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Dihydroartemisinin inhibits lung cancer bone metastasis by modulating macrophage polarization

Guihua Hang[1], Xia Gu[2], Yuanyuan Gu[3], Ping Gan[1], Chunwang Hua[1] and Aiming Chen[3*]

Abstract

The high metastasis rate of lung cancer contributes to its low 5-year survival rate. Bone metastasis is a common complication in advanced lung cancer, adversely affecting postoperative recovery. This study investigates the effects of DHA on macrophage polarization and its underlying mechanisms. In vitro, DHA was found to inhibit M2 polarization while promoting M1 polarization of macrophages, thereby reducing the invasion and migration of lung cancer cells. In vivo, DHA inhibited lung cancer growth and bone metastasis by modulating macrophage M1/M2 polarization in both lung cancer tissues and bone metastatic sites. In addition, through the CCL2/CCR2 pathway, DHA decreased macrophage recruitment and accumulation. These results suggest that DHA is effective in inhibiting lung cancer growth and bone metastasis, offering promising research and application prospects.

Keywords Lung cancer, Bone metastasis, Dihydroartemisinin, Macrophage polarization, Tumor microenvironment

Introduction

The leading cause of cancer-related deaths worldwide is lung cancer, posing a significant threat to public health worldwide [1]. It accounts for about 11.4% of new cancer cases and 18% of cancer-related deaths worldwide. The two main types of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC making up the majority of cases [2]. In general, lung cancer is classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC accounting for approximately 80–85% of cases. An array of multidisciplinary treatments is currently available for NSCLC, including surgery, chemotherapy, targeted therapy, immunotherapy, and radiotherapy [3]. However, the 5-year survival rate for lung cancer remains dismal, with distant metastasis being the primary factor affecting patient survival [4]. Factors such as the tumor microenvironment, epithelial–mesenchymal transition, tumor angiogenesis, immune evasion, and cell adhesion molecules collectively influence lung cancer invasion and metastasis [5]. It is crucial that the tumor microenvironment be altered to promote lung cancer metastasis and invasion.

Macrophages are essential to the innate immune system, demonstrating significant functional versatility [6]. Originating from bone marrow monocytes, these cells transform into different subsets of macrophages upon reaching tissues, each showcasing distinct traits and roles influenced by the surrounding microenvironment [7]. In general, macrophages fall into two primary categories: M1 and M2 [8, 9]. M1 macrophages are known

*Correspondence:
Aiming Chen
camlyq@sina.com
[1] Department of Pharmacy,Taizhou Second People's Hospital Affiliated to Yangzhou University, Taizhou Second People's Hospital, Taizhou 225500, Jiangsu, China
[2] Department of Emergency Medicine, Taizhou Second People's Hospital Affiliated to Yangzhou University, Taizhou Second People's Hospital, Taizhou 225500, Jiangsu, China
[3] Medical Affairs Section, Taizhou Second People's Hospital Affiliated to Yangzhou University, Taizhou Second People's Hospital, Taizhou 225500, Jiangsu, China

for their ability to present antigens and produce vital cytokines, fostering an anti-tumor response. Conversely, M2 macrophages have restricted antigen presentation abilities, promoting tumor growth and progression by suppressing immune responses [10]. Moreover, M2 macrophages facilitate tumor progression by promoting tissue reorganization, angiogenesis, and metastasis [11, 12]. Given these implications, manipulating macrophages could prove beneficial in controlling tumor growth and metastasis, potentially opening up new avenues for therapeutic interventions [13]. Ultimately, understanding the dynamic interplay between the diverse roles of macrophages in the immune response to cancer could offer valuable insights for developing effective treatments.

The exploration of the therapeutic effects and mechanisms of natural products is of great significance [14, 15]. Dihydroartemisinin (DHA), a first-generation derivative of artemisinin, is well-known for its anti-malarial properties [16, 17]. Recent studies have demonstrated that DHA exhibits significant anti-cancer effects in vitro and in vivo, inhibiting the proliferation and migration of various tumor cells and promoting apoptosis [18, 19]. Moreover, some studies suggest that DHA may directly inhibit angiogenesis, but whether it indirectly affects tumor angiogenesis by influencing the paracrine function of tumor cells remains unclear [20]. Previous studies have primarily focused on the anticancer effects of DHA through inducing cell apoptosis and inhibiting the cell cycle. This study further reveals that DHA enhances the inhibition of lung cancer growth and lung cancer bone metastasis through macrophage polarization, providing a new dimension to the understanding of DHA's anticancer mechanisms.

In this study, we used a lung cancer A549 mouse model to evaluate the effects of DHA on tumor growth and metastasis. We also investigated the regulatory role of DHA in macrophage polarization and further explored the mechanisms underlying macrophage polarization and recruitment. These findings provide a theoretical basis and guidance for the clinical application of DHA.

Materials and methods

Reagents

Dihydroartemisinin (DHA) was purchased from Shanghai National Medicines. DMEM medium, anti-CD206, anti-CD86, PBS, and related assay kits were obtained from Beyotime Biotechnology.

Cell culture

RAW 264.7 mouse macrophages and A549 lung cancer cells, sourced from the Shanghai Institute of Biochemistry and Cell Biology, were maintained in DMEM medium enriched with 10% serum in a humidified incubator at 37 °C with 5% CO_2.

qRT-PCR

Cells were plated at 5×10^5 cells per well in 6-well plates and incubated at 37 °C until reaching ~ 80% confluence. IFNγ and LPS were introduced to induce M1 polarization, while IL-13 stimulated M2 polarization, with or without DHA pretreatment. Following a 24-h incubation, total RNA was extracted from RAW 264.7 cells and A549 cells using TRIzol. cDNA synthesis involved a premix reagent and gDNA removal kit (Toyobo, Osaka, Japan). Real-time PCR amplification was conducted using SYBR Green PCR master mix and a real-time thermal cycler.

Flow cytometry

RAW 264.7 cells were processed for flow cytometry after harvesting and BSA (BSA; Sigma-Aldrich, St Louis, MO, USA) blocking. They were incubated with fluorescently labeled antibodies (phycoerythrin-conjugated anti-mouse CD86 (BioLegend, San Diego, CA, USA), allophycocyanin-conjugated anti-mouse CD206 (BioLegend, San Diego, CA, USA), fluorescein isothiocyanate-conjugated anti-mouse F4/80 (BioLegend, San Diego, CA, USA)) for 24 h, and the proportion of each cell subset was analyzed using a FACSVerse machine (Becton Dickinson, San Jose, CA, USA).

Immunofluorescence staining

RAW 264.7 cells were induced to polarize into M1 or M2 macrophages and co-cultured with DHA for 24 h. Cells were then fixed, permeabilized, and blocked before immunostaining with specific antibodies against F4/80 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD86 (Cell Signaling Technology, USA), and iNOS (BioLegend, San Diego, CA, USA) or Arg-1 (BioLegend, San Diego, CA, USA). Secondary antibodies labeled with Alexa Fluor 488 or 647 (Invitrogen, Carlsbad, CA, USA) were used, and nuclei were counterstained with DAPI. Cells were observed under a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Cell migration assay

Using sterile pipette tips, A549 monolayer cells in 6-well plates were scratched at 90% confluence, with non-adherent cells removed. The cells were then incubated for 12 h with supernatants from macrophages cultured under different conditions. Images were captured at 0 and 12 h, and the scratch area measured to determine the average migration rate.

Transwell assay

RAW 264.7 cells were induced to differentiate and cultured with DHA in 6-well plates. After 24 h, we collected the conditioned medium. Next, A549 cells (1×10^5 cells/well) were seeded in the upper chamber of the Transwell. Cells were incubated for 24 h, fixed in 4% paraformaldehyde, permeabilized with 0.1% BSA, and stained. Cells were observed and photographed under a microscope after washing with PBS.

Animal model

Female Balb/c mice aged 6–8 weeks were housed under specific pathogen-free conditions for 1 week prior to experimentation. Tumor inoculation was conducted under isoflurane anesthesia by injecting 100μL of cell suspension through a vertical subcutaneous incision. The incision was sutured, and mice were administered with 50 mg/kg of meloxicam intraperitoneally for analgesia. Post-inoculation, tumor growth was monitored, and mice were grouped once tumors reached 50mm^3. Daily mouse body weights and tumor volumes, measured every other day using calipers, were recorded throughout the experiment. Two weeks after inoculation, primary tumors were excised, sutured with 5 mg/kg of meloxicam for analgesia, and stored at −80 °C in liquid nitrogen. Prior to storage, excised tumors were washed twice with PBS, weighed, and measured for volume. These tumor tissues were later used for histological and immunohistochemical analyses, including H&E staining. To obtain bone metastasis-bearing tumor mice, 100 μL of a suspension containing 1×10^7 A549 cells was injected into the tibial bone marrow cavity of female Balb/c mice. The analysis of DHA's inhibitory effect on lung cancer bone metastasis was then performed according to the aforementioned method.

Statistical analysis

GraphPad Prism 8.0 (La Jolla, CA, USA) was utilized for all statistical computations. The statistical relevance was assessed through either a Student's *t* test or a one-way ANOVA. The data are shown as the average ± standard deviation (SD), with a *P* value of less than 0.05 indicating statistical significance.

Results

DHA inhibits macrophage M2 polarization

Macrophage polarization plays a crucial role in tumor growth and metastasis. To elucidate the regulatory effect of DHA on macrophage polarization, RAW264.7 cells were treated with a series of DHA concentrations for 72 h, and cell viability was assessed using the CCK-8 assay. The results indicated that 10 μM DHA and honokiol did not affect cell proliferation (Additional file 1: Figure S1). Subsequently, RAW264.7 cells were treated with IL-13, a stimulus for M2 polarization, and various concentrations of DHA for 24 h. Flow cytometry analysis showed that IL-13 treatment significantly upregulated the expression of CD206, a marker for M2 polarization, in RAW 264.7 cells (Fig. 1A, B and Additional file 1: Figure S2). However, in the presence of DHA, CD206 expression was dose-dependently downregulated. Consistent with this, qRT-PCR analysis revealed a significant downregulation of Arg1, MRC1, Fizz1, and CCL2 at the mRNA level in cells co-cultured with IL-13 and DHA compared to those treated with IL-13 alone. Immunofluorescence staining also confirmed that IL-13 treatment increased CD206 expression, which was significantly abrogated by DHA treatment (Fig. 1D, E). These findings demonstrate that DHA inhibits macrophage M2 polarization.

DHA promotes macrophage M1 polarization

The impact of DHA on promoting macrophage M1 polarization was studied through a series of experiments involving RAW 264.7 cells. These cells were subjected to different treatments including IFNγ + LPS, as well as varying concentrations of DHA for a period of 24 h. Analysis using flow cytometry and PCR revealed noteworthy insights into M1 polarization. Upon stimulation with IFNγ + LPS, there was a noticeable increase in the expression of the M1 marker CD86 in the RAW 264.7 cells (Fig. 2A, B). Interestingly, treatment with DHA led to a further enhancement of CD86 expression, with higher DHA concentrations resulting in a more pronounced effect. PCR analysis demonstrated a significant upregulation of CD11c, IL12p40, TNFα, and Stat1 at the mRNA level in the presence of DHA, compared to the IFNγ + LPS group (Fig. 2C). Immunofluorescence analysis corroborated these findings, showing a substantial increase in CD86 expression after DHA treatment (Fig. 2D, E). This suggested that DHA has the ability to promote M1 polarization of macrophages, as evidenced by the increased expression of M1-related markers. Overall, these results shed light on the potential immunomodulatory effects of DHA in enhancing macrophage polarization towards the M1 phenotype.

DHA inhibits tumor cell migration and invasion by regulating macrophage behavior

The impact of DHA on tumor cell migration and invasion was explored in relation to macrophage behavior. RAW 264.7 cells were exposed to IL-13 and varying doses of DHA for 24 h, and the resulting supernatant was collected for analysis. Through cell scratch and Transwell assays, the migration and invasion abilities of A549 cells were evaluated after co-culturing with the supernatant. Notably, the migration of cells increased when treated

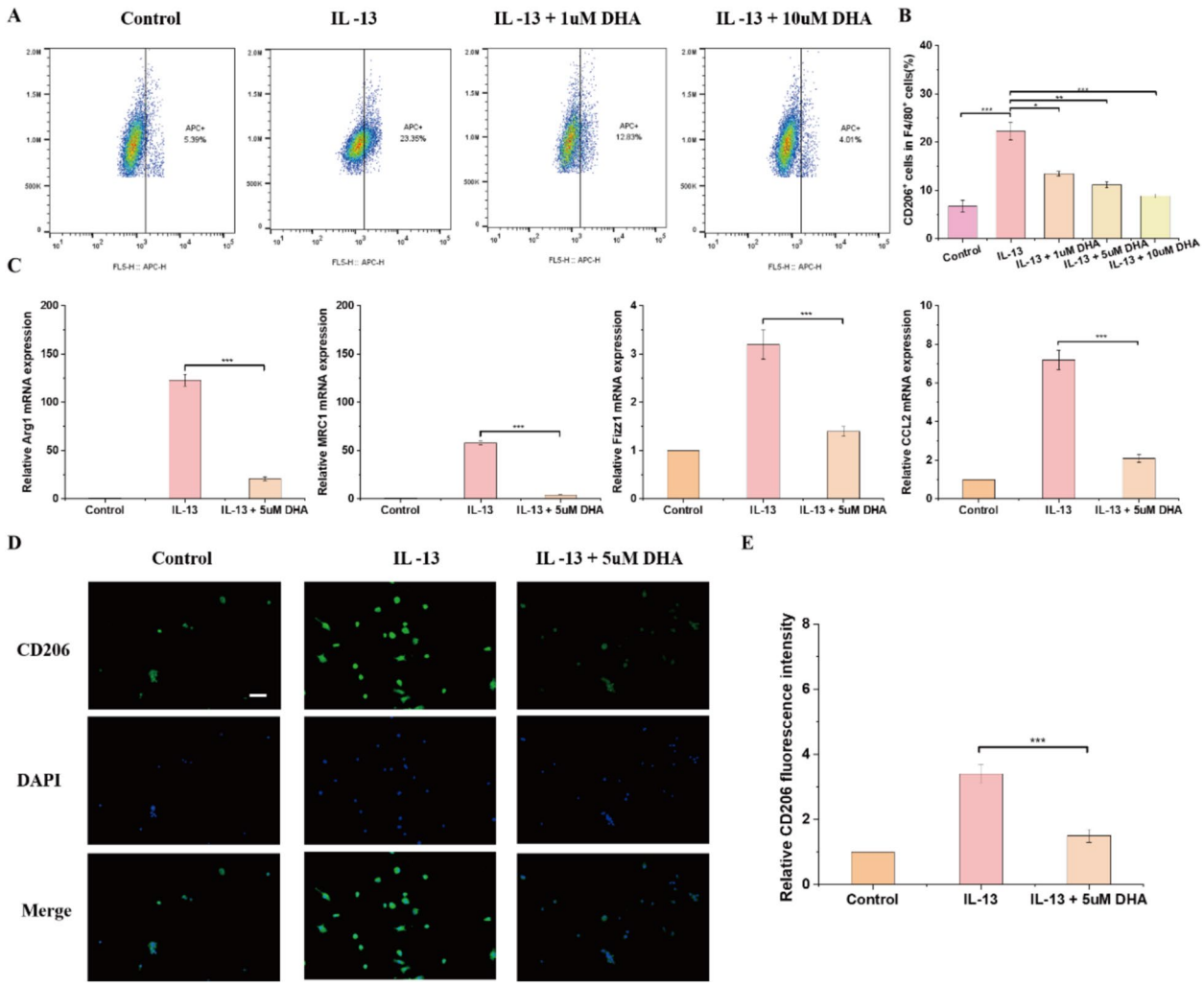

Fig. 1 Effect of DHA on M2-like polarization of IL-13-treated macrophages. **A** Expression of the M2 marker CD206 in RAW264.7 cells. **B** Quantitative analysis of CD206 expression. **C** Real-time RT-PCR was used to measure the mRNA levels of M2 marker genes. **D** Immunofluorescence analysis of CD206 expression. **E** Quantitative analysis. Scale bar, 25 μm. ***$p < 0.001$

with IL-13 alone, but DHA treatment effectively hindered A549 cell migration (Fig. 3A, B). Furthermore, the invasion capability of the cells was notably diminished as well (Fig. 3C, D). These findings indicate that DHA plays a pivotal role in suppressing the aggressive nature of tumor cells by impeding M2 polarization of macrophages, showcasing its potential as an anti-tumor metastasis agent in vivo. These results shed light on the promising therapeutic implications of DHA in combating cancer progression.

DHA inhibits lung cancer growth and metastasis in vivo

DHA has shown promising results in inhibiting lung cancer growth and metastasis in a recent in vivo study. Researchers established a subcutaneous lung cancer model using A549 cells and treated the animals with DHA to observe its effects on tumor progression. The results were astounding—the tumor volume and mass in the DHA treatment group were significantly reduced compared to the control group, indicating a powerful inhibitory effect on tumor growth (Fig. 4A–C). Importantly, as shown in 4D, there was no difference in body weight between the treatment and control groups, showing that DHA was well-tolerated by the animals. Surprisingly, the number of bone metastases in the DHA treatment group was significantly lower than in the control group, suggesting that DHA may also play a role in preventing cancer spread (Fig. 4E, F). Moreover, compared to the control group, the treatment group showed a significant reduction in tumor cell atypia, with nuclear size and nuclear-to-cytoplasm ratio approaching normal levels, and the cell arrangement becoming more organized (Fig. 4E). These findings shed light on the potential of DHA as a therapeutic agent for lung cancer.

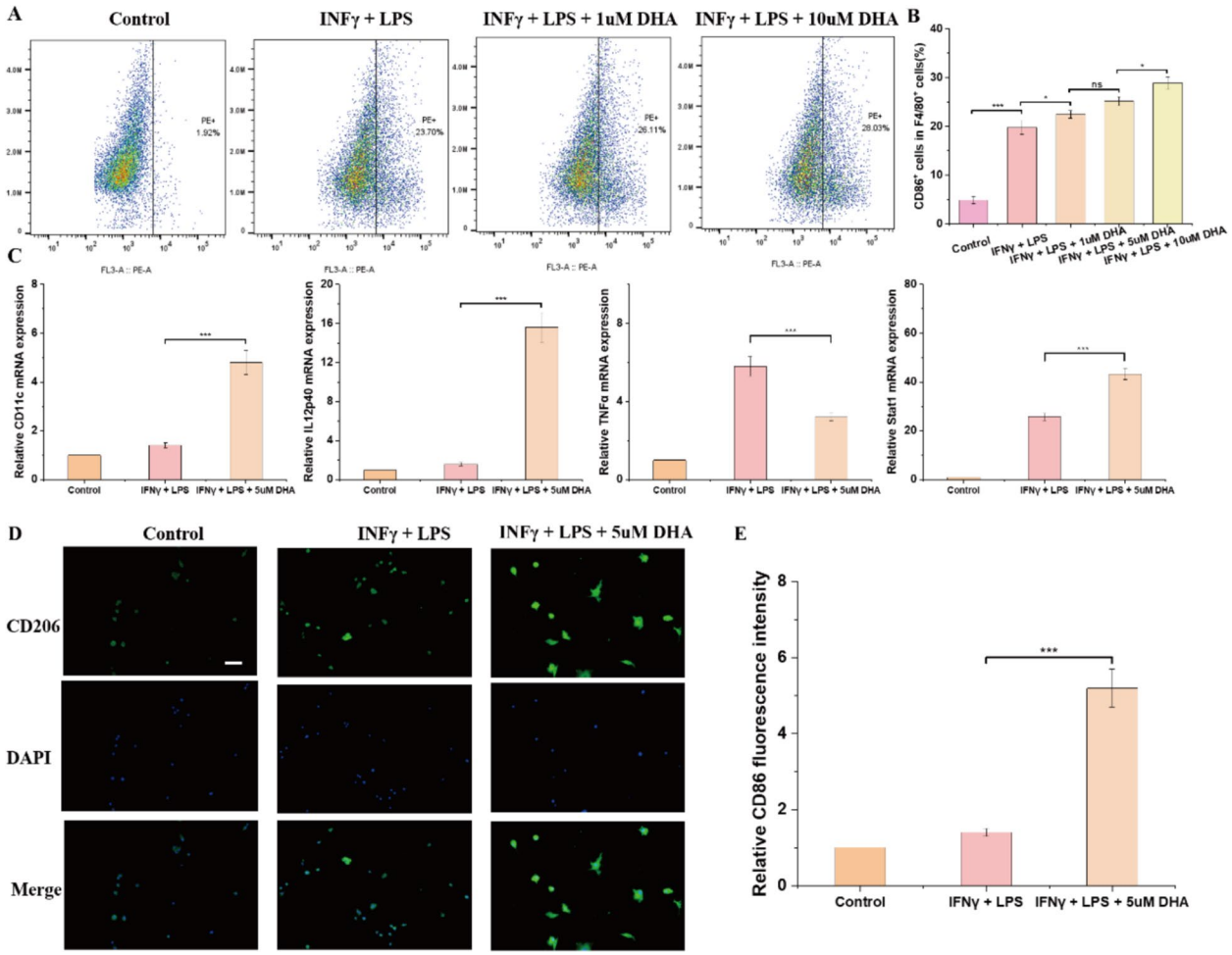

Fig. 2 Effect of DHA on M1 polarization of LPS/IFNγ-treated macrophages. **A** Expression of the M1 marker CD86 in RAW264.7 cells. **B** Quantitative analysis. **C** Real-time RT-PCR was used to measure the mRNA levels of M1 marker genes. **D** Immunofluorescence analysis of CD86 expression. **E** Quantitative analysis. Scale bar, 25 μm. ***$p < 0.001$

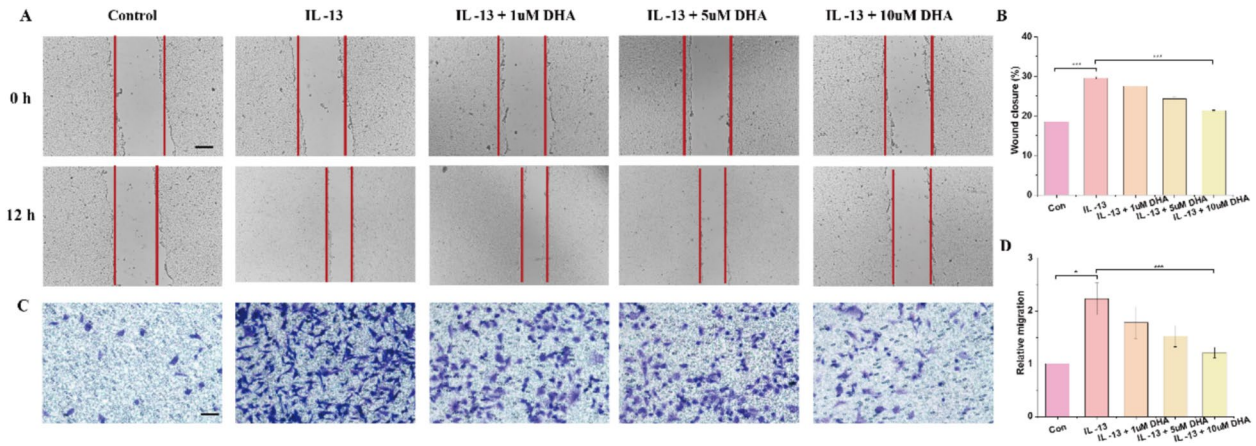

Fig. 3 Effect of DHA on the malignant behavior of lung cancer cells A549. **A** Representative images of A549 cell migration after co-culture with different concentrations of DHA for 24 h. **B** Quantitative analysis. **C** Representative images of A549 cell invasion after co-culture with different concentrations of DHA for 24 h. **D** quantitative analysis. Scale bar, 100 μm. ***$p < 0.001$

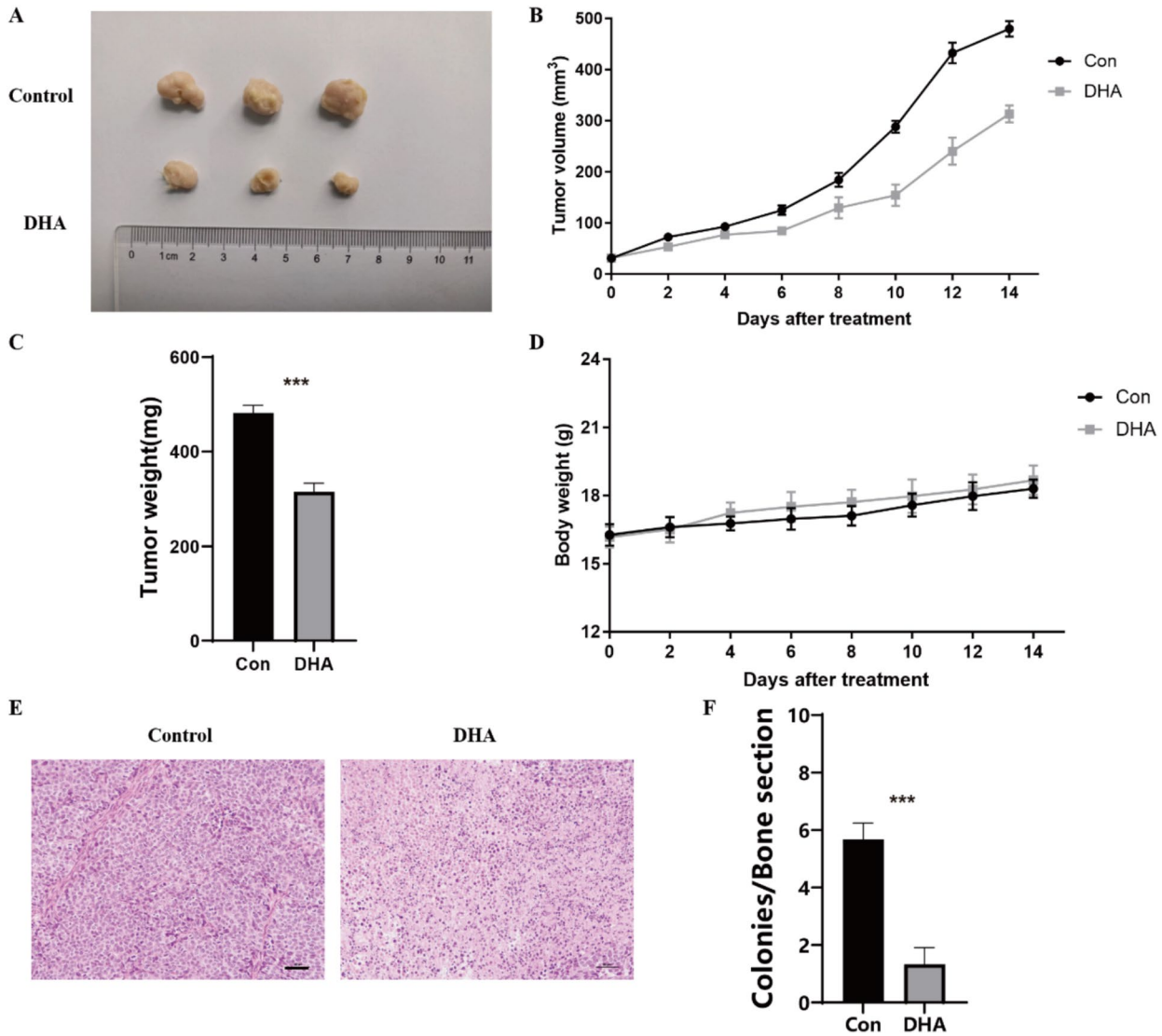

Fig. 4 Inhibitory effect of DHA on lung cancer in vivo. **A** Representative images of tumors in mice after treatment. **B** Changes in tumor volume during the treatment period. **C** Tumor mass at the end of the treatment. **D** Changes in mouse body weight during the treatment period. **E** Representative images of H&E staining of primary lung cancer in mice at the end of the treatment. **F** Statistics of bone metastasis

The study aimed to investigate the effect of DHA on lung cancer bone metastasis by analyzing macrophage polarization in primary lung cancer and metastatic sites. Through PCR analysis, the expression of key factors related to macrophage polarization was measured at bone metastasis sites. As shown in 5A and 5B, the results revealed a significant upregulation of M1-related genes and a downregulation of M2-related genes in the DHA treatment group, indicating a shift towards M1 polarization. Immunohistochemical analysis further supported these findings, showing a decrease in CD206 and an increase in CD86 at bone metastasis sites upon DHA treatment (Fig. 5C, D). Moreover, similar changes were observed in macrophages at the primary tumor site, suggesting a consistent pattern of M1 polarization and M2 inhibition due to DHA treatment (Fig. 5E, F). These results imply that DHA can effectively inhibit lung cancer bone metastasis by promoting M1 polarization and suppressing M2 polarization of macrophages at both primary and metastatic sites. Overall, the data underscores the potential of DHA as a therapeutic agent for targeting macrophage polarization in the context of lung cancer metastasis, offering new insights into the mechanisms underlying its anti-metastatic effects.

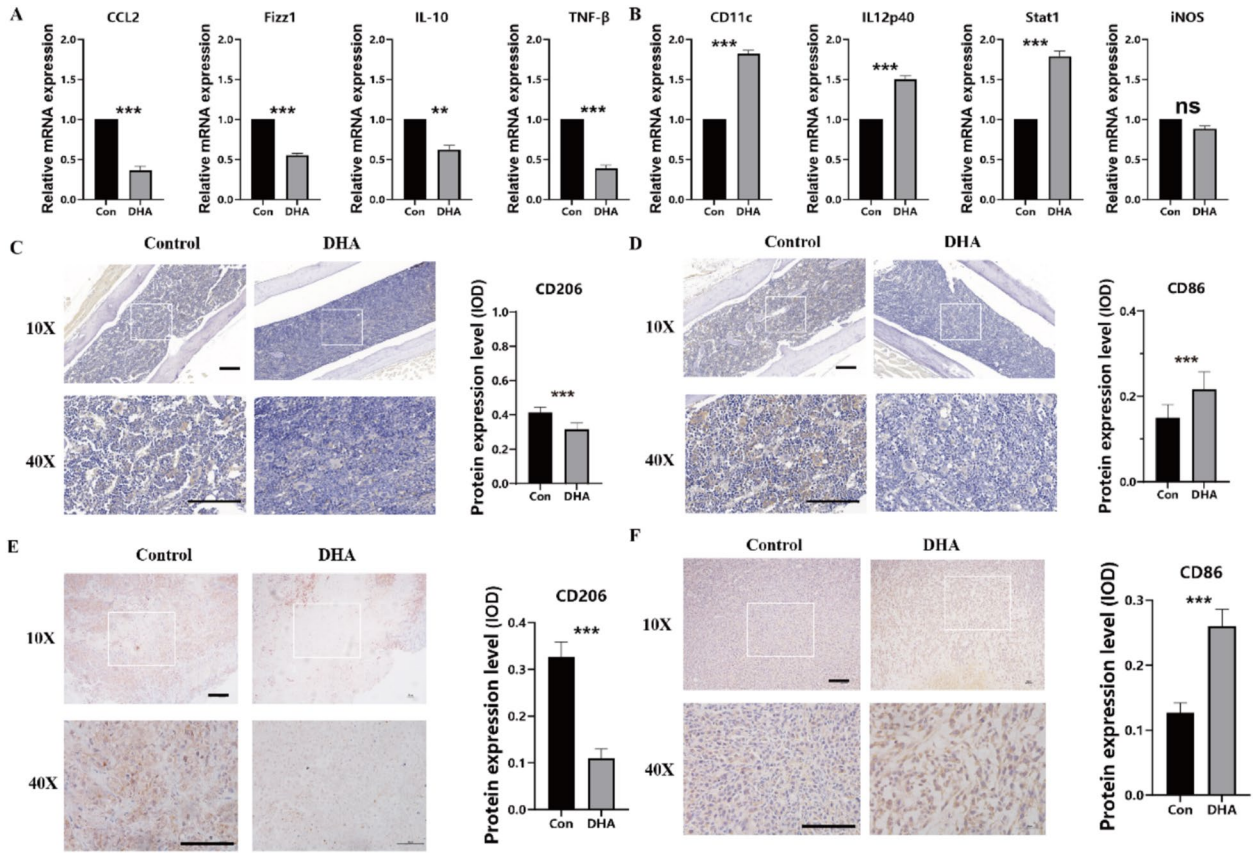

Fig. 5 Regulatory effect of DHA on macrophage polarization in vivo. **A** mRNA expression levels of M2-related markers in bone metastasis tissues. **B** M1-related markers. **C** Immunohistochemical staining and quantitative analysis of CD206 in bone metastasis tissues. **D** Immunohistochemical staining and quantitative analysis of CD86 in bone metastasis tissues. **E** Immunohistochemical staining and quantitative analysis of CD206 in primary lung cancer tumors. **F** Immunohistochemical staining and quantitative analysis of CD86 in primary lung cancer tumors. Scale bar, 100 μm. ***$p < 0.001$

Mechanism of DHA regulation of macrophage polarization

The regulation of macrophage polarization by DHA was examined in detail to understand its mechanism. Initially identified as a potent chemokine produced by tumors, CCL2 has been known to attract various immune cells and promote inflammation and angiogenesis, ultimately leading to tumor progression and a poor prognosis (Fig. 6A). This study focused on the expression of CCL2 and its receptor, CCR2, in the primary tumor site. As shown in 6B, the results revealed a significant decrease in

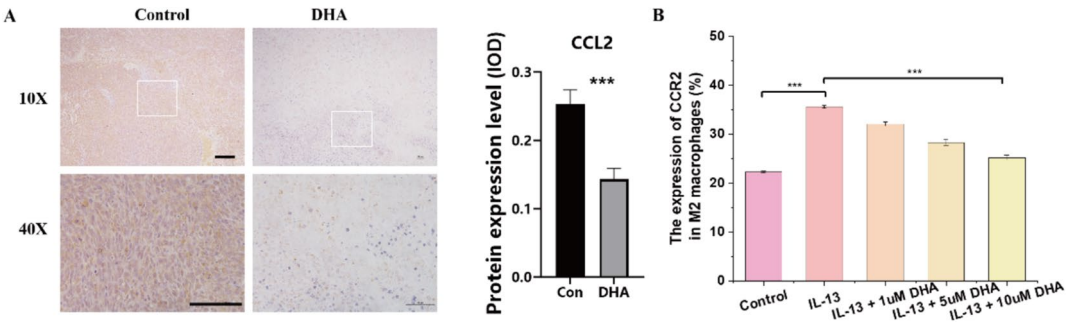

Fig. 6 DHA regulates macrophage recruitment to lung tissues through the CCL2/CCR2 pathway. **A** Expression and quantitative analysis of CCL2 in primary lung cancer tissues. **B** PCR quantification of CCR2 in primary lung cancer tissues. ***$p < 0.001$

CCL2 expression and CCR2 expression in all treatment groups. This indicates that DHA effectively inhibits the recruitment and accumulation of macrophages in tumor tissues by targeting the CCL2/CCR2 signaling pathway. By modulating the expression of these key molecules, DHA plays a crucial role in controlling the inflammatory response and ultimately affecting the progression of the tumor microenvironment. These findings shed light on the potential therapeutic benefits of DHA in cancer treatment.

Discussion

Malignant tumors are a global threat to human life, ranking alongside cardiovascular and respiratory diseases as a leading cause of death. Research has shown that DHA has promising anti-tumor properties across various types of cancer, without harming normal cells at appropriate doses. DHA serves as a valuable adjunct to traditional chemotherapy, boosting resistance and enhancing tumor destruction. In addition, DHA has standalone capabilities in inhibiting tumor growth effectively. It has demonstrated anti-cancer effects in lung, breast, prostate, ovarian, and gastrointestinal cancers. These findings highlight the potential of DHA as a natural treatment option for combating malignant tumors, offering hope for improved outcomes in cancer patients worldwide.

Tumor metastasis is a complex process, where cancer cells break away from the original tumor, travel through the body, and establish new growth in other locations. Despite advancements in cancer treatment, the high mortality rate of cancer is largely attributed to metastasis. Therefore, finding effective ways to prevent tumor metastasis is crucial in improving cancer outcomes. Research indicates that Docosahexaenoic acid (DHA) has the potential to inhibit the spread of cancer cells in gastric cancer by targeting key pathways involved in metastasis. Studies have shown that DHA can hinder the growth and epithelial–mesenchymal transition of gastric cancer cells by interfering with signaling pathways, such as snail and PI3K/AKT. In addition, DHA has been found to suppress the activation of cancer-associated fibroblasts (CAFs) in both human and mouse models by disrupting interactions between tumors and the tumor microenvironment. This inhibition of CAFs through the regulation of transforming growth factor-beta (TGF-β) signaling ultimately leads to the suppression of tumor metastasis. While numerous studies have highlighted the anti-metastatic properties of DHA in various cancers, limited research has explored its role in preventing lung cancer bone metastasis. Further investigation into the mechanisms by which DHA inhibits metastasis in different types of cancer could pave the way for novel therapeutic approaches in cancer treatment.

The TME is known to significantly impact immune regulation in lung cancer [21]. This environment can be categorized as either immunoreactive or immunosuppressive, depending on its role in the immune response [22]. In lung cancer, tumor-infiltrating lymphocytes (TILs) are the primary cell types present in the TME, showing diversity in their composition within the tumor nest and stroma. Various subtypes of TILs play vital roles in immune regulation through different mechanisms, affecting immune modulation in lung cancer [23]. Within the immunoreactive TME, M1 macrophages, also referred to as classical macrophages, exhibit pro-inflammatory effects that aid in activating immune responses against tumor formation. Recent research in our laboratory has shown that DHA has the potential to hinder M2 macrophage polarization while promoting M1 polarization. This process has been found to regulate tumor growth and metastasis in lung cancer.

DHA, a compound found in fish oil, has been found to reduce the recruitment and accumulation of inflammatory monocytes in tumor tissues, leading to decreased tumor metastasis. This is achieved by targeting the CCL2/CCR2 signaling pathway, which is responsible for mediating tumorigenesis and metastasis [24, 25]. CCL2 recruits monocytes to the tumor microenvironment by binding to CCR2, where they differentiate into pro-tumor M2 macrophages (TAMs). TAMs promote tumor growth, angiogenesis, and metastasis by secreting pro-inflammatory cytokines (such as IL-6 and TNF-α) and growth factors (such as VEGF and EGF). By inhibiting this pathway, DHA shows promising potential in preventing the spread of cancer cells in the body [26]. Research indicates that DHA can impact the growth of lung cancer and bone metastasis by influencing the polarization and recruitment of macrophages. DHA suppresses M2 polarization and enhances M1 polarization, as well as modulating tumor recruitment through the CCL2/CCR2 pathway. These discoveries could have important implications for the use of DHA in clinical settings to treat cancer effectively.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40001-025-02467-w.

Additional file 1: Fig. S1. CCK-8 assay for the cytotoxicity of DHA against RAW264.7 cells. Fig. S2. Expression of the M1 marker CD86 and M1 marker CD206 in RAW264.7 cells treated with 5 μM DHA.

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Author contributions
G.H. prepared the original draft of the manuscript and X.G. developed the software necessary for the research and Y.G. assisted in acquiring resources,

curated the data sets, and created visualizations to represent the research findings and P.G. provided extensive review and editing of the manuscript, ensuring clarity and accuracy, and supervised the writing process and C.H. managed the project 's administrative aspects and successfully acquired funding to support the research endeavors and A.C. played a pivotal role in conceptualizing the study, designing the methodology, overseeing the research team, providing critical review and editing of the manuscript, managing the project's administrative aspects, and securing financial support for the research. All authors reviewed the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The Animal Care and Use Committee of Nanjing Medical University approved all animal experiments (Permit number: IACUC-2105056).

Consent for publication
N/A.

Competing interests
The authors declare no competing interests.

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