CDDO-ME Activates NRF2 to Inhibit The Pro-Invasion Ability of TAMs

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Research Article

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CDDO-ME activates NRF2 to inhibit the pro-invasion ability of TAMs

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Abstract:

Background: Tumor-associated macrophages can account for more than 50% of the cells in the tumor immune microenvironment of breast cancer patients. A high TAM density is related to a poor clinical prognosis. Targeting TAMs is a promising therapeutic strategy since TAMs promote tumor growth, development and metastasis.

Results: We found that CDDO-ME significantly inhibited the tumor invasion-promoting ability of TAMs in the coculture system and further showed that CDDO-ME functioned by reducing ROS production in TAMs. The orthotopic 4T1 cell inoculation model and spontaneous MMTV-PyMT tumor model were used to evaluate the antitumor effect of CDDO-ME. The results showed that CDDO-ME significantly inhibited tumor metastasis and increased T cell infiltration into the tumor microenvironment. Mechanistically, NRF2 activation was necessary for CDDO-ME to exert its function.

Conclusions: Overall, CDDO-ME can play a role in breast cancer as an anticancer drug targeting TAMs.

Keywords*:* CDDO-ME, NRF2, TAMs, anti-cancer drug, breast cancer

Introduction

Breast cancer is a malignant tumor with high incidence among women all over the world, and it is a heterogeneous disease. According to the expression differences of Estrogen receptor (ER), Progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), breast cancer can be divided into four molecular subtypes. That is, Luminal A(ER^+/PR^+HER2^-), Luminal B(ER^+/PR^+HER2^+ or ER^+/PR^+ Ki67 > 14%), HER2($ER^-PR^-HER2^+$), TNBC(ER^-PR^-HER2). There are obvious differences in the incidence, treatment response and related risk factors of different subtypes[1]. According to the treatment guidelines, endocrine intervention, chemotherapy and other drugs are used as the first-line treatment for breast cancer patients in China, and selective estrogen receptor modulators (SERMs), selective estrogen receptor down-regulation (SERDs), aromatase inhibitors (AI), docetaxel or synergistic drug regimen are mainly used for treatment. Although these treatment strategies are the most important means in the comprehensive treatment of breast cancer patients, and have remarkable curative effect, greatly improving the clinical evaluation endpoints such as OS and PFS, but because the drug resistance rate reaches 30-40%,

the recurrence and metastasis of drug-resistant related tumors are still the bottleneck in the clinical treatment of various subtypes of breast cancer [2]. Therefore, it is obviously urgent to formulate new, innovative and positive methods to deal with this potential disease.

A large number of studies have shown that tumor microenvironment has obvious malignant and non-malignant cell types[3]. Tumor-associated macrophages (TAMs) can account for up to 50% of tumor mass [3, 4]. High TAM density is associated with poor clinical prognosis of patients with solid tumors including breast cancer, prostate cancer, cervical cancer and ovarian cancer [5]. TAMs are the key cells connecting inflammation and tumor, which can directly promote the occurrence, development and metastasis of tumor by releasing various inflammatory factors, growth factors and matrix proteases, or indirectly promote tumor progression by mediating tumor angiogenesis and tumor immunosuppression. TAM has become an important target for cancer treatment. Conditional M-CSF gene knockout of mammary epithelial cells leads to TAMs deletion, which leads to the obvious delay of tumor progression and inhibition of lung metastasis in mouse model of T-type oncoprotein (PyMT) in ER mammary epithelial cells[6, 7]. These findings suggest that TAM phenotype and function are the key factors to promote tumor growth.

Triterpenoids are widely used in Asian medicine, including oleanolic acid (OA) and ursolic acid (UA), which have weak anti-inflammatory and anti-cancer effects[8]. Triterpenoids have multidirectional effects. At low dose, they show anti-inflammatory and anti-oxidative stress, while at moderate dose, they can induce cell differentiation, while at high dose, they play the roles of cytotoxicity, anti-proliferation and apoptosis. CDDO-Me is a synthetic oleanane triterpenoid (SOS), which is derived from natural pentacyclic triterpenoid oleanolic acid, and its anti-inflammatory ability is more than 10,000 times that of its parent OA. CDDO-ME is an inhibitor of kinase IKKα which phosphorylates IκBα, and cause inhibition of NF-κB[9]. CDDO-Me is also an activator of NRf 2, which can cause protective response to stress caused by injury and oxidation[10]. It has been proved that CDDO-ME can delay the development of breast tumor and inhibit the growth of established tumor in the transgenic model of mouse breast tumor virus (MMTV-neu). In the invasive PyMT model of ER breast cancer, recent studies have shown that CDDO-Me not only delays the occurrence of tumors, but also inhibits TAM infiltration of breast tumors [11]. CDDO-Me reduces the expression of proinflammatory cytokines of various cell types (including but not limited to

tumor necrosis factor-α, interleukin-1β, IL-6 and interferon-γ) [12-15]. It is worth noting that CDDO-Me has an opposite effect on M2 macrophages because it reduces anti-inflammatory cytokines such as IL-10 and increases the production of TNF-α and IL-6[16].

Here, we have now proved for the first time that CDDO-ME inhibited the invasion of tumor cells by acting on TAMs at a lower concentration. In further study, it was found that CDDO-ME can increase the proportion of $CD8^+$T cells in tumor and inhibit the invasion of breast cancer. Mechanically, we found that CDDO-ME reduced the release of ROS in macrophages by activating NRF2, thus weakening the invasion ability of tumor cells. These results indicate that CDDO-Me plays an important role in the treatment of breast cancer.

Material and Methods

Cells

4T1 cells and RAW264.7 cells were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. 4T1 cells were cultured in RPMI 1640, RAW264.7 cells were cultured in DMEM, supplemented with 10% FBS (Gibco), 2 mM l-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. The cells were kept in a humidified atmosphere of 5% CO_2 at 37 °C.

Reagent

CDDO-ME(purity>99%) was dissolved in 100% DMSO, The final DMSO concentration in cell culture did not exceed 0.1% throughout the study. PE-conjugated anti-mouse CD206 (Clone M1), PE-conjugated anti-mouse CCR7, PE-conjugated anti-mouse MGL1/2, PE-conjugated anti-mouse MHCII and corresponding isotype controls were purchased from BD Pharmingen (San Diego, CA). enicillin and streptomycin, DCFH-DA, HRP-conjugated goat anti-mouse IgG (H+L), MTT are from Beytotime (Haimen, Jiangsu, China). Annexin-V/PI apoptosis kit was purchased from BD Pharmingen (San Diego, CA). Matrigel and N-acetyl cysteine (NAC) were purchased from Sigma (St. Louis, MO).

Macrophage/Tumor Cell Coculture

Coculture experiments were performed according to the methods used by Qingshang Wang, et al[17]. A PET film 6-hole hanging cell culture chamber (Millipore, Billerica, MA) was used.

RAW264.7 cells were co-cultured with 4T1 cells at a 1: 4 ratio in complete medium (CM) for 72 h. The serum concentrations of the two cell types were kept consistent.

Cell Invasion Assay

The cell invasion assay in this study was performed according to the methods used by Xinyu He, et al[18].The ability of liver cancer cells to migrate through Matrigel-coated filters was measured using Transwell chambers (Costar, Cambridge, MA) with polycarbonate membranes (8.0-μm pore size) coated with 100 μl of Matrigel (BD Biosciences) on the top side of the membrane. The upper surface of the matrix was challenged with 40,000 4T1 cells, and cells were kept in serum-free medium. The lower chamber contained medium supplemented with 10% serum. After 24 h, the cells were stained with 0.1% crystal violet solution. Cells and Matrigel on the upper surface of the membrane were removed carefully with a cotton swab.

Gene Expression Analysis

TRIzol reagent (Invitrogen) was used to prepare total RNA from macrophages or tissues. Total RNA (1.5μg) was reverse transcribed using a first strand cDNA synthesis kit (Biot-eke, Beijing, China). Primers used in the real-time PCR were GAPDH, forward 5'-AACT TTGGCATTGTGGAAGG-3', reverse, 5'-ACACATTGGGGGTAGGAACA-3'; G6PD,forward, 5'-CACAGTGGACGACATCCGAAA-3', reverse, 5'-AGCTACATAGGAATTACGGGCAA-3'; IDH1, forward, 5'-ATGCAAGGAGATGAAATGACACG-3', reverse, 5'-GCATCACGAT TCTCTATGCCTAA-3'; GCLC, forward, 5'-GGGGTGACGAGGTGGAGTA-3', reverse, 5'-GTTGGGGTTTGTCCTCTCCC-3'; Txn1, forward, 5'-CATGCCGACCTTCCAGTTTTA-3', reverse, 5'-TTTCCTTGTTAGCACCGGAGA-3'; PRDX1, forward, 5'-AATGCAAAAATTG GGTATCCTGC-3', reverse, 5'-CGTGGGACACACAAAAGTAAAGT-3'. GAPDH was used as the normalized gene. Q-PCR assays were carried out on the CFX96 real-time PCR de -tection system (Bio-Rad), using the Q-PCR kit (Bio-Rad). The comparative threshold meth -od for relative quantification was used, and results are expressed as -fold change. The pri -mers were synthesized by Invitrogen.

Western blotting

The cell protein was extracted by whole cell lysis or a nuclear protein extract kit purchased from Beytotime (Haimen, Jiangsu, China), which contained protease and phosphatase inhibitors. The cell debris was removed by centrifugation at 4℃, and the supernatants were collected and

stored at -70℃ until use. The protein amounts were determined using the BCA protein assay (Pierce) according to the manufacture's instruction. For western blot analysis, the proteins were electrophoresed on a 10% SDS-PAGE gel, followed by immunoblotting on PVDF membrane (American Biosciences). Immune complexes were incubated with peroxidase–labelled anti-rabbit or anti-mouse antibody (Kangchen, China) for 2h at room temperature. The blots were visualized with an enhanced chemiluminescent method kit (Sino-American Biotechnology, PR China).All the uncropped data was shown in Supplementary Fig.1.

Flow Cytometry Analysis

For ROS analysis [19]: The ROS production was examined by DCFH-DA probe. After the stimulation medium were removed, cells were washed twice with 2 ml warmed PBS. 1 mL PBS containing 10 μM DCFH-DA was added to the cells and incubated for 20 min at 37 °C. Cells were then washed with PBS and then subjected to the flow cytometry analysis.

For apoptosis analysis[20]: cells were stained with Annexin V–FITC in the presence of propidium iodide (PI) using Annexin V-FITC apoptosis detection kit according to the manufacturer's instruction (BD, America).

For evaluation of macrophages phenotypes:RAW264.7 in different treated condition were incubated with PE-conjugated anti-mouse CD206 (Clone M1) antibody, PE-conjugated anti-mouse CCR7 antibody, PE-conjugated anti-mouse MGL1/2 antibody, PE-conjugated anti-mouse MHC Ⅱ antibody for 30 min on ice followed by flow cytometry detection.

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry[21]. The cells were immobilized overnight with 75% ethanol at 20℃ and stained with 0.1% TritonX-100, 100 ng/ml PI and 10 mg/ml RNase at 4℃ for 30 min. The proportion of cells in G1, S and G2 phases was expressed by DNA histogram.

MTT

MTT assay was performed according to methods in previous study[22]. 5000 cancer cells were inoculated into 96-well plates, and treated with different concentrations of CDDO-ME for 24h and 48h, with 4 replications, including untreated control groups. MTT assay was used to detect

adherent cells according to the manufacturer's protocol (Beytotime, Haimen, Jiangsu, China). The average optical density of the control cells was 100%, and the treatment results were expressed as the percentage of the control.

In vivo assays

Six-week-old female BALB/c mice from Gempharmatech (Nanjing, China) were used for all animal experiments. All mice were housed in our Laboratory Animal Centre at the Southeast University. All protocols involving animal experiments were approved by the Ethics Committee of Southeast University(20200624007), and all methods were carried out in accordance with relevant guidelines and regulations. When the cell concentration reached 80%, the cultured 4T1 cells were isolated from monolayer culture, washed with serum-free medium, resuspended in RPMI 1640, 1×10^7 cells/ml, and then the 20 μl cell suspension was injected into the inguinal mammary fat pad of BALB/c mice with syngeneic immunological activity. The mice were euthanized by CO2 asphyxiation, and tumour were collected for experimental assays.

Statistical analysis

The data were expressed as the mean ± SEM. The statistical analysis was performed by the Student's *t*-test when only two value sets were compared. A one-way ANOVA followed by a Dunnett's test were used when the data involved three or more groups. $P < 0.05$, $P < 0.01$, $P < 0.001$ or $P < 0.0001$ were considered statistically significant and indicated by *, ** , *** or **** respectively.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Results

1. CDDO-ME inhibited pro-invasion ability of TAMs

First, we analyzed the effect of CDDO-ME on the viability of RAW264.7 macrophages at different concentrations by MMT assay. The results showed that CDDO-ME had significant inhibitory effect on the viability of RAW264.7 macrophages when the concentration was higher than 100 nM (figure 1A and 1B). Additionally, 100 nM CDDO-ME had no effect on the viability

of 4T1 tumor cells (figure 1C), so 100nm CDDO-ME is a suitable dose for culturing cell models in vitro. Further, we constructed co-culture system of macrophages and tumor cells in vitro for inducing macrophages to differentiate into TAM. In the co-culture system, the presence of macrophages significantly enhanced the invasion and migration ability of 4T1 tumor cells (figure 1D and 1E). However, it was found that CDDO-ME did not affect TAM pro-migration ability (figure 1D), but significantly inhibited TAM pro-invasion ability (figure 1E). In addition, we examined the effect of CDDO-ME on the proliferation, apoptosis and cell cycle of 4T1 cells in the co-culture system (figure 1F-H). The results showed that there was no difference between proliferation, apoptosis and cell cycle. The results showed that CDDO-ME specifically affected the invasion promoting ability of TAMs.

2. CDDO-ME inhibits pro-invasion ability of TAMs by reducing ROS production.

In order to assess the effect of CDDO-ME on macrophage phenotype, flow cytometry was used to analyze the expression of membrane molecules (MHCII, CCR7, CD206, MGL1/2) in macrophages after co-culture. We found that CDDO-ME had no effect on macrophage membrane molecules expression (figure 2A). At the same time, phagocytosis assay using fluorescent red latex beads showed that CDDO-ME didn't affect the phagocytosis (figure 2B) of macrophages. Furthermore, we evaluated the level of inflammatory cytokines co-culture system in presence of CDDO-ME. The results showed that there was no difference in the secretion of IL-6, IL-10, IL-12 and TNFα (figure 2C). Reactive oxygen species assay using DCFH-DA probe showed CDDO-ME decreased ROS production of macrophage (figure 2D). ROS plays an important role in the initiation and progression of cancer [23] and stimulate tumor progression by promoting cell proliferation, survival, invasion and metastasis[24]. In the co-culture system, N-acetyl cysteine (NAC), an antioxidant agent to inhibit the production of ROS. The results showed that the invasion ability of tumor cells was significantly inhibited (figure 2E). Further, we added H_2O_2 to simulate ROS while CDDO-ME was treated. The results showed that the invasion promotion ability of TAM was increased (figure 2F). In short, these results show that CDDO-ME inhibits TAM's invasion promoting ability by reducing ROS production.

3. CDDO-ME inhibit tumor growth and metastasis in 4T1 orthotopic inoculation model of

breast cancer

In order to further evaluate the effect of CDDO-ME on tumor in vivo, we constructed a 4T1 orthotopic inoculation model of breast cancer. One week after 4T1 cells were injected into the inguinal mammary fat pads of Balb/c mice, CDDO-Me with different concentrations (2.5 μg/kg, 5μg/kg, 10μg/kg) or vehicle was given intragastrically once every 2 days (n=5), and paclitaxel (a chemotherapeutic drug for breast cancer in clinical treatment) was used as positive control. The experiment was terminated on the 28th day. To detect the effect of CDDO-Me on tumor growth and progression, the tumor size (length x width 2) was measured by caliper, and the tumor volume was calculated according to the formula (length x width 2)/2. As shown in FIG. 3A and 3C, CDDO-ME significantly inhibit that growth of 4T1 tumor compared to control animal, but there was no significant difference in body weight (figure 3B). In addition, we also observed that lung metastasis of 4T1 tumor was significantly inhibited by CDDO-ME (figure 3D and 3E). Further, we analyzed the infiltration of T cells in tumor microenvironment. The results showed that after CDDO-ME administration, the infiltration of T cells was significantly up-regulated (figure 3F-I). These data prove the in vivo therapeutic effect of CDDO-ME on breast tumors.

4. CDDO-ME inhibits tumor metastasis in MMTV-PyMT spontaneous breast cancer model

MMTV-PyMT transgenic mice is a kind of spontaneous breast cancer tumor model[25]. From the perspective of tumor occurrence, the model is very similar to human breast cancer, and the experimental results are more beneficial to serve as the basis for clinical research in the future. Therefore, we further carried out efficacy evaluation of CDDO-ME under this model. The administration process was consistent with 4T1 orthotopic inoculation model. The results showed that although CDDO-ME had little effect on tumor weight (figure 4A-C), lung staining showed that CDDO-ME significantly inhibited tumor lung metastasis (figure 4D and 4E). Further, we observed an increase in $CD8^+$ T cell infiltration in tumor microenvironment from CDDO-ME-treated mice (figure 4F-I), but there was no difference in tumor size, indicating that the increase in $CD8^+$ T cells was not sufficient to inhibit tumor growth.

5. CDDO-ME inhibits TAM pro-invasion ability via the activation of NRF2.

CDDO-ME is a pharmacological activator of NRF2. We first speculated whether CDDO-ME

can activate the activity of NRF2. Western blotting showed that the expression of NRF2 in co-cultured macrophages increased after CDDO-ME stimulation (figure 5A), and Q-pcr showed that the expression of downstream genes of NRF2 was significantly up-regulated (figure 5B). Furthermore, we constructed NRF2 knockout macrophages. The results showed that NRF2 knockout significantly enhanced ROS production in macrophages after co-culture with tumor cells in the presence of CDDO-ME (figure 5C). And CDDO-ME did not inhibit the ability to promote invasion of TAM (figure 5D), indicating that CDDO-ME weakened the ability to promote invasion of TAM by activating NRF2.

Discussion

In this study, it was proved for the first time that CDDO-ME significantly reduce the pro-invasion ability of TAMs, and inhibited tumor metastasis in 4T1 orthotopic breast cancer and MMTV spontaneous breast cancer models. Although the direct cytotoxic effect of CDDO-ME on tumor cells has been reported[8], immune function is very important for CDDO-ME to inhibit tumor growth, since tumors growing in SCID mice injection model lacking functional lymphocytes do not respond to CDDO-ME treatment [26]. In addition, Nagaraj et al demonstrates that CDDO-ME inhibited the activation of myeloid-derived suppressor cells (MDSCs) by blocking ROS production. It is suggested that innate immune cells may be an additional target of CDDO-ME.

In the phase III clinical trial of treating chronic kidney disease caused by type 2 diabetes mellitus, CDDO-ME was forced to stop because of its abnormal mortality. Although CDDO-ME has strong anti-inflammatory activity, it also has the problem of toxic and side effects. Therefore, reducing the therapeutic concentration of CDDO-ME can effectively reduce its toxic and side effects. In our research, we chose a suitable concentration of CDDO-ME, which has no toxicity to tumor cells and macrophages. At this lower concentration, it effectively inhibited pro-invasion ability of TAMs.

The tumor immunosuppressive microenvironment not only promote the development of tumor, but also be the main obstacle to the effect of tumor immunotherapy. TAMs is the dominant myeloid cell group in breast tumors and the main source of immunosuppression. Altering the activation of TAMs may be a means to alleviate this obstacle. CDDO-ME is an oral drug with

good tolerance in cancer patients [27]. We found that CDDO-ME not only inhibit TAMs pro-invasion ability, but also increase T cell infiltration in tumor microenvironment to improve tumor immune microenvironment, which indicates that CDDO-ME combined with other immunotherapy strategies (immune checkpoint blocking, immune activator, etc.) may eliminate cancer cells more effectively with minimal side effects.

Tumor cell invasion, angiogenesis and metastasis are interrelated processes, representing the final and most destructive malignant stage. This process includes cell growth, proliferation and migration. Evidence accumulated from in vitro and in vivo studies in the past few years shows that ROS is the signal medium of angiogenesis and metastasis[28, 29]. ROS has been proved to mediate these effects by inducing transcription factors and genes to participate in angiogenesis and metastasis. However, the role of ROS in regulating tumor cell metastasis and angiogenesis seems to be contradictory: the high level of ROS inhibits tumor formation and metastasis by destroying cancer cells, while the suboptimal concentration helps cancer cell metastasis [30]. ROS also shows its potential in promoting angiogenesis and metastasis of tumor cells in animal models of breast cancer, bladder cancer, lung cancer, melanoma, sarcoma, colon cancer and prostate cancer. Catalase can significantly reduce the invasive behavior of tumor cells in the transgenic mouse model with metastatic breast cancer (MMTV-PyMT)[31]. In mouse bladder cancer model, ROS induced metastasis by stimulating NF-κB. Lung metastasis induced by RAS is also proved to be caused by ROS production and up-regulation of NF-κB and MMP-9 in mouse model [32]. It is worth noting that in the mouse melanoma model, surgical methods for tumor resection have been proved to induce ROS production and promote the growth of metastatic tumors. Similarly, our research shows that H_2O_2 increase the invasion ability of tumor cells, while CDDO-ME effectively reduce ROS production in TAM, thus weakening the invasion ability of tumor cells.

In summary, our results showed that CDDO-ME, an antioxidant, reduce ROS production of TAMs at low concentration, which leads to lower invasion ability of cancer cells. Therefore, reducing the production of ROS as a cancer treatment may be a very attractive idea, and CDDO-ME may be a promising anticancer agent.

Abbreviations

CDDO-ME: CDDO Methyl ester

ROS: Reactive Oxygen Species

TAM: Tumor-associated Macrophages

MMTV-PyMT: Murine Mammary Tumor Virus- Polyoma Middle T Antigen

CON: Control

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Contributions

Y L designed the experiments and YX J and YR Xu performed the experiments. Y L and YX J wrote the main manuscript text and YR X prepared figures 1-5. All authors have read and approved final manuscript for publication.

Corresponding authors

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Ethics declarations

Ethics approval and consent to participate

All animal use and experimental procedures were conducted with the approval and oversight of the Ethics Committee of Southeast University (20200624007) under rules and regulations.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

Additional information

Publisher's Note

Figure legend:

Fig 1. CDDO-ME inhibited pro-invasion ability of TAMs

A. The viability of RAW264.7 cells was detected by MTT after different concentration of CDDO-ME treated for 24 h; B. The viability of RAW264.7 cells was detected by MTT after different concentration of CDDO-ME treated for 48 h; C. The viability of 4T1 tumor cells was detected by MTT after 100 nM CDDO-ME treated for 48 h; D-H. In the presence or absence of CDDO-ME, 4T1 tumor cells were cultured alone or co-cultured with RAW264.7 cells for 48 h, scratch migration assay(D), invasion assay(E), proliferation assay(F), cycle analysis(G) and apoptosis analysis(H) were performed. Data are presented as the mean±SEM and represent at least three independent experiments with three replicates. ns, no significance. ****, $P<0.0001$ as determined by the one-way ANOVA test.

Fig 2. CDDO-ME inhibits pro-invasion ability of TAMs by reducing ROS production.

A. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and the expression of membrane surface molecules (MHCII, CCR7, CD206, MGL1/2) of RAW264.7 cells was detected by flow cytometry; B. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and the phagocytosis of RAW264.7 cells was detected by flow cytometry. C. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and the secretion levels of TNFa, IL-6, IL-12 and IL-10 of RAW264.7 cells were detected by ELISA. D. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and ROS production in RAW264.7 cells was detected by flow cytometry. E. in the presence or absence of NAC, 4T1 cells and RAW264.7 cells were co-cultured for 48 hours to detect the invasion ability of 4T1; F. In the presence or absence of CDDO-ME or H_2O_2, 4T1 cells were co-cultured with raw264.7 cells for 48 hours to detect the invasion ability of 4T1. Data are presented as the mean±SEM and represent at least three independent experiments with three replicates.

Fig 3. CDDO-ME inhibit tumor growth and metastasis in 4T1 orthotopic inoculation model of breast cancer

A. Tumor growth of mice in each group was analyzed by measuring volume every 2 days(n=5); B. Weight of each group of mice is measure once every two days(n=5); C. The tumor weight of each group of mice was analyzed(n=5); D-E, resection of lung tissue and counting of nodules to evaluate the anti-metastasis effect of oral CDDO-ME; F. Flow cytometry analysis and quantification of $CD3^+$ T cells in tumor microenvironment of each group; G. Flow cytometry analysis and quantification of $CD8^+$ T cells in tumor microenvironment of each group. Data are presented as the mean±SEM. ns, no significance. *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001 as determined by the one-way ANOVA test.

Fig 4. CDDO-ME inhibits tumor metastasis in MMTV spontaneous breast cancer model

A. Tumor growth of mice in each group was analyzed by measuring volume every 2 days(n=5); B. Weight of each group of mice is measure once every two days(n=5); C. The tumor weight of each group of mice was analyzed(n=5). D-E, resection of lung tissue and counting of nodules to evaluate the anti-metastasis effect of oral CDDO-ME; F. Flow cytometry analysis and quantification of $CD3^+$ T cells in tumor microenvironment of each group; G. Flow cytometry analysis and quantification of $CD8^+$ T cells in tumor microenvironment of each group. Data are presented as the mean±SEM. ns, no significance. *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001 as determined by the one-way ANOVA test.

Fig 5. CDDO-ME inhibits TAM pro-invasion ability via the activation of NRF2.

A-B, in the presence or absence of CDDO-ME, RAW264.7 cells 4T1 cells were co-cultured for 48 hours. Western was used to detect the expression of NRF2 in raw cells, and Q-pcr was used to detect the mRNA levels of G6PD, IDH1, GCLC, Txn1 and PRD1 in raw cells. C, control RAW264.7 and NRF2 knockout RAW264.7 were cultured with 4T1 tumor cells for 48h, and ROS production was detected by flow cytometry; D. In the presence of CDDO-ME, 4T1 tumor cells were co-cultured with control RAW264.7 and NRF2 knockout RAW264.7 for 48h to detect their invasion ability. Data are presented as the mean±SEM and represent at least three independent

experiments with three replicates. ns, no significance, **, P<0.01 as determined by Student's *t*-test.

Supplementary Fig.1 Uncropped data was shown.

Figures

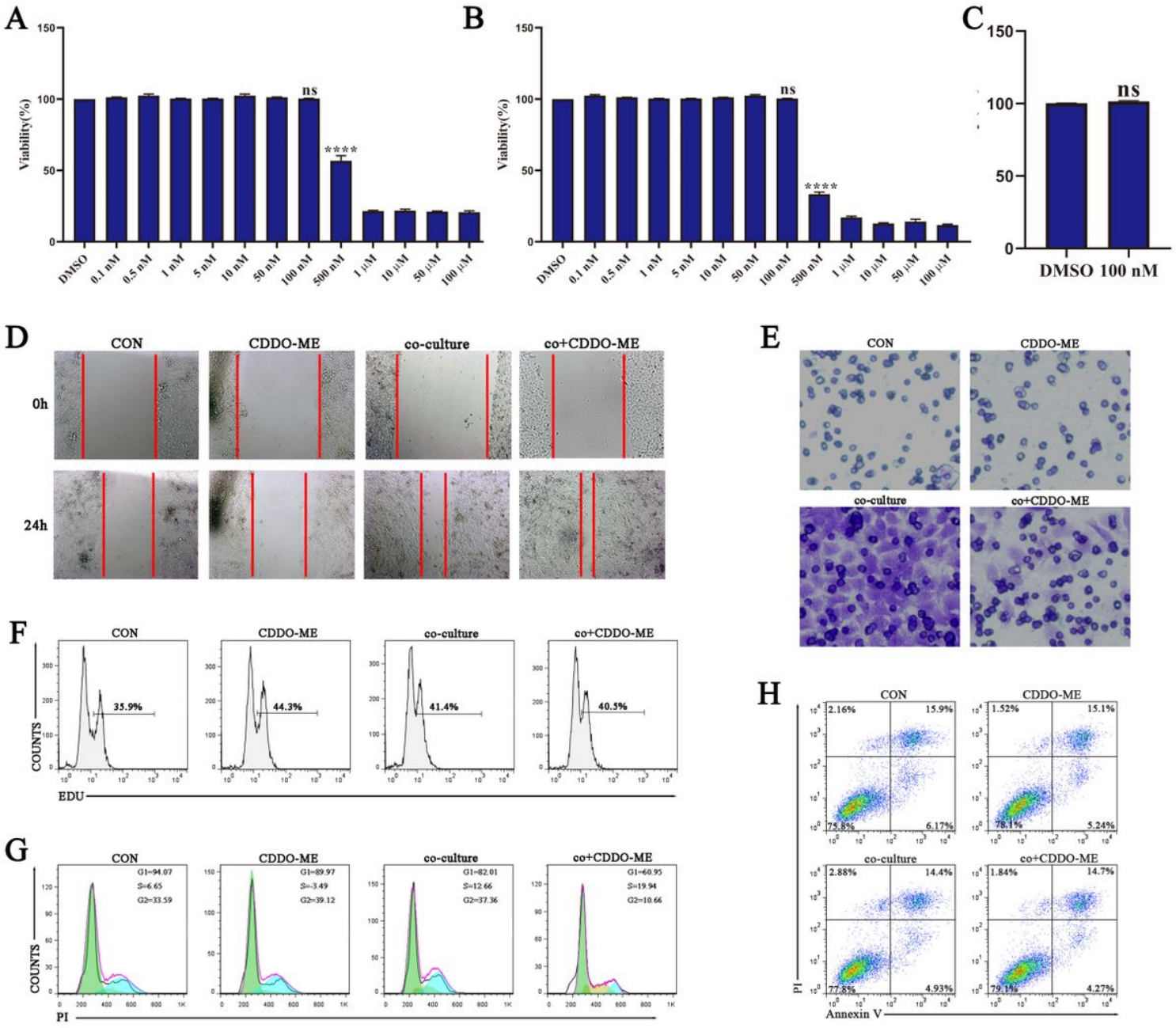

Figure 1

CDDO-ME inhibited pro-invasion ability of TAMs A. The viability of RAW264.7 cells was detected by MTT after different concentration of CDDO-ME treated for 24 h; B. The viability of RAW264.7 cells was detected by MTT after different concentration of CDDO-ME treated for 48 h; C. The viability of 4T1 tumor cells was detected by MTT after 100 nM CDDO-ME treated for 48 h; D-H. In the presence or absence of CDDO-ME, 4T1 tumor cells were cultured alone or co-cultured with RAW264.7 cells for 48 h, scratch migration assay(D), invasion assay(E), proliferation assay(F), cycle analysis(G) and apoptosis analysis(H) were performed. Data are presented as the mean±SEM and represent at least three

independent experiments with three replicates. ns, no significance. ****, P<0.0001 as determined by the one-way ANOVA test.

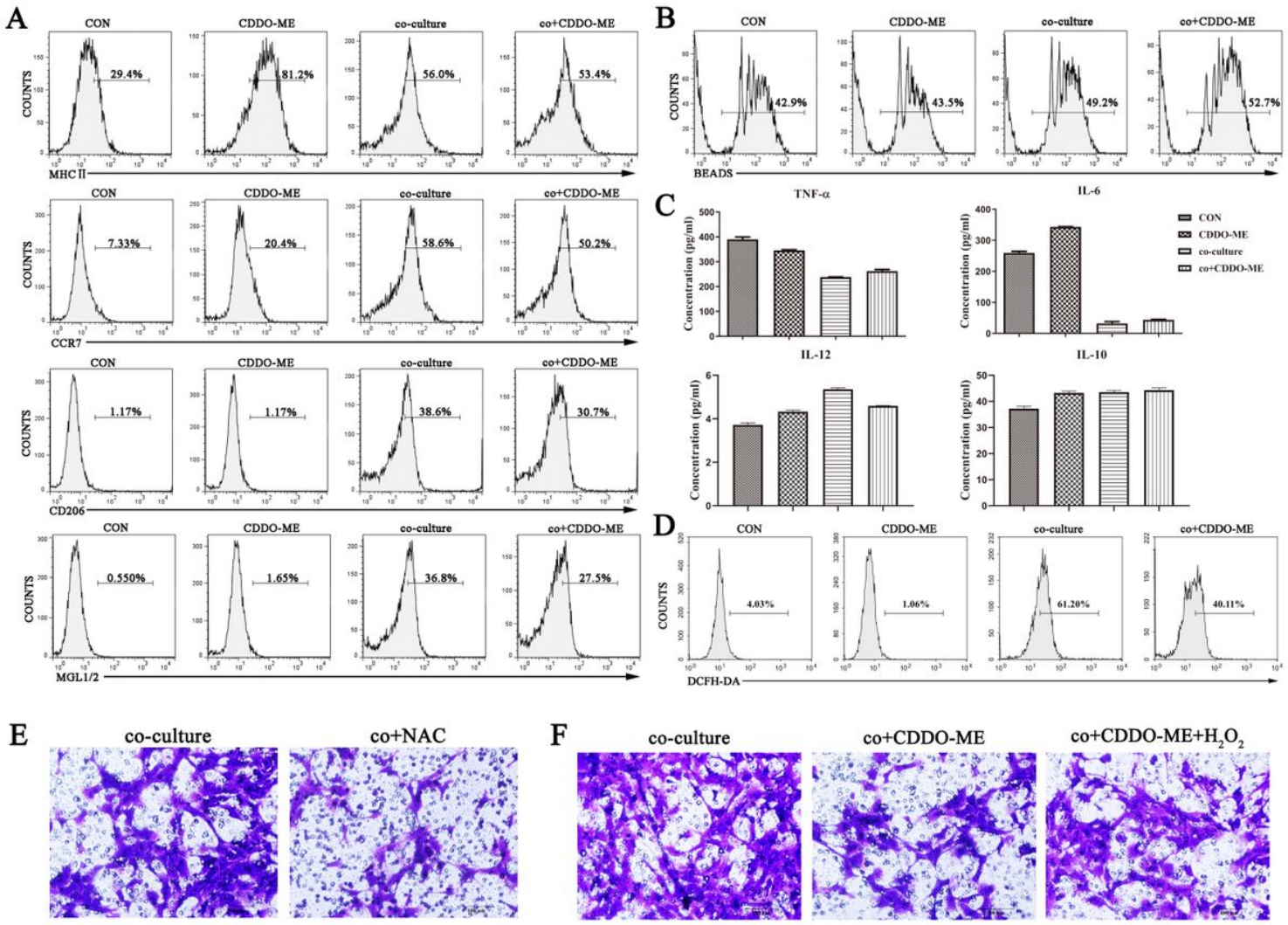

Figure 2

CDDO-ME inhibits pro-invasion ability of TAMs by reducing ROS production. A. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and the expression of membrane surface molecules (MHCII, CCR7, CD206, MGL1/2) of RAW264.7 cells was detected by flow cytometry; B. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and the phagocytosis of RAW264.7 cells was detected by flow cytometry. C. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and the secretion levels of TNFa, IL-6, IL-12 and IL-10 of RAW264.7 cells were detected by ELISA. D. In the presence or absence of CDDO-ME, RAW264.7 cells were cultured alone or co-cultured with 4T1 cells for 48 hours, and ROS production in RAW264.7 cells was detected by flow cytometry. E. in the presence or absence of NAC, 4T1 cells and RAW264.7 cells were co-cultured for 48 hours to detect the invasion ability of 4T1; F. In the presence or absence of CDDO-ME or H2O2, 4T1 cells were co-cultured with raw264.7 cells for 48 hours to detect the invasion ability of 4T1. Data are presented as the mean±SEM and represent at least three independent experiments with three replicates.

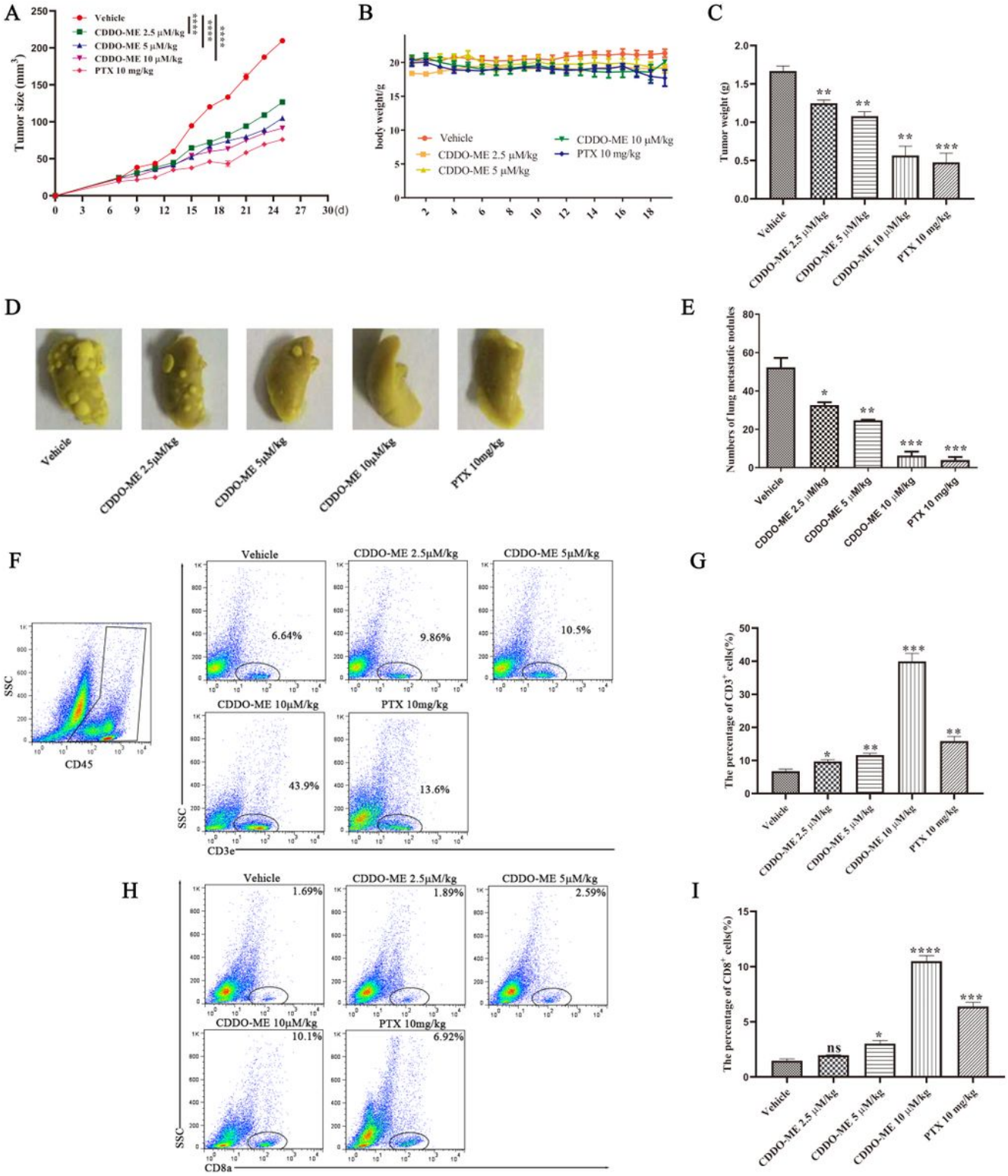

Figure 3

CDDO-ME inhibit tumor growth and metastasis in 4T1 orthotopic inoculation model of breast cancer A. Tumor growth of mice in each group was analyzed by measuring volume every 2 days(n=5); B. Weight of each group of mice is measure once every two days(n=5); C. The tumor weight of each group of mice was analyzed(n=5); D-E, resection of lung tissue and counting of nodules to evaluate the anti-metastasis effect of oral CDDO-ME; F. Flow cytometry analysis and quantification of CD3+ T cells in tumor

microenvironment of each group; G. Flow cytometry analysis and quantification of CD8+ T cells in tumor microenvironment of each group. Data are presented as the mean±SEM. ns, no significance. *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001 as determined by the one-way ANOVA test.

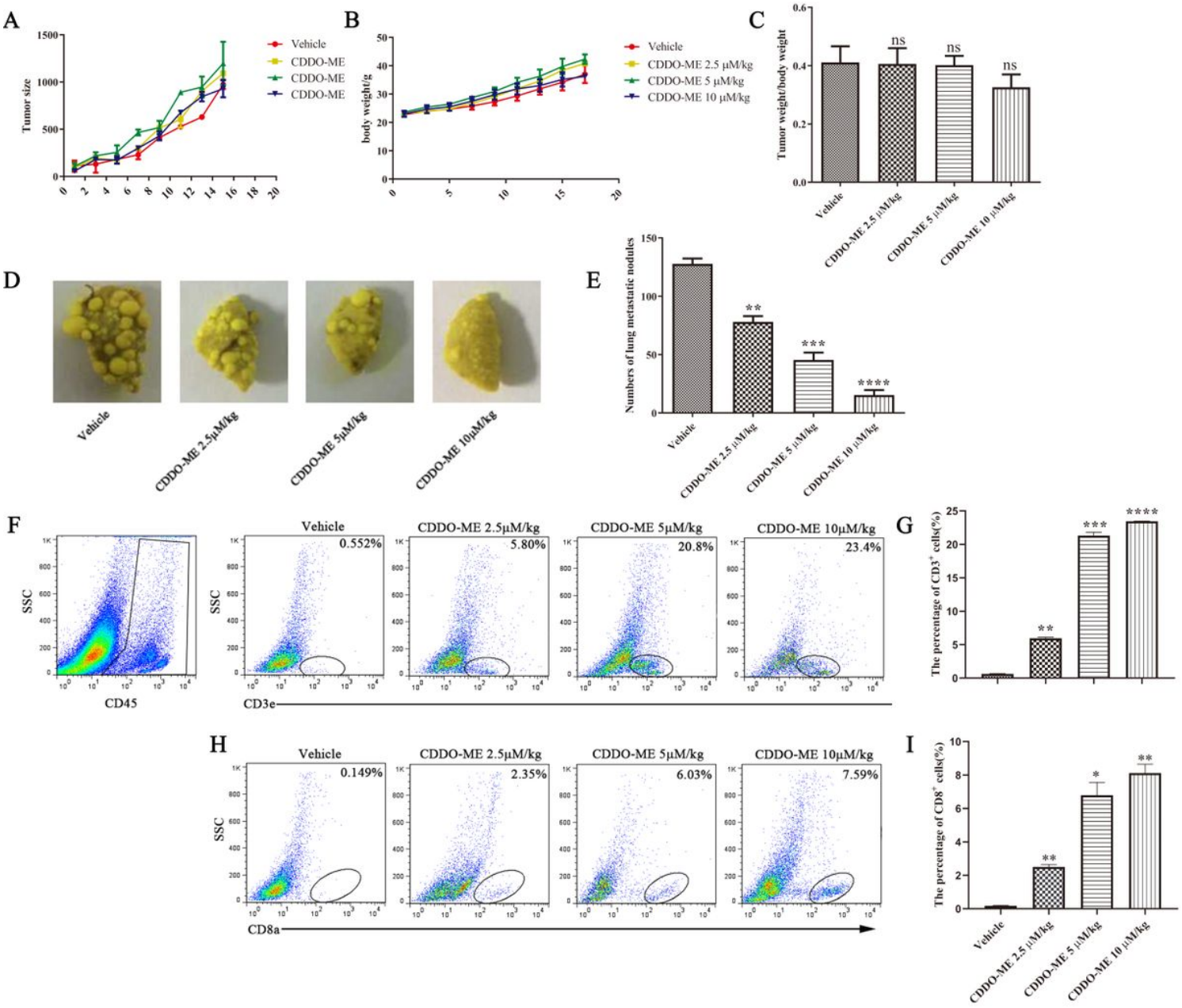

Figure 4

CDDO-ME inhibits tumor metastasis in MMTV spontaneous breast cancer model A. Tumor growth of mice in each group was analyzed by measuring volume every 2 days(n=5); B. Weight of each group of mice is measure once every two days(n=5); C. The tumor weight of each group of mice was analyzed(n=5). D-E, resection of lung tissue and counting of nodules to evaluate the anti-metastasis effect of oral CDDO-ME; F. Flow cytometry analysis and quantification of CD3+ T cells in tumor microenvironment of each group; G. Flow cytometry analysis and quantification of CD8+ T cells in tumor microenvironment of each group. Data are presented as the mean±SEM. ns, no significance. *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001 as determined by the one-way ANOVA test.

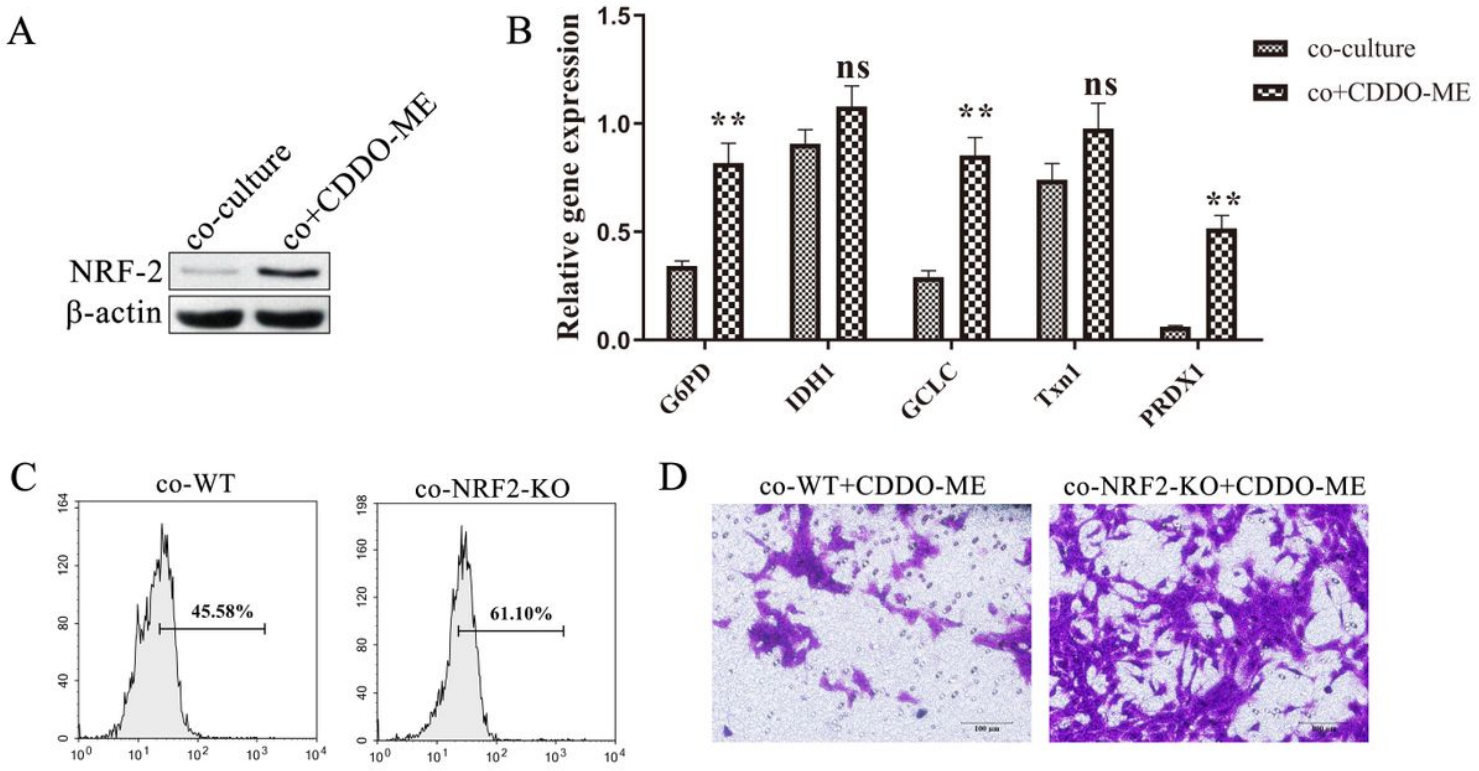

Figure 5

CDDO-ME inhibits TAM pro-invasion ability via the activation of NRF2. A-B, in the presence or absence of CDDO-ME, RAW264.7 cells 4T1 cells were co-cultured for 48 hours. Western was used to detect the expression of NRF2 in raw cells, and Q-pcr was used to detect the mRNA levels of G6PD, IDH1, GCLC, Txn1 and PRD1 in raw cells. C, control RAW264.7 and NRF2 knockout RAW264.7 were cultured with 4T1 tumor cells for 48h, and ROS production was detected by flow cytometry; D. In the presence of CDDO-ME, 4T1 tumor cells were co-cultured with control RAW264.7 and NRF2 knockout RAW264.7 for 48h to detect their invasion ability. Data are presented as the mean±SEM and represent at least three independent experiments with three replicates. ns, no significance, **, P<0.01 as determined by Student's t-test.

Supplementary Files

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