Emodin inhibits breast cancer growth by blocking the tumor-promoting feedforward loop between cancer cells and macrophages

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Running title: Emodin blocks cancer cell-macrophage interaction

Keywords: Emodin, breast cancer, tumor-associated macrophage, macrophage polarization, histone modification

Abbreviations list: MCP1 (Monocyte Chemoattractant Protein 1), CSF (Colony Stimulating Factor), IRF4 (Interferon Regulatory Factor 4), STAT6 (Signal Transducer and Activator of Transcription 6), CEBPβ (CCAAT/enhancer binding protein beta), H3K27M3 (Histone 3 Lysine 27 Tri-methylation), MDSC (Myeloid Derived Suppressor Cell), TME (Tumor Microenvironment), TAM (Tumor-Associated Macrophage), IL (Interleukin), TGFβ (Transforming Growth Factor beta), PPARγ (Peroxisome Proliferator-Activated Receptor gamma), Arg1 (Arginase 1), iNOS (Inducible Nitric Oxide Synthase), TNFα (Tumor Necrosis Factor alpha), JMJD3 (Jumonji Domain Containing-3), MMP (Matrix Metalloprotease), ICAM1 (Intercellular Adhesion Molecule 1), IFNγ (Interferon gamma), THY1 (Thymocyte antigen 1), DMSO (Dimethyl Sulfoxide), LDH (Lactate Dehydrogenase), DMEM (Dulbecco's Modified Eagle Medium), TCM (Tumor Conditioned Medium), SF (Serum Free), PBS (Phosphate-Buffered Saline)

Financial support: National Institutes of Health Grants HL116626 and AT003961-8455 to D. Fan.

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Conflict of interest: Authors declare no conflicts of interest.

236-word abstract

4992 total words of text (exclusive of references)

50 total references

6 total figures
ABSTRACT

Macrophage infiltration correlates with severity in many types of cancer. Tumor cells recruit macrophages and educate them to adopt an M2-like phenotype through the secretion of chemokines and growth factors, such as MCP1 and CSF1. Macrophages in turn promote tumor growth through supporting angiogenesis, suppressing anti-tumor immunity, modulating extracellular matrix remodeling, and promoting tumor cell migration. Thus tumor cells and macrophages interact to create a feedforward loop supporting tumor growth and metastasis. In this study, we tested the ability of emodin, a Chinese herb-derived compound, to inhibit breast cancer growth in mice and examined the underlying mechanisms. Emodin was used to treat mice bearing EO771 or 4T1 breast tumors. It was shown that emodin attenuated tumor growth by inhibiting macrophage infiltration and M2-like polarization, accompanied by increased T cell activation and reduced angiogenesis in tumors. The tumor inhibitory effects of emodin were lost in tumor-bearing mice with macrophage depletion. Emodin inhibited IRF4, STAT6, and C/EBPβ signaling and increased inhibitory histone H3 lysine 27 tri-methylation (H3K27m3) on the promoters of M2 related genes in tumor-associated macrophages. In addition, emodin inhibited tumor cell secretion of MCP1 and CSF1, as well as expression of surface anchoring molecule Thy-1, thus suppressing macrophage migration towards and adhesion to tumor cells. These results suggest that emodin acts on both breast cancer cells and macrophages and effectively blocks the tumor-promoting feedforward loop between the two cell types, thereby inhibiting breast cancer growth and metastasis.
INTRODUCTION

In spite of the many advances that have been made in treatment, breast cancer is still the second leading cause of cancer deaths among women (1). Tumor growth and metastasis depend on the support from stromal cells, including macrophages, fibroblasts, and myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment (TME) which promote angiogenesis, matrix remodeling, and immunosuppression (2, 3). Recently, there has been interest in immunotherapies for the treatment of breast cancer because of their low toxicity and extended duration of action (4, 5). However, the immunosuppressive microenvironment of tumors greatly diminishes the effectiveness of these therapies (6). MDSCs, M2-like tumor-associated macrophages (TAMs), and regulatory T cells have all been shown to repress an effective anti-tumor immune response through the production of anti-inflammatory cytokines and growth factors such as IL10 and TGFβ. Therapies targeting the immunosuppressive microenvironment have shown great potential on their own or in combination with other therapies in experimental models (7, 8).

Macrophages are the most abundant tumor infiltrating immune cells in breast cancer, and their number correlates with increased breast cancer aggressiveness and reduced survival (2, 9, 10). Macrophages are capable of adopting a spectrum of phenotypes, at either end of which are pro-inflammatory M1 and anti-inflammatory M2 macrophages. The phenotype is dependent on the signals in their environment. During the initiation and development of solid tumors, including breast cancer, tumor cells recruit and educate macrophages toward an M2-like phenotype through the secretion of soluble mediators such as CSF1, MCP1 (CCL2), IL10 and TGFβ, and through direct cell-cell contact (3, 11, 12). These tumor cell-derived signals trigger M2-like polarization of TAMs through activating several signaling pathways including STAT6, C/EBPβ, PPARγ, and IRF4. TAMs in turn secrete various cytokines and growth factors which promote tumor cell growth and migration toward and into blood vessels, enhance
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angiogenesis, and inhibit effector T cell response (13, 14). Therapies which target this cross-talk between tumor cells and macrophages have shown great potential in experimental models. For example, CSFr1 inhibition greatly decreased macrophage infiltration into mouse mammary tumors and thus led to an increased T cell response (15). Blocking CCL2 signaling resulted in significantly reduced macrophage infiltration and tumor size; however, cessation of treatment resulted in a rebound with increased macrophage infiltration and tumor growth (16).

Emodin is the active ingredient of several Chinese herbs including Rhubarb (*Rheum palmatum*), and it has generated much research interest recently because of its many biological properties (17, 18). Emodin has been shown to inhibit the growth of a variety of tumors and enhance the responsiveness of tumors to chemotherapy agents (19-21). Most of these studies have focused on emodin’s direct toxicity to cancer cells. However, emodin has also been shown to have strong anti-inflammatory properties and is able to inhibit macrophage activation in response to a variety of signals (22-24). Our laboratory has previously shown that emodin inhibited the lung metastasis of established breast tumors through inhibition of macrophage recruitment and M2-like polarization in the lungs (24). In this study we propose that emodin may affect breast cancer growth through modulating the TME if it is administered earlier in the cancer development. We provide data showing that emodin blocks the feedforward loop between the breast cancer cells and TAMs, thereby inhibiting breast cancer growth.

**MATERIALS AND METHODS**

**Emodin**

Emodin, a trihydroxy-anthraquinone (19), was purchased from Nanjing Langze Medicine and Technology Co. Ltd (Nanjing, China) and verified by NMR spectroscopy and mass spectrometry as we previously described (22).
Tumor cell culture and conditioned medium collection

The 4T1 cells, obtained directly from the American Type Culture Collection (ATCC) in 2013, and EO771 cells, developed from an ER+ spontaneous mammary adenocarcinoma (25, 26), were obtained in 2012 and maintained in culture as previously described (24). These two cell lines were authenticated in 2016 by IDEXX Laboratories (IDEXX BioResearch Case #7479-2016). The samples were confirmed to be of mouse origin and no mammalian interspecies contamination was detected. A genetic profile was generated for each sample using a panel of microsatellite markers for genotyping. The 4T1 cells were confirmed to match identically to the genetic profile established for this cell line. The genetic profile for EO771 cell line is more consistent with having been derived from a mouse with a mixed/stock genetic background. A very similar profile has been seen in other sources of EO771 cell lines (27).

For tumor conditioned medium (TCM) collection, cells were grown until they were 80-90% confluent. Then the medium was replaced with serum free (SF) DMEM and the cells were cultured for 48 h. The medium was then collected and passed through a 0.45 µm filter (Millipore Corp., Bedford, MA). The medium was concentrated 10 times using centrifuge filters with a 3,000 MW cutoff (Millipore). Before use the concentrated conditioned medium was diluted 1:2 with fresh SF DMEM.

Tumor cell viability assay

Tumor cells (2x10^4 cells) were seeded into 96 well culture plates in DMEM with 10% FBS and incubated overnight at 37 °C. The cells were then washed with phosphate-buffered saline (PBS), and DMEM containing emodin (0-100 µM) or an equal volume of DMSO was then added to the cells which were then incubated for 24-48 h. The viability of the cells was determined using a Lactate Dehydrogenase (LDH) Cytotoxicity Detection Kit (Clontech Mountain View, CA) according to the manufacturer’s instructions. Briefly, the supernatant was transferred from each well to a new well on the same plate. The cells were then lysed and the reaction mixture was
added to each well and incubated for 5 min. The absorbance was measured at 490 nm on a Spectra Max M5 Microplate Reader (Molecular Devices, Sunnyvale, CA). The percent viability was calculated as the ratio of LDH in the cell lysate to the total amount of LDH in the lysate plus the supernatant. The viability of each group was compared to the control.

**Tumor models**

C57BL/6 and BALB/c mice (8-12 weeks, female) were purchased from Jackson Labs (Bar Harbor, Maine). They were housed at the University of South Carolina Animal Research Facility, and all procedures were approved by the Institutional Animal Care and Use Committee. EO771 or 4T1 cells (2x10^5) in 20 µL of PBS were injected into the 4th pair of mammary glands on C57BL/6 or BALB/C mice, respectively, on Day 0. Starting on Day 1, emodin (40 mg/kg) or vehicle (2% DMSO) was injected intraperitonealy in 1 ml PBS once daily. The tumor size was measured using a caliper every 2-4 days, and the tumor volume was calculated using the formula: V (mm^3) = L (major axis) x W^2 (minor axis)/2. Mice were sacrificed at various time points. Lung’s were fixed and stained with Hematoxylin and eosin to detect metastatic nodule as previously described (24).

Systemic macrophage depletion was performed as previously described (28) with modifications. Briefly, clodronate liposomes (CLIP), purchased from Clodronateliposome.com (Amsterdam, Netherlands), were administered intraperitoneally (1 mg/20 g body weight) on Day -1 and then three times per week until sacrifice. Starting on Day 1, emodin (40 mg/kg) or vehicle (2% DMSO) was injected intraperitonealy in 1 ml PBS once daily.

**Flow cytometry**

The tumor draining lymph nodes and tumors were collected, and cell populations were analyzed using flow cytometry as previously described (29). Briefly, cells were stained with anti-CD3 FITC, anti-CD4 APC or anti-CD8 APC, and anti-CD25 PE (Biolegend) in PBS containing
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2% FBS for 30 min at 4 °C. Samples were washed twice with staining buffer and analyzed by flow cytometry using a BD FACS flow cytometer and CXP software version 2.2 (BD Biosciences, San Jose, CA). Data were collected for 20,000 live events per sample.

For Ki67 staining, 4T1 and EO771 tumor cells were seeded into 6 well plates and cultured in DMEM overnight. They were then treated with emodin (0-50 µM) for 24 h. The cells were resuspended with Trypsin plus EDTA and fixed with 1% paraformaldehyde. The cells were permeabilized with 0.25% Triton X-100 and stained with anti-Ki67 PE (Abcam). The cells were washed and incubated with goat anti-rabbit Alexa 488 (Invitrogen, Eugene, OR). After washing, the samples were analyzed by flow cytometry. Data were collected for 10,000 live events per sample.

Immunohistochemistry

At sacrifice, tumors were embedded in OCT. They were then cut into 8 µm thick frozen sections and placed on slides. For immunohistochemistry staining, the sections were fixed with 4% paraformaldehyde for 10 min, then blocked with 0.01 M glycine containing 0.1% Triton x-100. Next, the sections were blocked with 5% BSA. They were then incubated in primary antibody overnight at 4 °C: anti-F4/80 (1:50, Biolegend), anti-pSTAT6 (1:50, Cell Signaling), or anti-C/EBPβ (1:50, Santa Cruz). The sections were washed with PBS and then incubated with secondary antibodies for 1 h at room temperature. The sections were then stained with DAPI (1 µg/mL) and coverslipped with DABCO. Slides were imaged using a Zeiss LSM 510 Confocal microscope (Zeiss, Peabody, MA). For quantitative analysis, the number of positive cells was manually counted in six random fields of view per section. CD31 staining was performed as described previously (29). Slides were imaged on a Nikon ECLIPSE E600 microscope (Nikon, Melville, NY) at 200 X magnification (10 fields per section). The integrated optical density (IOD) of CD31 was quantified using Image-Pro Plus software.

Isolation of tumor infiltrating cells
Macrophages or T cells were isolated from 4T1 or EO771 tumors at the experimental end point using EasySep™ Mouse PE Positive Selection Kit (Stem Cell Technologies, Vancouver, BC) as previously described (29). For cell isolation, 1x10^7-1x10^8 cells were incubated with 20 µL PE conjugated anti-F4/80 or anti-CD3 (Biolegend) and 50 µL microbeads. The T cells and macrophages were lysed in Qiazol and used for RT-PCR analysis. For ChIP assays, 5-10x10^6 macrophages were fixed in 1% formaldehyde.

**Peritoneal macrophage isolation and treatment**

Mice were injected with 3 mL of 4% thioglycollate solution. After 3 d, macrophages were collected by peritoneal lavage with PBS. The cells were resuspended in DMEM with 10% FBS and cultured for 2 h. The non-adherent cells were then washed away and the remaining cells were cultured overnight in SF DMEM. The cells were then treated with TCM with or without emodin.

**Quantitative real-time PCR (qPCR)**

For qPCR, cells were lysed with Qiazol, and RNA was extracted using Zymo research Direct-zol RNA isolation kit. cDNA was then made from 1 µg of RNA using iScript cDNA Synthesis Kit (Bio-Rad Life Science, Hercules, CA). Primers are listed in Supplemental Table 1; run conditions were 95 °C for 10 s, 58 °C for 15 s, 70 °C for 15 s. Samples were run in duplicate on a Bio-rad CFX Real Time thermocycler.

**Chromatin immunoprecipitation and global histone analysis**

Macrophages were fixed in 1% formaldehyde. Excess formaldehyde was quenched with glycine, and the cells were collected in PBS by scraping. The cells were lysed (0.5% IGEPAL, 4 mM HEPES), and the nuclei were resuspended in nuclear lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1). The DNA was sheared by sonication using a Diagnode Bioruptor Pico (Diagenode, Denville, NJ) for 25 cycles of 30 s on/30 s off. Then 8 µg chromatin was diluted
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1:10 (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl), and 2% of the input was removed from each sample and saved for analysis. Anti-H3K27m3 (Abcam) was added to each sample along with 20 µL of protein A+G magnetic beads (Millipore), and the samples were incubated overnight at 4 °C. The beads were washed with low salt, high salt, LiCl, and TE wash buffers sequentially, and the DNA was eluted off the beads with Proteinase K at 62 °C for 2 h (elution buffer: 200 mM NaCl, 1% SDS and 50 mM Tris). The DNA was then analyzed by qPCR using primers listed in Supplemental Table 2.

In order to detect genome wide levels of histone H3 modifications, histones were isolated from macrophages treated with TCM with or without emodin for 24 h using EpiQuik total histone extraction kits (Epigentek, Farmingdale, NY) according to the manufacturer's instructions. Histones (100 ng) were then analyzed using an EpiQuik Histone H3 modification kits (EpiGentek) according to the manufacturer's instructions.

T cell proliferation and activation assays

Peritoneal macrophages were seeded into 10 cm plates and treated with EO771 conditioned medium with or without emodin for 24 h. The cells were then washed with PBS and resuspended. T cells were isolated from the spleens of C57BL/6 mice using EasySep T cell isolation kit (Stem Cell Technologies) according to the manufacturer's instructions. T cells for the proliferation assay were then labeled with CSFE (Biolegend). T cells were then mixed with macrophages and CD3/CD28 DYNA beads in a 1:1 ratio and added to 12 well plates. The cells were incubated for 24 h (activation assay) or 72 h (proliferation assay) and stained with anti-CD3, anti-CD4, and/or anti-CD69 for 30 min at 4 °C. The cells were then analyzed on a Beckman Coulter FC500.

Transwell migration assay
EO771 or 4T1 cells were treated with 0-50 µM emodin for 24 h. Then the cells were washed with PBS, further cultured, and TCM was collected as previously described. The TCM was placed in the bottom chamber of transwell inserts, and 2x10^5 macrophages were seeded into the top chamber in SF DMEM. The cells were incubated at 37 °C with 5% CO₂ for 4 h. The membranes were then fixed with 4% paraformaldehyde for 10 min. The cells were removed from the top chamber using cotton swabs, and the cells on the bottom chamber were stained with DAPI (1 µg/mL). The inserts were then cut out, mounted onto slides, and imaged under a Nikon Eclipse E-600 fluorescence microscope (Nikon Inc. Melville, NY) at 20 X magnification (5 fields/insert). DAPI stained cells were quantified using ImagePro Plus software.

**Adhesion assays**

For tumor cell adhesion assays, tumor cells were seeded into culture plates with emodin (0-50 µM). The cells were given 1-4 h to adhere to the plate, then plates were washed with PBS and adherent cells were collected with Trypsin and counted.

For macrophage adhesion assays, tumor cell monolayers (80-90% confluent) and macrophages were treated with emodin (0-25 µM) overnight. Then the cells were thoroughly washed with PBS and suspended in fresh SF DMEM with scraping. 5 x10^5 cells were then seeded onto the tumor cell monolayers. After 1 h the non-adherent cells were washed away, and the adherent cells were resuspended with Trypsin and mild scraping. The cells were counted, stained with anti-F4/80 FITC (Biolegend), and analyzed on a Beckman Coulter FC500.

**Statistical analysis**

For all experiments, data were presented as mean ± standard error of the mean (SEM). For two-group comparison, statistical significance was calculated by 2-tailed Student’s t test. For multiple group comparison, one-way ANOVA was used followed by Tukey multiple comparison
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Emodin inhibits breast tumor growth

In our previous study, when emodin treatment began after tumors were well established, it had no effects on the growth of the primary tumor but significantly reduced lung metastasis (24). We hypothesized that emodin might be most effective in the inhibition of primary tumor growth when administered in the early stages of tumor formation. Breast cancer EO771 and 4T1 cells were injected into the mammary glands of C57Bl/6 or Balb/c mice, respectively, and emodin treatment (40 mg/kg IP once daily) began 1 day after tumor cell injection. Emodin caused a significant inhibition of primary tumor growth (Figure 1A) and reduced tumor size (Figure 1B) and tumor weight (Figure 1C) at the endpoints in both EO771 and 4T1 models. We measured lung metastasis from EO771 tumors and found that emodin significantly reduced lung metastatic nodules (Supplementary Figure 1).

Emodin reduces macrophage infiltration and M2-like activation

Our previous study had shown that emodin could inhibit macrophage recruitment and M2-like polarization in metastatic breast cancer in the lungs. Here we investigate whether or not emodin also acts through macrophages in the inhibition of primary breast cancer growth. First we examined macrophage infiltration and phenotype in EO771 tumor bearing mice at the experimental endpoint. Immunohistochemical analysis revealed that emodin significantly reduced the number of tumor infiltrating macrophages (Figure 1E). We extracted F4/80+ cells from the tumors using magnetic beads and used qPCR to examine the expression levels of M1 or M2 macrophage markers. qPCR showed that TAMs in the emodin-treated mice had significantly lower M2 marker (Arg1 and CD206) expression but significantly higher M1 marker...
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(iNOS) expression and also had increased levels of inflammatory cytokines IL1β and TNFα, although without statistical significance (Figure 2A).

To exclude the possibility that reduced TAMs infiltration in emodin-treated mice was the result of halted tumor growth instead of it being the cause, we investigated emodin’s effects on macrophages in tumors at the time point when there was no difference in the size of the tumors between the two groups. Emodin significantly reduced the number of macrophages in 4T1 tumors 26 days post implantation; moreover, emodin significantly reduced the fraction of macrophages positive for transcription factors pSTAT6 and C/EBPβ (Figure 2B and Supplementary Figures 2A and B), indicating that emodin indeed directly inhibited macrophage infiltration and M2 polarization in the tumors independent of tumor size.

Furthermore, we isolated TAMs from the 4T1 tumors at the experimental endpoint and found that the TAMs in emodin-treated mice had significantly decreased expression of IRF4 compared to those in control mice (Figure 2C). IRF4 has previously been shown to play a major role in macrophage M2 activation and is regulated by removal of H3K27 tri-methylation (H3K37m3) by histone demethylase JMJD3 (30, 31). We found that emodin significantly decreased the expression of JMJD3 in TAMs (Figure 2C). Therefore we examined emodin’s effect on H3K27m3 using ChIP-qPCR and found that emodin significantly increased H3K27m3 levels on the IRF4 promoter but not on the CEBPβ promoter (Figure 2D). Taken together, these results indicate that emodin inhibited M2 like polarization in TAMs possibly through epigenetic mechanisms in the breast cancer TME.

To determine if emodin functions mainly through macrophages, we depleted macrophages systemically using clodronate liposomes (CLIP). CLIP treatment significantly decreased tumor volume (p<0.01, compared with Control; Figure 2E). While emodin significantly suppressed tumor growth in mice without macrophage depletion, the effects were
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lost in mice with macrophage depletion, indicating that emodin’s inhibition of tumor growth is predominantly mediated by its effects on TAMs.

Emodin suppresses macrophage response to tumor cell-derived soluble factors

Next we examined the effects of emodin on the response of macrophages to tumor cell-derived factors. Peritoneal macrophages from C57Bl/6 mice were treated with EO771 TCM, and gene expression was examined by qPCR. Emodin dose-dependently inhibited TCM-induced expression of Arg1 and transcription factors C/EBPβ and IRF4 (Figure 3A). Emodin also decreased the expression of CSF1r1 (Figure 3A), a key receptor on macrophages through which they are induced by tumor-secreted CSF1 towards M2-like activation (15). Moreover, emodin inhibited expression of MMP2 and MMP9 (Figure 3A), which have been shown to promote tumor growth through remodeling the extra cellular matrix (9). Interestingly, we also found that TCM treatment increased expression of ICAM1 in macrophages, and the effect was blocked by emodin, suggesting that emodin could interfere with macrophage adhesion to tumor cells or other cell types in the tumor. In agreement with the in vivo data, TCM treatment increased the expression of JMJD3, and emodin significantly attenuated the increase (Figure 3B). Both TCM and emodin had no effects on global levels of H3K27 methylation (Figure 3C). However, TCM decreased H3K27m3 on the promoters of IRF4, Arg1, and C/EBPβ; and emodin treatment reversed the reduction (Figure 3D). These results indicate that emodin epigenetically inhibits macrophage M2-like polarization in response to tumor-derived soluble factors.

Emodin increased T cell activation and decreased angiogenesis

TAMs substantially contribute to the immunosuppressive microenvironment in tumors. Since emodin inhibited TAM infiltration and M2-like polarization, we hypothesized that emodin treatment would lead to increased T cell activation in breast tumors. T cells were detected in the draining lymph nodes of mice bearing 4T1 tumors using flow cytometry. Emodin treated mice had increased activated CD4⁺ and CD8⁺ T cells (Figure 4A and Supplementary Figure 3). There
was a similar trend of increased activated T cells in the tumors of emodin treated mice (Figure 4B). We then isolated CD3+ cells from the tumors and analyzed them using qPCR. T cells from emodin treated mice had a two-fold increase in IFNγ expression compared to those from control mice (Figure 4C). Taken together, these data indicate that emodin treatment led to increased T cell activation in breast tumors.

Peritoneal macrophages were pre-treated with EO771 TCM with or without emodin for 24 h. Then the macrophages were incubated 1:1 with T cells stimulated with CD3/CD20 beads for 24 h. TCM treated macrophages reduced expression of activation marker CD69 by 70% on CD4 T cells compared to control macrophages; however, pre-treatment of macrophages with emodin along with TCM completely blocked the suppression of T cell activation and even increased CD69 expression on CD4 T cells above that of T cells co-cultured with control macrophages (Figure 4D). T cell proliferation was examined after 72 h by CSFE depletion analysis and revealed that TCM and emodin co-treated macrophages restored T cell proliferation which was suppressed by TCM only-treated macrophages (Figure 4E).

TAMs have also been shown to promote angiogenesis in breast tumors (32). Therefore, we examined the effects of emodin on angiogenesis in tumors by staining tumor sections with mouse endothelial antigen CD31 to detect microvessel density. Emodin significantly decreased CD31 staining in EO771 tumors to almost 50% of that in control (Figure 4F). Taken together, these data indicate that by regulating TAM infiltration and polarization, emodin was able to increase T cell activation and suppress angiogenesis in breast cancer.

Emodin affected gene expression in breast cancer cells

Tumor cell-TAM interaction has been shown to be a complex feedback loop that leads to a pro-tumor macrophage phenotype (14). Our results have shown that emodin can inhibit the macrophage response to tumor signals, but it is unknown if emodin could affect the ability of tumor cells to communicate with and therefore train macrophages. EO771 and 4T1 cells were...
treated with emodin in vitro and cell viability (LDH method) and proliferation (Ki67 staining) were determined. The results showed that emodin had low toxicity toward the two cell lines. Emodin only slightly decreased cell viability starting at 25 µM (Figure 5A) and had no significant effect on cell proliferation at concentrations less than 50 µM (Figure 5B and Supplemental Figure 4A). We further examined the effects of emodin on tumor cell adhesion in vitro. We found that emodin increased the time needed for 4T1 cells to adhere to the plate; it decreased the number of 4T1 cells adhered after 1 h but the effects diminished after 4 h (Supplemental Figure 4B). Emodin showed no effects on EO771 cell adhesion (Supplemental Figure 4C). These results are in agreement with our in vivo studies which showed no significant difference in tumor size at the early time point (16 d for EO771 cells and 19 d for 4T1 cells), indicating that emodin does not significantly disrupt the establishment of tumor grafts.

We then examined the effects of emodin on tumor cell gene expression. Emodin significantly inhibited the expression of MCP1, CSF1, and CSF2 in both 4T1 and EO771 cells (Figure 5C). Emodin treatment also significantly inhibited tumor cell expression of Thy1 (Figure 5D), which has been shown to help anchor macrophages to the tumor cells (12). These results indicate that emodin could interfere with the ability of tumor cells to signal to, attract, and polarize macrophages.

**Emodin blocks macrophage-tumor cell interaction**

We examined if emodin could inhibit tumor cell induction of macrophage migration. Conditioned medium was collected from tumor cells treated with various concentrations of emodin, and its ability to induce macrophage migration was examined. There was a decrease in macrophage migration toward the TCM collected from emodin treated cells (Figure 6A). These results suggest that emodin inhibits the ability of breast cancer cells to attract macrophages.

Recent studies have shown that tumor cells can use juxtacrine signaling to communicate with macrophages and induce them toward a pro-tumor phenotype (12, 13, 33). We therefore
examined the effects of emodin on tumor cell-macrophage adhesion by pre-treating macrophages, tumor cells, or both with emodin. We found that emodin treatment of either macrophages or tumor cells significantly inhibited the adhesion of macrophages to a monolayer of tumor cells, and treatment of both macrophages and tumor cells decreased the adhesion even further (Figure 6B). Taken together, these data show that emodin inhibits breast cancer cell-macrophage adhesion by acting on both cell types.

**DISCUSSION**

Our data show that emodin significantly inhibited the growth of breast cancer by modulating the tumor microenvironment. Emodin inhibited tumor cell-macrophage interactions through blocking the response of macrophages to tumor signals and by inhibiting the paracrine and juxtacrine signaling from tumor cells to macrophages. Through its effects on TAMs, emodin effectively increased T cell activation and inhibited angiogenesis in breast tumors. Our results indicate that emodin blocks the pro-tumor feedforward loop between breast cancer cells and macrophages by targeting both cell types (Figure 6C).

Our previous study showed that emodin had no effect on the growth of well-established breast tumors (24). Late stage tumors with established microenvironments already have high numbers of infiltrating macrophages as well as significant hypoxic areas (34, 35). It is possible that emodin might not be able to penetrate into the poorly vascularized regions. However, emodin treatment at the late stage can effectively suppress metastatic tumor growth (24). Taken together, our studies suggest that emodin may be most effective in treating early stage breast cancer, or inhibiting tumor recurrence or metastasis after surgical removal of primary tumors in patients. Since emodin’s poor oral bioavailability limits its effectiveness (36), intravascular delivery may be needed for patients. Certain formulations, for example, nanoparticles, may facilitate the delivery and improve bioavailability (36, 37).
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The majority of previous studies on emodin have been focused on emodin’s direct toxicity to tumor cells (19, 38). In this study, we used two orthotopic models of breast cancer, EO771 and 4T1. We found that emodin exhibited little or no direct toxicity towards either of the cell lines at concentrations lower than 25 µM which is the highest plasma concentration of emodin we achieved in vivo following intraperitoneal administration (22). Therefore, it is likely that emodin inhibits tumor growth predominately through modulating the tumor microenvironment in our study. Emodin decreased macrophage infiltration and M2 polarization in breast tumors by suppressing IRF4, STAT6, and C/EBPβ signaling. Here we are also the first to show that emodin epigenetically regulated TAM polarization by inhibiting IRF4, CEBPβ, and Arg1 expression through increasing H3K27m3 on their promoter regions, likely by decreasing the expression of H3K27 demethylase JMJD3.

Emodin has been reported to inhibit a wide variety of kinases, including several involved in macrophage activation (18, 39). Emodin could inhibit the JAK/STAT signaling pathways either directly though inhibiting JAK activity or indirectly through inhibiting CK2 (38, 40). It is also possible that emodin could inhibit the polarization of macrophages toward the TAM phenotype through regulating ROS production. Emodin has been shown to directly regulate ROS production in cells (18, 41). Zhang et al. reported that ROS production is necessary for alternative macrophage activation and that inhibition of ROS could block TAM generation in tumors (42). Therefore, emodin could inhibit TAM activation through blocking JAK/STAT signaling and/or through regulating ROS production in the macrophages. However, the direct molecular targets of emodin in macrophages in the context of cancer warrant further identification.

Emodin treatment increased T cell activation in tumors. Our data suggest that emodin activates T cells indirectly through inhibiting TAM-mediated immune suppression. TCM treated macrophages significantly inhibited T cell activation and proliferation; however, emodin
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treatment abrogated the suppressive ability of TCM treated macrophages. Nevertheless, we cannot completely exclude the possibility that emodin might also directly affect T-cell function in the context of breast tumor. The reduced angiogenesis in tumors of emodin-treated mice may be due to several mechanisms. Emodin has previously been shown to inhibit tumor angiogenesis by inhibiting tumor cell production of MMPs and VEGF (21, 43). However, we found that emodin had no effect on the expression of VEGF in 4T1 or EO771 cells or in macrophages (data not shown), but our results showed that emodin inhibited macrophage expression of MMP2 and MMP9 in response to tumor cell derived soluble factors. In addition, emodin has been reported to directly target endothelial cells to inhibit proliferation and vessel formation and reduce expression of cell adhesion molecules, ICAM-1, ELAM-1 and VCAM-1 (44-46). Therefore, it is likely that emodin inhibited angiogenesis through regulating the tumor microenvironment by targeting TAMs, as well as possibly targeting endothelial cells.

Although emodin did not cause direct cytotoxicity to breast cancer cells at low concentrations, it did significantly inhibit tumor cells to attract and polarize macrophages through blocking the secretion of MCP1 and CSF1, two chemokines that play important roles in the TME (33). In addition, emodin suppressed ICAM1 expression in macrophages and Thy-1 expression in breast cancer cells, thus blocking the direct adhesion between these two cell types. Emodin’s ability to inhibit cell-to-cell adhesion has been previously reported for different cell types (46, 47).

Our results indicate that emodin acts on both breast cancer cells and TAMs and thus ameliorates the immunosuppressive TME in breast cancer. There have been many obstacles for the development of immunotherapies, including the ability of tumors to adapt to immune attack, known as immunoediting, and the immunosuppressive environment supported by TAMs and other stromal cells (48, 49). Further, the development of TAM targeting therapies has been complicated by the diversity of TAM phenotypes within tumors (3, 49, 50). Emodin’s ability to
Emodin blocks cancer cell-macrophage interaction

interfere with the communication between tumor cells and TAMs, as demonstrated in our study, gives it great therapeutic potential to overcome these obstacles; therefore, emodin may be developed as a potent therapy for breast cancer, especially in combination with other immunotherapies or cancer cell targeting therapies.

REFERENCES

Emodin blocks cancer cell-macrophage interaction

Emodin blocks cancer cell-macrophage interaction

FIGURE LEGENDS

**Figure 1. Emodin inhibits growth of breast tumors.** C57Bl/6 (n = 7 or more) or Balb/c (n =7 or more) were injected with 2x10^5 EO771 or 4T1 cells, respectively. Emodin treatment (40 mg/kg IP once daily) began on Day 1 following injection of tumor cells. **A.** Tumor size was measured with calipers and volume was calculated using the following formula: V (mm^3) = L x W^2/2. **B.** Representative image of tumors. **C.** Weight of tumors. **D.** EO771 tumors were embedded in OCT (n = 5). Tumors were cut into 8-µm sections and stained for F4/80. Sections were imaged (200 X, 10 fields per section), and the number of positive cells was manually counted. Results are shown as means ± S.E. *p<0.05, **p<0.01, ***p<0.001 versus control.

**Figure 2. Emodin reduces macrophage infiltration into tumors and M2-like activation.** **A.** F4/80+ cells were isolated from the EO771 tumors of C57BL/6 mice (n = 5). Gene expression was detected using RT-qPCR. **B.** 4T1 tumors from Balb/c mice were collected 26 days post injection and embedded in OCT (n = 5 or more). Tumors were cut into 8-µm sections and stained for F4/80 and pSTAT6 or C/EBPβ. Sections were imaged (200 X, 5 fields per section), and the number of positive cells was manually counted. Results are shown as means ± S.E. **C.** F4/80+ TAMs were isolated from 4T1 tumors at the experimental endpoint using magnetic beads. Gene expression (n = 7 or more) was detected using RT-qPCR. **D.** A ChIP assay was used to detect H3K27m3 levels on the promoters of IRF4 and C/EBPβ (n = 6 or more). Results are shown as means ± S.E. **E.** Clodronate-liposomes (CLIP, 50 mg/kg) or PBS (control) were injected into Balb/c mice on day -1. On day 0, 4T1 cells (2x10^5) were injected into the mammary fat pads. Emodin (40 mg/kg) or vehicle control (2% DMSO) were administered IP once daily beginning on day 1. CLIP or PBS was then injected 3 times a week until the mice were sacrificed. Tumor size was measured with calipers and volume was calculated using the following formula: V (mm^3) = L x W^2/2. Results are shown as means ± S.E of each tumor, n=5 or more tumors per group. *p<0.05, ***p<0.001, versus control.
Figure 3. Emodin suppresses macrophage response to tumor cell secreted factors.

Peritoneal macrophages from C57Bl/6 mice were stimulated with EO771 TCM with or without emodin for 24 h. A and B. Gene expression was measured using RT-qPCR, n = 3. Results are shown as means ± S.E of one of two independent experiments. C. Histones were extracted from the macrophages and histone modifications were detected using an EpiQuick histone modification kit, n = 3. D. Gene specific H3K27m3 levels were examined using a ChIP assay. Results are shown as means ± S.E. of one of two independent experiments, n = 3. *p<0.05, **p<0.01, ***p<0.001.

Figure 4. Emodin attenuates the effects of macrophages on T cell activation and angiogenesis. A and B. The draining lymph nodes (A) and tumors (B) from mice bearing 4T1 tumors (n = 7 or more) were collected at the experimental endpoint. A single cell suspension was made, and the cells were stained with CD3, CD4 or CD8, and CD25 to detect activated T cells. Cells were analyzed using flow cytometry. Results are shown as means ± S.E. C. CD3+ T cells were isolated from the draining lymph nodes of mice bearing 4T1 tumors 6 weeks after tumor cell injections. Expression of IFNγ was detected using RT-qPCR. D. Peritoneal macrophages from C57Bl/6 mice were treated with EO771 TCM with or without emodin for 24 h. They were then washed and co-cultured with T cells isolated from the spleens of mice and stimulated with CD3/CD20 microbeads at a ratio of 1:1. After 24 h, the T cells were collected and stained with CD3, CD4, and CD69 and analyzed using flow cytometry. Left panel shows representative flow cytometry results. Right panel shows results as means ± S.E. (n = 3). E. Macrophages were pre-treated with TCM with or without emodin for 24 h. Then they were washed and co-cultured with CSFE-labeled T cells and stimulated with CD3/CD20 microbeads. After 72 h, the cells were collected and stained for CD3 and CD4, and CSFE depletion was detected as a measure of proliferation. Results are shown as means ± S.E. of one of two independent experiments, n = 3. F. EO771 tumors were collected from mice 5 weeks post
Emodin blocks cancer cell-macrophage interaction

injection and embedded in OCT. The tumors (n = 5) were cut into 8 μm thick sections, stained with CD31, and imaged (200 X, 10 fields per section). Images were quantified using ImagePro plus by calculating the IOD for CD31 positive areas. Results (right panel) are shown as means ± S.E. *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Effects of emodin on breast cancer cells. Tumor cells were treated with emodin (0-100 μM) for 24-48 h. A. Cell viability was determined using an LDH assay. Results are shown as means ± S.E. for one of two independent experiments, n = 4. B. Tumor cells were stained for Ki67 after 24 h culture. Cells were then analyzed using flow cytometry. Results are shown as means ± S.E., n = 4. C and D. After 24 h, gene expression was examined using qPCR. Results are shown as means ± S.E. of one of two independent experiments, n = 3. *p<0.05, **p<0.01, ***p<0.001, versus control.

Figure 6. Emodin inhibits macrophage migration and adhesion to tumor cells. A. 4T1 cells were treated with emodin for 24 h. The cells were then washed and cultured in fresh medium for 48 h. Conditioned medium was collected from the cells and placed in the bottom chamber of transwell inserts. Peritoneal macrophages were placed in the top and incubated for 4 h. The cells were then fixed and the membranes were mounted onto slides and imaged (200 X, 5 fields per membrane). The cells that migrated to the bottom chamber were counted. Results are shown as means ± S.E. of one of two independent experiments, n = 3. B. Peritoneal macrophages from Balb/c mice and/or 4T1 cells were treated with emodin for 24 h. Then the macrophages were collected and seeded onto 4T1 cell monolayers. After 1 h, non-adherent cells were washed away and the remaining cells were collected and counted. Cells were then stained with F4/80 and analyzed using flow cytometry. Results are shown as means ± S.E., n = 3. *p<0.05, **p<0.01, ***p<0.001. C. Illustration showing how emodin interferes with tumor cell-macrophage interactions and thus inhibits breast cancer growth. Red “X”s indicate the functions of emodin, including 1) suppressing M2-like polarization of TAMs by targeting signaling through
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IRF4, STAT6, and CEBPβ, 2) mitigating the immunosuppressive and pro-angiogenic activities of TAMs, 3) decreasing the expression and secretion of MCP-1 and CSF-1 in breast cancer cells, thus reducing the migration of macrophages towards cancer cells, and 4) decreasing the expression of ICAM-1 in TAMs and the expression of Thy-1 in breast cancer cells, thereby blocking the adhesion between the two cell types.
Figure 1 A: E0771 and 4T1 cell lines were inoculated into mice. Tumor volume (mm$^3$) and weight (g) were measured over time.

B: Images of tumor tissues from control and Emodin-treated groups.

C: Graphs showing tumor weight over time for control and Emodin-treated groups.

D: Immunofluorescence images of F4/80+ cells in tumor tissues.
Figure 2
**Figure 3**

(A) Relative Expression of Arg1, CEBPB, IRF4, CSFR1, MMP2, MMP9, and ICAM1 in Con, TCM, TCM-EM25, and TCM-EM50.

(B) Relative Expression of JMJD3 in Con, TCM, TCM-EM25, and TCM-EM50.

(C) Fold change of H3K27me1, H3K27me2, and H3K27me3 in Con, EM, TCM, and TCM-EM.

(D) Normalized to input levels of IRF4, Arg1, YM1, and CEBPB in Con, TCM, and TCM-EM.
Figure 4

A. % of total LN cells by CD4+CD25+ and CD8+CD25+ cells. 
B. Tumor T cells: CD4+CD25+ and CD8+CD25+ cells (x10^3)/mg. 
C. Relative Expression of IFN-γ in Tumor T cells. 
D. Unstained, Control MΦ, TCM MΦ, and TCM-EM MΦ cell number distribution. 
E. T cell proliferation: Con, TCM, and TCM-EM MFI (CD69). 
F. Tumor CD31: Control and Emodin stained sections.

**p=0.057

% of total LN cells

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Cells (x10^3)/mg

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Relative Expression

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T cell activation

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% of control

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Tumor CD31

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**p=0.057

Tumor T cell

IFN-γ

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Fold change

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Unstained

Control MΦ

TCM MΦ

TCM-EM MΦ

CD69

100 μm
Figure 5
Figure 6

A

Treatment of 4T1 cells

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MΦ migration

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MΦ-4T1 adhesion

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C

Immune suppression

MΦ migration

MΦ-4T1 adhesion

Breast Cancer cell

MΦ MΦ+ 4T1 4T1 4T1

Adhesion

MCP-1 CSF-1

Arg1

IRF4 STAT6 CEBPβ

ICAM1 Thy1

Immune suppression

Angiogenesis

Figure 6
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Molecular Cancer Therapeutics

Emodin inhibits breast cancer growth by blocking the tumor-promoting feedforward loop between cancer cells and macrophages

Stephen Iwanowycz, Junfeng Wang, Johnie Hodge, et al.

Mol Cancer Ther Published OnlineFirst May 18, 2016.

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Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0987

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