Article

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Formononetin Restrains Tumorigenesis of Breast Tumor by Restraining STING-NF-κB and Interfering with the Activation of PD-L1

Hongmei Liu[1,*], Zhipeng Wang[1], Zhigang Liu[1]

[1]Department of Chinese Medicine, Zhongda Hospital Southeast University, 210009 Nanjing, Jiangsu, China
*Correspondence: 17512502495@163.com (Hongmei Liu)
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Background: Breast cancer (BC), a common tumor in women, has high morbidity and mortality. Formononetin, an active ingredient in red clover and *Astragalus membranaceus*, has a wide range of pharmacological applications, including as an anticancer agent. Since immunotherapy is a hot topic in the treatment strategy of BC, it was dedicated to appraising the specific mechanism of formononetin in BC immunotherapy in this research.

Methods: Different formononetin concentrations (0, 20, 40, 60, 80, 100 μM) were used to treat BC cells transfected with pcDNA3.1-Programmed death ligand 1 (PD-L1) or Short-hairpin RNA (sh)-PD-L1. Cells were separated into four subgroups: CTRL, pcDNA3.1-PD-L1, sh-CTRL, and sh-PD-L1. Cell viability and cell cycle were assessed through Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and flow cytometry. Programmed death ligand 1 (PD-L1) mRNA concentration was validated via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Cell metastasis was evaluated via cloning assay and transwell assay. The p-STING/stimulator of interferon genes (STING), p-p65/p65, and PD-L1 concentrations were determined by western blot.

Results: Formononetin restrained the proliferation of MCF-7 and MDA-MB-468 cells, and reduced PD-L1 mRNA, p-STING/STING, and p-p65/p65 protein concentrations. Whereas PD-L1 inhibition restrained the viability of BC cells, pcDNA3.1-PD-L1 intervention had the opposite result. STING pathway inhibitor C-176 combined with formononetin treatment further restrained cell proliferation, colony formation, and cell invasion, in contrast to cells treated with formononetin alone.

Conclusion: Formononetin can restrain the proliferation of BC cells, which may be mediated through the interference of PD-L1 and suppression of the activation of the STING-NF-κB pathway.

Keywords: formononetin; breast tumor; PD-L1; STING-NF-κB pathway

Background

On a worldwide scale, with high morbidity and mortality [1], breast cancer (BC) has become the principal cause of tumor death among women [2,3]. At present, the conventional treatment of BC includes surgery, radiotherapy, and chemotherapy [4]. However, due to the existence of drug resistance and tumor metastasis and recurrence, the mortality rate of BC is still very high [5]. In recent years, researchers have focused on immunotherapy, including cell therapy, tumor vaccines, oncolytic viruses, and highly effective immune checkpoint inhibitors, confirming the potential of the immune system in a variety of new treatment strategies [6].

Programmed death ligand 1 (PD-L1) is a 33kDa-sized protein, which is located on the cell membrane or in the cytoplasm [7]. It restrains the proliferation and cytolytic activity of T cells, and promotes the immune escape of tumour cells by interacting with the receptor PD-1 on immune cells [7]. Immunotherapy targeting PD-1/PD-L1 has made positive progress in the clinical application of BC [8].

Formononetin is an isoflavone phytoestrogen widely found in legumes, such as red clover and *Astragalus membranaceus* [9]. A previous study has shown that formononetin has many pharmacological applications such as anti-proliferation, promoting apoptosis, inducing cell cycle arrest, antioxidation, regulating angiogenesis, anti-inflammation, and so on [10]. Formononetin has antitumour effects on a variety of tumor cells [9]. Formononetin may play an anti-tumor role in BC by acting on C-X-C motif chemokine ligand 12 (CXCL12), Estrogen receptor 1 (ESR1), and Insulin-like growth factor 1 (IGF1), and may have a potential synergistic function with immune checkpoint inhibitors [11]. However, the specific mechanism of formononetin in BC immunotherapy still needs to be further explored. Herein, we demonstrated that formononetin inhibits the tumorigenesis of BC by restraining stimulator of interferon genes (STING)-NF-κB and interfering with the activation of PD-L1.

Materials & Methods

Cell Culture and Treatment

Human BC cells were derived from Beijing Cell Bank (Batch number: MCF-7: H2-0201; MDA-MB-468: CL-0290A, China). Using the approved DNA-based method to confirm the origin of the cell line. The cell line name was checked against the cell line database for mistakenly identified cells, which was established by International Cell Line Authentication Committee (ICLAC). Short tandem repeat (STR) information was applied to build the genetic characteristics, subsequently, no cross-contamination was benchmarked. no contamination was detected via Mycoplasma testing. RPMI-1640 medium was utilized to cultivate cells (Hyclone, SH30809.01B, Logan, UT, USA), 10% fetal bovine serum was comprised in which (FBS, Gibco, 10099141, Erie, NY, USA), as well as 10^5 U/L penicillin and 100 mg/L streptomycin, in a humidified atmosphere containing 5% CO_2, at 37 °C [12]. Formononetin (0, 20, 40, 60, 80, 100 µM, MCE, HY-N0183, purity 99.92%, Trenton, NJ, USA) was applied to treat cells for 48 h.

Methylthiazolyldiphenyl-Tetrazolium Bromide (MTT)

Cells were collected during the logarithmic growth phase. Subsequently, at 5×10^3 cells per well concentrations, cell suspension concentration was modulated. In each subgroup, with 3 multiple holes, the cells were inoculated into 96-well plates. In a 37 °C 5% CO_2 incubator, cells were cultivated overnight, so as to make the cells adhere to the wall. Each well was complemented with 20 µL Methylthiazolyldiphenyl-tetrazolium bromide (MTT, 5 mg/mL, Bioswamp, C1736, Wuhan, China), taken out the culture plate soon afterwards, and cells were cultured for 4 h. Next, each well was complemented with 150 µL DMSO (Kangyang Chemical Co., Ltd., 67-68-5, Guangzhou, China), and the plates were shaken at a low speed for 10 min after removing the culture medium. Using an enzyme labeling instrument (CSB-E12912m, Huamei Biotechnology, Wuhan, China), the optical density (OD) value of each hole was appraised at 490 nm.

Flow Cytometry

Cells were centrifuged for 5 min at 400 ×g, and the cells of each subgroup were collected, subsequently, discarding the supernatant. Washed the cells twice with precooled PBS, and added precooled 75% ethanol. Cells were fixed for more than 24 h in the refrigerator. At 700 ×g for 5 min, the cells were centrifuged. Then, discarding the supernatant. The cells were washed twice with precooled PBS, 100 µL RNase A solution with 1 mg/mL concentration, and 400 uL PI solution with 50 µg/mL concentration were supplemented (Bangjing, BJ-KT77274, Shanghai, China). Cells were incubated at 4 °C without light for 10 min. The cell cycle was measured by flow cytometry (BeckmanCoulter, Brea, CA, USA).

Clone Formation Assay

Cells were collected during the logarithmic growth phase. RPMI 1640 medium was supplemented to cells to prepare the suspension, which contained 10% FBS. Each subgroup of cells was inoculated in dishes containing 37 °C preheated culture medium (10 mL) with a gradient density of 50, 100, and 200 cells per plate, and dishes were gently rotated to make the cells disperse evenly. In a cell incubator at 37 °C, 5% CO_2, Cells were cultivated, Cell culture was terminated when a clone visible to the naked eye appeared in the petri dish. Discarding the supernatant, using PBS to wash the cells twice. Fixing the cells with 4% tissue cell fixation solution (5 mL, Reagan Biology, BL539A, Beijing, China) for 1/4 h. Adding moderate crystal violet staining solution (Millipore Sigma, C8470, Burlington, MA, USA) for 1/4 h. The dye was washed off slowly with running water and the samples were air dried. Cell colonies were counted and photographed.

Transwell Assay

In the chamber before inoculation, 80 µL Matrigel glue was spread. The incubator was placed at 37 °C for half an hour. At 1×10^5 cells/mL concentrations of cell suspension, 0.5 mL of cell suspension was inoculated into the transwell chamber (Corning, 3401, Shanghai, China). 0.75 mL medium was supplemented to the lower 24-well plate and cultivated in 37 °C, 5% CO_2 incubator for 48 h, which contained 10% FBS. Took out the culture plate, added 4% formaldehyde solution and fixed it for 20 min. The fixed solution was discarded, and the crystal violet solution was added and allowed to stain for half an hour. After staining, under the microscope (Leica, Wetzlar, Germany), the cells were observed and counted.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Detection

From 1×10^6 cells, Trizol (Ambion, 15596026, Carlsbad, CA, USA) was applied to extract total RNA. Through reverse transcription, cDNA was synthesized and amplified by PCR. The reaction procedure was: 95 °C for 3 min; 95 °C for 5 s, 56 °C for 10 s, 72 °C for 25 s, 40 cycles in total. PCR primers were synthesized by Baori Medical Biotechnology Co., Ltd. (Beijing, China). *PD-L1*: Forward Sequence: 5′-GACCTATATGTGGTAGAGTATGG-3′, Reverse Sequence: 5′-GGCATTGACTTTCACAG-3′, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*): Forward Sequence: 5′-TATGATGATATCAAGAGGGTAGT-3′, Reverse Sequence: 5′-TGTATCCAAACTCATTGTCATAC-3′. At the end of the reaction, the Cycle threshold (Ct) value was obtained, and the GAPDH was applied as the internal reference, and the statistical analysis was carried out by the $2^{-\Delta\Delta Ct}$ method.

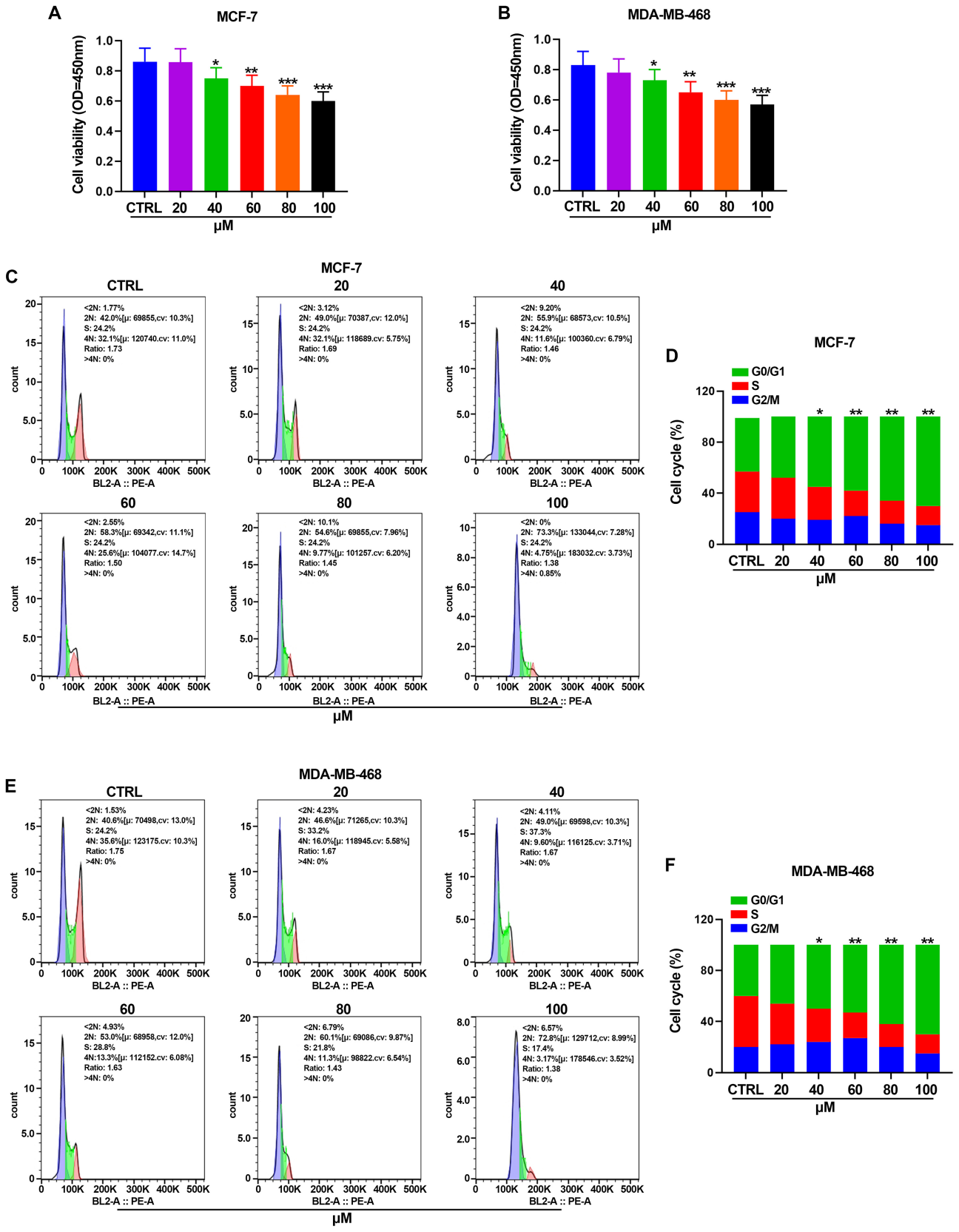

Fig. 1. Formononetin restrained the activity of human breast cancer (BC) cells. (A,B) Different concentrations of formononetin were applied to handle cells, and the cell viability was appraised via MTT assay. (C–F) Flow cytometry was applied to appraise cell cycle. N = 3. Versus CTRL subgroup, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test). OD, optical density; MTT, Methylthiazolyldiphenyl-tetrazolium bromide.

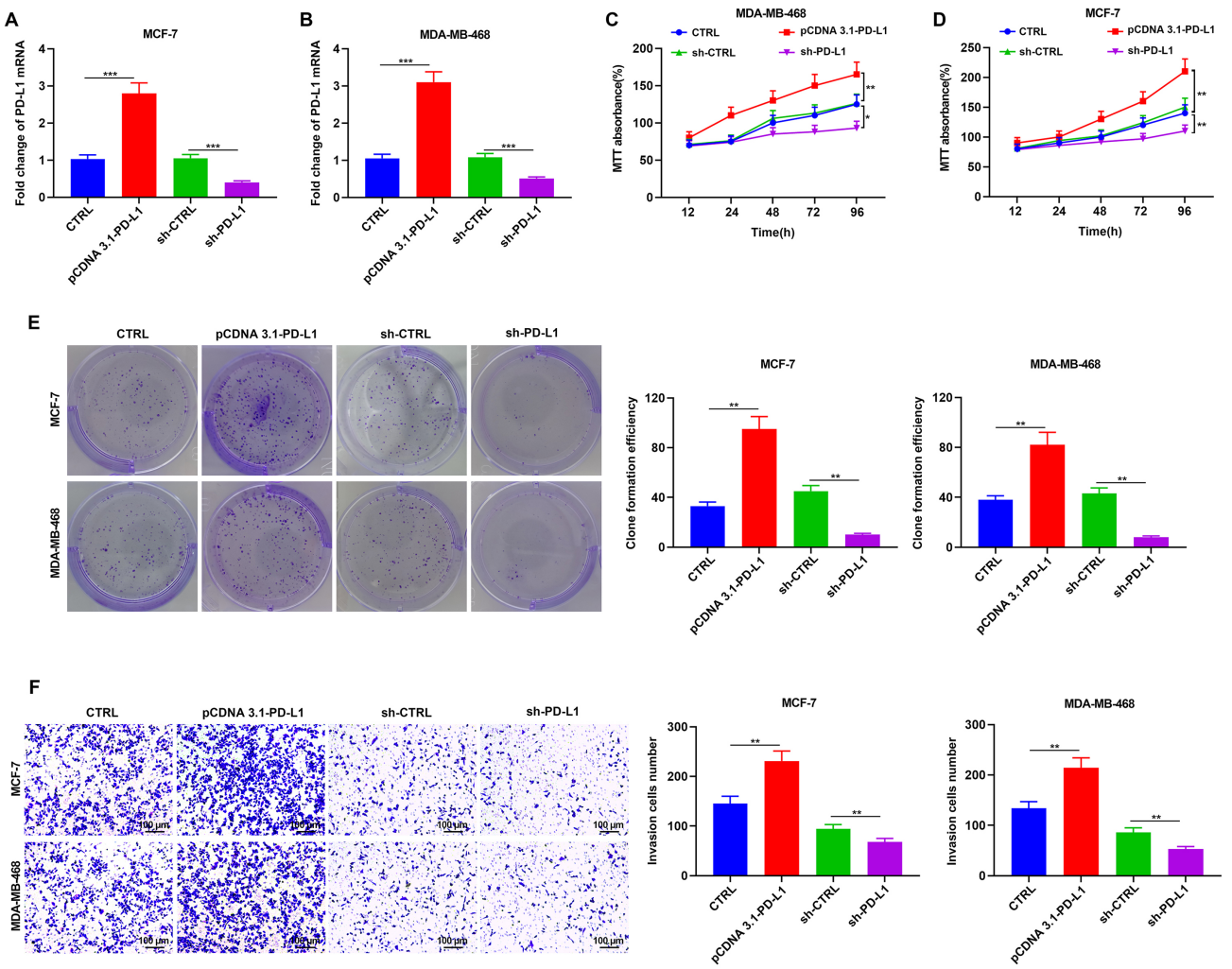

Fig. 2. PD-L1 facilitated tumor growth and metastasis *in vitro*. (A,B) The Programmed death ligand 1 (PD-L1) mRNA concentration was assessed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). (C,D) The outcome of down-regulation of PD-L1 on the growth rate of BC cells was assessed by MTT analysis. (E) Colony formation assay was applied to assess the outcome of PD-L1 suppression on the average colony number of BC cells. (F) Transwell invasion assay was applied to assess the outcome of PD-L1 suppression on the number of invasive cells. Scale, 100 μm. Magnification: 200×. N = 3. Versus CTRL subgroup, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's *t*-test).

Western Blot

Total protein was extracted using the radioimmunoprecipitation assay lysis buffer (MilliporeSigma, R0030, Burlington, MA, USA), and the bicinchoninic acid kit (Beyotime, PC0020, Shanghai, China) was used to quantify protein concentration. 20 μg of protein was extracted and transferred to polyvinylidene fluoride membranes (Beyotime, 24937-79-9, Shanghai, China). 5% skim milk powder (Beyotime, P0216-300g, Shanghai, China) was added and the samples were sealed overnight at 4 °C. GAPDH (1:2000, 5174), PD-L1 (1:1000, 15165), p-STING (1:1000, 50907), STING (1:1000, 13647), p-p56 (1:1000, 76778) and p56 (1:1000, 8242) were added and incubated for 12 h. All primary antibodies were from CST (MilliporeSigma, Burlington, MA, USA). Goat anti-rabbit IgG second antibody (Wuhan Sanying Biotechnology Co., Ltd., A11034, Wuhan, China) was incubated with GAPDH for 1 h as endogenous control. ImageJ software (ImageJ2, NIH, Bethesda, MA, USA) was used to analyze the grayscale of the results of western blot assay.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software (IBM, Amonk, NY, USA). Differences between subgroups were tested using one-way Analysis of Variance (ANOVA). The data were displayed as the average "±" standard deviation (n = 3) representing three independent experiments. p value < 0.05 was considered statistically significant.

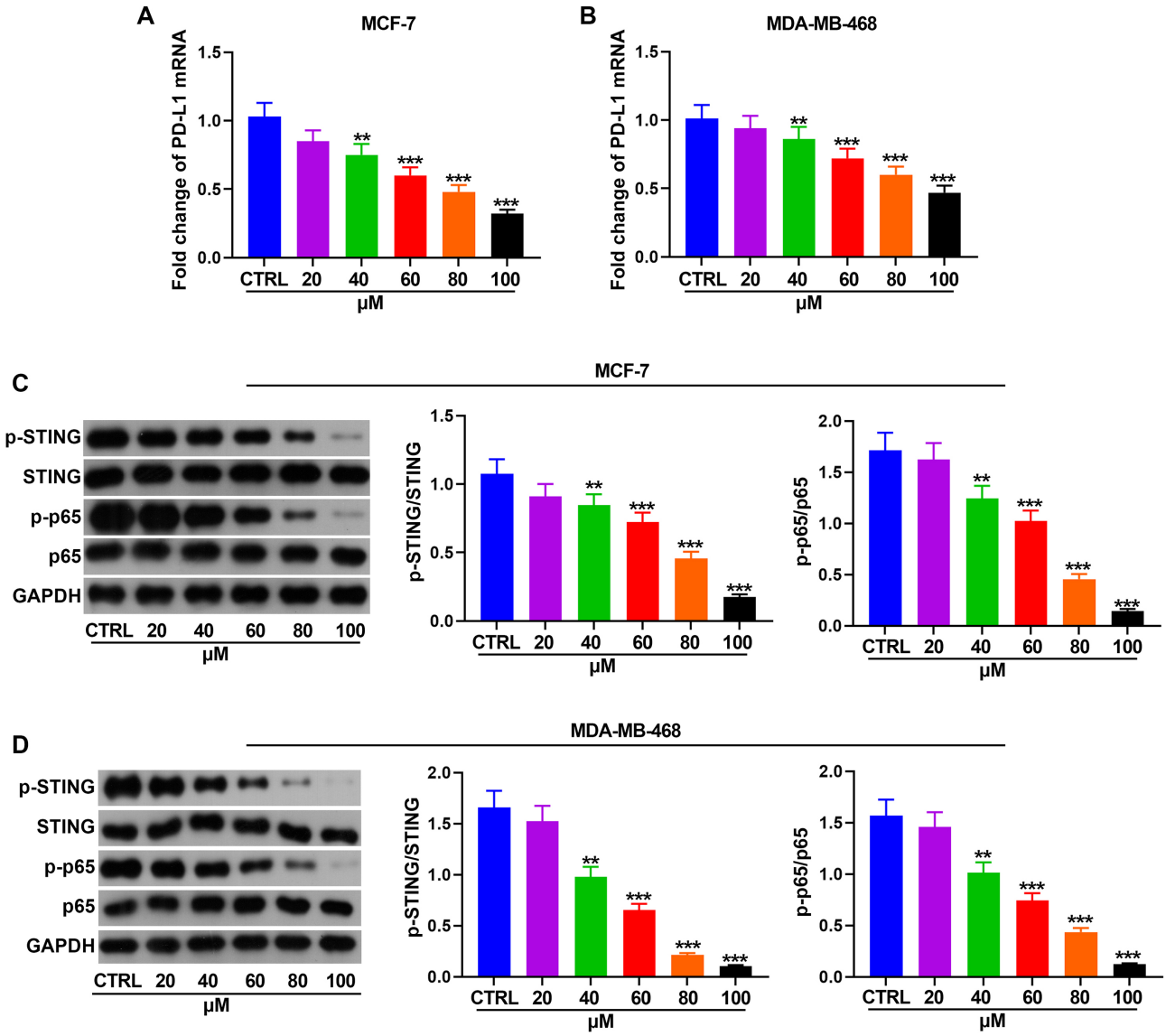

Fig. 3. Formononetin interfered with PD-L1 through the stimulator of interferon genes (STING)-NF-κB pathway. (A,B) Appraising PD-L1 mRNA level via qRT-PCR. (C,D) Evaluating p-STING/STING, p-p65/p65 concentrations via western blot. N = 3. Versus CTRL subgroup, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Results

Formononetin Restrained the Activity of Human BC Cells

To evaluate the effect of formononetin on the proliferation of human BC cells, formononetin (0, 20, 40, 60, 80, 100 μM) was applied to BC cells for 48 h. Formononetin restrained the cell viability versus CTRL subgroup, which was appraised via MTT assay (Fig. 1A,B). The proportion of cells in G0/G1 phase increased markedly in the formononetin subgroup, and G0/G1 phase arrest occurred versus the CTRL subgroup (Fig. 1C–F).

PD-L1 Facilitated Tumor Growth and Metastasis in Vitro

The effect of high and low PD-L1 concentration on BC cell activity was assessed (Fig. 2A,B). High concentration of PD-L1 accelerated the viability of BC cells versus the CTRL subgroup, whereas PD-L1 suppression restrained cell viability versus the sh-CTRL subgroup (Fig. 2C,D). High concentration of PD-L1 accelerated colony formation of BC cells versus the CTRL subgroup, whereas PD-L1 suppression restrained BC cells colony formation versus the sh-PD-L1 subgroup (Fig. 2E). The invasion number of PD-L1 high concentration in BC cells was notably increased versus the CTRL subgroup, whereas that of PD-L1 was notably reduced versus the sh-PD-L1 subgroup (Fig. 2F).

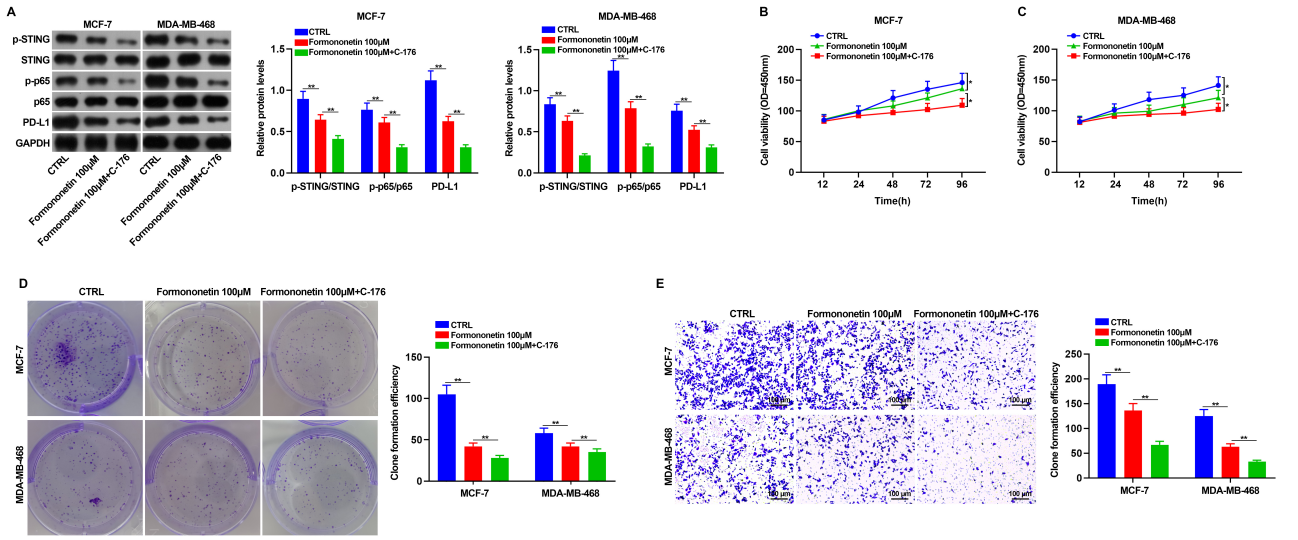

Fig. 4. Suppression of breast tumorigenesis by STING-NF-κB pathway-mediated formononetin. MCF-7 and MDA-MB-468 cells were treated with STING inhibitor C-176. (A) Assessing p-STING/STING, p-p65/p65, and PD-L1 concentrations via western blot. (B,C) MTT analysis was utilized to assess the outcome of formononetin and C-176, alone or in combination, on cell growth rate. (D) Assessing the outcome of formononetin and C-176, alone or in combination, on the average colony number via colony formation assay. (E) Transwell invasion assay was applied to show the outcome of formononetin and C-176, alone or in combination, on the cell invasion number. Scale, 100 μm. Magnification: 200×. N = 3. Versus CTRL subgroup, $*p < 0.05$, $**p < 0.01$ (Student's *t*-test).

Formononetin Interfered with PD-L1 through STING-NF-κB Pathway

Next, formononetin (0, 20, 40, 60, 80, 100 μM) was applied to treat BC cells for 48 h, and the effects on PD-L1 were determined. Formononetin restrained PD-L1 mRNA in BC cells versus the CTRL subgroup (Fig. 3A,B). Notably, formononetin reduced the p-STING/STING and p-p65/p65 concentrations in BC cells (Fig. 3C,D).

Suppression of BC by STING-NF-κB Pathway-Mediated Formononetin

STING pathway inhibitor C-176 combined with formononetin was applied to treat BC cells. The p-STING/STING, p-p65/p65 and PD-L1 concentrations in BC cells treated with formononetin were notably reduced versus the CTRL subgroup. C-176 further reduced concentrations of these proteins in BC cells versus the formononetin-treated subgroup (Fig. 4A). Formononetin restrained the viability, colony numbers, and invasion numbers of BC cells versus the CTRL subgroup. C-176 further restrained the viability, colony numbers, and invasion numbers of BC cells versus the formononetin-treated subgroup (Fig. 4B–E).

Discussion

BC is the most common invasive tumor among women and the leading cause of tumor-related death among women worldwide [13]. At present, the treatment of BC mainly includes chemotherapy, radiotherapy, and surgery [14]. However, chemotherapy and radiotherapy have serious side effects and poor prognosis. Therefore, it is necessary to find safer and more effective treatments to improve the survival rate of BC patients. In recent years, immunotherapy of BC has become a research hotspot. Immune checkpoint inhibitor therapy has been shown to produce a lasting response in a variety of tumors and has become the most successful immune-based intervention in tumour treatment [15]. Monoclonal antibodies against PD-1/PD-L1 and CTLA-4 have become powerful tools to release T-cell activation, suppression, and regulation from tumor cells or the tumor microenvironment [15]. Lotfinejad *et al.* [16] have reported that silencing PD-L1 in BC cells can effectively restrain their migration. Further studies revealed that silent PD-L1 induced T-cell toxicity by up-regulating the gene expression of pro-inflammatory cytokines (Interleukin-2 (IL-2), Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α)) and down-regulating the gene expression of anti-inflammatory cytokines (IL-10 and transforming growth factor-β (TGF-β)) [16].

Formononetin is a bioactive isoflavone isolated from different plants (*Trifolium pratense*, *Glycine max*, *Sophora flavescens*, *Pycnanthus angolensis*, *Astragalus membranaceus*). It has anti-inflammatory, anti-tumor, and antioxidant properties [17]. The application of formononetin in the treatment of BC has made positive progress. Wu *et al.* [18] have shown that formononetin can alleviate the malignant tumor progression and chemi-

cal resistance of triple-negative BC. In this study, the effects of formononetin on the activity of BC cells were explored first. Formononetin restrained the proliferation of BC cells and induced cell arrest in the G0/G1 phase. Formononetin was also found to reduce PD-L1 mRNA and protein concentrations in BC cells.

The sustained expression of PD-L1 in tumor cells is crucial for tumor immune escape and host T-cell failure. At present, the monoclonal antibody atezolizumab which targets PD-L1 has been approved in combination with nab-paclitaxel for unresectable, locally advanced, triple-negative breast tumor or metastatic triple-negative breast tumor patients expressing PD-L1 [19]. Next, we over-expressed and knocked down the PD-L1 concentration in BC cells. High concentration of PD-L1 promoted the proliferation, colony formation, and invasion of BC cells while knocking down the expression of PD-L1 restrained the viability, colony formation, and invasion of BC cells.

The coordinated interaction between tumor cells and the immune system is very important for tumor cells to evade host immune recognition [20]. Stimulator of interferon genes (STING), a cell membrane pattern recognition receptor, is very important for the spontaneous induction of anti-tumor T-cell immunity [21]. STING can sense double-stranded DNA of the cell membrane and trigger cascade signals, including TANK binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF-3) [22]. In the present study, formononetin reduced the p-STING/STING, p-p65/p65 protein concentrations in BC cells. In order to further determine the mechanism of the effects of formononetin on the malignant biological behavior of BC cells, formononetin and STING pathway inhibitor C-176 were applied, alone or in combination, to treat BC cells. C-176 further promoted the effects of formononetin on the proliferation, colony formation, and invasion of BC cells versus BC cells treated with formononetin alone. At the same time, C-176 further promoted the effects of formononetin on PD-L1 expression in BC cells. We also expected to conduct *in vivo* experiments; however, due to the limited experimental funds and time, we did not confirm these results through *in vivo* experiments. In the future, we will perform nude mouse tumorigenesis experiments to further confirm that formononetin, through effects on PD-L1 and through the STING-NF-κB pathway, affects BC progression.

Conclusion

To sum up, formononetin can restrain the proliferation and invasion of BC cells, and its mechanism may be related to the interference of PD-L1 expression and suppression of the activation of the STING-NF-κB pathway. However, the effects of formononetin on BC immune cells, and its specific role in immunotherapy, need to be further explored.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

HML and ZPW has been involved in drafting the manuscript and revising it critically for important intellectual content. HML and ZPW has made substantial contributions to the conception and design, acquisition of data, and analysis and interpretation of data. ZGL has helped perform the analysis with constructive discussions and supervision and has been involved in drafting the manuscript or revising it critically for important intellectual content. All authors have given final approval of the version to be published. Each author have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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