Tamoxifen Suppresses Brain Metastasis of Estrogen Receptor-deficient Breast Cancer by Skewing Microglia Polarization and Enhancing Their Immune Functions

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Abstract

Background: Brain metastasis of breast cancer exhibits exceedingly poor prognosis, and both triple negative and $Her2^+$ subtypes have the highest incidence of brain metastasis. Although estrogen blockers are considered to be ineffective for their treatment, recent evidences indicate that estrogen blockade using tamoxifen showed certain efficacy. However, how estrogen affects brain metastasis of triple negative breast cancer (TNBC) remains elusive.

Methods: To examine the effect of estrogen on brain metastasis progression, nude mice were implanted with brain metastatic cells and treated with either estrogen supplement, tamoxifen or ovariectomy for estrogen depletion. For clinical validation study, brain metastasis specimens from pre- and post-menopause breast cancer patients were examined for microglia polarization by immunohistochemistry. To examine the estrogen-induced M2 microglia polarization, microglia cells were treated with estrogen and the M1/M2 microglia polarization was detected by qRT-PCR and FACS. The estrogen receptor-deficient brain metastatic cells, SkBrM and 231BrM, were treated with conditioned medium (CM) derived from microglia that were treated with estrogen in the presence or absence of tamoxifen. The effect of microglia-derived CMs on tumor cells was examined by colony formation assay and sphere forming ability.

Results: We found that M2 microglia were abundantly infiltrated in brain metastasis of pre-menopausal breast cancer patients. A similar observation was made *in vivo*, when we treated mice systemically with estrogen. Blocking of estrogen signaling either by tamoxifen treatment or surgical resection of mice ovaries suppressed M2 microglial polarization and decreased the secretion of C-C motif chemokine ligand 5, resulting in suppression of brain metastasis. The estrogen modulation also suppressed stemness in TNBC cells *in vitro*. Importantly, estrogen enhanced the expression of signal regulatory protein α on microglia and restricted their phagocytic ability.

Conclusions: Our results indicate that estrogen promotes brain metastasis by skewing polarity of M2 microglia and inhibiting their phagocytic ability, while tamoxifen suppresses brain metastasis by blocking the M2 polarization of microglia and increasing their anti-tumor phagocytic ability. Our results also highlight a potential therapeutic utility of tamoxifen for treating brain metastasis of hormone receptor deficient breast cancer.

Background

Metastatic brain tumors are the most frequently occurring intracranial neoplasms in adults with the annual incidence of over 200,000 cases in the United States [1]. The majority of brain metastases originate from primary tumors of lungs, melanoma, and breast [2, 3]. Patients with brain metastasis of breast cancer have extremely poor prognosis, high mortality rate and frequent incidence of tumor recurrence. Even with aggressive treatments involving surgical intervention, irradiation, and chemotherapy, only a fraction of these patients with brain metastasis survives longer than 2 years after

diagnosis [4]. Therefore, understanding the pathological mechanism of brain metastasis of breast cancer is urgently needed to identify a novel and effective therapeutic strategy.

One of major risk factors for brain metastasis of breast cancer is age. A previous study showed that the cumulative 5-years incidence of brain metastasis is higher in younger patients (20.5%) than in older patients (7.5%) [5]. Importantly, premenopausal women have 1.5–2 times higher incidence of brain metastasis than postmenopausal women [6]. This difference is considered to be attributed to the female sex hormones, especially estrogen. Several studies have shown that the incidence of brain metastasis is associated with the expression of estrogen receptor (ER) in luminal subtype breast cancer [7-10]. Blocking ER by antagonist prolonged the onset of brain metastases from breast cancer [8-10]. Although the function of estrogen in promoting primary cancer is well documented, the physiological role of estrogen on brain metastasis and its tumor microenvironment is poorly understood.

Among all subtypes of breast cancer, triple-negative breast cancer (TNBC) has a significantly poorer outcome, and approximately 30 to 46% TNBC patients will eventually develop brain metastasis prior to death [11, 12]. Although tamoxifen is used as the first-line treatment for ER^+ breast cancer, it is not a standard treatment for ER negative or TNBC patients due to a lack hormone receptor [13-15]. Interestingly, however, recent studies showed that tamoxifen exhibited anti-tumor effect [16, 17] in TNBC, suggesting that tamoxifen may modulate estrogen-related tumor microenvironment that suppresses tumor progression. Tumor metastasis in the brain is a complex process that involves communication between the neoplastic cells and the normal brain cells. The non-neoplastic cells such as endothelial cells, macrophages, lymphocytes, astrocytes and microglial cells present in brain tumor microenvironment play a critical role in the formation of brain metastasis. Among these cells, microglia accounts for 30–50% of the total brain tumor mass [18, 19]. Previous *in vitro* and *in vivo* studies have demonstrated that activated microglia accelerate growth and invasion of brain tumors [18-20]. Depletion of microglia significantly inhibited metastatic spread of tumors [21-23], suggesting that microglia plays a critical role in the tumor invasion and metastasis in the brain.

Here, we investigated the effect of estrogen and tamoxifen on brain metastasis of hormone receptor deficient breast cancer and examined the role of estrogen-induced polarization of microglia in tumor progression. We found that estrogen strongly skewed microglia to M2 phenotype, which significantly suppressed anti-tumor immune functions. M2 microglia also promoted tumor stem cell growth by secreting C-C motif chemokine ligand 5 (CCL5) in response to estrogen. Blocking of estrogen signaling by tamoxifen and ovariectomy exhibited anti-tumor effect on hormone receptor-negative tumor growth by modulating M2 microglial polarization and suppressing their anti-tumor immune function.

Methods

Animal

Animals were treated in accordance with the US National Institutes of Health Animal

Protection Guidelines and approved by the Wake Forest Baptist Health Institutional Animal Care and Use Committee. 5-6 weeks old of female Nude mice were used. The mice were housed (five per cage) with a stable temperature (24 ± 1°C), a 12-h light/dark cycle, and unrestricted access to food and water. The housing environment and animal health were monitored by the laboratory animal center. To examine the role of estrogen on brain metastasis, nude mice were anesthetized and randomly divided into five groups: 1) tumor transplant only, 2) tumor transplant plus 17β-estradiol (E2), 3) tumor transplant plus ovariectomy (OVX), 4) tumor transplant plus OVX and E2 supplement and 5) Tamoxifen. For OVX, the mouse was anesthetized by inhalation of isofluorane. After wiping the injection site with three times of Betadine and 70% alcohol, a midline dorsal incision (Approx 1 cm) was made using a sharp scissor. A smaller incision (<1 cm) in the muscle layer on either side of midline incision was then made to allow entry into the peritoneal cavity. The ovaries and uterus were identified in a fat pad. The end of bilateral uterus were ligated by 6-0 polyproplene suture and the ovaries were removed. The sham group received the same incision but the ovaries were not removed. For E2 supplement, a 0.5 cm incision was made under the neck skin and implanted with 1.5mg/pellet E2 (Innovative Research of America, 60 day release) and incision was closed by 6-0 polypropylene suture. Finally, the 6-0 polyproplene suture was used to close the incision site of body wall and skin. One dose of buprenorphine (0.05 mg/kg) was subcutaneously administered and animals were allowed to wake up on the heated pad. The animals were positioned laterally and kept warm for 30 min until they recovered from anesthesia. One week later, mice received luciferase-labeled 231BrM cells by intracardiac injection (i.c.) at a concentration of 2×10^5 cells in 100 µL PBS into the left cardiac ventricle. After 3 days of tumor transplantation, mice in tamoxifen group received tamoxifen (20 mg/kg) treatment by i.p. injection every three days for 30 days. The whole body photon flux of mice was measured immediately after injection to confirm a successful injection using IVIS Xenogen bioimager (Caliper). Tumor growth was monitored by bioluminescence until day 30. For bioluminescent imaging, the mice were injected with D-luciferin intraperitoneally (100 mg/kg), followed by capturing images every week using IVIS Xenogen bioimager. The brain metastasis was monitored and the luminescence was quantified once per week. At the endpoint, whole brain was removed, incubated in PBS with 0.6 mg/mL luciferin for 5 minutes and ex vivo photon flux was measured by IVIS.

Human subject

Human breast cancer specimens were obtained from surgical pathology archives of the Wake Forest Baptist Comprehensive Cancer Center (WFBCCC), and Cooperative Human Tissue Network, and Pathology Shared Resource at WFBCCC. All tissue sections were obtained by surgical resection and the patient's information about age, cancer type and menstrual period and status were record by Wake Forest Baptist hospital. This study was approved by the Wake Forest School of Medicine Institutional Review Board and written informed consent was obtained from all participants.

Cell culture and reagents

Human breast cancer line, MDA-MB231BrM2a (231BrM), was a kind gift from Dr. Massague (Memorial Sloan-Kettering Cancer Center). SKBrM3 cell line was derived from parental SKBr3 cells through three rounds of *in vivo* selection [24, 25]. SkBrM and 231BrM cell lines were authenticated by using GenePrint® 10 STR System (Promega, # B9510). Human and mouse microglia lines, HMC3 and SIM-A9, were purchased from American Type Culture Collection (ATCC) and were authenticated by ATCC. The SkBrM and 231BrM were cultured in DMEM supplemented with 10% FBS, streptomycin (100 mg/mL) and penicillin (100 U/mL) and HMC3 and SIM-A9 cells were cultured in DMEM/F12 medium supplemented with 5% FBS. All cells were grown at 37 °C under 5% CO2. The HMC3 cells were seeded in a 10-cm dish. After reaching 70% confluence, cells were incubated with DMEM/F12 medium supplemented with 2% FBS or in medium containing 1 nM E2 (Sigma) or in 1 nM E2 plus the STAT3 inhibitor, STATTIC (Selleckchem.com) at 0.5 μM concentration or in 1nM E2 plus the estrogen receptor antagonist, Tamoxifen (Sigma) at 1 μM concentration. After 24 hours, cells were washed twice with PBS and then incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 hours. The conditioned medium (CM) harvested from the cell culture were centrifuged at 300 ×g for 10 minutes to remove the cells and stored at -80 °C. All cell lines were ensured to be mycoplasma negative by using universal mycoplama detection kit (ATCC, #30-1012k, Lot: 70008746). The cells were collected and qRT-PCR and western blotting were used to quantify protein and mRNA levels. In another round of cell culturing, E2-treated cells were washed again and cultured for an additional 24 hours in fresh medium. The CM were collected to identify cytokines using the cytokines array (Raybio). To examine the effect of microglia on T cell proliferation, the E2-treated SIM-A9 cells were cultured with fluorescent-labeled (CFSE Cell Proliferation Kit; Thermo Fisher) primary mouse T cell for 24 hours and the cell proliferation was measured by flow cytometry.

Immunohistochemistry

The human brain sections were stained using goat anti-CD206 (1:200, R&D systems) for M2 microglia and anti-CD47 (1:100, Invitrogen) for tumor cells and anti-SIRP (1:500, Cell signaling). Brain sections were then incubated with appropriate HRP-conjugated secondary antibodies using diaminobenzidine as the substrate. The signals were evaluated based on their intensities after subtracting the signals of the primary antibody-omitted negative controls. In some cases, the primary antibodies were replaced by isotype antibodies to control for non-specific binding of the antibodies. To determine the area of $CD206^{+}$ cells in brain metastasis, we chose 3 randomly selected fields in each tumor and measured staining intensity by using the Image-Pro software. We also chose and measured staining intensities of 3 non-tumor areas in the consecutive slide, and this background intensity was subtracted to normalize the tumor intensity in the specimens. The average normalized intensity of 3 fields was used as the score of that patient. The scoring range was set from 0 (lowest) to 3 (highest).

Western blot

The cultured microglia cells were homogenized (1:3, in cultured cells) in the RIPA buffer, and then centrifuged at 17,000 g for 30 min at 4°C. The protein concentrations of the supernatants were

determined and adjusted to the same concentration. Supernatants (30 g of total protein) were mixed with sample buffer containing 0.5 M of dithiothreitol, heated to 80°C for 10 min, loaded into each well of 4% polyacrylamide gel and resolved at 120 V for 2 h. The separated proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA), blocked in 5% milk, and probed with respective primary antibodies: JAK (1:1000, Cell signaling), STAT3 (1:1000, Cell signaling), p-STAT3 (1:3000, Cell signaling), GAPDH (1:10000) (Cell Signaling), SOX2 (1:500) (Cell Signaling), Oct4 (1:1000) (Cell signaling), Nanog (1:500) (Cell Signaling), PD-L1 (1:2000) (Cell Signaling). The bound antibodies were detected using an enhanced chemiluminescence detection kit (PerkinElmer, Boston, MA, USA). The band densities were measured using an imaging system (BioChemi; UVP, Upland, CA, USA) and analyzed using ImageJ (1.43u) (http://rsb.info.nih.gov/ij/). For gel loading control, membranes were stained with monoclonal-GAPDH antibody (1:50000, Cell signaling).

Flow Cytometry

Nude mice were anesthetized with an overdose of isoflurane and their brains were removed and placed in ice-cold PBS containing 2.5 mg/ml of trypsin and dissociated using mechanical shearing. The cell suspension homogenates were passed through a 40-μm nylon membrane (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and then centrifuged at 500 g for 10 min. The pellets were suspended in PBS, and microglial cells were isolated using a combination of protocols of differential density (stepwise Percoll) gradient centrifugation and immunomagnetic $Iba1^+$ cell separation. Purified microglial cells were fixed in 4% paraformaldehyde at 4°C for 1 h and then incubated with F4/80 antibody (1:250) (eBioscience), CD206 (1:250) (eBioscience), CD44 (1:10000) (BioLegend) and ESA (1:250) (Invitrogen) at 4°C for 16 h. After they were washed twice with PBS, the stained cells were subjected to flow cytometric analysis (FACScan; Becton-Dickinson, Mountain View, CA, USA). The percentage of cells was calculated using Cell-QuestTM software (Becton-Dickinson).

Phagocytosis assay

231BrM cells were labeled with PKH26 dye (Sigma, USA). The labeled cells were then washed three times and cultured overnight to reduce nonspecific leakage of dye during the assay. Labeled 231BrM cells were mixed with HMC3 microglia that were pre-treated with or without E2 (1 nM) or E2 plus tamoxifen at 1 μM concentration. Co-cultured 231BrM and HMC3 cells in the culture slides were harvested after 8 h and fixed with 4% paraformaldehyde. Microglial cells were counterstained with anti-Iba1 antibody overnight. Secondary antibody conjugated with fluorescent dye Alexa-fluor 488 (1:1000, Invitrogen) was used to detect the microglia. The phagocytic activities were measured by immunofluorescence microscope and flow cytometry. For quantification of phagocytosis, the phagocytic percentage was calculated as: 100 x [percent $Iba1^+/PKH26^+$ cells / (percent $Iba1^+/PKH26^-$ cells + percent $Iba1^+/PKH26^+$ cells)].

CCL5 and TNF-α ELISA

Microglial cells (HMC3) were treated with or without E2 (1 nM) or E2 plus tamoxifen (1 μM) for 24 hours. Cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2%

FBS for additional 24 hours. Conditioned medium was collected and analyzed by the human CCL5 and TNF-α ELISA kit (Sigma, USA). The HMC3 cells were homogenized in RIPA lysis buffer and centrifuged at 13,000g for 30 min at 4 °C. The supernatant were collected, and protein concentrations were measured and adjusted to 1 mg/mL. Briefly, 96-well plates were first coated with anti-CCL5 or TNF-α monoclonal antibodies followed by addition of 100 µL of the microglia conditional medium or the same volume of the CCL5 and TNF-α standards. The plate was incubated at 4 °C overnight on a rocking platform. After washing the plates, the detection antibody and streptavidin solution were added to each well. After incubation, the TMB and stop solution were added to each well. Finally, the plates were examined at an absorbance wavelength of 450 nm. Standard curves were obtained from values generated from known concentrations of mouse and human CCL5 or TNF-α provided by the kit.

Promoter reporter assay for Arginase-1 gene.

To examine the estrogen-induced M2 microglial polarization and the effect of tamoxifen on estrogen-induced microglial polarization, HMC3 cells were first infected with lentivirus containing green fluorescent protein (GFP) gene, and GFP^+ cells were sorted by FACS. The GFP^+ HMC3 cells were then seeded in 96-well plates for one day and followed by tranfecting them with the Arginase-1 promoter reporter plasmid (Addgene) using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA). After 24 hours of incubation, the cells were treated with only E2 or E2 plus tamoxifen in presence of 2% FBS and cultured for another 24 hours. Cells were washed twice and were treated with 100 µL D-luciferin for 5 min. The expression of luciferase was detected by using IVIS Xenogen bioimager. For arginase-1 promoter luciferase normalization, the photon flux was divided by GFP signal which was measured by Multi-Mode Reader (Biocompare). Each experiment was conducted a minimum of three times.

Statistical analysis

Data are represented as mean ± standard deviation (SD). Significance was set at $p < 0.05$. The estrogen effect on M1/M2 gene expression, effect of estrogen-derived microglial CM on tumor growth, and effect of estrogen on microglia phagocytosis ability were analyzed using unpaired Student's t test. One-way ANOVA was used to analyze the effect of estrogen, OVX, STATTIC or tamoxifen treatment on M1/M2 gene polarization and tumor progression, effect of different concentrations of TNF-α or CCL5 on cancer progression. Significance between groups was represented as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Results

Estrogen promotes brain metastasis of breast cancer

To test the effect of female hormones on brain metastasis of ER-negative breast cancer and understand how tamoxifen modulate the metastatic growth in the brain, we first performed bilateral ovariectomy (OVX) in 5-6 week-old female mice. One week after the surgery, mice were intracardially implanted with luciferase-labeled human ER-negative breast cancer cells, 231BrM. The mice were then implanted with or without 17β-estradiol (E2) by subcutaneous implantation of a disc or received tamoxifen treatment (20

mg/kg) by i.p. injection every three days for one month (**Fig. 1A**). Tamoxifen is known to be blood brain barrier (BBB) permeable [26, 27]. As shown in Figure **1A**, E2 significantly increased the incidence and growth of brain metastasis [Brain metastasis rate: Veh (33%) vs. E2 (89%)]. On the other hand, OVX dramatically suppressed brain metastasis progression [Brain metastasis rate: E2 (89%) vs. OVX (11%)]. However, the OVX-mediated suppression of brain metastasis was rescued by E2 treatment [Brain metastasis rate: OVX (11%) vs. E2+OVX (33%)]. Interestingly, tamoxifen significantly repressed brain metastasis growth to the similar level observed in the OVX group (**Fig. 1B-C**). Since MDA-MB-231 cells are resistant to tamoxifen [28], we sought of a possibility that the tumor suppressive effect is due to the tumor microenvironment. Previous studies showed that activation of microglia, especially pro-tumor M2 microglia, promoted the progression of glioma [29-32]. Therefore, we examined M1 and M2 microglia in the brain of OVX, E2 and tamoxifen treated mice by flow cytometry. The result of FACS analysis revealed that the population of M1 microglia cells ($F4/80^+$) was significantly decreased (**Fig. 1D**), and high levels of M2 microglial cells ($CD206^+$) accumulated in the lesions of E2-treated mice (**Fig. 1E**), suggesting that E2 induces microglia polarization to M2 phenotype in the brain metastatic lesions. In contrast, the effect of E2 in microglia polarization was abrogated by OVX or tamoxifen treatment (**Fig. 1D and 1E**). These results indicate that E2 promotes brain metastasis by skewing microglia polarization to M2 phenotype. To validate this notion in a clinical setting, we examined the status of M2 microglia activation in brain metastatic lesions of pre- and post-menopausal breast cancer patients (**Fig. 1F**). We observed that M2 microglia ($CD206^+$ cells) abundantly infiltrated in the brain lesions of pre-menopause patients compared to post-menopause patients (**Fig. 1F**). Microglia and peripheral macrophages are known to be recruited by brain metastasis lesion. Although microglia and macrophage share many common surface markers and similar physiological functions, recent studies have demonstrated that microglia and macrophages are two distinct myeloid populations with different developmental origins [33, 34], and they can be distinguished by CD45 macrophage marker [35-37]. We found that the macrophages were recruited in the brain metastasis lesion of breast cancer patients (**Fig. 1F**). However, the population of $CD45^+$ macrophage was significantly less than that of M2 microglia. Importantly, there was no difference in $CD45^+$ cells in the brain metastasis lesions of pre-menopause patients compared to post-menopause patient (**Fig. 1F**).

Estrogen skewed M2 microglial polarization

Because M2 microglia are abundantly infiltrated in brain metastatic lesions of pre-menopause patients, we examined whether estrogen promotes brain metastasis by polarizing microglia to M2 microglia. We examined the expression of estrogen receptors in HMC3 (Human) and SIM-A9 (Mouse) microglia cells and found that the expressions of estrogen receptor α and β are up-regulated by E2 treatment (**Supplemental Fig. 1**). To further investigate the effect of estrogen on microglia polarization, HMC3 and SIM-A9cells were treated with E2 followed by examining the expression of M1 and M2 markers. Our results showed that E2 significantly increased the mRNA expression of M2-related genes, arginase-1 (Arg1), arginase-2 (Arg2), and CD206, in both human and mouse microglial cells lines (**Fig. 2A** and **2B**). On the other hand, blocking of estrogen receptor (ER) signaling by tamoxifen treatment suppressed E2-

induced M2 microglia polarization (**Fig. 2A** and **2B**). Moreover, tamoxifen significantly suppressed the E2-mediated up-regulation and down-regulation of M2 and M1 microglia (**Fig. 2C and 2D**).

As the JAK-STAT3 pathway is known to induce M2 microglia polarization [38], we examined the status of phospho-STAT3 and JAK expression in microglia after E2 treatment. Indeed, E2 treatment strongly enhanced the activation of JAK/STAT3 in microglia, while tamoxifen suppressed E2-induced activation of JAK and STAT3 (**Fig. 2E**). We then treated microglia with E2 in the presence or absence of the STAT3 inhibitor (STATTIC) or tamoxifen and examined their effects on polarization of M1/M2 microglia using the Arg1 promoter reporter [39]. We found that E2 upregulated the Arg1 gene promoter activity, and this effect was significantly suppressed by STATTIC and/or tamoxifen treatment (**Fig. 2F**). Moreover, inhibition of STAT3 and estrogen receptor reversed the E2-mediated suppression of M1 ($Iba1^+/F4/80^+$) and promotion of M2 ($Iba1^+/CD206^+$) polarization (**Fig. 2G and 2H**). These results suggest that the E2 promotes M2 polarization of microglia via activation of the STAT3 pathway.

Conditioned medium (CM) of estrogen-treated microglia promotes tumor cell growth and stem cell self-renewal

To examine how E2 promotes brain metastasis by upregulating M2 microglia polarization, we treated breast cancer brain metastatic cells, SKBrM ($ER^-/Her2^+$) and 231BrM ($ER^-/PR^-/Her2^-$), with conditioned medium (CM) that was generated from human HMC3 microglia treated with or without E2. To avoid direct effect of E2 on tumor growth, microglia were treated with E2 (1 nM) for 24 hours, washed with PBS to remove E2, and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 hours. The CM was then collected and added to SKBrM and 231BrM cells. We found that the estrogen-free CM derived from E2-treated microglia significantly increased cell viability (**Fig. 3A**) and colony forming ability (**Fig. 3B**) of both SKBrM and 231BrM cells. The expression of stemness-inducing genes, SOX2, OCT4 and NANOG, were also significantly upregulated when the brain metastatic cells were treated with the CM derived from E2-treated microglia (**Fig. 3C and 3D**). Furthermore, treatment of SKBrM and 231BrM cells with CM generated from E2-treated microglia showed significant increase in their sphere forming ability (**Fig. 3E**) and $CD44^+/ESA^+$ cancer stem cell (CSC) population (**Fig. 3F**). In contrast, direct treatment of cancer cells with the same dose of E2 (1 nM) did not affect the stem cell renewal or expression of stemness genes (**Supplemental Fig. 2A-D**). Furthermore, E2-mediated promotion of the CSC population ($CD44^+/ESA^+$) was also significantly suppressed by STATTIC treatment (**Fig. 3G**). These results suggest that E2 induces secretory factor(s) from microglia, which in turn promotes tumor cell growth and self-renewal. We also examined the direct cytotoxic effect of tamoxifen on tumor cell viability *in vitro* (**Fig. 3H**). Our results showed that tamoxifen suppressed cell viability at the concentration of 100 μM, but not at 1 μM (the dose used throughout our microglia experiments) (**Fig. 3H**), indicating that the anti-tumor effect of tamoxifen is not mediated by direct cytotoxic effect on tumor cells, and it is rather mediated by the effect on microglia. To further investigate the effect of estrogen on microglia-derived tumor progression, 231BrM cells were treated with CM from microglia that were pre-treated with E2 or E2 plus tamoxifen. We found that CM from E2 plus tamoxifen treated microglia significantly suppressed the

tumor promoting effect of E2 treated microglia CM (**Fig. 3I and 3J**). Similarly, we found that CM derived from microglia that were treated with E2 plus tamoxifen significantly suppressed the expression of stemness-related genes SOX2, OCT4 and NANOG (**Fig. 3K-M**).

Estrogen induces secretion of CCL5 from microglia and promotes tumor growth and stemness.

To identify the factor(s) induced by E2 in microglia, we performed cytokine array analysis (consisting of 99 cytokines and growth factors) and found that E2 treatment significantly increased the secretion of C-C motif chemokine ligand 5 (CCL5) whereas it decreased tumor necrosis factor (TNFα) secretion in the microglial CM (**Fig. 4A**). The effect of E2 in increasing CCL5 and decreasing TNF-α secretion was further verified in human microglia by performing ELISA (**Fig 4B and 4C**). In contrast, blocking estrogen receptor by tamoxifen significantly suppressed E2-mediated secretion of CCL5 and up-regulated the production of TNF-α (**Fig. 4C**). TNFα and CCL5 are known to be associated with M1 and M2 phenotypes, respectively [40-42]. To examine the functional effects of TNF-α and CCL5, we treated breast cancer cells with recombinant TNF-α or CCL5 and examined their effect on these cells. We found that TNF-α treatment significantly decreased cell viability while CCL5 significantly increased viability of both SKBrM and 231BrM cells (**Fig. 4D** and **4E**). It should be noted that TNF-α has been shown to kill some tumor cells through activation of apoptotic signaling [43, 44]. We also found that the mRNA expression of CSC markers, SOX2, OCT4 and NANOG, was increased in 231BrM cells when they were treated with recombinant CCL5 (**Fig. 4F**). Similarly, CCL5 augmented the population of $ESA^+/CD44^+$ CSC (**Fig. 4G**). To examine whether the upregulation of stemness genes by the CM is indeed mediated by CCL5, CM of E2-treated microglia was treated with CCL5 neutralizing antibody followed by measuring the expression of CSC genes. Our result indicates that depletion of CCL5 in the CM significantly suppressed the expression of stemness genes SOX2, OCT4 and NANOG (**Fig. 4H**). Moreover, the stimulatory effect of CM on stem cell population ($CD44^+/ESA^+$ CSC) was also suppressed after the CCL5 depletion (**Fig. 4I**). In addition, the E2-mediated increase in the population of CSC ($CD44^+/ESA^+$) was significantly suppressed by tamoxifen treatment (**Fig. 4I**). These results further support our notion that E2 stimulates the secretion of CCL5 from microglia, which in turn promotes self-renewal of tumor cells.

Estrogen suppressed anti-tumor immune function of microglia

It is known that tumor cells express programmed death-ligand 1 (PD-L1) that binds PD-1 on T cell, resulting in dysfunction of cytotoxic T-cell activity [45]. Several other studies have also shown that microglia expresses PD-L1, which interacts with T cell PD-1 to suppress adaptive immune function and tumor infiltration of T cells [45-47]. We therefore examined whether E2 affects PD-L1 expression in microglia. As shown in **Fig. 5A**, both mRNA and protein expression of PD-L1 in microglia were significantly up-regulated by E2, and inhibiting STAT3 activation by STATTIC or blocking estrogen receptor by tamoxifen treatment significantly suppressed E2-induced up-regulation of PD-L1 (**Fig. 5A**). Moreover, the expression of PD-L1 was elevated in the membrane of microglia after E2 treatment (**Fig. 5B**). To investigate how upregulation of PD-L1 on microglia by E2 affects T cell function, T cells were incubated with microglia cells that were pre-treated with E2. We found that the proliferation of T cells was

significantly suppressed when T cell were incubated with E2-treated microglia (**Fig. 5C**). Blocking STAT3 activation in microglia by STATTIC treatment restored E2-mediated suppression of T cell proliferation (**Fig. 5C**).

Microglia cells are known to exhibit anti-tumor functions by phagocytosis and by releasing cytotoxic factors [48]. However, tumor cells express CD47 that interacts with signal regulatory protein α (SIRPα) on microglia [49] and triggers the 'do not eat me' signal which enables cancer cells to evade microglia's phagocytic ability. To examine if E2 functionally affects the "do not eat me" signal, we measured the expression of SIRPα on microglia and CD47 on tumor cells. We found that SIRPα was significantly upregulated in human and mouse microglia upon E2 treatment (**Fig. 5D**). Tamoxifen dramatically decreased E2-mediated up-regulation of SIRPα (**Fig. 5D**). We also found that E2 significantly increased the expression of CD47 in both SKBrM and 231BrM cells (**Fig. 5E**). Importantly, the expression of CD47 was shown to be strongly up-regulated in brain metastatic lesions of pre-menopause patients (**Fig. 5F**). We then examined the effect of E2 on phagocytic ability of microglia. 231BrM cells were pre-labeled with the red-fluorescent dye, PKH26 [50], and co-cultured with microglia that were treated with or without E2 or E2 plus tamoxifen. After 24 hour of incubation, small pieces of $PKH26^+$ cells were accumulated inside of microglia in control group, suggesting that tumor cells were rapidly degraded by microglia through phagocytosis (**Fig. 5G**). However, the microglia suppressed phagocytic activities toward tumor cells after E2 treatment (**Fig.5G and 5H)**. In contrast, tamoxifen reversed E2-mediated suppression of phagocytic activities **(Fig. 5Gand 5H)**. These results suggest that E2 suppresses microglia's immune function by blocking the phagocytic ability and up-regulating PD-L1, which in turn promotes brain metastasis progression.

Discussion

The tumor promoting effect of estrogen is well established; however, how estrogen affects tumor microenvironment and support tumor growth is still poorly understood. Our results indicate the critical roles of estrogen on tumor microenvironment in promoting brain metastasis by skewing microglia to M2 phenotype and inhibiting microglial phagocytic ability. This phenotypic conversion of microglia by estrogen is mediated through activation of STAT3, and that blocking STAT3 activation suppressed the M2 conversion, down-regulated the secretion of pro-tumor M2 cytokines and suppressed their phagocytic activity towards tumor cells. We also found that the population of M2 microglial cells were decreased in OVX and tamoxifen treated mice, while the estrogen-induced brain metastasis were suppressed in both OVX and tamoxifen treated mice. These results suggest that overall homeostasis of sex hormones play a key role in microglial polarization, which affects the progression of brain metastasis in women.

Skewing polarization of microglia by estrogen was reported by multiple previous studies [51-54], and several possible mechanisms were suggested. Saijo et. al. showed that estrogen could activate CCAAT/enhancer-binding proteins and inhibit the production of M1 pro-inflammatory cytokines in microglial cells [55]. It was also reported that estrogen suppressed the expression of M1 pro-inflammatory genes by blocking the nuclear translocation of NF-κB that suppressed DNA binding activity of NF-κB and

upregulated IκB, an inhibitor of NF-κB signaling [56, 57]. In addition, Vejito et al found that estrogen binding to estrogen receptor downregulated toll-like receptor 4 expression and inhibited M1 pro-inflammatory cytokines production [58]. These results suggest that estrogen modulates microglial polarization through multiple pathways. In the current study, we found a novel mechanism by which estrogen promotes M2 microglia by upregulating STAT3 expression. It should be noted that STAT3 activation has been associated with promotion of immunosuppressive microenvironment [59]. The activated STAT3 was known to reduce the expression of the genes that are necessary for antigen presentation such as MHC-II, CD80, and CD86 in microglia [60, 61]. Activation of STAT3 also promotes cell proliferation of microglia by enhancing cell survival genes [62]. Treating primary culture of microglia with the STAT3 inhibitor blocked their proliferation [63, 64]. We also observed that the number of M2 microglia was increased in the brain of premenopausal breast cancer patients as well as in the mouse supplemented with E2. On the other hand, the estrogen-associated proliferation of M2 microglia was blocked by the OVX and Tamoxifen treatment in mice, suggesting that estrogen deficiency suppresses microglia proliferation, which may well explain why the number of microglia is lower in brain metastasis of older breast cancer patients and in female mice with an OVX or tamoxifen treatment.

We have shown that estrogen decreased the secretion of M1-associated TNF-α and increased the M2-associated CCL5 in the conditioned medium of microglial cells. TNF-α is known to inhibit cell survival and proliferation by activating apoptosis signaling [43, 44]. The CCL5 and its receptor CCR5 have been detected in hematological malignancies and lymphomas. The interaction of CCL5 and CCR5 has been shown to promote tumor development by multiple mechanisms including acting as growth factors [65], enhancing migration and invasiveness [66], stimulating angiogenesis [67], and recruitment of macrophage [68]. Furthermore, knockdown or knockout of CCL5 in tumor cells was shown to suppress metastatic capability *in vivo* and *in vitro* of 4T1 breast cancer model [65, 69]. In our study, we found that stem cell genes, NANOG/OCT4/SOX2, were significantly up-regulated in CCL5-treated breast cancer cells, suggesting that E2-induced CCL5 promotes brain metastasis by enhancing the stemness of breast cancer brain metastatic cells. The depletion of CCL5 from CM of E2-treated microglia significantly suppressed E2-induced stemness genes and decreased the population of stem cells. Consistent with these results, CM from microglia treated with tamoxifen repressed the population of cancer stem cells by downregulating CCL5. These results also suggest that CCL5 in serum may serve as a novel therapeutic target as well as a biomarker for brain metastasis of breast cancer.

The current study focused on the effect of estrogen on tumor microenvironment; however, there are abundant reports showing direct effect of estrogen on tumor cells. There are mainly three known mechanisms for the pro-tumor actions of estrogen. First, estrogen promotes cell survival by increasing anti-apoptotic genes and by enhancing pro-survival factors such as PI3K-Akt signaling [70, 71]. In addition to the pro-survival effect, estrogen induces activation of Akt that may also increase the secretion of CCL5 [72]. Second, estrogen increases the activity of electron transport chain complexes I and II that increases mitochondrial bioenergetics [73, 74]. Finally, estrogen elevates anti-oxidative capacity in tumor region by increasing the glutathione levels in mitochondria [75]. Our study here showed that estrogen promotes brain metastasis by suppressing the phagocytosis function of microglia through activation of

the “don’t eat me” signal. Our results showed that the estrogen promoted the expression of CD47 on tumor cells and SIRPα on microglia. Interestingly, we found that brain metastasis lesion of pre-menopause breast cancer patient had higher expression of CD47 in tumor and SIRPα in microglia than post-menopausal patients. These results suggest that estrogen-induced up-regulation of tumor CD47 and SIRPα compromises phagocytic function of microglia, resulting in further tumor growth. In this context, it is noteworthy that high CD47 expression in breast cancer tissues showed significant association with reduced 5-year disease-free survival [76]. Therefore, CD47 may serve as a biomarker to predict breast cancer progression. We found that tamoxifen suppressed estrogen-induced up-regulation of SIRPα expression, resulting in enhanced phagocytic ability. In addition to this suppressive function of estrogen on innate immune function, we found that the expression of PD-L1 was increased in the estrogen-treated microglia. In contrast, a combination of estrogen and tamoxifen suppressed E2-induced elevation of PD-L1 expression. The proliferation of T cells decreased when T cells were co-cultured with microglia cells that were pretreated with E2. Since PD-L1 on microglia interacts with PD-1 on T cells, it also decreases T cell proliferation and their cytotoxic functions as we have shown. These results suggest that estrogen promotes brain metastasis by modulating both innate and adaptive immune functions in the brain.

Patients with TNBC generally show poor outcomes including early CNS metastasis and a short survival [11, 77]. Hormonal therapy is a standard of care for ER^+ tumors, however, it has been considered to be not useful for hormone receptor negative patients. Consequently, TNBC has limited treatment approaches. We found that tamoxifen did not directly affect tumor progression on ER-negative breast cancer or TNBC; however, tamoxifen exhibited strong anti-tumor ability *in vivo* by skewing microglial polarization to M1 and enhancing their phagocytic functions. This result strongly suggests that the effect of tamoxifen on hormone receptor deficient brain metastasis is mediated via modulation of brain microenvironment such as reprograming the function of microglia. Previous study demonstrated that treatment of recurrent malignant gliomas with chronic oral high-dose tamoxifen prolonged the patient survival [78], suggesting that tamoxifen can readily cross the BBB [26, 27] and affect brain tumor microenvironment. Moreover, Yang et. al. showed that tamoxifen increased the overall survival and disease-free survival rate in ER^-/PR^+ breast cancer patients [79]. In addition, treatment with combination of tamoxifen and radiation increased the overall survival rate and reduced the mortality rate of patients with brain metastasis of triple negative tumors [80]. It is noteworthy that Phase I/II clinical trial for testing efficacy of Tamoxifen and anastrozole on TNBC is currently ongoing (NCT01194908 and NCT01234532). Tamoxifen has been shown to up-regulate autophagy and inhibit the AMPK/mTOR pathway, resulting in tumor suppression in TNBC [81]. Previous studies also showed that tamoxifen suppressed ER-negative breast cancer cell invasion and metastasis by modulating miR-200c and transcription factor Twist1 [16, 17], suggesting that tamoxifen can suppress progression of ER negative breast cancer by ER-independent pathways.

Conclusion

Our results showed that estrogen promotes TNBC-brain metastasis by skewing microglia polarization and diminishing their innate and adaptive immune functions. We also found that the M2 microglia and tumor

progression were suppressed by OVX and tamoxifen treatment. Considering the inhibitory effect of tamoxifen on estrogen-related M2 microglial activation and their minimum toxicity, tamoxifen may serve as a potential treatment option for the brain metastasis, particularly for patients with ER-negative subtype.

Abbreviations

TNBC: Triple negative breast cancer

ER: Estrogen receptor

CCL5: C-C motif chemokine ligand 5

OVX: Ovariectomy

CM: Conditioned medium

GFP: Green fluorescent protein

E2: 17β-estradiol

BBB: Blood brain barrier

Arg1: Arginase-1

Arg2: Arginase-2

PD-L1: Programmed death-ligand 1

SIRPα: signal regulatory protein α

FACS: Flow cytometry

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Authors' Contributions

SW and KW designed the study and wrote the manuscript. SW, SS and KWu conducted experiments and acquired, analyzed and interpreted the data. SS, AT and KWu performed animal experiment. SW and KWu performed intracardiac injections. DZ and RPD performed clinical sample analysis, SW, SS, KWu, AT, DZ, RPD, and KW reviewed and edited manuscript and interpreted the data. KW supervised the study.

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Ethics declarations

Ethics approval and consent to participate: This study was approved by the institutional review board of the Wake forest University.

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Figures

Figure 1

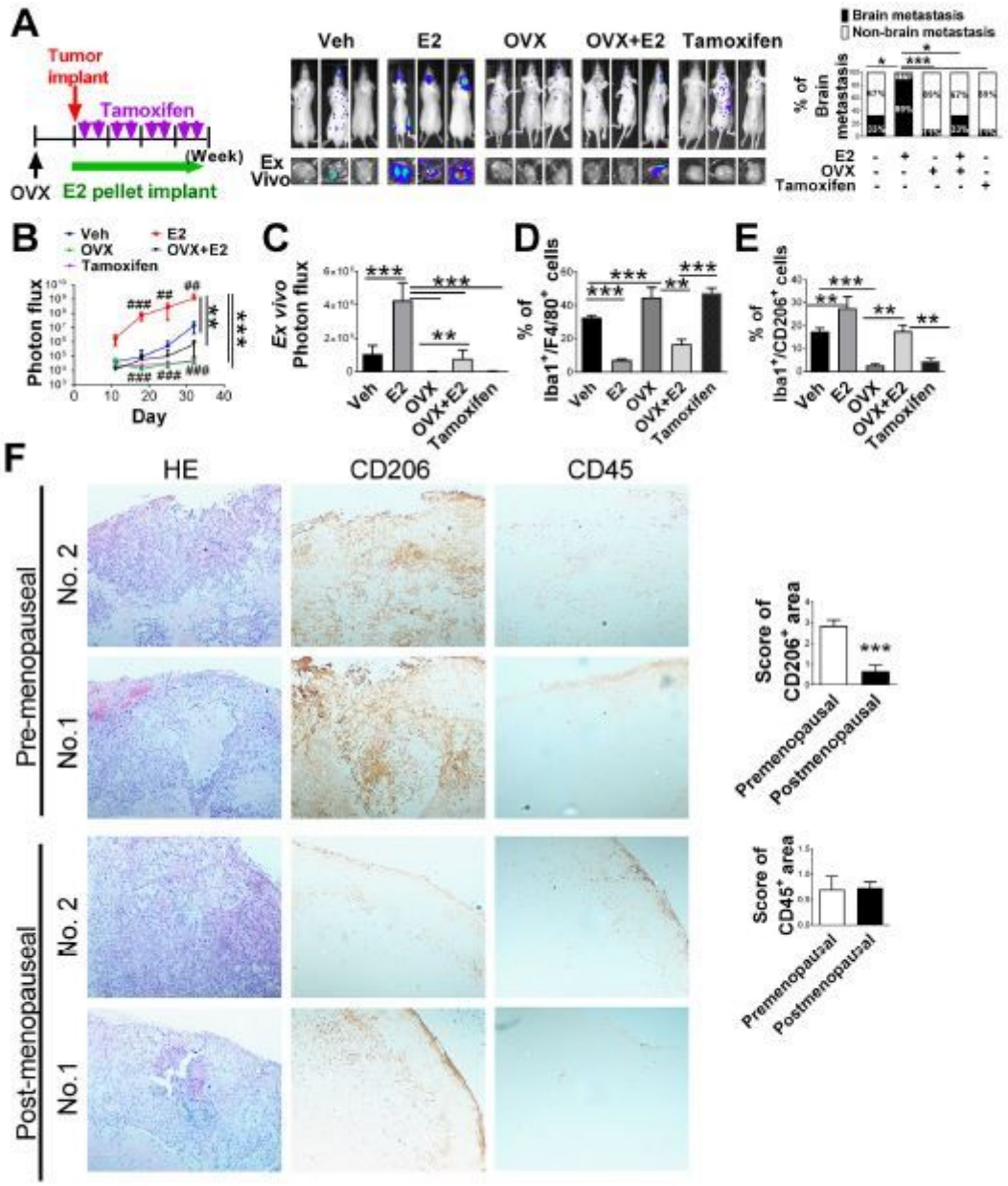

Figure 1

Estrogen promotes brain metastasis by activating microglia in vivo. A. Scheme of the experimental design is shown in the left panel. The OVX and OVX+E2 groups received the OVX treatment one week before the tumor injection. 231BrM (2×105 cells) were intracardially injected into nude mice (n = 9) followed by administration of E2 pellet. After 3 days of intracardiac transplantation of the tumor cells, mice in tamoxifen group received tamoxifen (20 mg/kg) treatment by i.p. injection every three days till

day 32. Middle upper panel: Representative BLI images of mice from each experimental group (vehicle alone or E2, OVX, OVX+E2 or tamoxifen treatment). Middle lower panel: Representative images of total ex vivo photon flux of brain metastatic lesions measured by BLI at the end point (Day 32). Right panel: Percentage of brain metastasis of breast cancer in nude mice with or without estrogen and/or tamoxifen treatment. Chi square test, *: $p < 0.05$; ***: $p < 0.001$. B. Quantitative data of in vivo brain metastasis of breast cancer. Two-way ANOVA, **: $p < 0.01$; ***: $p < 0.001$ versus the opposite group; ##: $p < 0.01$. ###: $p < 0.001$versus related Veh group. C. Quantitative data of ex vivo signals in the brain at the endpoint. One-way ANOVA, **: $p < 0.01$; ***: $p < 0.001$. D and E. Metastatic brain tumors from (A) were isolated and examined by flow cytometry for M1 (Iba1+/F4/80+) and M2 (Iba1+/CD206+) microglial polarization. One-way ANOVA, **: $p < 0.01$; ***: $p < 0.001$. F. Representative images of immunohistochemical analysis for CD206+ (M2 microglia) and CD45+ (Macrophage) cells in the brain metastatic lesions of pre-menopause (n=7) and post-menopause (n=7) breast cancer patients. Right panels: CD206 and CD45 staining cells were quantified in premenopausal and postmenopausal patients. t-test, ***: $p < 0.001$. The data are presented as the mean ± SD.

Figure 2

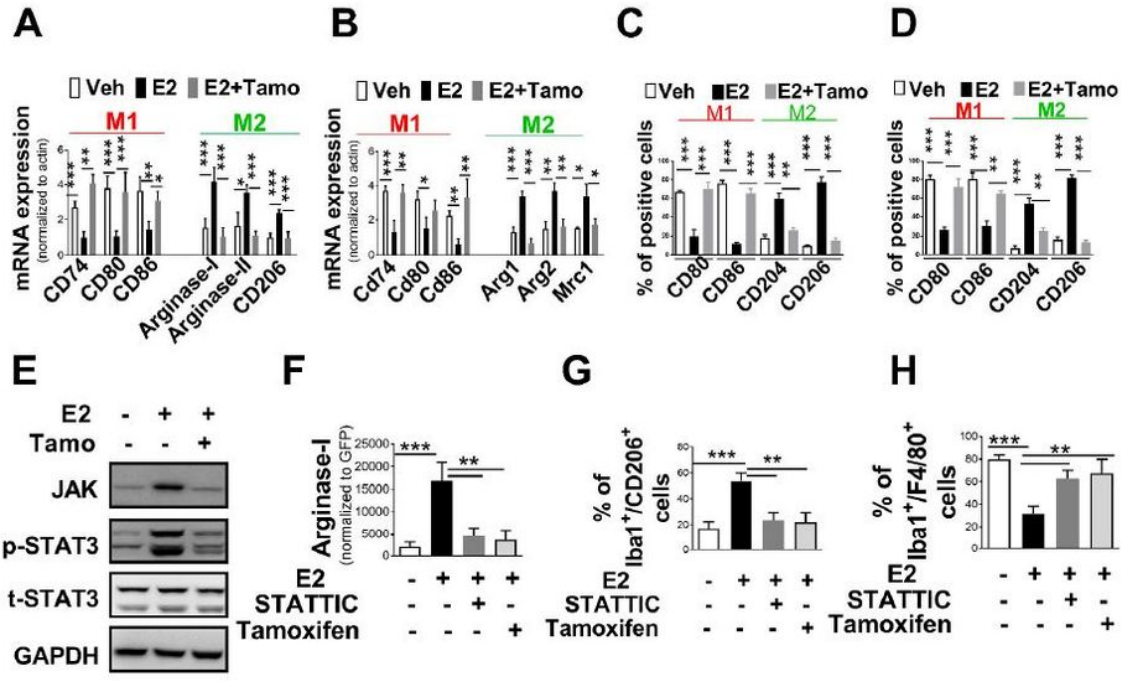

Figure 2

Estrogen promotes M2 microglial polarization through activation of STAT3. A and B. Expressions of M1/M2 microglia markers were examined after the vehicle or E2 (1 nM) or E2 plus tamoxifen (1 μM) treatment of human microglia (HMC3) (A) and mouse microglia (SIM-A9) (B). The value of qRT-PCR in each Figure was normalized using actin as a control. The Y-axis indicates arbitrary unit. C and D. Population of M1 and M2 cells was examined by FACS after the treatment of human microglia (HMC3) with vehicle alone, E2 (1 nM) or E2 plus tamoxifen (1 μM) (C) and SIM-A9 (D) cells. E. HMC3 cells were treated with or without E2 (1 nM) or Tamoxifen (1 μM) for 24 hours and the expressions of JAK and STAT3 were examined by western blot. F. The HMC3 cells were infected with lentivirus containing green fluorescent protein (GFP) gene and GFP+ cells were sorted by flow cytometry (FACS). The GFP+ HMC3 cells were seeded in 96-well plates for one day and they were transfected with the Arginase-1 promoter reporter plasmid using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA). After 24 hours, the cells were treated with only estrogen (1 nM) or estrogen plus STATTIC (0.5 μM) or tamoxifen (1 μM) and cultured for another 24 hours. The expression of luciferase was measured by using IVIS Xenogen bioimager. The Arginase-1 reporter luciferase activity (M2) was normalized with the expression of GFP+. G and H. Human microglia (HMC3) were treated with or without E2 (1 nM) and the STAT3 inhibitor (0.5 μM) or tamoxifen (1 μM) for 24 hours. Cells were then subjected to flow cytometry for quantifying Iba1+/CD206+ (M2 cell) (G) and Iba1+/F4/80+ (M1 cell) (H). The data are presented as the mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. ($n = 4$).

Figure 3

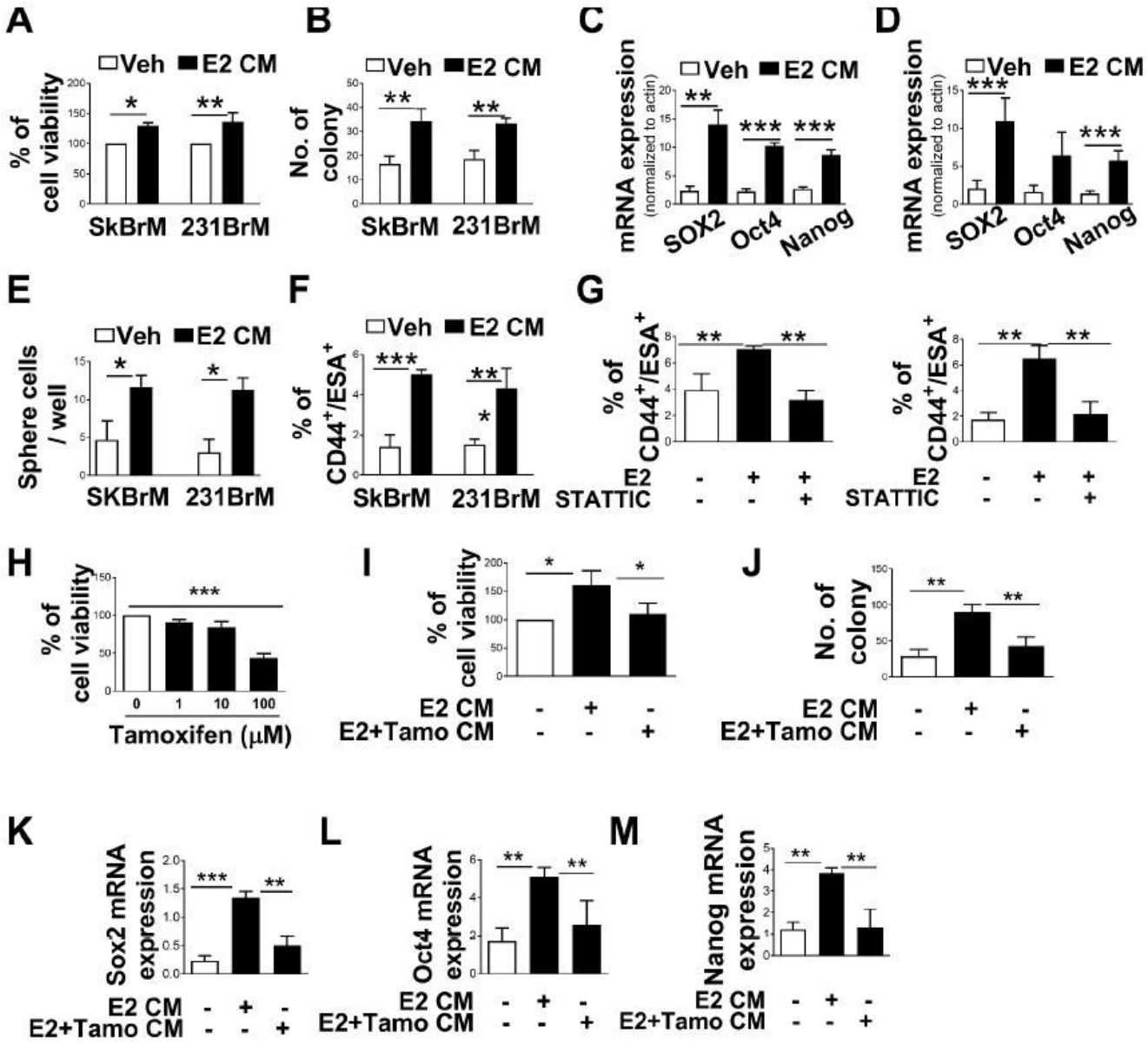

Figure 3

Estrogen-mediated M2 microglial polarization promotes tumor growth and stemness. A. SKBrM and 231BrM cells were incubated with the CM derived from E2-treated microglia for 24 hours. They were then examined for cell viability. Veh or E2 CM: microglia cells were treated with PBS or E2 (1 nM) for 24 hours, and they were washed with PBS and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 hours. B. Colony forming abilities of SKBrM and 231BrM cells were measured in the presence or absence of the CM derived from E2-treated human microglia. C and D. SKBrM and 231BrM cells were incubated with the CM derived from E2-treated microglia for 24 hours, and the expressions of stemness genes were examined by qRT-PCR. E. Brain metastatic breast cancer cells (SKBrM and 231BrM) were treated with the non-E2 or E2 CM for 24 hours and their sphere forming abilities were measured. F. Effect

of the CM derived from E2-treated microglia on cancer stem cell markers, CD44+/ ESA+ were examined using flow cytometry. G. SKBrM (Left) and 231BrM (Right) cells were examined for CD44+/ESA+ cancer stem cell population by FACS. H. 231BrM cells were incubated with tamoxifen at different concentration for 24 hours. They were then examined for cell viability. I. 231BrM cells were incubated with the CM derived from microglia that were treated with E2 alone or E2 plus tamoxifen for 24 hours. They were then examined for cell viability. E2 or E2+Tamo CM: microglia cells were treated with E2 (1 nM) or E2 plus tamoxifen (1 μM) for 24 hours, and they were washed with PBS and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 hours. J. Colony forming abilities of 231BrM cells were measured in the presence or absence of the CM derived from E2- or E2 plus tamoxifen-treated human microglia. K-M. 231BrM cells were incubated with the CM derived from E2- or E2 plus tamoxifen-treated microglia for 24 hours, and the expressions of stemness genes were examined by qRT-PCR. The data are presented as the mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. (n = 3).

Figure 4

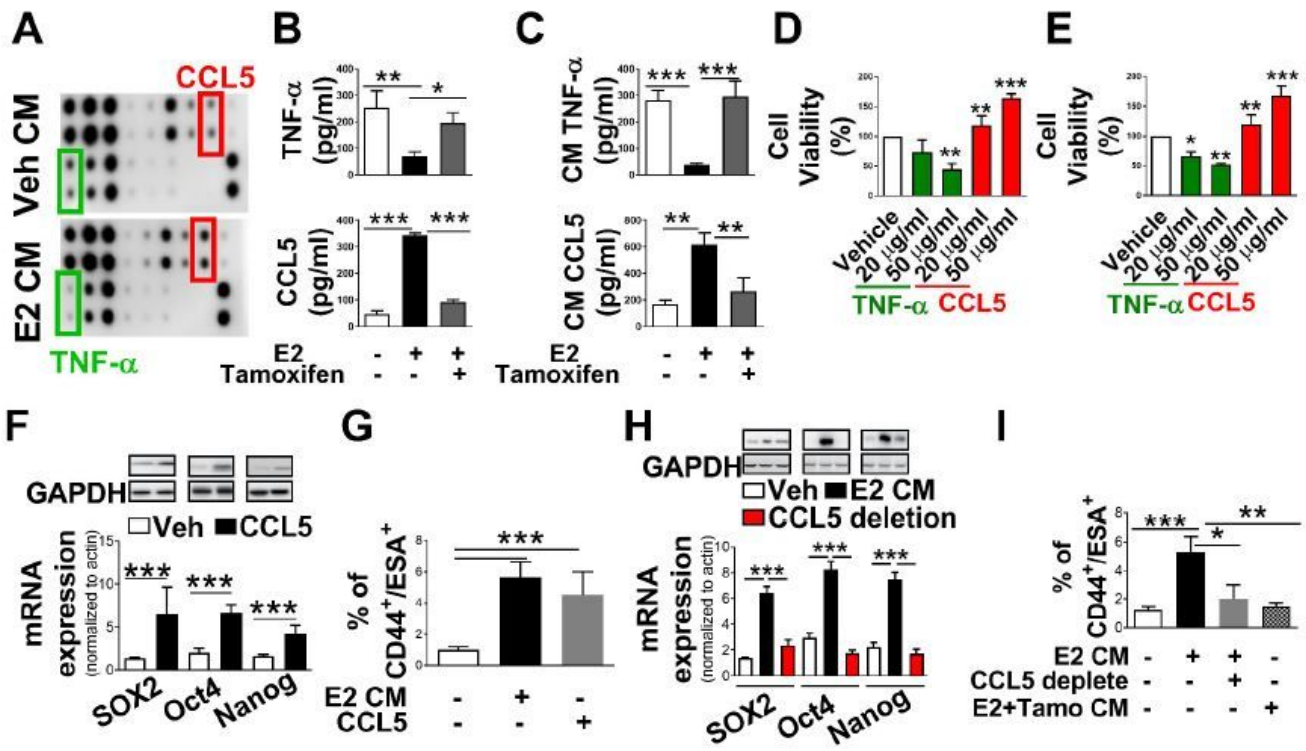

Figure 4

Estrogen induces secretion of CCL5 from microglia and promotes tumor growth and stemness. A. Human microglial (HMC3) cells were treated with PBS or E2 (1 nM) for 24 hours and they were washed with PBS and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 hours. CM was collected and analyzed by using the cytokine/growth factor array (Raybio). B and C. Human microglial (HMC3) cells were treated with or without E2 (1 nM) or E2 plus tamoxifen (1 μM) for 24 hours and cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS

for additional 24 hours. Cell lysates (B) and CM (C) were examined for the amount of TNF-α and CCL5 by ELISA (n = 5/group). D and E. SKBrM (D) and 231BrM (E) cells were treated with the indicated concentrations of recombinant TNFα and CCL5 for 24 hours. They were then examined for cell viability by the MTS assay. F and G. 231BrM cells were treated with CCL5 for 24 hours followed by measuring the stemness genes by qRT-PCR (F) and ESA+/CD44+ stem cell population by flow cytometry (G). H and I. Human microglia were treated with or without E2 (1 nM) for 24 hours, and their CM was prepared. The CM was then added with anti-CCL5 antibody to deplete CCL5. 231BrM cells were then treated with the CCL5-depleted CM followed by assaying stemness-related genes by qRT-PCR (H) and ESA+/CD44+ stem cell population by FACS (I). The data are presented as the mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. (n = 4).

Figure 5

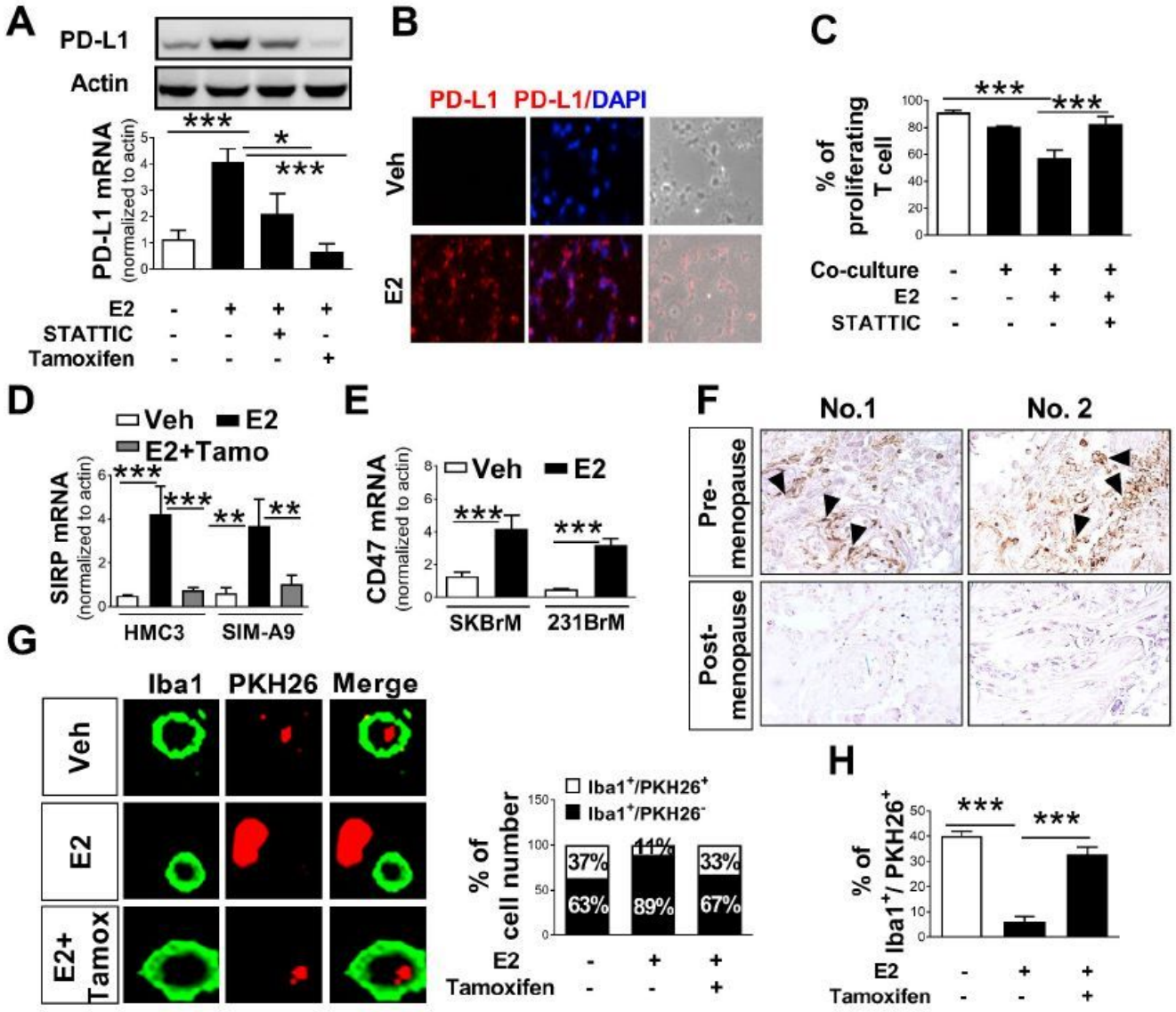

Figure 5

Estrogen suppresses innate anti-tumor immune function of microglia. A. PD-L1 expression was examined in human microglia (HMC3) cells after treating them with or without E2, STAT3 inhibitor (0.5 μM STATTIC) or tamoxifen (1 μM) by RT-PCR and western blot. B. Representative images of immunohistochemical analysis of PD-L1 in human microglia (HMC3) cells that were treated with or without E2 for 24 hours. C. T cells were incubated with E2- or E2 plus STATTIC-treated microglia. Proliferation of T cells was measured by flow cytometry. D. Human (HMC3) microglia cells were treated with or without E2 (1 nM) or E2 plus tamoxifen (1 μM) for 24 hours, and the expression of SIRPα was examined by qRT-PCR. E. SKBrM and 231BrM cells were treated with or without E2 for 24 hours, and the expression of CD47 was examined by qRT-PCR. F. The brain metastatic lesions from pre- or post-menopause patients in Fig. 1F were sectioned and they were subjected to immunohistochemical analysis for CD47+. Arrows indicate CD47+ cells (Brown) in pre-menopause (n=7) or post-menopause (n=7) patients. G. Phagocytic activities of HMC3 human microglial cells (green labeled) with or without E2 or E2 plus tamoxifen treatment were examined using PKH26-labeled 231BrM cells (red labeled). Both cells were incubated together for 24 hours, photographed (left panels), and the number of microglia that phagocytosed tumor cells were counted to quantify microglial phagocytic activity as shown in the right panel. H. A parallel set of cells in (G) was analyzed by flow cytometry to quantify phagocytic abilities of microglia after E2 or E2 plus tamoxifen treatment. The data are presented as the mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (n=4-5).

Figure 6

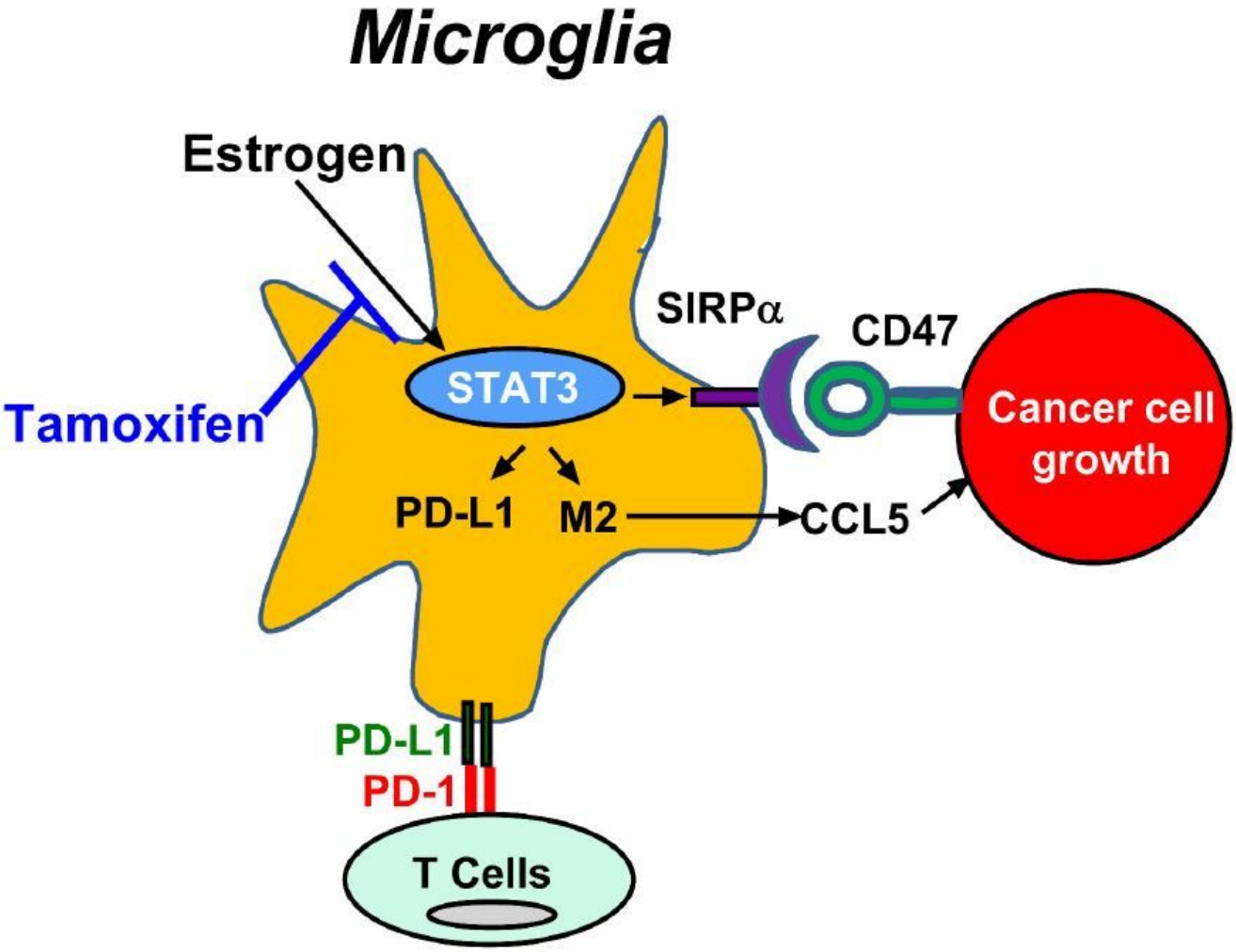

Figure 6

A proposed model illustrating an estrogen-induced brain metastasis.

Supplementary Files

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