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Original Article

Baicalein suppress EMT of breast cancer by mediating tumor-associated macrophages polarization

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Abstract: Tumor associated macrophages (TAMs) are the main infiltrating component in the tumor microenvironment and play an important role in cancer progression. Baicalein has a wide range of pharmacological properties. This study explores the potential effect of baicalein on macrophages polarization and epithelial-mesenchymal transition (EMT) of breast cancer. Co-culture system was established to evaluate the interaction between TAMs and breast cancer cells. Then the role of baicalein in modulating TAMs function was assessed. Finally, breast cancer mouse model was established to study the underlying mechanism. In vitro experiments showed that co-culture with M2 macrophages significantly enhanced EMT of both MDA-MB-231 and MCF-7 breast cancer cells. Baicalein could regulate polarization of M2 and attenuate TGF-β1 secretion. In vivo experiments showed that compared with the MDA-MB-231 + M2 group, tumor growth and metastasis of baicalein + MDA-MB-231 + M2 group was significantly inhibited, with smaller tumor size and decreased lung metastasis lesions. Our findings suggest that the regulation of TAMs may be a novel mechanism underlying the anti-tumor effects of baicalein in breast cancer.

Keywords: Baicalein, TAMs, TGF-β1, EMT, breast cancer

Introduction

Breast cancer is the most common cancer type and the second leading cause of cancer death for females worldwide and is expected to account for 29% of all new cancer diagnoses [1]. Recent advances in diagnosis, chemotherapies, hormonal therapies and anti-HER2 therapies have significantly improved the prognosis of breast cancer. However, metastasis is still main cause of cancer death [2]. Traditionally, breast cancer is regarded as a low immunogenic carcinoma, but the impact of local immunologic factors has attracted more and more attention [3]. Our group has shown that TAMs are associated with poor OS (overall survival) and RFS (relapse-free survival) in breast cancer [4]. As the association between tumor cells and TAMs becomes clear, TAMs are starting to be regarded as potential biomarker for diagnosis and prognosis of cancer, and even a therapeutic target [5, 6].

Recent studies have shown that the functions of macrophages change as tumor progress. Macrophages acquire different phenotypic characteristics through different activation mechanisms, including classical activated macrophages (M1-like) and alternatively activated macrophages (M2-like) [7]. M1 and M2 macrophages have distinct cytokines and receptors expression. CD86 and CD80 are used as M1 macrophages marker, and CD206 and CD163 are used as M2 macrophages marker [8, 9]. CD68 is a pan macrophage marker [9]. Tumor associated macrophages (TAMs) mainly are of the M2 phenotype and are the main infiltrating component in tumor microenvironment with content varying from 50% to 80% in human cancers [10-12]. Accumulated evidence indi-

cates TAMs play a critical role in cancer and that, more importantly, TAMs can be reprogrammed to anti-tumor phenotype (M1), which may provide a perfect target for suppressing tumor progression [13, 14].

Tumor metastasis is a complex multistep process and EMT is an early and important step of metastasis. It has been reported that TAMs are associated with EMT and that cytokines secreted by TAMs in the tumor microenvironment may promote cancer stem cell-like properties and EMT [15, 16]. TGF-β1 is a strong EMT inducer and can activate Smad signaling and non-Smad signaling pathway, such as MAPK, PI3K, and AKT signals [17]. Akt, also named protein kinase B or PKB, is a serine/threonine kinase that plays vital role in phosphoinositide 3-kinase (PI3K)-Akt signaling pathway. This cascade exerts a crucial role in oncogenesis, drug resistance and EMT [18, 19].

Baicalein is a widely used Chinese herbal medicine derived from the root of scutellaria baicalensis that possesses many biochemical and pharmacological benefits including anti-fibrosis, anti-microbial, anti-inflammation and anti-tumor properties [20, 21] and has a defined chemical structure. Emerging studies have demonstrated that Chinese medicinal herbs can exert their anti-tumor effects through targeting of the tumor microenvironment [22, 23]. However, there are still no studies about the role of baicalein on TAMs regulation in breast cancer.

Material and methods

Reagents and cell lines

Human breast cancer cell lines of MDA-MB-231 and MCF-7, and human acute leukemia mononuclear cell line of THP-1 were obtained from Shanghai Cell Biological Institute of the Chinese Academy of Science (Shanghai, China). MDA-MB-231 cells were cultured in DMEM-F12 (Hyclone, USA), MCF-7 cells were cultured in DMEM (Hyclone, USA) and THP-1 cells were cultured in RPMI 1640 (Hyclone, USA) according to instructions. All the medium were supplied with 10% fetal bovine serum (Gibco, Life Technologies, USA) and 1% penicillin-streptomycin solution (Thermo Fisher Scientific, USA). Cells were cultured at 37°C in moist atmosphere containing 5% CO_2.

The stock solution of baicalein (465119, Sigma-Aldrich) was diluted with dimethyl sulfoxide (DMSO) and stored in -20°C in the dark. Recombinant human TGF-β1 (240-B, R&D Systems, USA) was used with condition of 5 ng/ml for 24 h. Anti-TGF-β1 neutralizing antibody (MAB240, R&D Systems, USA) 1.0 μg/m was used 1 h before other treatment [24]. TGFβR-I/II inhibitor (LY2109761, Selleck Chem) 10 μM was used to block action of TGF-β1 two hours ahead of other treatment as described previously [25]. Baicalein treatment and extraction of RNA and protein were all performed during the logarithmic growth phase of cells.

Co-culture procedure

Breast cancer cells and THP-1-derived macrophages were co-cultured using cell culture insert (Corning, NY, USA) with 0.4 μm porous membrane to separate the upper and lower chambers. THP-1 monocytes (5×10^5 cells/ml) were seeded into the upper chamber of the transwell apparatus, stimulated to differentiate into macrophages by the addition of 100 ng/ml PMA (phorbol myristate acetate) (Sigma-Aldrich) for 24 h and IL4/IL13 (Sino Biological, China) for another 24 h as described before [26]. Then the chambers were washed three times with phosphate-buffered saline (PBS). The MDA-MB-231 and MCF-7 cells were placed in the lower chamber at a density of 2.5×10^5 cells/ml for 24 h to allow their adherence to the walls. After that, chambers with the THP-1-derived macrophages were placed directly on top of the six-well plates containing the MDA-MB-231 and MCF-7 cells. The co-culture systems were incubated for 24 h in serum-free RPMI 1640. Breast cancer cells and THP-1 derived macrophages alone were also cultured for 24 h in serum-free RPMI 1640 as controls.

Flow cytometry

Monocytes with different treatments were collected and washed twice in PBS, then cells were stained with FITC-CD68 (eBioscience, 11-0689) or FITC-CD86 (eBioscience, MHCD8601) antibody for 30 min at room temperature using the recommended dilution of instruction. As to CD206 detection, cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature and next permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 5 min, then stained

Table 1. RT-PCR primers

Gene	Primer sequence	
	Forward (5'-3')	Reverse (5'-3')
TGF-β1	TCCTGGCGATACCTCAGCAA	GCTAAGGCGAAAGCCCTCAA
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
Arginase-1	GTGGAAACTTGCATGGACAAC	AATCCTGGCACATCGGGAATC
IL-12 (p35)	CCTTGCACTTCTGAAGAGATTGA	ACAGGGCCATCATAAAAGAGGT
TNF-α	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG

with PE-CD206 (eBioscience, 12-2069-42) antibody for 30 min at room temperature using the recommended dilution. After incubation, cells were washed twice for examination and analyzed with FlowJo software.

CCK-8 assay

Effects of baicalein on the vitality of MDA-MB-231, MCF-7 and induced TAMs were analyzed by CCK-8 assay (7 sea biotech, China). 5 × 10^3 cells/well were seeded in 96-well plates and treated with different concentrations of baicalein for 24 h, 48 h and 72 h at 37°C in a moist atmosphere containing 5% CO_2. Then the medium was removed and replaced with fresh medium containing 10% CCK-8. After incubating 3 h, the optical density of each well was measured at a wavelength of 450 nm and recorded with a standard ELISA microplate reader (BioTek). The inhibition rate was calculated as follows: Inhibition rate = (1 - (OD drug treated - OD blank)/(OD control - OD blank)) × 100% [27].

RT-PCR assay

Total RNA was obtained using MiniBEST Universal RNA Extraction Kit (TaKaRa, Tokyo, Japan). cDNA was synthesized with PrimeScriptTM RT Master Mix (TaKaRa). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) reaction system was prepared following the instructions of SYBR® Premix Ex TaqII (Tli RNaseH Plus) RT-PCR Kit (TaKaRa). GAPDH was used as endogenous control. Each sample was analyzed in triplicate by using a StepOne™Real-Time PCR System (Thermo Fisher Scientific). Data of gene copy number was analyzed in accordance with the $2^{-\Delta\Delta Ct}$ method relative to expression of GAPDH and compared with control. Primers for specific genes in this study were listed in **Table 1**.

Western blotting and ELISA

Cells were collected and cells lysis was used for Western blot analysis. Antibodies used for Western blot analysis were purchased from Cell Signaling Technology (Danvers, MA, USA): E-cadherin (rabbit mAb, 24E10), N-cadherin (rabbit mAb, D4R-1H), vimentin (rabbit mAb, D21-H3), AKT (pan, rabbit mAb, C67E7) and p-AKT (Ser473, rabbit mAb, D9E). Tubulin (ab7291, mouse mAb) and TGF-β1 (ab179695, Rabbit monoclonal) was bought from Abcam (Cambridge, MA, USA). Peroxidase-conjugated anti-mouse or rabbit antibody (CST) was used as secondary antibody and the antigen-antibody reaction was visualized by enhanced chemiluminescence assay (ECL, Thermo). After centrifugation, culture supernatant was collected and level of TGF-β1 secreted by macrophages and breast cancer cells was qualified using active TGF-β1 ELISA kits (R&D Systems) according to the manufacturer's instructions.

Immunofluorescence

Immunofluorescence assay was used to identify the expression of Ki67. Cells were seeded in 24-well plates with PLL-coated coverslips at density of 2 × 10^4/well. After cells adhered overnight, 4% paraformaldehyde (PFA) was used to fix cells on coverslips for 30 min at room temperature. Then cells were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) for 5 min and blocked with goat serum (CWBIO, China) for 1 h. Primary antibodies rabbit anti-Ki67 (1:200; Abcam) were added on the coverlips and incubated overnight at 4°C. After that, second antibodies cy3 goat anti-rabbit IgG (1:200; CWBIO) was used for 2 h in the dark at room temperature. All antibodies were diluted with PBS. Controls for non-specific binding included omitting primary or secondary antibodies. Then cells were washed and stained with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/mL) for 5 min. After coverslips were placed on glass slides using glycerinum, images were taken by Olympus BX51 fluorescence microscope equipped with a DP71 digital camera connected to computer (Olympus). Then cell numbers of Ki67 expression and DAPI expression were counted and averaged in five random fields.

Immunohistochemistry and scoring

Tumor tissues and metastasis lesions were collected and paraffin-embedded. Then paraffin-embedded tissues were sliced into 5 μm sections. The sections were incubated with F4/80 (GB11077-1, 1:1000), CD206 (GB110-62, 1:500), TGF-β1 (GB11179, 1:500), iNOS (GB11119, 1:1000), N-cad (GB11135, 1:2000), Vimentin (GB11192, 1:1000), E-cad (GB13083, 1:400) and p-AKT (GB13012-3, 1:100) overnight at 4°C. Primary antibodies for IHC were all purchased from Servicebio technology (China). Images were taken using Zeiss microscope (Zeiss Microscopy, Jena, Germany). Stained slides were scored by a pathologist who was blind to the grouping. Staining, scoring and qualification was carried out as described in a recent study [28]. Staining intensity was graded as negative (0-5%), weak (6-25%), moderate (26-50%), intense (51-75%) or very intense (76-100%) according to the intensity of each slide, with H score ranging from 0-4.

Invasion assay

The invasion assay was conducted using 24-well transwell chambers of 8 μm pore size with Matrigel (BD Biosciences, 1:8 dilution). MDA-MB-231 (3×10^4 cells, 30 h) and MCF-7 (1×10^5 cells, 70 h) under different treatment were seeded in serum free medium. The lower chambers were filled with medium supplemented with 10% FBS as chemo-attractant. Untreated MDA-MB-231 and MCF-7 were taken as controls. Then the upper chambers were washed with PBS and then fixed in 100% methanol. Cells remained in the upper chamber were wiped off gently and those passed through pores to the lower surface were stained using 0.3% crystal violet solution and washed with PBS. After that, cell numbers were counted and averaged in five random fields at magnification of × 200.

In vivo studies

BALB/c nude mice of 6-8 weeks were divided into three groups (N = 5/each group), the first group and second/third group were subcutaneously injected with MDA-MB-231 cancer cells alone (2×10^6 cells) and MDA-MB-231 cancer cells (2×10^6 cells) mixed with M2 macrophages (4×10^6 cells) respectively into right side of axillary. Cells were resuspended in 50% serum-free RPMI1640 and 50% Matrigel medium with total volume of 200 μl [29]. Tumor size was measured every three days. Tumor volume was calculated using formula: V (mm^3) = L (major axis) × W^2 (minor axis)/2. The third group was treated with baicalein (dissolved in corn oil, 50 mg/kg, every other day) by gavage after 1 week since inoculation and the other two groups were treated with vehicle meantime. To obtain tumor tissues, mice were sacrificed after 5 weeks.

Statistical methods

All results of quantitative assays were shown as mean ± SD from three independent experiments. Statistical significance was determined using Student's t-test with SPSS statistical software (version 12.0). $P<0.05$ was considered statistically significant. And all graphs were made using GraphPad Prism 6 software (GraphPad Software, Inc.).

Results

Morphology of cancer cells changed after co-culture with TAMs

After treating THP-1 with PMA for 24 h and IL4/IL13 for another 24 h, results showed that cell morphology changed from round to multiple apophysis (**Figure 1A**) and there was a significant increase of CD68 (pan macrophage marker) and CD206 (M2 specific marker) (**Figure 1B**) expression, which indicated that PMA and IL-4/IL-13 treatment in vitro could induce THP-1 to differentiate to M2 macrophages, as mentioned in lots of literature [8, 30].

Besides, morphology of both MDA-MB-231 and MCF-7 changed after co-culture with M2 macrophages. The shape of MDA-MB-231 tended to be more fusiform (**Figure 1C**) and shape of MCF-7 changed from epithelioid phenotype to mesenchymal phenotype (**Figure 1C**) with cell gap enlarged, which was more prone to metastasis [31, 32].

TGF-β1 secretion promotes MDA-MB-231 and MCF-7 cellular proliferation and metastasis in vitro

Active TGF-β1 secretion in mono-culture and co-culture supernatant of M2, MDA-MB-231 and MCF-7 was tested. M2 macrophages cultured alone secreted more active TGF-β1 than

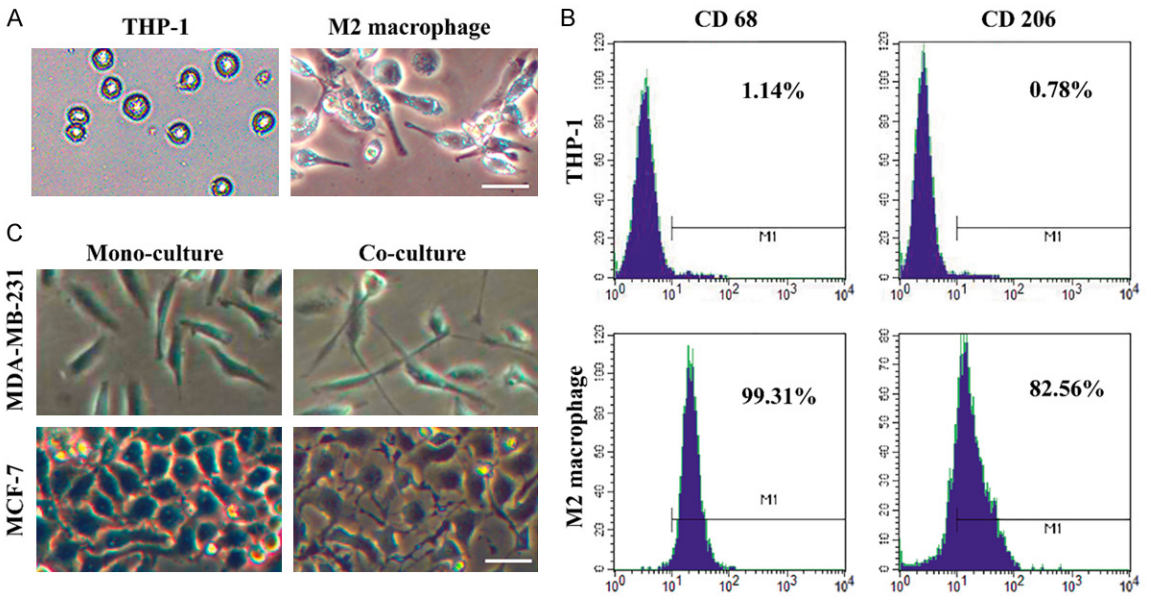

Figure 1. M2 macrophages induction and morphology change of cancer cells after co-culture. A. Morphology of THP-1 after treatment of PMA for 24 h and IL-4/IL-13 for anther 24 h. B. CD68 and CD206 expression of THP-1 after treatment with PMA and IL-4/13 was analyzed by FACS. C. Morphology of MDA-MB-231 and MCF-7 after co-culture with M2 macrophages for 24 h. Scale bar = 50 μm.

MDA-MB-231 and MCF-7 breast cancer cells cultured alone (P<0.001, P<0.001) (**Figure 2A**). Besides, active TGF-β1 secretion of co-culture system increased significantly (P<0.001, **Figure 2A**). Moreover, mRNA level of TGF-β1 was analyzed. Results showed that TGF-β1 mRNA level of M2 macrophages co-cultured with MDA-MB-231 and MCF-7 increased nearly eight to nine times compared with M2 macrophages cultured alone (**Figure 2B**). And TGF-β1 mRNA level of MDA-MB-231 and MCF-7 after co-culture increased about three to four times (**Figure 2C**). From the above, TGF-β1 secretion in co-culture medium was significantly increased and TGF-β1 mRNA levels of M2, MDA-MB-231 and MCF-7 after co-culture were obviously upregulated. We next analyzed metastasis and proliferation ability of cancer cells before and after co-culture.

After co-culture with TAMs, invasion cell numbers of both MDA-MB-231 and MCF-7 were significantly increased (P<0.01, P<0.05). And rhTGF-β1 (recombinant TGF-β1) treatment alone achieved the same effect for MDA-MB-231 and MCF-7 cancer cells (P<0.05, P<0.01). Besides, addition of anti-TGF-β1 neutralizing antibody before co-culture could reverse invasion ability of both MDA-MB-231 and MCF-7 cancer cells compared with breast cancer cells co-cultured with M2 macrophages (P<0.01, P<0.05) (**Figure 2D**). Immunofluorescence of Ki67 indicated that proliferation of both MDA-MB-231 and MCF-7 was enhanced after co-culture (P<0.01, P<0.05). And rhTGF-β1 treatment alone also obtained the same result for MDA-MB-231 and MCF-7 cancer cells (P<0.01, P<0.05). Besides, addition of anti-TGF-β1 neutralizing antibody before co-culture could reverse proliferation ability of both MDA-MB-231 and MCF-7 cancer cells compared with breast cancer cells co-cultured with M2 macrophages (P<0.01, P<0.05) (**Figure 2E**).

Baicalein suppress viability of M2, MDA-MB-231 and MCF-7 dose- and time-dependently and induce phenotype and function skewing of M2 macrophage to M1 macrophage

The viability of MDA-MB-231, MCF-7 and TAMs was inhibited by baicalein (0-140 μmol/L) in a dose- and time-dependent manner (**Figure 3A**). IC_{50} of baicalein for MDA-MB-231 at 24 h, 48 h and 72 h was 79.12/50.10/34.77 μmol/L, for MCF-7 at 24 h, 48 h and 72 h was 49.76/43.73/39.44 μmol/L, for TAM at 24 h, 48 h and 72 h was 191.5/107.1/41.78 μmol/L respectively, which suggested that baicalein had much more biological toxic effect on breast

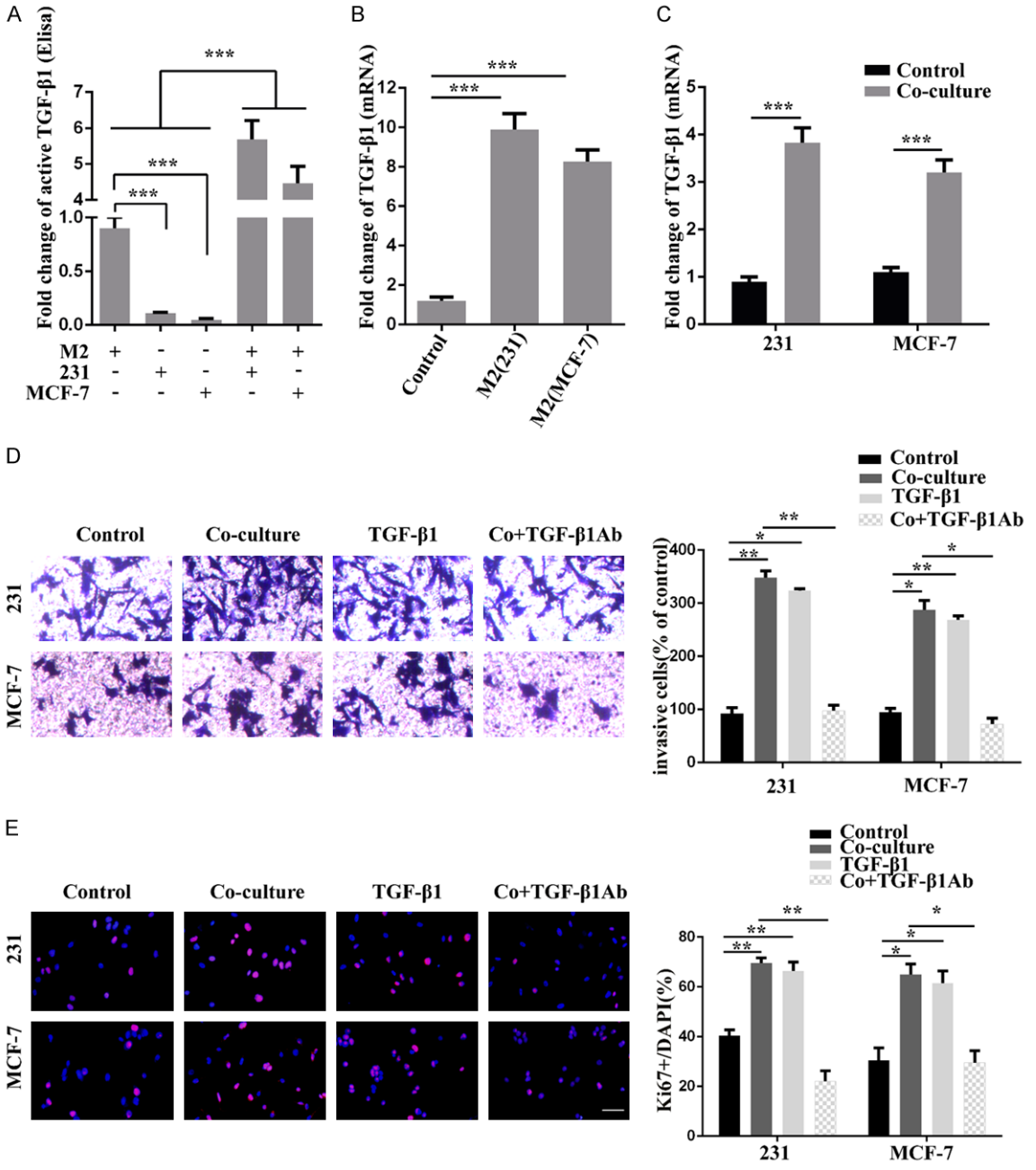

Figure 2. TGF-β1 secreted by M2 macrophage improve the invasion and proliferation of breast cancer cells. A. Active TGF-β1 expression levels in mono-culture medium of M2, MDA-MB-231 and MCF-7 and in co-culture medium of M2/MDA-MB-231 and M2/MCF-7 were analyzed using Elisa assay. B. TGF-β1 mRNA levels of M2 macrophages before and after co-culture with MDA-MB-231 and MCF-7 breast cancer cells respectively. C. TGF-β1 mRNA levels of MDA-MB-231 and MCF-7 before and after co-culture with M2 macrophages. D. Invasion assay of MDA-MB-231 and MCF-7 of mono-culture, co-culture, TGF-β1 treatment, co-culture + neutralizing TGF-β1 antibody. Results were qualified. E. Ki67 immunofluorescence staining of MDA-MB-231 and MCF-7 of mono-culture, co-culture, TGF-β1 treatment, co-culture + neutralizing TGF-β1 antibody. Results were qualified. Values were represented as mean ± SD, *P<0.05, **P<0.01, ***P<0.001. Scale bar = 50 μm. 231, MDA-MB-231. Co, co-culture. TGF-β1Ab, neutralizing TGF-β1 antibody.

cancer cells directly than on TAMs. Synthesized IC_{50} of all three cell lines above and excluded the obvious suppressing effect of baicalein on MDA-MB-231 and MCF-7, the concentration

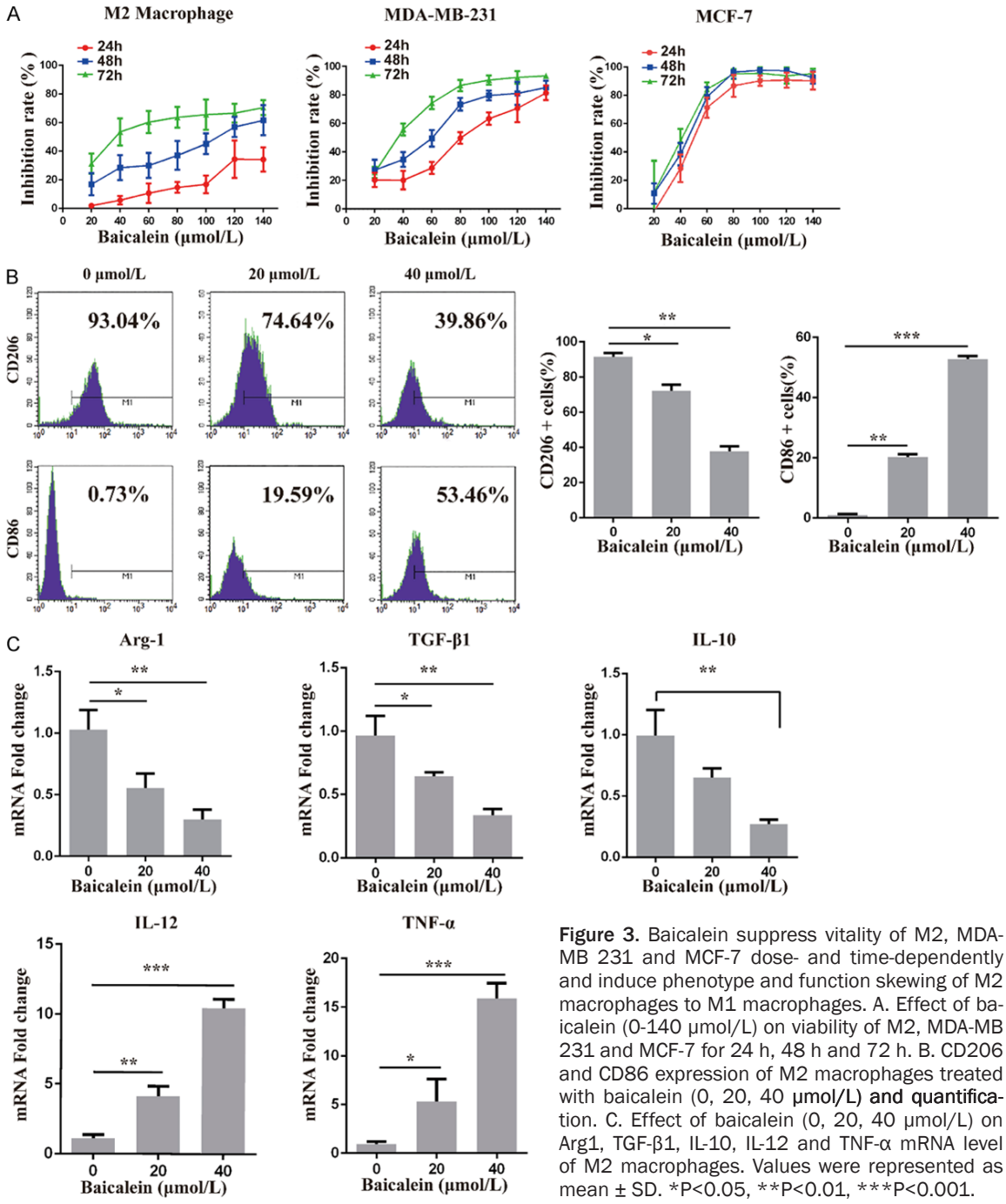

Figure 3. Baicalein suppress vitality of M2, MDA-MB 231 and MCF-7 dose- and time-dependently and induce phenotype and function skewing of M2 macrophages to M1 macrophages. A. Effect of baicalein (0-140 μmol/L) on viability of M2, MDA-MB 231 and MCF-7 for 24 h, 48 h and 72 h. B. CD206 and CD86 expression of M2 macrophages treated with baicalein (0, 20, 40 μmol/L) and quantification. C. Effect of baicalein (0, 20, 40 μmol/L) on Arg1, TGF-β1, IL-10, IL-12 and TNF-α mRNA level of M2 macrophages. Values were represented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

less than or equal to 40 μmol/L for 48 h was adopted to verify whether baicalein could induce skewing of M2 macrophages to M1 phenotype.

After treatment of baicalein (0, 20, 40 μmol/L), M2 macrophages were collected and used for Flow cytometry. Results showed M2 specific marker CD206 decreased and M1 specific marker CD86 increased significantly, which indicated that baicalein could change the phenotype of macrophages from M2 to M1 (**Figure 3B**). Further test was performed to confirm whether macrophages polarization induced by baicalein was functional. Results showed that baicalein could lead to not only phenotype shift but also functional change with mRNA expression of M2 associated cytokines (TGF-β1, Arg1,

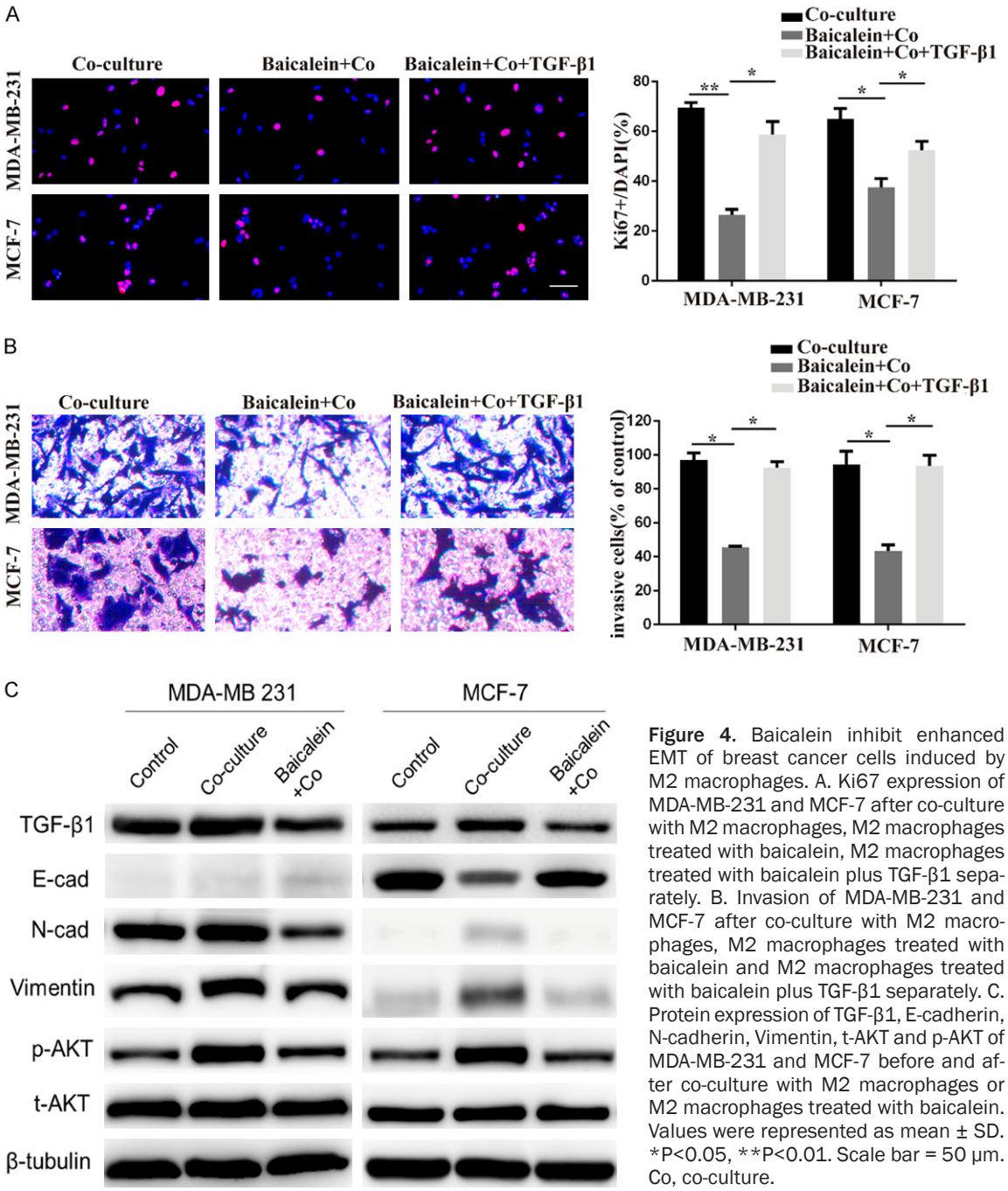

Figure 4. Baicalein inhibit enhanced EMT of breast cancer cells induced by M2 macrophages. A. Ki67 expression of MDA-MB-231 and MCF-7 after co-culture with M2 macrophages, M2 macrophages treated with baicalein, M2 macrophages treated with baicalein plus TGF-β1 separately. B. Invasion of MDA-MB-231 and MCF-7 after co-culture with M2 macrophages, M2 macrophages treated with baicalein and M2 macrophages treated with baicalein plus TGF-β1 separately. C. Protein expression of TGF-β1, E-cadherin, N-cadherin, Vimentin, t-AKT and p-AKT of MDA-MB-231 and MCF-7 before and after co-culture with M2 macrophages or M2 macrophages treated with baicalein. Values were represented as mean ± SD. *P<0.05, **P<0.01. Scale bar = 50 μm. Co, co-culture.

IL-10) decreasing and M1 associated cytokines (IL-12 and TNF-α) increasing significantly at 40 μmol/L (**Figure 3C**). Based on the above, baicalein concentration of 40 μmol/L was used for further experiment.

Baicalein inhibit enhanced EMT of breast cancer cells induced by co-culture with TAMs

TAMs were divided into three groups according to different treatments and then co-cultured with cancer cells. Pretreatment of baicalein for 48 h before co-culture could significantly reduce proliferation (**Figure 4A**) and migration (**Figure 4B**) of both MDA-MB 231 and MCF-7 compared with control group. And addition of rhTGF-β1 in co-culture medium could reverse inhibiting effects of baicalein (**Figure 4A** and **4B**).

Since TGF-β1 is a strong EMT inducer, we next analyzed the protein expression involved in

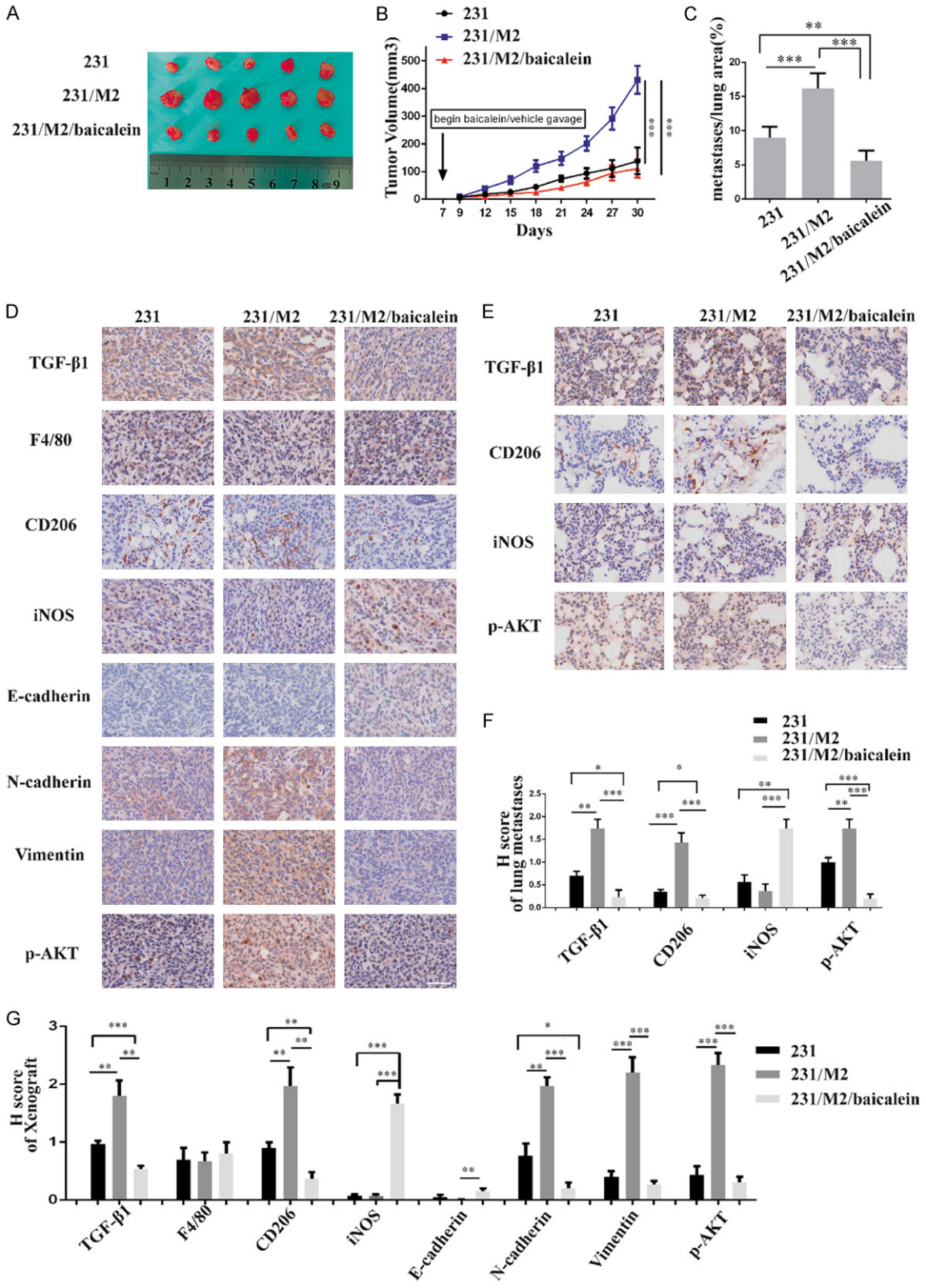

Figure 5. Baicalein suppress breast cancer in vivo. A. Primary tumors were collected from MDA-MB-231, MDA-MB-231/M2 and MDA-MB-231/M2/baicalein groups. B. Growth curve of primary tumors in BALB/c nude mice. Tumor volume was described as mean ± SD (mm^3). C. Quantification of lung lesions for each group, data was represented as percentage of lesion area of lung tissue. D. TGF-β1, F4/80, CD206, iNOS, E-cadherin, N-cadherin,

Vimentin and p-AKT expression of primary tumors by immunohistochemistry. E. TGF-β1, CD206, iNOS and p-AKT expression of lung lesions by immunohistochemistry. F. Qualification of TGF-β1, CD206, iNOS and p-AKT expression of lung lesions. G. Qualification of TGF-β1, F4/80, CD206, iNOS, E-cadherin, N-cadherin, Vimentin and p-AKT expression of primary tumor. Scale bar = 50 μm. *P<0.05, **P<0.01, ***P<0.001.

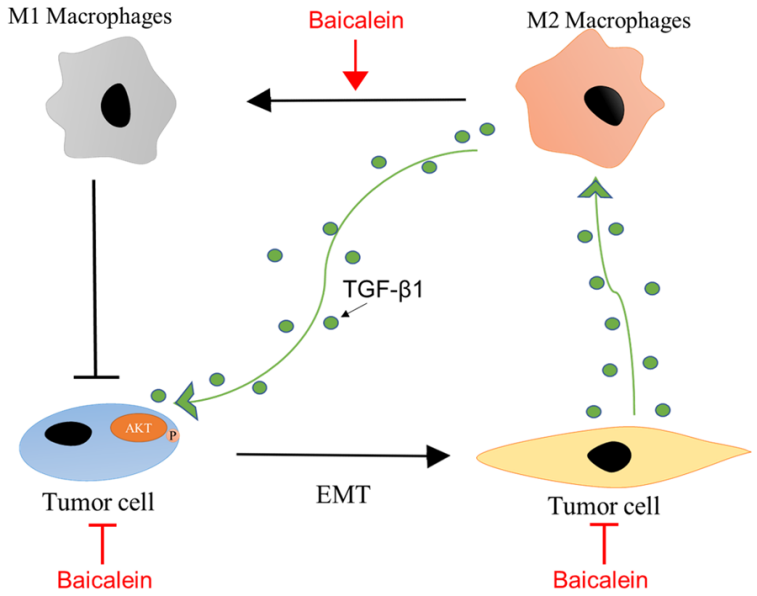

Figure 6. Schematic diagram of baicalin in inhibiting breast cancer by regulating tumor microenvironment.

EMT process. Co-culture with M2 macrophages could significantly upregulate TGF-β1, N-cadherin and Vimentin expression in both MDA-MB-231 and MCF-7, and downregulate E-cadherin expression in MCF-7 cancer cells. And baicalein pretreatment before co-culture could reverse this effect (**Figure 4C**). Besides, the role of inhibiting EMT shift after co-culture of baicalein could be targeting PI3K-AKT signal pathway (**Figure 4C**).

Baicalein inhibit growth and metastasis of breast cancer in vivo

To further confirm our results, BALB/c nude mice was used for in vivo analysis. After co-culture with M2 macrophages, MDA-MB-231 breast cancer cells had stronger ability in promoting tumor growth (**Figure 5A** and **5B**) and lung metastasis (**Figure 5C**) and baicalein treatment could effectively prevent this effect and exerted anti-tumor role (**Figure 5A-C**). Potential mechanism was explored using immunohistochemical method. For primary tumor tissues, expression of TGF-β1, CD206, N-cadherin, Vimentin and p-AKT expression was significantly upregulated after co-culture. And compared with co-culture group, additional baicalein treatment could significantly increase expression of iNOS, E-cadherin and decrease expression of TGF-β1, CD206, N-cadherin, Vimentin and p-Akt. There was no statistical difference of F4/80 expression among three groups (**Figure 5D** and **5G**). For metastatic lung lesions, expression of TGF-β1, CD206, p-Akt and iNOS were consistent with primary tumor tissues (**Figure 5E** and **5F**).

Discussion

During carcinogenesis, the interplay between cancer cells and various tumor microenvironment components shapes the phenotype and physiology of TAMs that may lead to promotion or inhibition of tumor progression [33]. It has been reported that IL-4 and IL-13 are strong inducers of M2-like macrophage activation [14, 34]. TGF-β also induces an M2-like functional phenotypes that share properties with IL-4 or IL-13 activated macrophages [34]. Since macrophages in tumor microenvironment mainly are of the M2 phenotype, we use THP-1 derived M2 macrophages to mimic TAMs. In this study, we showed that TAMs produced plenty of TGF-β1 to activate PI3K-Akt signal pathway and induced EMT of breast cancer cells. In turn, breast cancer cells after co-culture secreted TGF-β1 to keep macrophages as a TAM-like phenotype, forming a positive feedback loop. Moreover, baicalein induced skewing of M2-like TAMs to M1-like phenotypes and decreased TGF-β1 expression, thus breaking the vicious circle (**Figure 6**).

The molecular mechanism that polarize TAMs towards the M2 pro-tumor phenotype have become research hot spot recently. TGF-β1 has been reported to promote the differentiation of non-activated macrophages into a TAM-like (M2-like) phenotype [35] and plays a funda-

mental role in multiple steps of cancer progression, including EMT [36]. PI3K-Akt signals, as non-Smad signaling pathways, are activated in TGF-β induced EMT [17]. Our study showed that the addition of neutralizing TGF-β1 antibody in co-culture medium blocked the tumor promoting effect of TAMs. Besides, we showed that addition of rhTGF-β1 in baicalein pretreatment co-culture medium could reverse the inhibiting effects of baicalein, which indicated that TGF-β1 might be the main factor for TAMs to execute tumor supporting function.

It has been reported that baicalein may be an immunomodulatory agent through regulating Th17 cells and dendritic cells and that baicalein can increase expressions of IFN-γ and IL-12, thus boosting immune response [37]. So far, there are no studies investigating the role of baicalein on the interaction between breast cancer cells and TAMs. Our results indicated that baicalein could directly inhibit viability of breast cancer cells and TAMs, but the concentration needed for TAMs inhibition was higher than directly targeting breast cancer cells. However, we found that baicalein regulated macrophages polarization at lower concentrations. Our in-vivo immunohistochemical staining of F4/80 showed that baicalein didn't change the total number of macrophages, but increased the amount of M1 phenotype (iNOS+) and decreased the amount of M2 phenotype (CD206+) in primary tumor. Besides, higher expression of TGF-β1, CD206 and p-Akt could be seen in lung lesions after MDA-MB-231 co-cultured with M2 macrophages, which was consistent with in vitro results that co-culture with M2 could enhance proliferation and invasion of breast cancer cells. Most importantly, baicalein could block the effect of M2 macrophages by repolarization and inhibit TGF-β mRNA of M2 macrophages. Many studies showed that P38 MAPK, ERK, and JNK were involved in TGF-β transcription [38, 39]. And baicalein, as a multi-target drug, could block MAPK signal pathways, which maybe the potential mechanism of inhibiting TGF-β mRNA level [40-42]. In summary, our study showed that baicalein had dual roles in tumor suppression at lower concentration: one was the direct role in poisoning cancer cells, and the other was through the regulation of macrophage polarization.

AKT isoforms have been reported to play key roles in a wide variety of cellular processes including anti-apoptosis, proliferation, polarity, migration, DNA repair, angiogenesis, and stem cell self-renewal [43]. Our study showed that co-culture with TAMs could enhance EMT of breast cancer cells possibly through TGF-β1-Akt signal pathway and that baicalein could regulate TAMs polarization, thus blocking this effect. PI3K/Akt pathway has been reported to involve in inflammatory signals and can regulate activation phenotype of macrophages [44]. PI3K activation is an essential step towards M2 macrophages activation in response to IL-4 [45]. And Akt activation is needed for M2 activation, because Akt inhibition abolish the up-regulation of M2 genes [45]. It is possible that baicalein may regulate M2 macrophages repolarization through inhibiting PI3K/Akt signal pathway.

Taken together, our study shows an important role of the positive feedback loop between breast cancer cells and TAMs in cancer growth and metastasis. Baicalein, as an inhibitor of breast cancer through regulating the polarization and function of TAMs, provides novel mechanism for cancer therapy. Considering that baicalein can also directly target cancer cells under same condition, our study provides a new insight regarding the therapeutic potential of baicalein for breast cancer, and possibly for other cancers as well.

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Disclosure of conflict of interest

None.

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