in vivo. A, Percentage of CD4<sup>+</sup> T cells in lymphocytes. B, Percentage of CD8<sup>+</sup> T cells in lymphocytes. C, Proliferation of mouse splenic lymphocytes. D, NK cell activity in mouse spleen. ( $^{ns}p > 0.05$ ,  $^{*}p < 0.05$ ,  $n = 6$ ). C group, mouse blank control; 0.5 g/kg GanoOil group, mice were administered 0.5 g/kg GanoOil daily. NK = natural killer; ns = no significant.

mitigated CTX-induced weight loss in mice ( $^{*}p < 0.05$  vs  $^{ns}p > 0.05$ ) (Fig. 4D).

**3.5. GanoOil can assist CTX with the reduction of the L/W and S/W ratio and the increase in the HGB content of mice**  
Lung cancer metastasis is associated with an elevation in the ratio of lung/spleen weight to body weight.<sup>21,22</sup> Treatment with GanoOil effectively mitigated the reduction in the L/W ratio and

S/W ratio induced by CTX ( $^{ns}p > 0.05$ ,  $^{*}p < 0.05$ , see Fig. 5A, B). The L/W and S/W ratios in the CTX + GanoOil-treated group approached those of the control group ( $^{ns}p > 0.05$ ), whereas a statistically significant difference was observed between the CTX-treated group and the control group ( $^{*}p < 0.05$ ). Various indicators in the mice's blood were analyzed using a hematology analyzer. GanoOil effectively mitigated the decrease in HGB content induced by CTX ( $^{ns}p > 0.05$ ,  $^{*}p < 0.05$ , see Fig. 5G).



**Fig. 4** GanoOil assisted CTX with the reduction of lung cancer metastasis. A, Images of the lung tissue in the four treatment groups; red box indicates obvious lung metastasis. B, H&E-stained lung tissue sections from the five treatment groups (amplification  $\times 10$  [the left] and amplification  $\times 40$  [the right]); arrows indicate obvious metastasis in the lung. C, Number of pulmonary metastatic nodules in the four treatment groups. D, Body weight of mice in the five groups. ( $^{ns}p > 0.05$ ,  $^{*}p < 0.05$  vs control group,  $^{**}p < 0.01$  vs control group,  $^{\#}p < 0.05$  vs CTX-treated group,  $n = 6$ .) Normal control group, blank control; 4T1 group, mice administered 4T1 cells ( $10^5$ /each) alone (ie, no CTX or GanoOil); GanoOil group, mice administered 4T1 cells ( $10^5$ /each) and 0.3 g/kg GanoOil, but no CTX; CTX group, mice administered 4T1 cells ( $10^5$ /each) and 0.05 g/kg CTX, but no GanoOil; and CTX + GanoOil group, mice administered 4T1 cells ( $10^5$ /each), 0.05 g/kg CTX, and 0.3 g/kg GanoOil. CTX = cyclophosphamide; H&E = hematoxylin and eosin; ns = no significant.

However, the groups treated with CTX and CTX + GanoOil exhibited no statistically significant differences in WBC count, GRA count, LYM count, LYM percentage, PLT count, or RBC count ( $^{ns}p > 0.05$ , see Fig. 5C–F and Fig. 5H, I).

### 3.6. GanoOil promotes NK cell activity and stimulates cytokine IL-6 to assist CTX with the prevention of tumor metastasis

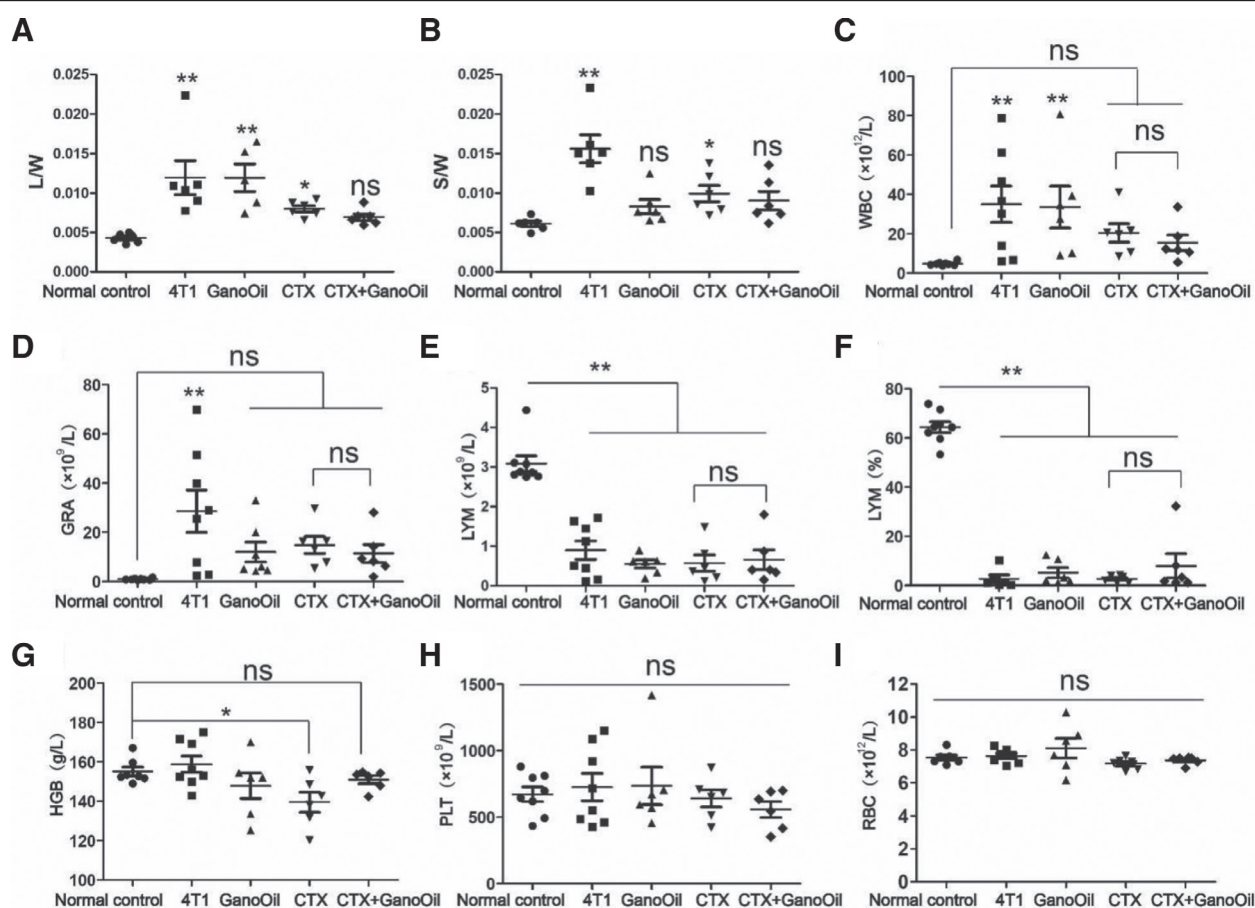
The percentage of CD4<sup>+</sup> Tcs did not differ significantly between the CTX-treated and CTX + GanoOil-treated groups (Fig. 6A). In contrast, compared to the untreated 4T1 group, the CTX + GanoOil-treated group exhibited a greater increase in the proportion of CD8<sup>+</sup> Tcs than the CTX-treated group ( $^{**}p < 0.01$  vs  $^{*}p < 0.05$ ) (Fig. 6B). In addition, we assessed the proliferation capacity of splenic LYM, NK cell activity, and the expression of the cytokines IL-6 and TNF- $\alpha$ , with the standard curves for IL-6 and TNF- $\alpha$  presented in Figure S3, <http://links.lww.com/JCMA/A229>, within the treatment groups. Fig. 6C reveals no significant difference in splenic LYM proliferation between the CTX-treated and CTX + GanoOil-treated groups. In contrast, the CTX + GanoOil-treated group exhibited significantly higher NK cell activity and IL-6 content compared to the CTX-treated group ( $^{\#}p < 0.05$ , see Fig. 5D–F). Therefore, GanoOil might aid

in the prevention of tumor metastasis to the lung by augmenting CD8<sup>+</sup> Tcs and NK cell activity, as well as promoting the secretion of IL-6, albeit not TNF- $\alpha$ .

## 4. DISCUSSION

Natural products have garnered significant attention in the field of cancer prevention and treatment. *G. lucidum* is among the extensively researched traditional Chinese medicines. Several studies have highlighted the significant role of natural medicinal compounds derived from *G. lucidum* in treating various human diseases.<sup>23–25</sup> The GLS powder obtained from mature *G. lucidum* plays a crucial role in preventing cancer cell invasion and metastasis.<sup>26</sup> This is achieved by modulating the immune system and inhibiting tumor angiogenesis.<sup>27,28</sup> GanoOil is derived from GLS powder through supercritical CO<sub>2</sub> extraction. GanoOil comprises active ingredients such as triglycerides, unsaturated fatty acids, triterpenes, and sterols. Jiao et al<sup>16</sup> investigated the mechanism of GanoOil in tumor prevention, however, the impact of GanoOil on chemotherapeutic drugs and its underlying tumor prevention mechanisms remain unclear.

Natural products are comprised of a variety of components. This study confirmed the abundance of ergosterol in GanoOil (Fig. 1A, B), which has been shown to possess anticancer properties.<sup>29</sup>

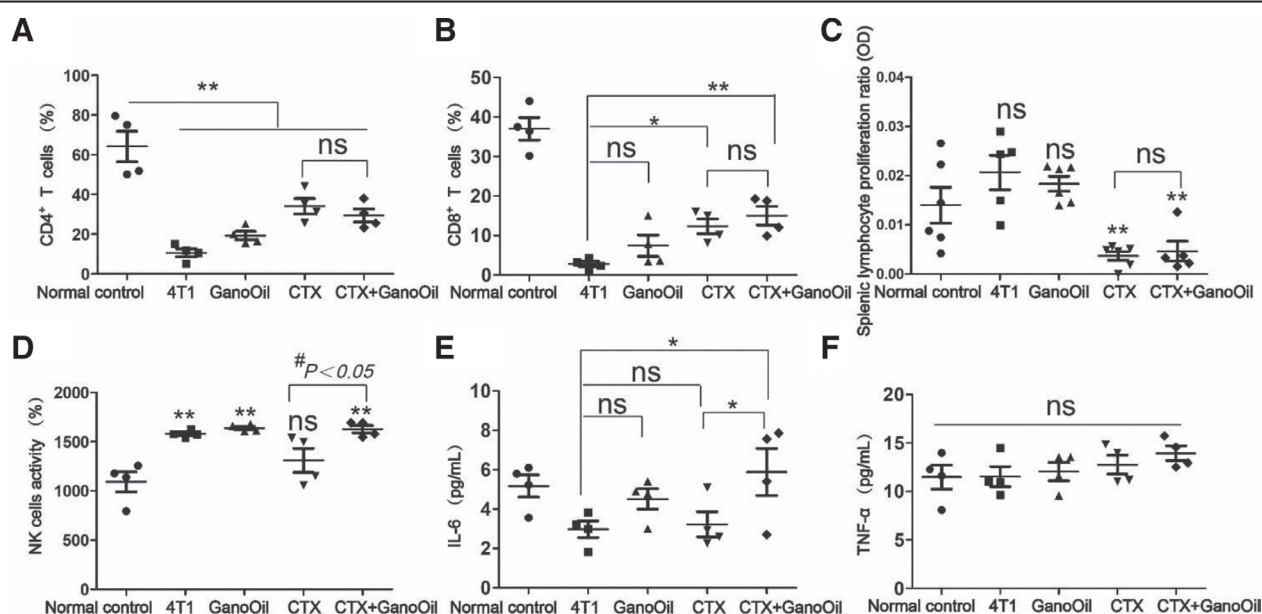


**Fig. 5** GanoOil reduced the proportion of L/W and S/W in mice and increased the content of HGB to assist CTX with the prevention of tumor metastasis to the lung. A-B, Lung weight/body total weight (L/W) and spleen weight/body total weight (S/W) of mice in the treatment groups. C-I, Number of WBC, LYM, LYM (percentage), HGB, PLT, and RBC in blood samples collected from the five groups. (<sup>ns</sup> $p > 0.05$ , <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ ,  $n = 6$ .) Normal control group, mouse blank control; 4T1 group, mice administered 4T1 cells ( $10^5$ /each) alone (ie, no CTX or GanoOil); GanoOil group, mice administered 4T1 cells ( $10^5$ /each) and 0.3g/kg GanoOil, but no CTX; CTX group, mice administered 4T1 cells ( $10^5$ /each) and 0.03g/kg CTX, but no GanoOil; and CTX + GanoOil group, mice administered 4T1 cells ( $10^5$ /each), 0.03g/kg CTX, and 0.3g/kg GanoOil. CTX = cyclophosphamide; GRA = granulocyte; HGB = hemoglobin; L/W = lung-to-body weight; LYM = lymphocyte; ns = no significant; PLT = platelet; RBC = red blood cell; S/W = spleen-to-body weight; WBC = white blood cell.

Furthermore, GanoOil contains a diverse array of triglycerides that have been documented to enhance growth performance, immune and antioxidant functions, and intestinal health in weaned rabbits.<sup>30</sup> Additionally, they have been found to possess pain-relieving properties through hypnotic and analgesic actions.<sup>31</sup> In vivo studies have demonstrated the absence of toxicity in GanoOil, including no impact on mouse body weight, tissues, and blood monocytes (Fig. 2 and Figure S1, <http://links.lww.com/JCMA/A229>). These findings confirm the safety of GanoOil, endorsing its broad utilization. CD8<sup>+</sup> Tcs, referred to as cytotoxic T LYM, are the primary immune cells used in cancer targeting. Additionally, CD4<sup>+</sup> Tcs, known as helper Tcs, aid in cellular immune killing alongside CD8<sup>+</sup> Tcs.<sup>32,33</sup> These findings indicate that GanoOil has the potential to elevate the proportion of CD8<sup>+</sup> Tcs in mouse blood samples and enhance NK cell activity. Given the significant roles of CD8<sup>+</sup> Tcs and NK cells in tumor immunotherapy, the observed increase in their proportions after GanoOil treatment in mice (Fig. 3) signifies the immune-enhancing effect of GanoOil. Previous studies have demonstrated that *G. lucidum* activates NK cells through the NGK2D/NCRs, as well as the p38, JNK, ERK, and MAPK signaling pathways, elucidating the underlying mechanism of NK cell activation. The enhanced NK cell cytotoxic activity induced by *G. lucidum* proves effective against tumor cells.<sup>34</sup> Wang et al<sup>35</sup> from Peking University Health Science Center demonstrated that the broken-spore of *G. lucidum* could enhance NK cell cytotoxic

activity, stimulate splenic LYM proliferation, increase the proportion of neutral red phagocytosis by macrophages, and elevate the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells by upregulating serum levels of interferon (IFN)- $\gamma$ , TNF- $\alpha$ , and nitric oxide, which corroborate our findings. Furthermore, our results also provide evidence that treatment with GanoOil promotes NK cell activity. These activated NK cells play a critical role as primary effector LYMs in effectively eliminating circulating tumor cells within the vasculature.<sup>36</sup> These findings demonstrated the cancer metastasis prevention ability of GanoOil.

The treatment of cancer has a significant impact on the quality of life for patients. Moreover, the administration of chemotherapeutic drugs can lead to weight loss and compromised immune function. Hence, our investigation aimed to assess the impact of conventional chemotherapeutic drugs, CTX and GanoOil, on inhibiting lung metastasis in 4T1 breast cancer. The findings demonstrate that GanoOil can mitigate CTX-induced weight loss and decrease the ratios of lung weight/body weight and spleen weight/body weight. This effect is achieved by enhancing the activity of CD8<sup>+</sup> Tcs and NK cells, as well as stimulating the secretion of IL-6 cytokine, whereas not affecting TNF- $\alpha$  secretion (Figs. 4–6). These mechanisms collectively support CTX's role in preventing lung metastasis in breast tumors. In line with our findings, Li et al<sup>37</sup> reported that GLS powder enhances the phagocytic



**Fig. 6** Effect of GanoOil on CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, splenic lymphocytes, NK cell activity, and IL-6 and TNF- $\alpha$  contents in vivo. A-B, Flow cytometry was performed to detect the percentage of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. C, Proliferation ability of splenic lymphocytes in the treatment groups. D, Activity of NK cells in the treatment groups. E-F, Contents of IL-6 and TNF- $\alpha$  based on ELISA. (<sup>ns</sup> $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ ,  $n = 4$ .) Normal control group, mouse blank control; 4T1 group, mice administered 4T1 cells ( $10^5$ /each) alone (ie, no CTX or GanoOil); GanoOil group, mice administered 4T1 cells ( $10^5$ /each) and 0.3g/kg GanoOil, but no CTX; CTX group, mice administered 4T1 cells ( $10^5$ /each) and 0.03g/kg CTX, but no GanoOil; and CTX+GanoOil group, mice administered 4T1 cells ( $10^5$ /each), 0.03g/kg CTX, and 0.3g/kg GanoOil. CTX = cyclophosphamide; IL = interleukin; NK = natural killer; ns = no significant; TNF = tumor necrosis factor.

capability of zebrafish macrophages, and exhibits an antitumor effect in mice with tumors by bolstering the immune system.<sup>38</sup> *G. lucidum* is a traditional Chinese medicine known for its lack of side effects. In a randomized, double-blind, placebo-controlled study, it was found that a traditional Chinese medicine complex, which incorporates the complete extract of *G. lucidum*, significantly enhanced the immune function of cancer patients undergoing chemotherapy and/or radiotherapy.<sup>39</sup> Despite numerous preclinical studies demonstrating the anticancer effects of *G. lucidum* extracts, clinical research in this field remains insufficient. GanoOil exhibits synergistic effects with chemotherapy drugs, reducing the likelihood of tumor metastasis and mitigating chemotherapy-induced side effects. These findings suggest promising applications of GanoOil in pain management for cancer patients and other clinical scenarios.

In conclusion, GanoOil is an extract derived from the spores of *G. lucidum*, a traditional Chinese herbal medicine widely used in Asia. This study aimed to investigate the mechanisms and adjuvant therapeutic effects of GanoOil in conjunction with the chemotherapeutic drug CTX for preventing breast cancer metastasis. The results demonstrated that GanoOil alone significantly promoted splenocyte proliferation and enhanced NK cell activity in the spleens of the experimental mice. Moreover, in a lung metastasis model, GanoOil demonstrated notable effectiveness in conjunction with CTX in preventing breast cancer lung metastases. This effect was achieved by increasing the percentage of CD8<sup>+</sup> Tcs in the blood samples of mice and enhancing the activity of NK cells in the spleen. These significant findings suggest that GanoOil has the potential to serve as an immune-enhancing drug and can be used as a complementary therapy in chemotherapeutic anticancer strategies.

#### APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://links.lww.com/JCMA/A229>.

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