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ARTICLE

Ganoderic acid alleviates chemotherapy-induced fatigue in mice bearing colon tumor

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Chemotherapy-related fatigue (CRF) is increasingly being recognized as one of the severe symptoms in patients undergoing chemotherapy, which not only largely reduces the quality of life in patients, but also diminishes their physical and social function. At present, there is no effective drug for preventing and treating CRF. Ganoderic acid (GA), isolated from traditional Chinese medicine *Ganoderma lucidum*, has shown a variety of pharmacological activities such as anti-tumor, anti-inflammation, immunoregulation, etc. In this study, we investigated whether GA possessed anti-fatigue activity against CRF. CT26 tumor-bearing mice were treated with 5-fluorouracil (5-FU, 30 mg/kg) and GA (50 mg/kg) alone or in combination for 18 days. Peripheral and central fatigue-related behaviors, energy metabolism and inflammatory factors were assessed. We demonstrated that co-administration of GA ameliorated 5-FU-induced peripheral muscle fatigue-like behavior via improving muscle quality and mitochondria function, increasing glycogen content and ATP production, reducing lactic acid content and LDH activity, and inhibiting p-AMPK, IL-6 and TNF-α expression in skeletal muscle. Co-administration of GA also retarded the 5-FU-induced central fatigue-like behavior accompanied by down-regulating the expression of IL-6, iNOS and COX2 in the hippocampus through inhibiting TLR4/Myd88/NF-κB pathway. These results suggest that GA could attenuate 5-FU-induced peripheral and central fatigue in tumor-bearing mice, which provides evidence for GA as a potential drug for treatment of CRF in clinic.

Keywords: ganoderic acid; 5-fluorouracil; chemotherapy-related fatigue; tumor-bearing mouse; muscle; hippocampus; energy metabolism; proinflammatory cytokine

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INTRODUCTION

Colorectal cancer (CRC) is the fourth most common cancer in the world; there are more than 1.2 million patients with CRC, and 600,000 individuals die from the disease every year [1, 2]. 5-FU, one of the most widely used first-line chemotherapy agents for the treatment of CRC [3, 4], frequently causes fatigue and loss of appetite [5–8]. Chemotherapy-related fatigue (CRF) is increasingly recognized as a severe symptom in patients undergoing chemotherapy, and it can persist for years after treatments [9–12]; this condition not only largely reduces the quality of life of patients but also diminishes physical and social function, resulting in treatment limitations and increased morbidity [13].

CRF can result from both muscle dysfunction, termed 'peripheral fatigue', and decreased capacity of the central nervous system (CNS), termed 'central fatigue' [14]. Some studies have reported that chemotherapy leads to muscle fatigue by decreasing lean body and muscle mass [15, 16]. In addition, chemotherapy damages the skeletal musculature, inducing mitochondrial damage [17, 18], and leads to energy metabolism alterations and distinct metabolic derangements, including decreased energy supply and ATP production and increased lactate content [19]. Moreover, there is increasing evidence that proinflammatory cytokines, especially IL-6, TNF-α and IL-1β, in muscle and the nervous system significantly contribute to the occurrence of CRF [20–24], indicating that both muscle and central disorders are likely to play an essential role in CRF.

At present, there is no FDA-approved drug that can effectively prevent and treat CRF. Recently, increasing evidence has revealed that natural products derived from a variety of sources may be novel and promising alternative drugs for preventing and treating CRF [25–27] owing to their multiple pharmacological effects. *Ganoderma lucidum* (*G. lucidum*), a well-known traditional Chinese medicine [28], has various therapeutic properties, such as the ability to improve immunity, hepatic and renal function, as well as increase energy production, promote health and protect against the aging [29–32]. Clinical use of *G. lucidum* has revealed that it exerts beneficial effects in the context of CRF and on the overall quality of life in patients with cancer undergoing endocrine treatment by decreasing the concentrations of IL-6 and TNF-α [33]. Bioactive compounds that can be isolated from the mycelia and fruiting bodies of *G. lucidum* include triterpenes, polysaccharides, and other constituents, such as alkaloids, flavonoids, proteins,

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amino acids, mannitol, and steroids [34]. It has been reported that ganoderic acid (GA), the major component of *Ganoderma triterpenes*, reduces LPS-induced expression of IL-1β, IL-6 and TNF-α and alters mitochondrial metabolic activity [35] and neuronal inflammation [36], which may contribute to occurrence of CRF [20].

In this study, we investigated the effects of GA on 5-FU-induced peripheral muscle fatigue and central fatigue in colon tumor-bearing mice by performing a series of behavioral tests and evaluating biological parameters. Our experimental results showed that GA alleviated CRF by improving muscle quality, increasing energy metabolism and suppressing peripheral and neuronal inflammation, which indicates that GA may be developed as a novel drug for CRF treatment.

MATERIALS AND METHODS

Animals

Female BALB/c mice (6–7 weeks, 18–22 g body weight) were purchased from the Animal Center of Peking University Health Science Center (Beijing, China). Female mice were used in this study because tumor-bearing female mice maintain food intake and body weight better than male mice [37] and male mice often bite the tumor site [38]. The experiments were performed according to the National Institutes of Health Guidelines on the Use of Laboratory Animals. The University Animal Care Committee for Animal Research of Peking University Health Science Center approved the study protocol (Beijing, China). The mice were maintained in cages at 25 ± 1 °C on a 12 h light/dark cycle and provided free access to water and food throughout the experimental period. The animals were randomly divided into experimental groups ($n = 10$/group).

Drugs

GA was extracted and purified from the dried fruiting bodies of *G. lucidum* as reported previously [39, 40]. The concentrations of GA-A, GA-B, and GA-C2 were 16.101, 10.586, and 5.404 μg/mL, respectively. These three monomers accounted for 16.1% (GA-A), 10.6% (GA-B), and 5.4% (GA-C2) of crude GA. The purity of these three monomers was >98%, as determined by HPLC [39, 40]. GA was dissolved in saline with 5% Tween 80 and injected intraperitoneally at a dose of 50 mg/kg.

5-FU, which was purchased from Sigma (MO, USA), was dissolved in saline with 5% Tween 80 and injected intraperitoneally at a dose of 30 mg/kg according to previous research [41].

Tumor cell culture

The mouse colon adenocarcinoma cell line CT26 was provided by the Cell Resource Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). The cells were cultured in RPMI-1640 medium (M&C, Beijing, China) with 10% fetal bovine serum (FBS; Gibco, Australia), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37 °C. The cells were resuspended in PBS for injection.

Experimental tumor-bearing mouse model

Mice were injected subcutaneously over the scapula with 200 μL of suspended tumor cells (2.5×10^5 cells/mouse) or PBS as described previously [42]. This site was selected as the tumor site instead of the right flank or an orthotopic site to avoid movement constraints that could interfere with the behavioral evaluations. It has been reported that this tumor model exhibits fatigue [37, 42, 43]. A treadmill adaptation test (TAT) was carried out before the tumors were palpable. Based on tumor size, the tumor-bearing mice were divided into four groups: the CT26 group, chemotherapy (5-FU, 30 mg/kg/2day) group, GA (GA, 50 mg/kg/day) group and chemotherapy combined with GA (5-FU + GA, 30 mg/kg/2day + 50 mg/kg/day) group. Tumor size was monitored twice a week using a digital caliper and calculated by the following formula: $(\text{width in mm})^2 \times (\text{length in mm})/2$. Body weight was recorded at the same time. As shown in Fig. 1a, the mice were subjected to behavioral tests on the drug intervention days.

Grip strength test (GST)

The muscle strength of the mice was assessed weekly using a commercial digital grip strength meter with an attached metal grid (YLS-13A, Shanghai, China). The mice were lifted and held by the tail so that the forepaws could grasp the wire grid, which was connected to a dynamometer. The mice were then gently pulled backward by the tail until they released their grip. The peak force of the forelimbs was recorded. Three trials were performed for each mouse, and the results were used for statistical analysis.

Treadmill fatigue test (TFT)

The TFT was performed according to a previously described method [42] with minor modifications. In brief, a mouse treadmill (Zhongshidichuang, Beijing, China) with 10-degree incline and a grid that delivered 1.2 mA electric shocks at 2 Hz was used for training and testing. The mice were acclimated for 3 consecutive days before the tumors were palpable. The fatigue test was conducted on day 8 and day 15, and the following program was used: 12 m/min for 5 min, 14 m/min for 5 min, 17 m/min for 3 min, 20 m/min for 3 min, 23 m/min for 3 min, 26 m/min for 5 min, and 29 m/min until the end of the test. The experimenter did not interact with the animals until they met the criterion for fatigue-like behavior, which was defined as remaining in the designated fatigue zone of the treadmill on the electric grid for 5 s. Fatigue-like behavior in each run-to-exhaustion trial was estimated using the running distance.

Open field test (OFT)

Central fatigue-like behavior was measured by using the open field test (OFT) as reported previously [44]. Mice were individually placed in the center of the open field apparatus (50 cm × 50 cm × 50 cm) and observed for 5 min. Locomotor activity, such as distance traveled, rest time, speed while mobile, ratio of time spent in the central zone and the number of entries into the central zone was assessed with an animal tracking system (Zhongshidichuang, Beijing, China).

Tail suspension test (TST)

It has been reported that the tail suspension test (TST), which is typically used to evaluate depression-like behavior, can measure fatigue-like behavior in mice [45–47], and recently, researchers have used the TST to assess mental fatigue-like behavior related to cancer-related fatigue [48]. The TST was performed according to the method reported by Streru [37]. The mice were hung 50 cm above the floor with adhesive tape, which was placed ~1 cm from the top of the tail. The total time of immobility over 6 min was recorded.

Biochemical assessments

After the behavioral tests, the mice were deeply euthanized by inhaled isoflurane. Serum was collected by centrifuging whole blood at 3500 r/min for 15 min. The gastrocnemius muscles, tibialis anterior muscles, quadriceps muscles and tumors were excised and weighed, frozen in liquid nitrogen, and kept at −80 °C for further studies. Serum samples were subjected to biochemical assays, including assays of lactate dehydrogenase (LDH) and lactic acid (LD) (NJJC Bio, Nanjing, Jiangsu, China), ATP (Vigorous Biotechnology, Beijing, China), IL-6, IL-1β and IL-15 (Applygen, Beijing, China) levels with biochemical kits. Glycogen in the liver and muscle was hydrolyzed in aqueous alkali solution, and the glycogen content was determined with a biochemical kit (NJJC Bio, Nanjing, Jiangsu, China). All assays were carried out according to the manufacturers' instructions.

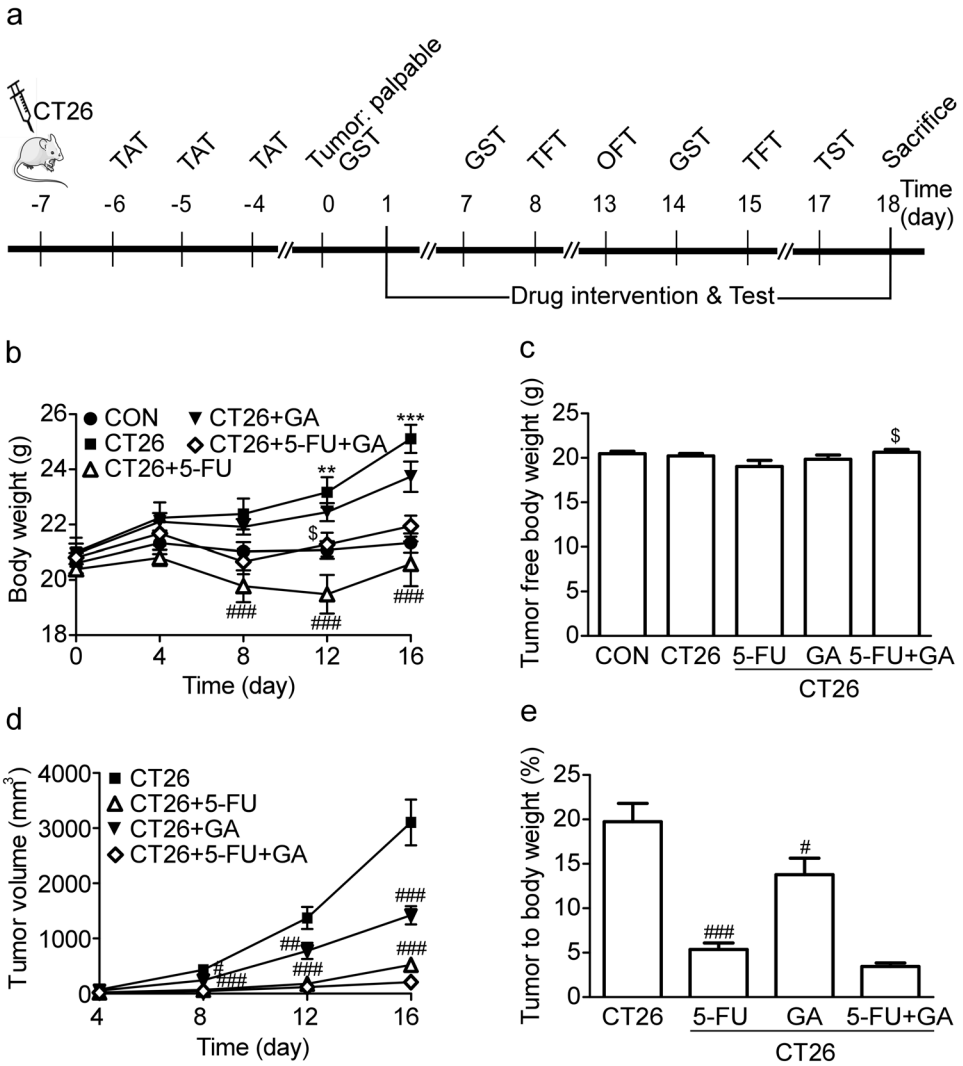

Fig. 1 GA significantly inhibited colon tumor growth in CT26 tumor-bearing mice. GA (50 mg/kg), 5-FU (30 mg/kg) or normal saline with 5% Tween 80 was intraperitoneally administered to female BALB/c tumor-bearing mice for 18 days after grouping. **a** Study design. **b** Body weight. **c** Tumor free body weight. **d** Tumor volume. **e** Tumor to body weight. The values are shown as the mean ± SEM ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the CT26 group. $^{\$}P < 0.05$ vs the CT26 + 5-FU group. TAT treadmill adaptation test, GST grip strength test, TFT treadmill fatigue test, OFT open field test, TST tail suspension test, CON control, CT26 CT26 tumor-bearing mice, 5-FU 5-fluorouracil, GA ganoderic acid, 5-FU + GA 5-fluorouracil combined with ganoderic acid.

Histological staining and ultrastructural examination

Skeletal muscles were carefully collected, minced, fixed in 10% formalin in 0.01 M phosphate-buffered saline (PBS), dehydrated, embedded in paraffin, and cut into 5 μm-thick slices. After deparaffinization, the skeletal muscle sections were rehydrated, stained with hematoxylin and eosin, and examined under a microscope for morphological and pathological evaluation.

For ultrastructural evaluation, skeletal muscles that were cut into 1 mm^3 pieces were fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, and stained with uranyl acetate and lead citrate. The samples were thinly sectioned and observed under a transmission electron microscope, and ultrastructural changes in mitochondria were examined.

Western blot analysis

Protein was extracted from the skeletal muscles and hippocampi of mice from different groups by homogenization using tissue protein lysis buffer (Mei5, MF188-01, Beijing, China) supplemented with protease inhibitor cocktail (Roche, Basel, Kanton Basel-Stadt, Switzerland). The concentrations of the protein samples were determined by using a Bicinchoninic Acid Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were loaded on gels, separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Boston, MA, USA). After blocking, the membranes were incubated overnight at 4 °C with homologous primary antibodies against β-actin (1:10000, ABclonal, AC026, Wuhan, China), p-AMPK (1:2500, CST, 2535, MA, USA), AMPK (1:2500, CST, 2523, MA, USA), IL-6 (1:1000, ABclonal, A0286, Wuhan, China), IL-1β (1:1000, ABclonal, A11370, Wuhan, China), TNF-α (1:1000, ABclonal, A0277, Wuhan, China), COX2 (1:1000, ABclonal, A1253, Wuhan, China), iNOS (1:1000, ABclonal, A0312, Wuhan, China), Myd88 (1:1000, ABclonal, A0980, Wuhan, China), TLR4 (1:1000, ABclonal, A11226, Wuhan, China), and NF-κB (1:1000, Immunoway, YM3111, TX, USA), and the blots were developed with an ECL plus kit (Biodragon, Beijing, China). The protein bands were visualized with a chemiluminescence detection system (Syngene, GeneGnome XRQ, Cambridge, Cambridgeshire, UK) and analyzed by ImageJ software (NIH, MD, USA).

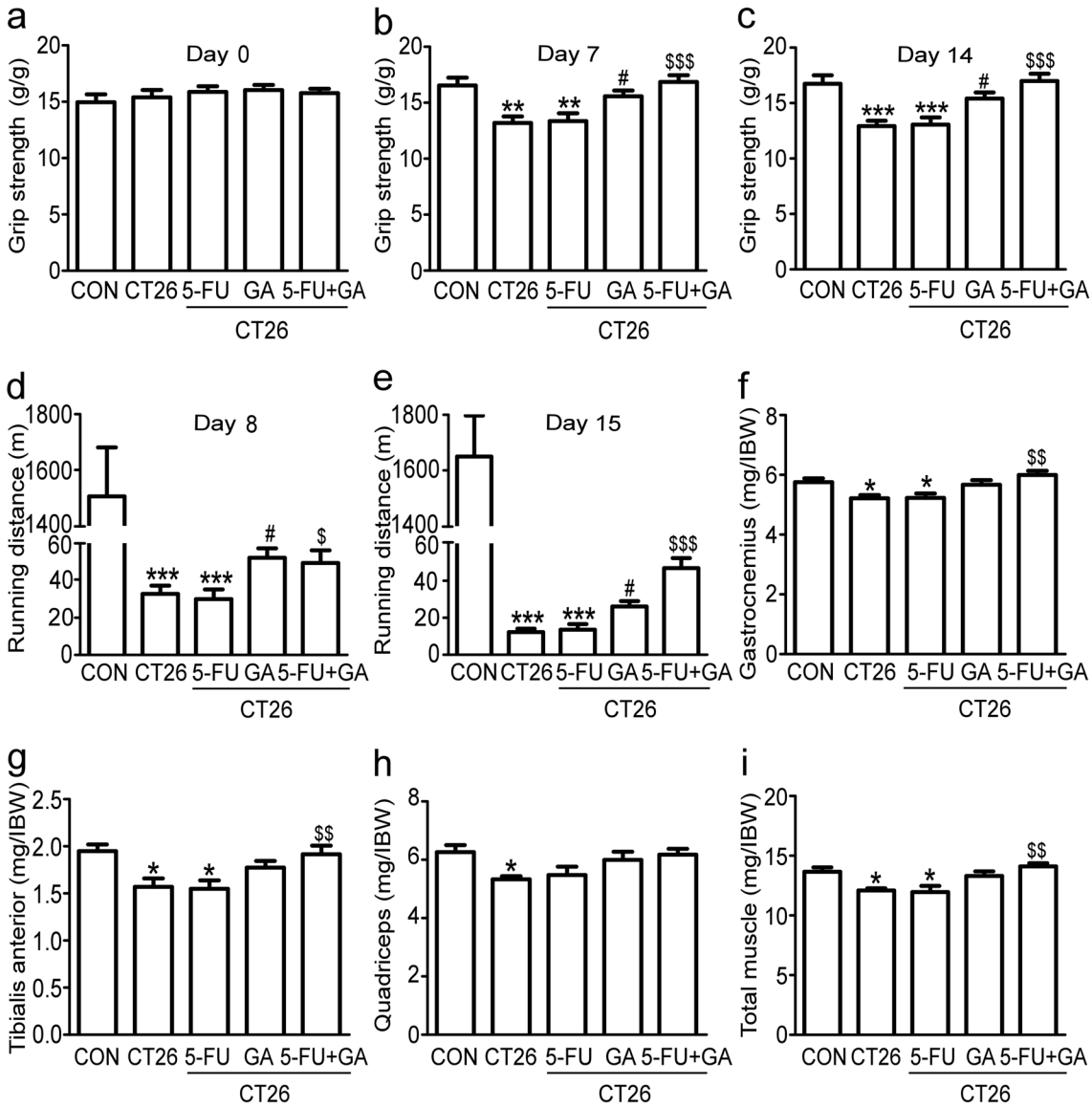

Fig. 2 GA attenuated peripheral muscle fatigue-like behavior in chemotherapy-treated CT26 tumor-bearing mice. a Grip strength on day 0. **b** Grip strength on day 7. **c** Grip strength on day 14. **d** Running distance on day 8. **e** Running distance on day 15. **f** Gastrocnemius muscle weight to initial body weight (IBW) ratio. **g** Tibialis anterior muscle weight to IBW ratio. **h** Quadriceps muscle weight to IBW ratio. **i** Total skeletal muscle weight to IBW ratio. The values are shown as the mean ± SEM ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\#}P < 0.05$ vs the CT26 group. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ vs the CT26 + 5-FU group. CON control, CT26 CT26 tumor-bearing mice, 5-FU 5-fluorouracil, GA ganoderic acid, 5-FU + GA 5-fluorouracil combined with ganoderic acid.

Statistical analysis

The statistical data were plotted with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). For multiple comparisons, statistical analysis was performed using Student's *t* test or one-way ANOVA or two-way ANOVA. $P < 0.05$ was considered statistically significant. All data are expressed as the mean ± SEM.

RESULTS

GA significantly inhibited colon tumor growth in CT26 tumor-bearing mice

As shown in Fig. 1b, compared to control group, body weight of CT26 tumor-bearing mice was significantly increased from day 12 to day 16, which may be due to the increased tumor burden, because the tumor free body weight of CT26 tumor-bearing mice did not differ from the mice in control group (Fig. 1c). Co-administration of GA increased the body weight and tumor-free body weight of chemotherapy-treated mice compared to the CT26 + 5-FU group (Fig. 1b, c). Furthermore, in mice that subcutaneously received CT26 tumor cells, the tumor burden rapidly increased from day 8 in the absence of drug intervention, while tumor growth was significantly inhibited (Fig. 1d) and tumor to body weight was reduced (Fig. 1e) following the treatment with GA and 5-FU. During the experiment, none of the mortality and macroscopic metastases were found upon gross visual examination of the livers, lungs, hearts, and kidneys of tumor-bearing mice treated with or without GA.

GA attenuated peripheral muscle fatigue-like behavior in chemotherapy-treated CT26 tumor-bearing mice

The grip strength test (GST) showed that muscle strength was identical among all groups on day 0 (Fig. 2a) but that muscle strength in the CT26 group and 5-FU-treated group were

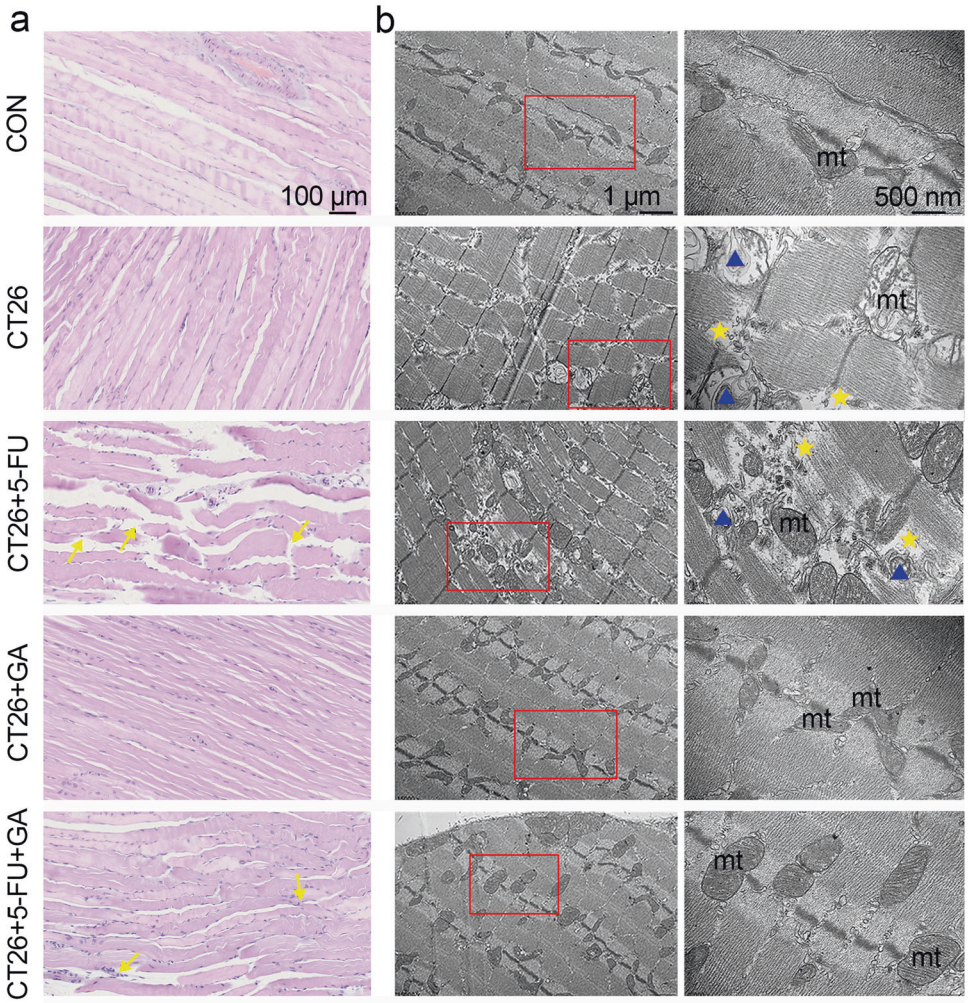

Fig. 3 GA alleviated skeletal muscle injury in chemotherapy-treated tumor-bearing mice. a Representative micrographs of H&E staining (magnification of ×200; typical myofibrillar fragmentation is indicated by the yellow arrows). **b** Representative transmission electron microscopy (TEM) micrographs (magnification of ×15,000 or ×40,000, typical mitochondria, dissolving myofilaments and mitochondria swelling are indicated by mt, the yellow stars and the blue triangles, respectively). CT26 CT26 tumor-bearing mice, 5-FU 5-fluorouracil, GA ganoderic acid, 5-FU + GA 5-fluorouracil combined with ganoderic acid.

significantly decreased on day 7 (Fig. 2b) and more evidently decreased on day 14 compared to control group (Fig. 2c). Muscle strength was markedly improved in the GA-treated and GA plus 5-FU-treated groups compared to the CT26 group and 5-FU-treated group. Running distance on the treadmill was significantly decreased in the CT26 group and 5-FU-treated group compared to the control group on day 8 and day 15, while this change was significantly alleviated in the GA-treated and GA plus 5-FU-treated groups (Fig. 2d, e). Muscle mass, including gastrocnemius muscle (Fig. 2f), tibialis anterior muscle (Fig. 2g), quadriceps muscle (Fig. 2h) and total skeletal muscle mass (Fig. 2i), was reduced in CT26 tumor-bearing mice and 5-FU-treated mice compared to control mice. GA significantly rescued the depletion of these muscles' mass in 5-FU-treated mice.

GA alleviated skeletal muscle injury in chemotherapy-treated tumor-bearing mice

H&E staining (Fig. 3a) revealed a long column of skeletal muscle fibers with regular morphology, uniformly distributed nuclei under an intact myolemma, and clear periodic streaks of light and dark fibers in the control group and GA-treated group. There was no obvious change in muscle sections from CT26 mice, but 5-FU-treated tumor-bearing mice exhibited characteristic muscle pathology, such as disorganized skeletal muscle fibers, irregular cell morphology, broken muscle fibers, and inflammatory cell infiltration. Interestingly, GA reversed 5-FU-induced skeletal muscle pathological alterations in tumor-bearing mice, as shown in Fig. 3a.

TEM assessment of the morphology of skeletal muscles (Fig. 3b) also showed that CT26 tumor-bearing mice and 5-FU-treated mice exhibited a series of alterations, such as disordered myofibril arrangement; shortened sarcomere length; dissolved or disappeared myofilaments; sarcoplasmic reticulum expansion; intracellular myelocystic bodies; and swollen, deformed, and even vacuole-like mitochondria. These mitochondrial changes were more pronounced in the 5-FU-treated mice than in the CT26 tumor-bearing mice, and GA effectively protected mitochondria from tumor- or 5-FU-induced dysfunction.

GA improved energy metabolism abnormalities and the levels of inflammatory cytokines in skeletal muscle in 5-FU-treated tumor-bearing mice

To evaluate the effect of GA on energy metabolism, we measured the serum ATP, LDH, LD, liver, and muscle glycogen contents and

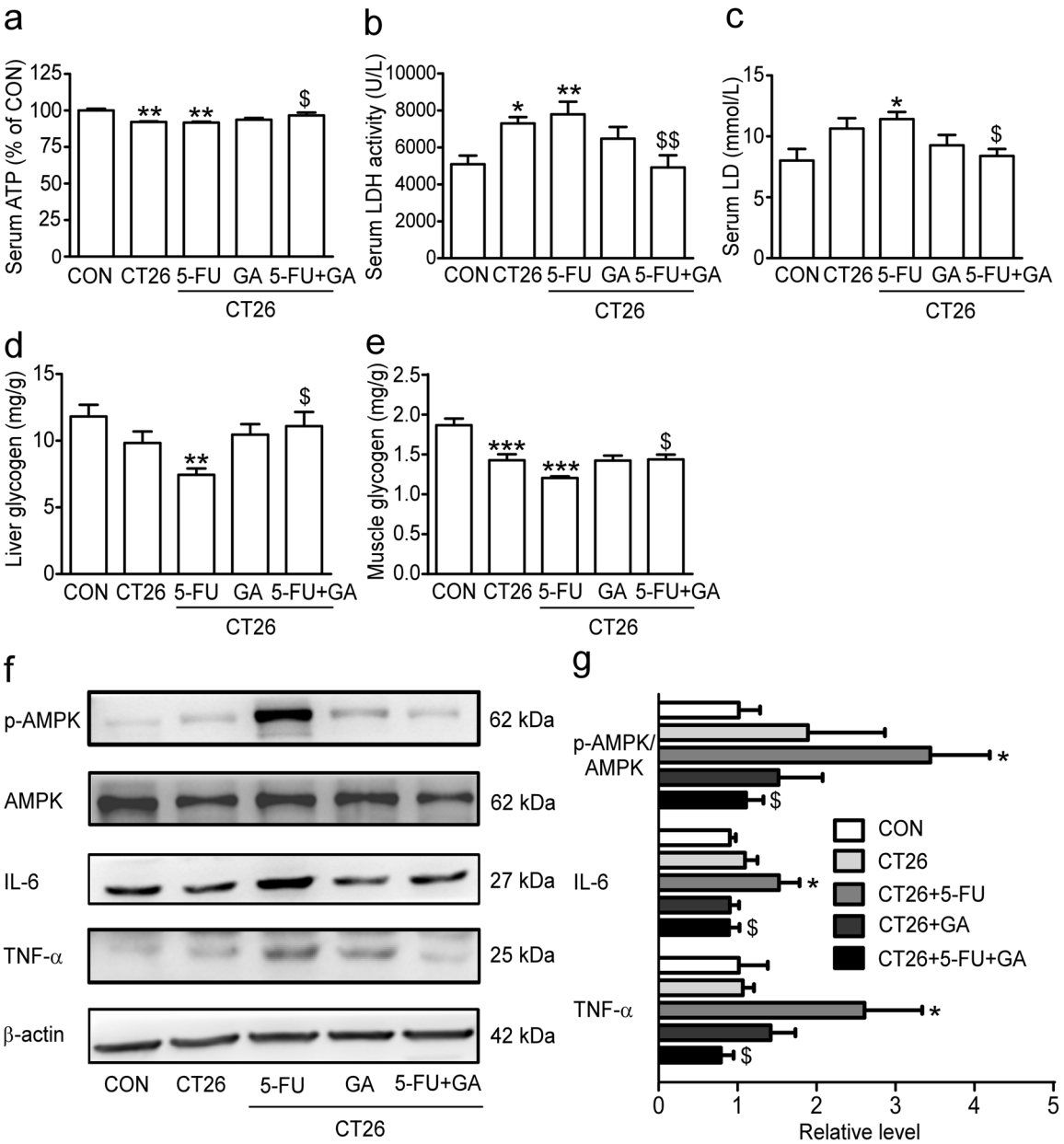

Fig. 4 GA improved energy metabolism abnormalities and skeletal muscle inflammatory cytokine levels in 5-FU-treated tumor-bearing mice. a Serum ATP content (% of CON). **b** Serum LDH activity. **c** Serum LD content. **d** Liver glycogen content. **e** Muscle glycogen content. **f** Representative Western blots showing p-AMPK, AMPK, IL-6 and TNF-α protein expression in skeletal muscle. **g** Relative protein levels in the experiments shown in (**f**). The data were normalized to the intensity of β-actin or the nonphosphorylated form of the protein of interest and are expressed relative to the value of the control group. The values are shown as the mean ± SEM ($n = 5–10$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs the CT26 + 5-FU group. CON control, CT26 CT26 tumor-bearing mice, 5-FU 5-fluorouracil, GA ganoderic acid, 5-FU + GA 5-fluorouracil combined with ganoderic acid, ATP adenosine-triphosphate, LDH lactate dehydrogenase, LD lactic acid, p-AMPK phosphorylated 5′-AMP-activated protein kinase, IL-6 interleukin-6, TNF-α tumor necrosis factor alpha.

the relative protein expression levels of p-AMPK, AMPK, TNF-α, and IL-6. The results showed that while serum ATP content decreased (Fig. 4a), serum LDH activity and LD content increased in the CT26 group or the 5-FU-treated group (Fig. 4b, c). Fortunately, these alterations were significantly alleviated in the GA combined with 5-FU group. Muscle and liver glycogen contents were decreased in the 5-FU-treated group and CT26 group, but liver glycogen content was only significantly reduced in the 5-FU-treated group. Furthermore, GA rescued the decreases in muscle and liver glycogen contents in 5-FU-treated tumor-bearing mice (Fig. 4d, e). Furthermore, we measured the protein expression of p-AMPK, an energy stress sensor, to better understand energy balance and found that the relative expression of p-AMPK/AMPK was markedly upregulated in 5-FU-treated tumor-bearing mice. GA successfully downregulated the expression of p-AMPK/AMPK in chemotherapy-treated tumor-bearing mice (Fig. 4f, g).

To determine whether 5-FU could activate skeletal muscle inflammation in CT26 tumor-bearing mice and whether GA could modulate chemotherapy-related inflammatory activation in mice with CRF, we analyzed the expression of proinflammatory cytokines in skeletal muscle, including IL-6 and TNF-α. As shown

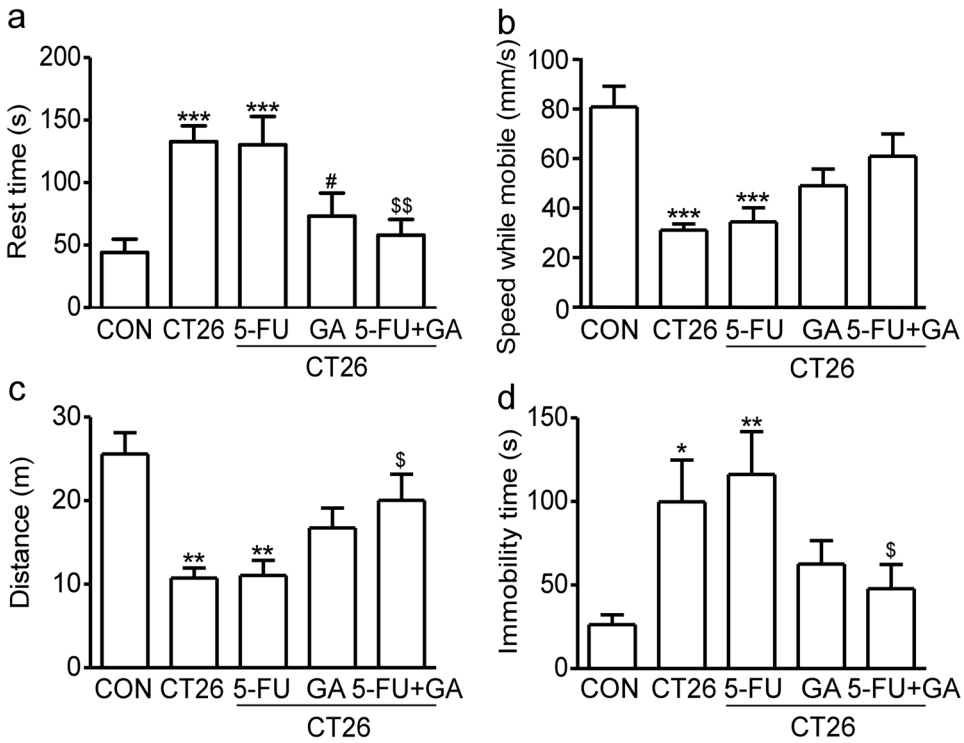

Fig. 5 The central fatigue-like behavior of chemotherapy-treated tumor-bearing mice was attenuated by GA. a Rest time in the open field test (OFT). **b** Speed while mobile in the OFT. **c** Total distance traveled in the OFT. **d** Immobility time in the tail suspension test (TST). The values are shown as the mean ± SEM ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\#}P < 0.05$ vs the CT26 group. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs the CT26 + 5-FU group. CON control, CT26 CT26 tumor-bearing mice, 5-FU 5-fluorouracil, GA ganoderic acid, 5-FU + GA 5-fluorouracil combined with ganoderic acid.

in Fig. 4f, g, the expression of IL-6 and TNF-α in skeletal muscle was significantly upregulated in tumor-bearing mice treated with 5-FU, and fortunately, we found that GA significantly downregulated the expression of these proinflammatory cytokines in 5-FU-treated tumor-bearing mice.

The central fatigue-like behavior of chemotherapy-treated tumor-bearing mice was attenuated by GA

The results of the open field test (OFT) showed that the rest time (Fig. 5a) was obviously increased while speed while mobile (Fig. 5b), traveled distance (Fig. 5c), the time spent in the central zone (Supplementary Fig. S1a) and the number of central zone entries (Supplementary Fig. S1b) were significantly decreased in the CT26 group and 5-FU-treated group compared to the control group. GA reversed the abnormal changes in the rest time and traveled distance, but not increased the speed while mobile, time spent in the central zone or the number of central zone entries in the 5-FU-treated tumor-bearing mice. The immobility time (Fig. 5d) in the TST was increased in both CT26 tumor-bearing mice and 5-FU-treated tumor-bearing mice; furthermore, GA-treated mice showed no obvious change in immobility time, but the immobility time of the GA combined with 5-FU-treated mice was significantly lower than that of the 5-FU-treated mice.

GA hindered the expression of inflammatory cytokines and mediators in the hippocampi of colon tumor-bearing mice treated with 5-FU

We found that the serum contents of IL-6 (Supplementary Fig. S2a) and IL-1β (Supplementary Fig. S2b) were increased in 5-FU-treated tumor-bearing mice and that the serum content of IL-15 (Supplementary Fig. S2c) was markedly decreased in the CT26 group and 5-FU-treated group. GA suppressed the expression of IL-6 and IL-1β in the CT26- or 5-FU-treated group but did not increase the content of IL-15. Moreover, 5-FU treatment not only increased the levels of the proinflammatory cytokine IL-6 and the inflammatory response factors iNOS and COX2 (Fig. 6a, b), but also upregulated the expression of the innate immune response molecules TLR4, Myd88 and the transcription factor NF-κB in the hippocampus (Fig. 6c, d). Interestingly, GA effectively downregulated the expression of these over-activated inflammatory cytokines and mediators in the GA combined with 5-FU-treated group, suggesting that GA could relieve the central fatigue-like behavior of 5-FU-treated tumor-bearing mice by inhibiting neuroinflammation.

DISCUSSION

Fatigue is one of the most prevalent symptoms in cancer patients that complain of tiredness before, during, and after cancer treatment [9, 10, 49], and it decreases quality of life [13]. CRF prevents patients from making full use of adequate cytotoxic chemotherapy to improve life span. Previous studies have shown that GA, the major component of *Ganoderma triterpenes*, not only exhibits anti-tumor effects but also displays anti-inflammatory, anti-oxidative and immunomodulatory activities [50], which may affect the pathogenesis of CRF [20], suggesting that GA may have effects against CRF. In the present study, we found that GA effectively hindered fatigue-like behavior in 5-FU-treated mice, as evidenced by improvements in muscle mass and energy metabolism and modulation of peripheral muscle inflammation and neuroinflammation.

The etiology of CRF is complex, encompassing both peripheral and central mechanisms [20, 22, 23], which have been hypothesized to contribute to overall fatigue in cancer patients [51]. Generally, cancer-related fatigue is mainly induced by energy metabolism disruption in skeletal muscles [19, 23] and neuroinflammation in the CNS [22, 37, 52].

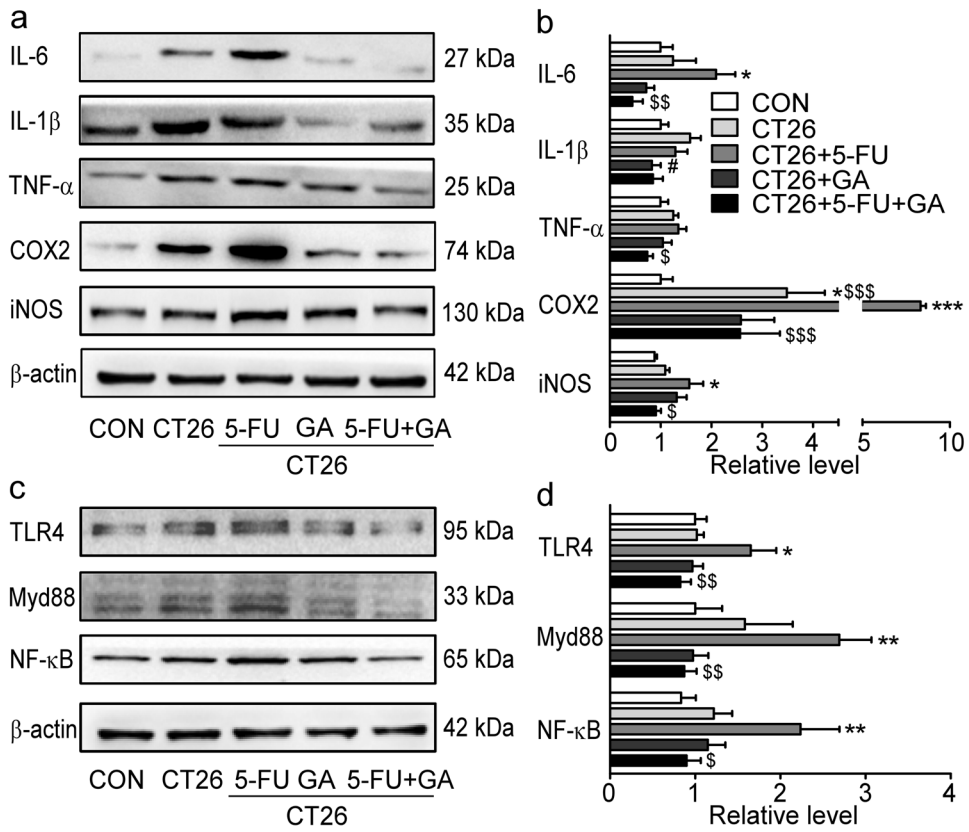

Fig. 6 GA hindered the expression of inflammatory cytokines and mediators in the hippocampi of colon tumor-bearing mice treated with 5-FU. a Representative Western blots showing the expression of key proteins, namely, IL-6, IL-1β, TNF-α, COX2 and iNOS in the hippocampus. **b** Relative protein levels in the experiments shown in (**a**). The data were normalized to the intensity of β-actin and are expressed relative to the value of the control group. **c** Representative Western blots showing the protein expression of TLR4, Myd88 and NF-κB in the hippocampus. **d** Relative protein levels in the experiments shown in (**c**). The data were normalized to the intensity of β-actin and are expressed relative to the value of the control group. The values are shown as the mean ± SEM ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\#}P < 0.05$ vs the CT26 group. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ vs the CT26 + 5-FU group. CON control, CT26 CT26 tumor-bearing mice, 5-FU 5-fluorouracil, GA ganoderic acid, 5-FU + GA 5-fluorouracil combined with ganoderic acid, IL-6 interleukin-6, TNF-α tumor necrosis factor alpha, IL-1β interleukin-1beta, COX2 cyclooxygenase 2, iNOS inducible nitric oxide synthase, TLR4 Toll-like receptor 4, Myd88 myeloid differentiation factor 88, NF-κB nuclear factor kappa B.

Peripheral fatigue refers to skeletal muscle fatigability or an inability to sustain force over time [14], most notably due to reductions in muscle strength and muscle mass, energy source and ATP depletion, and excess metabolite accumulation [53]. Some researchers have also reported that loss of muscle mass is common before [54] and after cancer treatment [19]. Recently, a clinical study also suggested that improving muscle mass is an effective strategy for ameliorating fatigue in patients with advanced cancer [16]. In this study, we found that GA could improve muscle mass, including the mass of the gastrocnemius, tibialis anterior and quadriceps muscles, and alleviate the decreases in grip strength and treadmill running in 5-FU-treated tumor-bearing mice, which may have been induced by reduced muscle mass.

Skeletal muscle is a highly metabolic organ that requires generation of a sufficient amount of ATP. Therefore, decreased ATP production eventually results in skeletal muscle dysfunction and fatigue [55]. In this study, we found a series of mitochondrial structural impairments, decreased muscle and liver glycogen content, decreased circulating ATP levels, and elevated LD content and LDH activity in 5-FU-treated tumor-bearing mice, indicating that balance of energy metabolism is clearly impacted by CRF. Furthermore, under glycolytic conditions, glycogen is rapidly consumed to produce a large amount of LD [56] and leads to muscle fatigue [57].

The energy-sensing molecule 5′-AMP-activated protein kinase (AMPK), as an indicator of cellular energy levels in response to metabolic stress [58], is activated under conditions of energy stress when intracellular ATP levels decline and intracellular AMP levels increase (high intracellular AMP/ATP ratio) [59]. Recently, researchers found that inflammation, such as elevated IL-6 and TNF-α levels, is linked to cachexia-induced mitochondrial dysfunction in skeletal muscle [60, 61]. It was also reported that an increase in IL-6 content in cachexia mice can lead to the activation of AMPK, whereas blockage of IL-6 ameliorates cachexia-induced AMPK activation [62], and reported that the activated AMPK causes decreased muscle mass [61], suggesting that chronic activation of AMPK by cytokines results in mitochondrial dysfunction, leading to lack of an appropriate energy supply for cell function, and loss of muscle mass and strength. Interestingly, GA significantly hindered 5-FU-induced mitochondrial dysfunction, depletion of energy sources and ATP generation, glycolytic metabolite accumulation, and upregulation of p-AMPK expression, which indicates that enhancing energy metabolism may be involved, at least in part, in the anti-peripheral muscle fatigue effects of GA.

Increasing evidence indicates that enhanced levels of inflammatory cytokines induced by chemotherapy may be closely associated with CRF [10, 63, 64]. Cytokines, such as IL-6, IL-1β and TNF-α, play important roles in the development of CRF [20, 21, 41]. These cytokines act as cell-to-cell mediators in response to

external immunologic stressors, trigger immune-to-brain communication signaling in the CNS and alter behaviors [65]. Peripherally released cytokines are able to activate immune-to-brain communication pathways [66], enter the brain through various routes to activate astrocytes and microglia, and eventually trigger the generation of cytokines, inflammatory mediators and other neurotoxins, which induce neuroinflammation in the CNS [67].

Some researchers have demonstrated that neuroinflammation, especially that caused by proinflammatory cytokines in the hippocampus, contributes to decreased voluntary activity and fatigue [37, 47, 68]. Although TLR4 and the downstream molecule NF-κB are widely investigated as the mediators of inflammatory response and cytokine-mediated processes, limited studies have focused on the role of the TLR4 signaling pathway in chemotherapy-induced fatigue. It was reported that after stimulation of TLR4 by LPS, the Myd88-dependent pathway activates NF-κB, which induces the production of proinflammatory cytokines and mediators, including IL-6, iNOS and COX2 [69, 70]. Some researchers have also reported that COX2 inhibitors not only increase the effectiveness of chemoradiation [71] but also improve cancer patients' quality of life via suppression of the proinflammatory cytokine pathway [72]. Others have found that Myd88, a key component of both the innate and adaptive immune systems and a universal adaptor protein for TLR4, plays a crucial role in mediating cancer-induced inflammation and that blocking this pathway alleviates pancreatic ductal adenocarcinoma-associated fatigue [73].

Surprisingly, we found that 5-FU led to significant overactivation of IL-6, iNOS and COX2 via the TLR4/Myd88/NF-κB-dependent pathway, whereas GA prevented the overactivation of the TLR4/Myd88/NF-κB signaling pathway in the hippocampus to alleviate central fatigue in chemotherapy-treated tumor-bearing mice. Abnormal activation of these cytokines was not found in the tumor-bearing mice, although there was an increasing trend in the expression of these cytokines and mediators in hippocampus; these changes are in line with previous research [74, 75]. Other researchers have also reported that chemotherapy triggers the production and secretion of inflammatory cytokines by tumor or immune cells [76]. Therefore, we hypothesized that inoculation of female BALB/c mice with CT26 cells in the subcutaneous space over the scapula might not be directly involved in cytokine abnormalities but that chemotherapy may contribute to the induction of inflammatory cytokines in tumor-bearing mice.

The limitations of this study must be acknowledged. To avoid possible behavior constraints due to tumor growth, we injected tumor cells into the subcutaneous space above the scapula instead of the right flank or an orthotopic site. Mice bearing subcutaneous tumors over the scapula present fatigue-like behavior with no movement restriction [37, 42, 72], but the microenvironment in this model may differ from that in models injected into the right flank or an orthotopic site. Therefore, it is imperative to study orthotopic mouse models, which better recapitulate the conditions of patients in the clinic.

In summary, as shown in Fig. 7, our study provides the first evidence that GA exerts a therapeutic effect against CRF in tumor-bearing mice. Based on our experiment, GA not only has good anti-tumor activity but also alleviates peripheral muscle fatigue and central fatigue-like behavior by improving muscle quality and emotional impairment. Moreover, GA can alleviate peripheral muscle fatigue by relieving decreased glycogen content, enhanced glycolysis, decreased ATP production and chronic activation of AMPK via inhibition of IL-6 and TNF-α in 5-FU-treated tumor-bearing mice. Finally, GA effectively inhibits TLR4-mediated Myd88 activation, which decreases the release of proinflammatory cytokines and mediators through the NF-κB pathway. Therefore, our study suggests that GA can be developed as a promising auxiliary therapeutic agent for CRF and is an alternative treatment for improving the quality of life of 5-FU-treated patients with colon cancer.

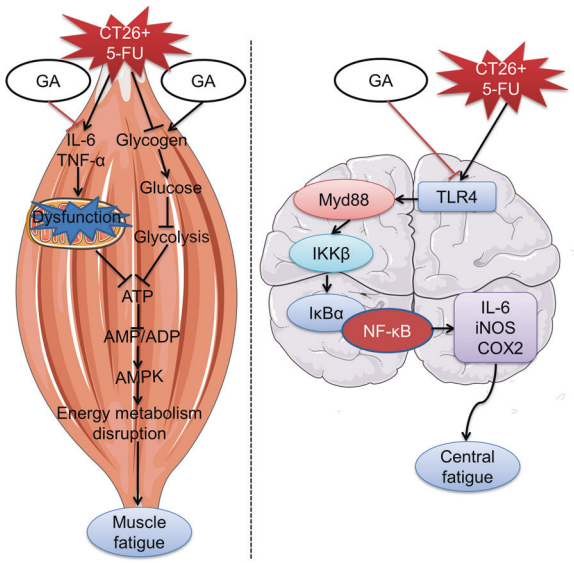

Fig. 7 Schematic diagram of the proposed mechanisms underlying the anti-fatigue activity of GA in chemotherapy-treated mice bearing colon tumor. GA ameliorates 5-FU-induced peripheral muscle fatigue (left) and central fatigue (right) in mice bearing colon tumor by improving energy metabolism and inhibiting neuroinflammation, respectively.

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AUTHOR CONTRIBUTIONS

AA, BXY, and ML conceptualized and designed this study. AA, LH, AM, FYS, HZZ, SML, GYS, YX, JHR, JL, DML, and LFW performed the experiments and analyzed the data. AA and BXY wrote the paper. HZ, ML, and BXY reviewed and revised the paper.

ADDITIONAL INFORMATION

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